TARGET ORGAN TOXICOLOGY SERIES

Adrenal Toxicology

Edited by
Philip W. Harvey
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For my family in England and Slovakia, especially my wife Daniela and our children Jessica Ruth, Rebecca Eve, and William Jozef.

P.W.H.

For my father, John.

D.J.E.

For colleagues at Covance whose support has allowed us the time to undertake this project.

C.J.S.

Preface

Despite the adrenal gland being the most common target within the endocrine system (Ribelin, 1984), adrenal dysfunction is poorly recognized in toxicology. The adrenal is a defined vital organ with a primary role in the adaptation to stressful circumstances (indeed adrenocortical glucocorticoid production is the single most important physiological response for survival of an organism post-injury or infection, e.g., Munck et al., 1984), and toxicological pathology in regulatory studies often disregards adrenocortical histological findings as secondary to stress, which can be inappropriate without evidence that the adrenal cortex is functionally competent. For example, adrenal hypertrophy may certainly be due to stress-induced oversecretion of adrenocorticotropic hormone (ACTH), but this condition may also arise from more serious adrenocortical steroidogenic enzyme inhibition, the consequent loss of glucocorticoid (cortisol or corticosterone) secretion, and reduced or abolished feedback control of pituitary ACTH secretion. The resultant uncontrolled ACTH hypersecretion can then overstimulate the cortex to produce hypertrophy. Genuine stress-related changes encountered in toxicity studies are generally considered to be of minimal toxicological consequence, being physiologically adaptive responses that are reversible upon withdrawal of treatment. By contrast, pharmacotoxicological suppression of steroidogenesis can be a serious condition leading to Addisonian crisis (adrenal insufficiency characterized by lethargy, hemodynamic instability, and cardiovascular collapse) and death. Indeed, there are many examples of drugs and chemicals known to inhibit critical adrenocortical enzymes, potentially producing adrenal incompetence, insufficiency, or suppression, and several examples have been discovered in patients following unexpected side-effects and fatalities apparently not adequately detected, or indeed ignored as inconsequential, in preclinical toxicology.

The adrenal medulla and cortex, respectively have acute and prolonged adaptive functions in the stress response, but it is the cortex that has additional important roles in regulating water and electrolyte balance, metabolism, inflammation, immune function, and various reproductive and developmental processes depending on life stage and species. It is therefore surprising that the adrenal has been neglected in endocrine toxicology and this has been pointed out as a critical omission in a regulatory toxicology context (Harvey and Johnson, 2002; Harvey and Everett, 2003; Harvey and Everett, 2006; Hinson and Raven, 2006; Harvey, Everett, and Springall, 2007). The United States Environmental Protection Agency (USEPA), Endocrine Disrupter Screening and Testing Advisory

Committee (EDSTAC), in not incorporating adrenal evaluation studies in its endocrine disruption strategy, failed to recognize the adrenal as an important endocrine gland influencing health, development, and survival fitness, or the unique vulnerability of the adrenal to toxic insult (see chap. 1) compared with other endocrine organs. Further, the adrenal cortex is also unique in possessing almost universal steroidogenic capability, and this was also overlooked in the recommendations for the development of models/assays to examine the effects of chemicals on steroidogenesis. The human adrenocortical carcinoma derived H295R cell line has been suggested as a useful system to address both issues (e.g., Harvey and Everett, 2003; Harvey, Everett, and Springall, 2007). Other regulatory bodies have since partially rectified this situation and recognized the importance of the adrenal in toxicology, or at least the utility of adrenocortical cells as a model for steroidogenesis as a whole, and the OECD has recently undertaken a program to validate the H295R cell line as a universal model to evaluate steroidogenic toxicity (e.g., Hecker et al., 2007). Oskarsson et al. (2006) report that steroidogenic gene expression following chemical challenge is comparable between the H295R cell line and the normal human adrenal demonstrating the validity of this cell line. The consensus is that the H295R cell line is currently the best available model, and even though ACTH receptors are not well expressed, effective downstream challenge augmentation can be achieved by drugs, such as forskolin.

The number of literature studies on adrenocortical toxicity has risen markedly over the past decade, which indicates the growing scientific and regulatory interest in the adrenal as a target for "endocrine disruption," with the majority of studies using in vitro techniques. Numerous laboratories are now using the H295R cell line to investigate the diverse effects of chemicals on molecular mechanisms of adrenocortical toxicity, and/or the general process of steroidogenesis, using steroid production, gene regulation, and enzyme expression as endpoints see Sanderson et al., 2001; Muller-Vieira et al., 2005; Gracia et al., 2006; Hecker et al., 2006; Oskarsson et al., 2006; Furuta et al., 2008 and Stigliano et al., 2008 for recent examples of the range of endpoints that can be assessed in this cell line, and chapters 7 and 8 for thorough reviews). While this is an important step forward, in vitro systems will not detect drugs or chemicals that affect adrenal function higher in the hypothalamo-pituitary axis, or effects on carrier proteins, and therefore there is also a need to validate in vivo models. A strategy for evaluation of drug and chemical effects on adrenocortical function has been proposed (Harvey, Everett, and Springall, 2007, and also see chap. 1 for further details) and involves a short-term in vivo adrenal challenge test in rodents (or other laboratory species if indicated—see Colagiovanni et al., 2006, and in this volume, for information on adrenal function and mechanism evaluation in the dog) coupled with the H295R in vitro assay to shed light on molecular sites of toxicity/functional inhibition.

The primary purpose of this text is to review the scope of, and developments in, adrenal toxicology. It is specifically designed to be both complementary to, and an update of, the first text in this area *The Adrenal in Toxicology: Target*

Organ and Modulator of Toxicity (Harvey, 1996a). The present text has minimal overlap with the former, but reviews the major developments in the field over the last decade, and identifies research requirements including the validation of standardized models and methods. The focus of this new text is on the adrenal as a target organ, both in the main mammalian species used in pharmaceutical and chemical regulatory toxicology and environmental sentinel species, and also on the advances in identifying molecular mechanisms of action. Most toxicological research has focused on the cortex rather than the medulla, which reflects its wider and more complex role in physiological processes, and this is also by necessity reflected in this text, although pathology of the medulla is examined in the rodent and human.

Following an introduction and overview of adrenal endocrine control and toxicology covering the range of toxicants, targets, mechanisms, interactions, models, and species differences (see chap. 1), there is a section covering the endocrinology and pharmacology of the adrenal in health and disease. An exemplary chapter on human adrenal dysfunction (see chap. 2) reinforces the critical importance of the correct function of this gland, both medulla and cortex, and from this, the potential consequences of drug or chemical-induced dysfunction may be inferred. For example, many adrenal diseases and syndromes are generally due to faults in gene, enzyme, or receptor expression, and the knowledge derived from human medicine of the molecular basis of dysfunction can assist in identifying the significance and consequences of pharmacotoxicologically induced effects on these targets. Further, the fact that many of the enzymes involved in human adrenocortical disorders can be pharmacologically manipulated (Hakki and Bernhardt, 2006) raises the real possibility that these adrenal conditions could occur as a consequence of off-target toxicity from drugs and chemicals, both in normal subjects, but especially in patients predisposed to develop the natural etiological factors of the disease, where additional insult may accelerate or precipitate onset. In addition, this section also provides a highly authoritative review (see chap. 3) of the endocrinology, pharmacology and pathophysiology of the hypothalamo-pituitary-adrenal (HPA) axis, including new emerging knowledge of developmental effects of HPA hormones. The early life influence of glucocorticoid hormones is an important pharmacological field with immense potential application; adrenal glucocorticoid analogues have well documented uses to accelerate maturation of premature/fetal lung, but have also long been known to have adverse effects on general growth and development in terms of teratogenicity (Hawkins, 1983). New evidence suggests they may also be associated with adult susceptibility to major diseases such as hypertension, type 2 diabetes, coronary heart disease, hyperlipidemia and nervous system disturbance following early life exposure. The pharmacology of glucocorticoids and effects on immune and inflammatory responses is introduced (properties which have been exploited in one of the most important classes of medicines in the past 50 years, for example, in asthma and allergy—see also Harvey, 1996a, and chapters therein for accounts of the well-known classical pharmacology of adrenal glucocorticoids and their synthetic analogues).

Thus, inappropriate adrenocortical steroid secretion due to pharmacotoxicological action, either oversecretion or suppression, can have far reaching effects on organ systems and tissues throughout the body. Understanding molecular mechanisms of drug action is fundamental to both pharmacology and toxicology, especially if the toxicity is due to suprapharmacological effects, and thus the purpose of this section is to outline the importance of the adrenal and its physiology in health and disease, and thereby identify potential pharmacotoxicological targets and the consequences of their manipulation and dysfunction.

The main section of the book details mammalian adrenal toxicology in the rodent, dog, and primate, both medulla and cortex. There are two definitive reference chapters on adrenal pathology, one on toxicopathology of the rodent adrenal medulla (see chap. 4) and the other on pathology of the primate adrenal (see chap. 5), and both provide microscopic histopathological examples and expert descriptions of the conditions affecting the adrenal. There is a chapter on evaluation of HPA function in the dog and in vivo mechanism elucidation (see chap. 6), which is a unique case illustration of a strategy for investigation of the site of toxicity within the integrated endocrine axis, developed to identify a mechanism and solve a specific regulatory preclinical toxicology question. There are two state-of-the-art chapters on in vitro mechanistic approaches from leading researchers in the field, one covering a comparison of the strengths and weaknesses of the various mammalian and human cell lines available (see chap. 8), and the second focusing exclusively on the use of the human H295R cell line (see chap. 7). This cell line is becoming the standard research system for toxicological evaluation of both adrenocortical function and the process of steroidogenesis as a whole (discussed earlier) and, having the advantages of being derived from human tissue and retaining complete steroidogenic functionality, this cell line can be used in mechanistic research with in vivo models to evaluate the majority, if not all, of the theoretically possible biochemical mechanisms of toxicity. The final chapter in this section is an extensive review of pharmacotoxicological interactions of adrenal hormones and the toxic response at the cellular and molecular levels (compare this with Harvey, 1996b, which discussed such interactions at the target organ and whole body level), detailing the molecular mechanisms influencing these effects including regulation of genes, cell cycle pathways and apoptosis (see chap. 9). Also discussed are the interesting and complex circumstances where these responses may be either beneficial or detrimental depending on the toxic insult and a variety of other interactive variables and conditions (see also chap. 1 on how coincidental physical stress as an interactive variable at the whole body level uncovered occult subclinical enzyme inhibition and severe adrenal suppression in patients treated with etomidate). Essentially, this section is designed to cover the major principles of adrenal toxicology at the tissue, cellular, and molecular levels; reinforce the complexity of adrenocortical hormone actions and interactions; and provide a primary reference source of examples, strategies, and methods for toxicologists and pathologists in both regulatory and research fields.

The final section covers adrenal dysfunction in environmental species and draws on field leaders who have documented adverse effects in fish (see

chap. 10) and birds (see chap. 11) from insidious ambient environmental exposures to chemical pollutants. Such species can be considered as sentinel or indicator species, and the fact that real environmental exposures have been shown to cause adrenal endocrine disruption in wildlife prompts the question of whether human adrenal function could also be compromised by low-level chemical exposures, producing occult subclinical effects. In such a case, further adrenal insult may combine with any preexisting functional deficiency to have interactive, additive, or disproportionally pronounced effects (especially in sensitive or predisposed individuals as discussed earlier), or indeed to overtly precipitate a toxicological reaction comprising an adrenal insufficiency crisis (although there is currently no evidence of adrenal suppression in humans from environmental chemical exposure, this is mainly because it has not been studied). If continuous or combined low-level exposures are indeed influencing adrenal function in a variety of wildlife species, this could ultimately affect survival fitness especially in adverse environmental circumstances. While endocrine disruption involving estrogenic/antiandrogenic effects often produces gross macroscopic morphological evidence in target/sentinel species, for example, to reproductive organs, this is generally not the case with adrenal dysfunction, and indeed the ultimate endpoint of adrenal dysfunction is death without obvious cause if there has been rapid onset of contributory factors (and as detailed above, these may be complex interactions where no single factor is an obvious cause). As with adrenal toxicity evaluation in mammalian regulatory toxicology, the challenge in environmental or ecotoxicology is to find reliable and accurate methods of identifying effects, made more difficult with wildlife species requiring minimal disturbance in situ. This section therefore extends the scope of adrenal toxicology into the field of "environmental endocrine disruption," and although the specific examples of fish and birds are of primary interest to the ecotoxicologist, humans are also environmentally exposed to combinations of a variety of chemical pollutants, and the toxicological hazards and potential consequences to human health can be at least recognized in principle (for example, see Furuta et al., 2008, on the effects of diesel exhaust chemicals on adrenocortical cell function in vitro). From this, there is a research need for properly controlled studies into human exposures to known adrenal toxicants, and for potential effects to be evaluated from which risk assessments can be developed. Human studies are notably lacking in the "endocrine disruption" literature in general, and addressing this should be considered a research priority.

Adrenal dysfunction can lead to significant morbidity and mortality across species. This has been well documented for humans in the medical literature, and even though drugs such as the anesthetic agent etomidate have been known for more than 20 years to inhibit critical enzymes on the cortisol pathway as an off-target side effect, new cases of etomidate-induced Addisonian crisis continue to be described, even after a single dose and as new variables come to light (Lundy et al., 2007). There is clearly a need to develop an assessment strategy to assess potential effects on adrenal function, both in mammalian regulatory toxicology programs of drugs and chemicals for human risk extrapolation, and also in environmental toxicology to assess the potential effects on wildlife and

humans from low-level chemical exposures. In regulatory toxicology of drugs and chemicals, Harvey, Everett, and Springall (2007) have suggested that a rodent in vivo adrenal function test is developed where, for example, rats treated with a test compound are also challenged with ACTH, and corticosterone secretion is assessed as an indicator of adrenal competence (see also chap. 1). This in vivo ACTH challenge test could also be applied to cortisol secretion in the dog (see chap. 6) or the primate to assess adrenal function in these species, if indicated by toxicological findings. An in vitro test using H295R cells and cortisol secretion and enzyme expression analyses was also suggested to examine and characterize mechanisms of toxicity (Harvey and Everett, 2003; Harvey, Everett, and Springall, 2007). This cell line is currently undergoing validation under OECD protocols as a method of examining the effects of chemicals on steroidogenesis (Hecker et al., 2007), but at present this appears to only involve sex steroid production. It would be prudent to extend this validation to include glucocorticoid and mineralocorticoid secretion with appropriate positive- and negative-control compounds. There is a similar need to develop and validate laboratory ecotoxicology tests in representative and ecologically important species such as fish. Thus, while the primary purpose of this text has been to highlight the importance of the adrenal gland in health and disease and review the scope and developments in adrenal toxicology, another major goal is to call for a strategy of standardized adrenal toxicology methodology applicable to regulatory toxicology, for the initiation and validation of such protocols in both mammalian and environmental toxicology, and for research into human exposures and assessment of their potential effects.

Finally, this text is designed for toxicologists and pathologists working in research and regulatory fields and pharmaceutical and chemical industry laboratories, requiring a reference source in an area rapidly gaining prominence in a regulatory context, namely adrenal toxicology and adrenal endocrine disruption. It will also be of interest to those working in in vitro, mechanistic and molecular toxicology, and to pharmacologists, environmental health scientists, ecotoxicologists, risk assessors, and endocrinologists.

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REFERENCES

Colagiovanni DB, Drolet DW, Dihel L, et al. Safety assessment of 4-thio-beta-D-arabinofuranosylcytosine in the beagle dog suggests a drug-induced centrally mediated effect on the hypothalamic-pituitary-adrenal axis. Int J Toxicol 2006; 25:119–126.

Furuta C, Noda S, Li C, et al. Nitrophenols isolated from diesel exhaust particles regulate steroidogenic gene expression and steroid synthesis in the human H295R cell line. Toxicol Appl Pharmacol 2008; 229:109–120.

Gracia T, Hilscherova K, Jones PD, et al. The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicol Environ Saf 2006; 63:293–305.

Hakki T, Bernhardt R. CYP17- and CYP11B-dependent steroid hydroxylases as drug development targets. Pharmacol Ther 2006; 111:27–52.

Harvey PW. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996a.

Harvey PW. Glucocorticosteroid modulation of toxicity. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996b:185–204.

Harvey PW, Everett DJ. The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: Critical omissions from regulatory endocrine disrupter screening strategies for human health? J Appl Toxicol 2003; 23:81–87.

Harvey PW, Everett DJ. Regulation of endocrine-disrupting chemicals: Critical overview and deficiencies in toxicology and risk assessment for human health. Best Pract Res Clin Endocrinol Metab 2006; 20:145–165.

Harvey PW, Everett DJ, Springall CJ. Adrenal toxicology: A strategy for assessment of functional toxicity to the adrenal cortex and steroidogenesis. J Appl Toxicol 2007; 27:103–115.

Harvey PW, Johnson I. Approaches to the assessment of toxicity data with endpoints related to endocrine disruption. J Appl Toxicol 2002; 22:241–247.

Hawkins DF. Drugs and Pregnancy: Human Teratogenesis and Related Problems. Longman, Edinburgh: Churchill Livingstone, 1983.

Hecker M, Hollert H, Cooper R, et al. The OECD validation program of the H295R steroidogenesis assay for the identification of in vitro inhibitors and inducers of testosterone and estradiol production. Phase 2: Inter-laboratory pre-validation studies. Env Sci Pollut Res 2007; 14(Special Issue 1):23–30.

Hecker M, Newsted JL, Murphy MB, et al. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: Hormone production. Toxicol Appl Pharmacol 2006; 217:114–124.

Hinson JP, Raven PW. Effects of endocrine-disrupting chemicals on adrenal function. Best Prac and Res Clin Endocrinol Metab 2006; 20:111–120.

Lundy JB, Slane ML, Frizzi JD. Acute adrenal insufficiency after a single dose of etomidate. J Intensive Care Med 2007; 22:111–117.

Muller-Vieira U, Angotti M, Hartman RW. The adrenocortical tumor cell line NCI-H295R as an in vitro screening system for the evaluation of CYP11B2 (aldosterone synthase) and CYP11B1 (steroid-11-beta-hydroxylase) inhibitors. J Steroid Biochem Mol Biol 2005; 96:259–270.

Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological action. Endocrine Rev 1984; 5:25–44.

Oskarsson A, Ulleras E, Plant KE, et al. Steroidogenic gene expression in H295R cells and the human adrenal gland: adrenotoxic effects of lindane in vitro. J Appl Toxicol 2006; 26:484–492.

Ribelin WE. The effects of drugs and chemicals upon the structure of the adrenal gland. Fund Appl Toxicol 1984; 4:105–119.

Sanderson JT, Slobbe L, Lansbergen GW, et al. 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin and diindolylmethanes differentially induce cytochrome P450 1A1, 1B1, and 19 in H295R human adrenocortical carcinoma cells. Toxicol Sci 2001; 61:40–48.

Stigliano A, Cerquetti L, Borro M, et al. Modulation of proteomic profile in H295R adrenocortical cell line induced by mitotane. Endocr Relat Cancer 2008; 15:1–10.

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Part I

Introduction to Adrenal Toxicology

1

Adrenal Toxicology: Molecular Targets, Endocrine Mechanisms, Hormonal Interactions, Assessment Models, and Species Differences in Toxicity

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INTRODUCTION

The adrenal gland is the most common toxicological target in the endocrine system (Colby and Longhurst, 1992; Ribelin, 1984; Rosol *et al*., 2001). In surveys based on in vivo toxicology studies, the order of endocrine organ toxicity by frequency of reported effects was adrenal> testes> thyroid> ovary> pancreas> pituitary> parathyroid (Colby and Longhurst, 1992; Ribelin, 1984) with the adrenal cortex, rather than the medulla, being the most frequent site of toxicity within the adrenal gland. Despite this, there has been a lack of recognition of the importance of adrenal function in a regulatory endocrine disruption evaluation context, and the need for an adrenal toxicology assessment strategy has been pointed out (Harvey and Everett, 2003; Harvey and Everett, 2006; Harvey and Johnson, 2002; Harvey *et al*., 2007; Hinson and Raven, 2006; Oskarsson *et al*., 2006). The lack of a regulatory strategy is surprising, given the experience in human medicine of the impact of adrenocortical suppression due to unrecognized drug side effects, the emerging evidence of the large range of compounds that produce adrenocortical toxicity, and also because environmental sentinel species, such as fish and birds, are showing evidence of adrenal dysfunction (Baos *et al*., 2006; Bisson and Hontela,

2002; Champoux *et al*., 2006; Dorval *et al*., 2005; Hontela *et al*., 1992; Norris *et al*., 1999; Quabius *et al*., 1997). Further, adrenocortical function is also known to modulate the response to, and tolerance of, toxic insult (Harvey *et al*., 1999; Harvey *et al*., 1994; Harvey, 1996a; Harvey, 1996b) and indeed glucocorticoid production is the single most important physiological response for survival of an organism postinfection or injury (Munck *et al*., 1984), including the stress resulting from chemical intoxication. Any impairment of this response is detrimental to health, and potentially, survival.

The adrenal is a vital organ, and inadvertent iatrogenic pharmacotoxicological inhibition of normal function of the cortex has been documented to be fatal in humans, following single exposures to some compounds at remarkably low dose levels. For example, etomidate (an anesthetic induction agent administered intravenously as a single dose of approximately 0.3 mg/kg bodyweight) and aminoglutethimide caused fatal adrenocortical suppression (Addisonian crisis), unpredicted and unrelated to the primary prescribed action of the compounds, (Camacho *et al*., 1967; Goldberg, 1983; Hinson and Raven, 2006; Leddingham and Watt, 1983; Leddingham *et al*., 1983; Raven and Hinson, 1996; Vermeulen *et al*., 1983). Aminoglutethimide, originally prescribed as a sedative and anticonvulsant, is now known to inhibit CYP11A1 cholesterol side chain cleavage (Johansson *et al*., 2002) and suppress the expression of the adrenocorticotropin receptor (Fassnacht *et al*., 1998), and is used in the treatment of Cushing's syndrome. Similarly, etomidate inhibited 11β/18 hydroxylase (cytochrome P450 11B1, CYP11B1, CYP11β/18) and blocked cortisol production with devastating consequences in trauma patients postsurgery, when a functioning hypothalamo-pituitary-adrenocortical axis (HPA) response was absolutely vital for survival. Indeed, this adverse effect only became apparent in conditions of coincident stress, which challenged the competence of the adrenal, illustrating the importance of hidden subclinical effects and the interactions of toxicological responses with physiological state.

Although medicinal use is usually a worst-case human exposure scenario (compared with environmental chemical exposures), the fact that etomidate has prolonged adrenocortical suppressant activity at low doses (in the microgram per kilogram bodyweight dose range) after only a single dose/exposure, raises the question of whether low-level, long-term exposures to environmental chemicals could produce unrecognized subclinical adrenal effects in the human population. For example, if the dose of etomidate that produced such marked unexpected off-target adrenocortical suppression was 0.3 mg/kg as a *single dose,* it is interesting to speculate that if it was a nonpharmaceutical chemical, and if the "no observed effect level" for this endpoint was assumed to be only 10-fold lower for oral chronic *repeat dose* exposures (0.03 mg/kg/day), then regulatory convention for a chemical (e.g., pesticide or agrochemical) would set the acceptable daily intake (ADI, representing safe human exposure) based on adrenocortical effects at 100-fold below this. This places the ADI at 0.0003 mg/kg/day (300 ng/kg/day), which is very low. Further, humans are exposed not to a single

chemical, but to many environmental industrial contaminants, agrochemical and pesticide residues from a variety of sources, both simultaneously and consecutively, and although these exposures are low in isolation, a combined multiple exposure scenario exists leading to additive action. Furthermore, as cortisol production can be affected by chemical action at a number of sites along the steroidogenic pathway (Harvey and Everett, 2003; Harvey *et al*., 2007), these sequentially dependent and obligate steps are particularly vulnerable and therefore combined exposures to chemicals or mixtures, potentially affecting different sites along a common pathway, could precipitate adrenocortical dysfunction at even lower exposure rates. The lack of a regulatory framework to study adrenal toxicity means that there is a paucity of quality standardized data, that data acquisition is slow, and that there are no recommended standardized regulatory protocols. However, an assessment strategy to evaluate adrenocortical toxicology in vivo and in vitro that could be developed in a regulatory toxicology context has recently been proposed (Harvey *et al*., 2007).

As well as direct action on the adrenal cortex, drugs can also suppress adrenocortical function by inhibition of hormones higher in the endocrine axis at the level of the hypothalamus or pituitary. However, the end result of deficits in glucocorticoid secretion is the same as a direct-acting adrenocortical enzyme inhibitor. For example, valproic acid, bromocriptine, cyproheptadine, ketanserin, ritanserin, somatostatin analogues, and glucocorticoids are drugs that suppress pituitary adrenocorticotrophic hormone (ACTH; adrenocorticotropin) secretion, and in turn adrenal glucocorticosteroid output, in both humans and rats; valproic acid also suppresses hypothalamic corticotropin-releasing hormone (CRH) (Kasperlik-Zaluska *et al*., 2005; Mercado–Asis *et al*., 1997; Sonino *et al*., 2005; Tringali *et al*., 2004). Additionally, 4-thio-β-D-arabinofuranosylcytosine (a structural analogue of gemcitabine and cytarabine) has recently been reported to markedly reduce cortisol and ACTH in dogs, suggesting centrally mediated impairment of the HPA axis (Colagiovanni *et al*., 2006). Furthermore, hexachlorobenzene reduces both plasma corticosterone and hepatic glucocorticoid receptors in vivo and in vitro in rat adrenals (Lelli *et al*., 2007), demonstrating that glucocorticoid receptors (required for correct control and function of the HPA axis) are also a target for toxicity. Conversely, ACTH secretion can be pharmacologically stimulated. For example, caffeine (Spindel *et al*., 1983), 3,4-methylenedioxymethamphetamine (Williams *et al*., 2005), and di-2-ethylhexyl phthalate (Supornsilchai *et al*., 2007) stimulate ACTH and corticosterone in the rat, and excess adrenocortical activity can also contribute to toxicopathological responses. Further, Chandra et al. (2007) showed that chromium increased corticosterone secretion and caused testicular toxicity in rats and concluded that adrenocortical hyperactivity accompanied by testicular oxidative stress may have a crucial role in chromium-induced male reproductive impairment (however, Harvey et al. (1992) showed that corticosterone alone did not produce testicular toxicity). The importance of these examples is that these compounds affect the adrenal by a mechanism only likely to be detected by in vivo studies, with intact HPA axis function,

which in turn affects any proposed assessment strategy for adrenal toxicology evaluations.

ADRENAL ENDOCRINOLOGY RELEVANT TO TOXICOLOGY

Reviews of the general endocrinology of the HPA axis and ACTH secretion are provided elsewhere (e.g., See Refs. Buckingham *et al*., 1992 and Philip *et al*., 1999) as are discussions of adrenal hormone synthesis, metabolism, transport, and action related to toxicology (See Refs. Raven and Hinson, 1996 and Hinson and Raven, 1996; Gumbleton and Nicholls, 1996; Hinson and Raven, 1999). However, the major activator of the HPA axis is stress, which may be psychological or physical such as cold, exercise, anesthesia, infection, hypoglycemia, injury or, indeed, toxic insult (in rodents, cage/bedding disturbance, noise, irritation, pain, arousal, strange environments, and smells can also stimulate the HPA axis). The release of ACTH from the pituitary is controlled by hypothalamic CRH and arginine vasopressin (AVP), although various other hormones can affect ACTH secretion (Philip *et al*., 1999); control of the HPA axis is shown in Figure 1.ACTH is released into the blood to stimulate the adrenal cortex to produce and secrete

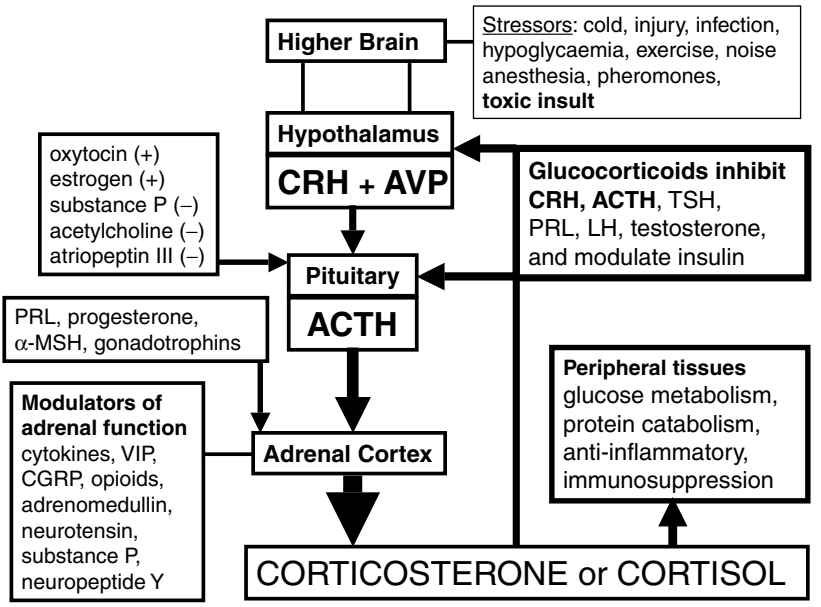

Figure 1 Control of the hypothalamo-pituitary-adrenocortical axis. *Abbreviations*: CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; ACTH/Corticotropin, adrenocorticotropic hormone; TSH/Thyrotropin, thyroid stimulating hormone; PRL, prolactin; α-MSH, α-melanocyte stimulating hormone; LH, luteinizing hormone; VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related protein.

glucocorticoids (corticosterone in rodents and cortisol in higher mammals and humans), and a variety of other steroids along the glucocorticoid pathway are also secreted. The glucocorticoids produce negative feedback inhibition at the level of both pituitary and hypothalamus to reduce ACTH output, thereby controlling the axis. The production and secretion of aldosterone is not under the control of ACTH; aldosterone is an endpoint response in the renin–angiotensin cascade, where angiotensin-II is potent stimulator of aldosterone in human and rat (the gene induction profile of angiotensin-II in both human and rat has recently been described by Nogueira et al. (2007) also, refer to Healing (1999) for overview of control of the renin–angiotensin system).

In considering an integrated endocrine system (Harvey and Rush, 1999), it is important to recognize that there are other endocrine interactions that affect HPA function, providing a range of additional toxicological targets and effects that may subsequently affect the adrenal. Estrogen is known to stimulate ACTH (Barrett, 1960) and therefore, adrenocortical function, while progesterone is reported to inhibit adrenocortical function in the rat (Rodier and Kitay, 1974). Gonadotropins, melanocyte stimulating hormone, and prolactin, both alone and synergistically with estrogen, have been reported to stimulate corticosterone in the rat (Ogle and Kitay, 1979; Sugihara *et al*., 1982; Vasquez and Kitay, 1978; Vinson *et al*., 1976). Prolactin and growth hormone are reported to stimulate chromaffin cells in the adrenal medulla and produce hyperplasia (Colby and Longhurst, 1992; Rosol *et al*., 2001). The adrenal medulla produces neuropeptides that influence the adrenal cortex (Hinson *et al*., 2000; Toth and Hinson, 1995; Whitworth *et al*., 2003). Indeed, within the adrenal, a diverse range of regulatory neuropeptides influence adrenal function including, adrenomedullin, calcitonin gene-regulated peptide, vasoactive intestinal peptide (VIP), neuropeptide Y, substance P, neurotensin, enkephalins, and other opioids (Hinson *et al*., 2000; Kapas *et al*., 1995; Renshaw and Hinson, 2001; Whitworth *et al*., 2002; Whitworth *et al*., 2003) with some peptides inducing proliferative responses as well as affecting steroidogenesis (Whitworth *et al*., 2002), which is of interest in a mechanistic toxicology context. Thus, in addition to the primary hormones of the HPA axis, and enzymes of adrenal steroidogenesis (Fig. 2),adrenal function can be affected by a range of other more subtle biochemical pathways and receptors, which theoretically may also present as pharmacological or toxicological targets.

Further, endogenous glucocorticoids have a number of endocrinological effects other than controlling ACTH release, and are reported to suppress prolactin in rats (Gala *et al*., 1981) and humans (Bratusch-Marrain *et al*., 1982), acutely inhibit insulin in rats (Billaudel and Sutter, 1982), and suppress thyroid stimulating hormone (Pamenter and Hedge, 1980). Corticosterone also inhibits luteinizing hormone (LH) and testosterone in vivo and in vitro in the male rat (Kamel and Kubajak, 1987; Sankar *et al*., 2000; Srivastava *et al*., 1993), and corticosterone treatment decreases prostate and seminal vesicle weights attributed to suppressed LH and testosterone (Harvey *et al*., 1992). The purpose of reviewing

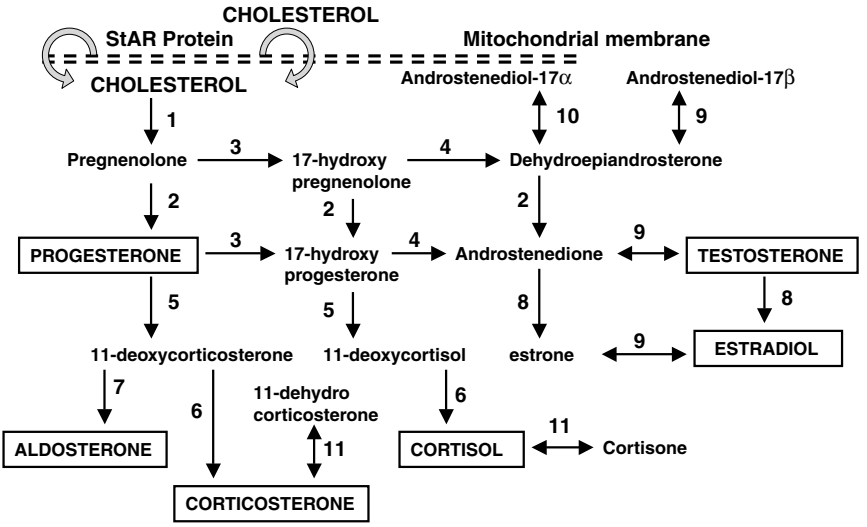

Figure 2 Adrenocortical steroidogenesis pathways. *Abbreviation*: StAR, steroid acute regulatory protein. Key to enzymes: 1 = CYP11A1 (cholesterol side chain cleavage). 2 = 3b-hydroxysteroid dehydrogenase-D 4,5 isomerase. 3 = CYP17 (17a-hydroxylase). 4 = CYP17 (17, 20 lyase). 5 = CYP21 (21 hydroxylase). 6 = CYP11B1 (11B/18-hydroxylase). 7 = CYP11B2 (aldosterone synthase). 8 = CYP19 (aromatase). 9 = 17b-hydroxysteroid dehydrogenase. 10 = 17a-hydroxysteroid dehydrogenase. 11 = 11b-hydroxysteroid dehydrogenase. Adapted from Harvey et al., (2007). Copyright John Wiley and Sons Limited. Reproduced with permission.

these endocrinology examples is to draw attention to the fact that influences on adrenal function can occur in a number of diverse ways, and altered adrenal function may have far reaching repercussions physiologically, not least to the function of thyroid, testes, and pancreas. Understanding the endocrinology of rodents is important for understanding mechanisms of toxicity in regulatory studies, and adrenal toxicity may be direct or secondary to altered endocrine function, and occur within or across the HPA axis (Harvey *et al*., 1999; Harvey, 1996a; Harvey, 1996b).

THE ADRENAL AS A TARGET ORGAN: MECHANISMS AND EXAMPLES IN CORTEX AND MEDULLA

Previous reviews have collectively introduced knowledge of the range of structurally diverse compounds known to induce toxicity in vivo to the adrenal cortex (Colby and Longhurst, 1992; Colby, 1996; Harvey and Everett, 2003; Harvey *et al*., 2007; Hinson and Raven, 1999; Raven and Hinson, 1996; Ribelin, 1984; Rosol *et al*., 2001; Szabo and Lippe, 1989) and to the adrenal medulla (Hinson and

Raven, 1999; Rosol *et al*., 2001; Tucker, 1996). Hinson and Raven (2006) summarize the factors predisposing the adrenal cortex to toxic insult in vivo which include:

- The large number of potential toxicological targets such as receptors, enzymes, and peripheral hormone-carrier molecules (the number of sequentially dependent steroidogenic steps in cortisol or aldosterone production/secretion also adds to their vulnerability)
- High vascularity and disproportionately large blood volume received per unit mass of adrenal tissue
- The high content of unsaturated fatty acids in adrenocortical cell membranes that are susceptible to lipid peroxidation
- Lipophilicity due to rich cholesterol and steroid content
- The high content of cytochrome P450 (CYP) enzymes present in the adrenal cortex, that normally catalyze steroidogenesis, but which can also produce both reactive metabolites of toxicants and hydroxylation reactions that may generate free radicals.

Added to this is the reliance of the adrenal cortex on the trophic support of hormones of the hypothalamus and pituitary (and the side effects of drugs such as valproic acid, bromocriptine, ketanserin, and numerous other compounds on HPA activity have been previously mentioned) and on interactions with other hormones and glands, not least local adrenomedullary neuropeptide effects on the adrenal cortex (Hinson *et al*., 2000; Toth and Hinson, 1995; Whitworth *et al*., 2003), such as the stimulatory effect of adrenomedullin on aldosterone and cortisol secretion (Thomson *et al*., 2001).

The adrenal medulla is directly dependent on nervous system function (and the adrenal cortex is ultimately dependent on hypothalamus and other structures in the brain), and this illustrates how distal neuropharmacological actions could ultimately influence the adrenal, both medulla and cortex. Indeed, adrenocortical cells (human adrenocortical carcinoma derived H295R cells) have β-adrenergic receptors [the synthetic catecholamine isoproterenol provokes aldosterone, cortisol, and dehydroepiandrosterone secretion (Kosti *et al*., 2002)] and dopamine receptors, where the D2 receptor is involved in inhibiting aldosterone release whereas the D4 receptor augments aldosterone secretion (Chang *et al*., 2008). H295R cells also have γ-aminobutyric acid (GABA) receptors, and produce GABA with a suggested paracrine/autocrine role (Metzeler *et al*., 2004). These examples illustrate the responsiveness of the adrenal cortex to neuropharmacological stimulation.

Mechanisms of Adrenomedullary Toxicity

There are fewer examples of chemicals affecting the adrenal medulla and consequently there is less information concerning mechanisms. Tucker (Tucker, 1996) reviewed the chemicals reported to induce general toxicity and proposed mechanisms (e.g., cysteamine hydrochloride, acrylonitrile, tamoxifen, reserpine, and

mannitol) and also adrenomedullary tumourigenesis (e.g., polyols, growth hormone). Phaeochromocytoma is a common finding in the rat (Tischler *et al*., 2004), particularly the male, and studies of chromaffin cell proliferation suggest excess growth hormone or prolactin stimulation of cholinergic nerves and dietary mechanisms such as hypercalcemia as causes (Rosol *et al*., 2001; Tucker, 1996). Progress has been made in developing animal models of phaeochromocytoma and in comparing gene expression profiles of rat phaeochromocytoma material obtained from the National Toxicology Program, with both normal rat adrenal and human phaeochromocytoma (Elkahloun *et al*., 2006; Ohta *et al*., 2006; Tischler *et al*., 2004). Adrenal medullary lesions and toxicity are detailed elsewhere in this volume (Chapter 4).

Although more agents historically have been reported to affect the adrenal cortex compared with the medulla (Colby, 1996; Ribelin, 1984), implying direct adrenocortical toxicity and adrenocortical vulnerability; the proportion showing true direct adrenocortical toxicity compared with secondary effects (stress-induced–nonspecific adrenocortical changes due to administration of a compound at the maximum tolerated dose—MTD) is unknown. A variety of compounds have been shown to result in higher corticosterone secretion in rodents at dose levels approximating the MTD, indicating stress-induction of the HPA axis (Harvey, 1996a) and indeed administration of compounds at the MTD is, by definition, stressful (Miller, 1992). In these cases, the reported elevations of corticosterone at least establishes functionally competent adrenals.

Mechanisms of Adrenocortical Toxicity

Although the above mechanisms are largely relevant to in vivo adrenal toxicology, effective and relevant in vitro toxicology models, such as the H295R cell line, have recently been developed that are now expanding the range of known adrenocortical toxicants and their molecular mechanisms of action (Blaha *et al*., 2006; Canton *et al*., 2006; Gracia *et al*., 2006; Hecker *et al*., 2006; Hilscherova *et al*., 2004; Imagawa *et al*., 2006; Kau and Kan, 2005; Li and Wang, 2005; Li *et al*., 2004; Lin *et al*., 2006; Muller-Vieira *et al*., 2005; Ohno *et al*., 2002; Oskarsson *et al*., 2006; Sanderson *et al*., 2002; Sanderson *et al*., 2004; Sanderson, 2006; Voets *et al*., 2004; Xu *et al*., 2006; Zhang *et al*., 2005 and see discussion in Harvey et al., 2007). Figure 2 shows the general steroidogenic pathways in the adrenal cortex, identifying potential molecular targets for toxic action. The molecular steroidogenic steps within the adrenal cortex are generally similar among rat, mouse, and human supporting the relevance of the rodent as a predictive toxicological model. However, factors triggering activation and sensitivity of the HPA axis vary across species, and the dominant glucocorticosteroid in rodents is corticosterone, compared with cortisol in humans and other higher mammals. Table 1 lists more than 70 chemicals affecting adrenocortical function and steroidogenesis and their reported molecular targets including Steroid Acute Regulatory protein (StAR), and cytochrome P450 (CYP) and dehydrogenase enzymes.

Table 1 Examples of Compounds Inducing Functional Adrenocortical and Steroidogenic Toxicity and Targets Affected

Steroidogenic target	Compound	Reference
ACTH receptor	Aminoglutethimide	(Fassnacht *et al*., 1998)
Steroid acute regulatory (StAR) protein	Econazole, miconazole, lindane	(Oskarsson *et al*., 2006; Walsh and Stocco, 2000; Walsh *et al*., 2000)
	Glyphosate	(Walsh *et al*., 2000)
	Dimethoate	(Walsh *et al*., 2000)
	Carbachol	(Janossy *et al*., 2001)
	Ethanol	(Khisti *et al*., 2003)
	Arsenite, anisomycin	(Zhao *et al*., 2005)
	Bromocriptine	(Kan *et al*., 2003)
	Spironolactone	(Hilscherova *et al*., 2004)
	Helenalin (sesquiterpene lactone)	(Supornsilchai *et al*., 2006)
	Beta-napthoflavone (AhR ligand)	(Sugawara *et al*., 2001)
CYP11A1 (CYPscc)	Aminoglutethimide	(Camacho *et al*., 1967; Hecker *et al*., 2006; Johansson *et al*., 2002; Vermeulen *et al*., 1983)
	Dimethoate	(Walsh *et al*., 2000)
	Bromocripitine	(Kan *et al*., 2003)
CYP17	Spironolactone	(Kossor *et al*., 1991)
	Ketoconazole	(Johansson *et al*., 2002; Loose *et al*., 1983)
	Flavonoids (6-hydroxyflavone)	(Ohno *et al*., 2002)
	PCB126	(Li and Wang, 2005)
	Penta, octa, deca-brominated diphenyl ethers, tetrabromobisphenol-A, hexabromocyclododecane isomers	(Canton *et al*., 2006)
	Thiazolidinediones—pioglitazone	(Kempna *et al*., 2007)
	Salbutamol	(Gracia *et al*., 2007)
3-Hydroxysteroid dehydrogenase Δ 4,5 isomerase	Cyanoketone	(McCarthy *et al*., 1966)
	Trilostane	(Potts *et al*., 1978)
	6-hydroxyflavone, daidzein, genistein,biochanin A, formononetin	(Ohno *et al*., 2002)
	PCBs (101, 110, 126, 149) PAHs/PCBs	(Blaha *et al*., 2006; Xu *et al*., 2006)

(*continued*)

Table 1 (*continued*)

Steroidogenic target	Compound	Reference
	Thiazolidinediones—pioglitazone	(Kempna *et al*., 2007)
	Oestradiol	(Gell *et al*., 1998)
	Bromophenols, polybrominated biphenyls, 2, 3, 7, 8-tetrabromodibenzo-*p*-dioxin, 2, 3, 7, 8-terabromodibenzofuran (effects also on 17β-Hydroxysteroid dehydrogenase, StAR and CYPs 11A1, 11 B2, 17, 19 and 21)	(Ding *et al*., 2007)
17β-Hydroxysteroid dehydrogenase	Di (2-ethylhexyl) phthalate	(Akingbemi *et al*., 2001)
	PCBs (101, 110, 126, 149)	(Xu *et al*., 2006)
CYP21	RU486	(Albertson *et al*., 1994)
	Ketoconazole	(Johansson *et al*., 2002)
	Flavonoids	(Ohno *et al*., 2002)
	PCB126	(Li and Wang, 2005)
	PAHs/PCBs	(Blaha *et al*., 2006)
CYP11B1 (CYP11β/18)	Metyrapone	(Johansson *et al*., 2002; Liddle *et al*., 1958)
	Mitotane (*o*,*p*-DDD); MeSO2-DDE	(Hornsby, 1989; Johansson *et al*., 2002; Lindhe *et al*., 2002)
	Etomidate	(Hinson and Raven, 1996; Leddingham and Watt, 1983)
	Ketoconazole; aminoglutethimide	(Johansson *et al*., 2002)
	Flavonoids	(Ohno *et al*., 2002)
	PCB126	(Li and Wang, 2005; Lin *et al*., 2006)
	PCBs (101, 110, 126, 149)	(Xu *et al*., 2006)
	Efonidipine, mibefradil	(Imagawa *et al*., 2006)
	Potassium and potassium + PCB126	(Li and Lin, 2007)
CYP19 (Aromatase)	Prochloraz, imazalil	(Andersen *et al*., 2000)
	Prochloraz, fadrozole, epoxyconazole	(Heneweer *et al*., 2004)
	Diindolylmethanes	(Sanderson *et al*., 2001a)
	Triazines—atrazine, simazine, propazine	(Sanderson *et al*., 2001b)

Table 1 (*continued*)

Steroidogenic target	Compound	Reference
	Atrazine, simazine, benzopyrene (CYP19 induction via SF-1)	(Fan *et al*., 2007)
	Di-, tributyl and phenyltin chlorides	(Sanderson *et al*., 2002)
	Imidazoles, vinclozalin, fenarimol	(Sanderson *et al*., 2002)
	Flavonoids (7-hydroxyflavone, chrysin)	(Sanderson *et al*., 2004)
	Fadrozole	(Muller-Vieira *et al*., 2005; Hecker *et al*., 2006)
	PCBs (101, 110, 126, 149)	
	PCB 126 & 39	(Li, 2007)
	Bisphenol A diphenylalkanes (no effect)	(Letcher *et al*., 2005)
	Mono-(2-ethylhexyl) phthalate (inhibition via Nur77 gene expresion)	(Noda *et al*., 2007)
	Amoxicillin	(Gracia *et al*., 2007)
	Cyproterone + salbutamol binary	(Gracia *et al*., 2007)
CYP11B2 (Aldosterone synthase)	Guanabenz-related amidinohydrazones	(Hinson and Raven, 1996; Soll *et al*., 1994)
	PCB126	(Li and Wang, 2005; Lin *et al*., 2006)
	Fadrozole	(Muller-Vieira *et al*., 2005)
	PCBs (101, 110, 126, 149)	(Xu *et al*., 2006)
	Efonidipine, mibefradil	(Hecker *et al*., 2007)
	PAHs/PCBs	(Blaha *et al*., 2006)
	Amoxicillin, erythromycin	(Gracia *et al*., 2007)
Altered steroid output, enzyme activity, and gene expression in H295R cells	**Examples of steroid studies**	
	Ketoconazole, prochloraz, fadrozole, aminoglutethimide, vinclozalin (↓oestradiol, ↑progesterone, ↑pregnenolone, ↓testosterone: profile varies with chemical)	(Hierlihy *et al*., 2006)
	6-hydroxyflavone, 4-hydroxyflavone, apigenin, daidzein, genistein, formononetin (↓cortisol, ↓DHEA)	(Ohno *et al*., 2002)
	Procaine (↓HMG-coA/cholesterol)	(Xu *et al*., 2003)
	Efonidipine (↓cortisol, ↓aldosterone)	(Imagawa *et al*., 2006)
	Fadrozole (↓cortisol, ↓androgens, ↓aldosterone)	(Muller-Vieira *et al*., 2005)

(*continued*)

Table 1 (*continued*)

Steroidogenic target	Compound	Reference
	Digoxin, ouabain (↓cortisol, ↓aldosterone)	(Kau and Kan, 2005)
	Lindane (↓cortisol)	(Oskarsson *et al*., 2006)
	PCB126 (↑aldosterone)	(Li *et al*., 2004)
	PCB126 (↑aldosterone, ↑cortisol, ↓androgens)	(Li and Wang, 2005)
	Lipopolysaccharide endotoxin (↑cortisol,	(Vakharia and Hinson, 2005)
	no effect on aldosterone)	(Vakharia *et al*., 2002)
	Amoxicillin (↑oestradiol) erythromycin (↑oestradiol, progesterone) salbutamol (↓oestradiol) Ethynylestradiol + trenbolone (↑oestradiol)	(Gracia *et al*., 2007)
	Forskolin (↑testosterone, ↑oestradiol) Fadrozole, prochloraz (↓testosterone, ↓oestradiol)	(Hecker and Giesy, 2007; Hecker *et al*., 2007)
	Examples of enzyme studies	
	PAHs/PCBs	(Blaha *et al*., 2006)
	Triazines—atrazine, simazine, propazine	(Sanderson *et al*., 2001b)
	Di-, tributyl and phenyltin chlorides, imidazoles, vinclozalin, fenarimol	(Sanderson *et al*., 2002)
	Flavonoids	(Ohno *et al*., 2002; Sanderson *et al*., 2004)
	Thiazolidinediones—pioglitazone,	(Kempna *et al*., 2007)
	Amoxicillin, erythromycin, salbutamol, salbutamol + cyproterone	(Gracia *et al*., 2007)
	Examples of gene expression studies (expression of steroidogenic enzymes)	(Gracia *et al*., 2007; Hilscherova *et al*., 2004; Kau and Kan, 2005; Kempna *et al*., 2007; Li and Wang, 2005; Li, 2007; Noda *et al*., 2007; Oskarsson *et al*., 2006; Xu *et al*., 2006; Gracia *et al*., 2006; Zhang *et al*., 2005)

↑ = increased hormone secretion, ↓ = decreased hormone secretion

Source: Adapted from Harvey, Everett and Springall (2007). Copyright John Wiley and Sons Limited. Reproduced with permission.

The relatively large number of compounds known to affect adrenal steroidogenic targets should allow structure activity analyses to identify toxophores, and this would be a useful area of study. Further, the number of compounds reported to affect adrenocortical function is larger when compound class analogues and metabolites from the cited examples are included, or when data on common steroidogenic targets in nonadrenal cells are also considered, and in this case, the in vitro molecular regulation of the target in nonadrenal cells compared with adrenal cells (e.g., StAR) may not be identical, but vulnerability can be inferred. For example, Hierlihy et al. (2006) examined the effects of ciprofibrate on testicular and adrenal steroidogenic enzymes in rats and reported that 3β-hydroxysteroid dehydrogenase-isomerase, and to a lesser extent 17β-hydroxysteroid dehydrogenase, were reduced in the testes, but much less so in the adrenal. Hierlihy et al. (2006) interpreted this difference in tissue sensitivity with ciprofibrate to differences in enzyme control mechanisms; however, it is also worth noting that 3β-hydroxysteroid dehydrogenase-isomerase activity was 10-fold higher in the adrenal, which indicates a greater reserve and resistance to inhibition compared with the testes. Under normal physiological conditions, the adrenal cortex has a large functional reserve capacity, but inhibition of a single critical path enzyme, such as CYP11B1 by etomidate, can completely and rapidly abrogate this. Furthermore, the aforementioned studies in Table 1 have been selected to illustrate compounds that influence adrenocortical function via steroidogenesis, and there are other mechanisms of adrenal toxicity. These mechanisms include downregulation of the ACTH receptor, for example, by aminoglutethimide, (Fassnacht *et al*., 1998) and the induction of apoptosis and severe hemorrhagic necrosis by 7,12-dimethylbenz[a]anthracene (Fu *et al*., 2005).

Individual compounds may also induce adrenocortical toxicity by more than one mechanism. For example, aminoglutethimide both inhibits CYP11A1 cholesterol side chain cleavage (Johansson *et al*., 2002) and downregulates the ACTH receptor (Fassnacht *et al*., 1998) in H295R cells. Mixtures or combined exposures of different chemicals may also interact to affect adrenocortical function. Recently Li and Lin (2007) reported that potassium provokes both aldosterone and cortisol biosynthesis in H295R cells, and that pretreatment with polychlorinated biphenyl-126 synergistically increased the effects of potassium. The additive or synergistic responses to combined exposures are a particularly important consideration in toxicology, and real-life exposures of both humans and animals are to multiple chemical compounds. Even if combined exposures do not occur at the same time, prior or historical chemical exposures may prime tissues and predispose them to vulnerability to later toxic insult.

The Human Adrenocortical H295R Cell Line in Mechanism Elucidation

Much of the recent work to identify the molecular targets in adrenocortical toxicity has used the H295R cell line. In a regulatory toxicology and strategic evaluation

context, the H295R cell line has been suggested as a potentially useful tool for examining both adrenocortical toxicity and also the general process of steroidogenesis (Harvey and Everett, 2003; Harvey and Everett, 2006; Harvey *et al*., 2007; Hinson and Raven, 2006; Oskarsson *et al*., 2006; Sanderson, 2006), and is now in an OECD validation program as a primary method for evaluating endocrine disruption (Hecker and Giesy, 2007; Hecker *et al*., 2007). This cell line expresses all the enzymes necessary for steroidogenesis and the production of all major steroids such as progesterone, androgens, estrogens, glucocorticoids, and the mineralocorticoid aldosterone (Zhang *et al*., 2005). The H295R cell line compares favorably with normal human adrenal (Oskarsson *et al*., 2006) but has different levels of enzyme expression compared with other cell lines derived from the same adrenocortical tumor, such as H295A (Samandari *et al*., 2007). Numerous recent studies have now evaluated the H295R cell line and methodology as a rapid in vitro screening and mechanism elucidation tool, specifically for toxicant-induced effects on steroidogenesis (Gracia *et al*., 2006; Hecker *et al*., 2006; Hilscherova *et al*., 2004; Muller-Vieira *et al*., 2005; Oskarsson *et al*., 2006; Sanderson, 2006; Zhang *et al*., 2005) with the consensus that it is a relevant, suitable, and sensitive system for evaluating mechanisms of adrenocortical function. H295R cell systems can be used to assess the effects of compounds on steroid production and secretion (Hecker *et al*., 2006; Imagawa *et al*., 2006; Muller-Vieira *et al*., 2005; Oskarsson *et al*., 2006; Voets *et al*., 2004), on steroidogenic enzyme activity (Canton *et al*., 2006; Ohno *et al*., 2002; Oskarsson *et al*., 2006), and expression profiling of steroidogenic genes (Blaha *et al*., 2006; Gracia *et al*., 2006; Hilscherova *et al*., 2004; Oskarsson *et al*., 2006; Xu *et al*., 2006; Zhang *et al*., 2005). H295R cells have also been used to study the effects of drugs and chemicals on ACTH receptors which may be increased by chemicals to sensitize cells (Li and Wang, 2005) or downregulated producing insensitivity and adrenocortical insufficiency (Fassnacht *et al*., 1998).

H295R cells have been well described endocrinologically. They have functional ACTH receptors (Fassnacht *et al*., 1998; Li and Wang, 2005) but these are expressed at lower levels than normal tissue (See chap. 8 for further information), and functional CRH receptors (Willenberg *et al*., 2005). H295R cells respond to forskolin (Sanderson *et al*., 2002; Watanabe and Nakajin, 2004), isobutyl methylxanthine cAMP induction (Sanderson *et al*., 2002), and dibutyryl cAMP (Xu *et al*., 2003), which are standard molecular endocrine challenges. H295R cells also produce aldosterone following angiotensin II stimulation (Kau and Kan, 2005), and aldosterone is a critical and unique hormone of the zona glomerulosa of the adrenal cortex. Additionally, H295R cells respond to VIP by production of cortisol (Nicol *et al*., 2004), have functional atrial natriuretic peptide (ANP) receptors (Bodart *et al*., 1996), have functional luteinizing hormone (LH)/chorionic gonadotropin (hCG) receptors (Rao *et al*., 2004), have functional estrogen receptors (Montanaro *et al*., 2005), and respond to activin A by decreased sex steroid secretion (Vanttinen *et al*., 2003). H295R cells are also reported to produce adrenomedullin, which stimulates aldosterone and cortisol secretion (Thomson *et al*., 2001) and respond to

tumour necrosis factor, which increases steroidogenesis (Mikhaylova *et al*., 2007), transforming growth factor β-1 [which inhibits aldosterone and cortisol through inhibition of CYP11B1 and CYP11 B2 (Liakos *et al*., 2003)], epidermal growth factor [which increases cortisol and 3β-hydroxysteroid dehydrogenase and modulates CYP19 (Feltus *et al*., 2003; Watanabe *et al*., 2006)], and prostaglandins (Watanabe *et al*., 2006). The general nonsteroidogenic molecular biology of this cell line is also described in the literature and H295R cells also release a number of cytokines [IL-2, IL-4, IL-8, IL-10, IL-13, and TNFa, of which IL-8 is considered an important point of convergence between the adrenal and immune system, and angiotensin II stimulates IL-8 secretion in H295R cells (Romero *et al*., 2006)] and cytokines can also modulate adrenal steroid secretion. Although responsive to ACTH, H295R cells have relatively lower sensitivity to ACTH compared with normal adrenal, attributed to lower level of ACTH receptor expression (ACTH transmits its signal to activate steroidogenesis by a cAMP-dependent pathway), and challenge for steroidogenic capability is affected by forskolin (to activate adenylyl cyclase) or cAMP analogues.

As such, the H295R cell line is a well-characterized and versatile tool for endocrine toxicology (both the assessment of adrenocortical function and the process of steroidogenesis as a whole) supporting its proposal as a good standard model. As it is a human cell line, it is also particularly relevant for human toxicological hazard assessment and extrapolation, but it is important that this adrenocortical carcinoma–derived cell line has similar functionality to normal human adrenocortical cells. In fact, Oskarsson et al. (2006) have compared the H295R cell line with normal adult human adrenal over a number of endpoints and reported good correlation of response, which was improved with the addition of forskolin to H295R cells, and this, therefore, also supports the H295R cell line as a model relevant for general human extrapolation. Related to this is the equally important consideration of multidrug resistance (MDR) proteins that act as chemical efflux pumps particularly in cancer cell lines (from which the H295R line is derived). The overexpression of such proteins could result in the test compound being pumped out of the cell, leading to insensitivity and false-negative results. Little is known about multidrug resistance proteins specifically affecting the expression of toxicity in the H295R cell line, and none of the reports cited above or in Table 1 appear to have examined this, but Oskarsson et al. (2006) report good correlation of response to toxicity between the H295R cells and normal human adrenal, suggesting that the cell line itself is not inherently insensitive because of its derivation from a carcinoma. However, MDR genes/P-glycoprotein efflux transporter are known to express in both the normal human adrenal cortex (Srinivas *et al*., 2006) and human adrenocortical carcinoma (Ahlman *et al*., 2001) and as such, this is a variable requiring general consideration. Investigation of the multidrug resistance status in H295R cells under a number of different conditions and challenges would be a worthwhile area of research. Interestingly, the synthetic glucocorticoid dexamethasone has been shown to reduce P-glycoprotein mediated doxorubicin (adriamycin) efflux in rat hepatocytes (Fardel *et al*., 1993),

showing that glucocorticoids themselves modulate MDR efflux pumps, and raising the question of whether natural adrenal corticosteroids have similar actions in other cells including H295R. Glucocorticoid modulation of cellular chemical efflux pumps is one mechanism by which the adrenal may have a role in modulating the response to toxicity, as are the principal pharmacological actions of the glucocorticoids (e.g., anti-inflammatory, gluconeogenic, or catabolic actions) and this is briefly reviewed later in the chapter.

EXAMPLES OF PHARMACOLOGICALLY AND CHEMICALLY INDUCED HUMAN ADRENOCORTICAL DYSFUNCTION

There are several well-characterized conditions affecting humans that involve alterations to the control and function of various adrenocortical steroidogenic and enzyme pathways, and it is not only conceivable that such effects may also be chemically induced, but there is also clear evidence that the conditions have been toxicologically induced, for example, through enzyme inhibition (chemical inhibition of steroidogenic enzymes is reviewed in Table 1). The most toxicologically significant and frequent effect is adrenocortical suppression, iatrogenically induced by drugs (Goldberg, 1983; Harvey *et al*., 2007; Hinson and Raven, 2006; Leddingham and Watt, 1983; Raven and Hinson, 1996; Vermeulen *et al*., 1983) and particularly the corticosteroids used for anti-inflammatory action (Kaliner, 2006). Human adrenocortical suppression can result in Addisonian crisis and this has been inadvertently induced by the administration of drugs; the unexpected off-target side-effects of steroidogenic CYP enzyme inhibition by etomidate and aminoglutethimide have been previously mentioned, and the corticosteroids predictably suppress the adrenal cortex by pharmacological feedback inhibition of the hypothalamus and pituitary, which can then provoke an adrenocortical insufficiency crisis upon their withdrawal.

Adrenogenital syndrome is a congenital condition where a fault in enzyme activity in the adrenal produces an excess of androgen secretion that virilizes/masculinizes females during development. The ability to pharmacologically manipulate the enzymes leading to this condition (Hakki and Bernhardt, 2006) raises the possibility that chemical toxicity may also cause this syndrome. Similarly, salt-losing congenital adrenal hyperplasia, resulting in adrenal crisis and cardiovascular collapse in infants, typically presents within the first 2 weeks of life. However, a recent report has shown that this can also occur relatively late, outside the usually expected time frame, in 6- to 8-month-old children as a result of defects in steroidogenic acute regulatory protein (StAR) function, which is apparently much slower to express (Gassner *et al*., 2004). Gassner et al. (2004) report that two out of the three patients studied had mutations in the StAR gene, and no mutations were found in the third, suggesting a "novel disease"; as no mutation was found suggestive of an inborn error, transient environmental chemical factors could also be implicated since chemicals are known to inhibit StAR. Indeed, a variety of compounds including pesticides, fungicides, pharmaceuticals, and

ethanol impede normal StAR protein function (Table 1) and therefore, cholesterol transport into the primary and rate limiting step of steroidogenesis (Hilscherova *et al*., 2004; Janossy *et al*., 2001; Kan *et al*., 2003; Khisti *et al*., 2003; Oskarsson *et al*., 2006; Walsh and Stocco, 2000; Walsh *et al*., 2000; Walsh *et al*., 2000; Walsh *et al*., 2000; Zhao *et al*., 2005). These congenital endocrine conditions illustrate the developmental vulnerability of the adrenal and consequences of altered secretory function during critical early life stages, and the above example of salt-losing congenital adrenal hyperplasia may involve at least three different subtypes. However, the consequences of adrenal failure are equally serious in adulthood.

Cushing's syndrome is a condition of overproduction of adrenocortical steroids, particularly glucocorticoids, attributed in humans to overexpression of the ACTH receptor (Clark, 2006) and is also a common side effect of glucocorticoid therapy (Newell-Price *et al*., 2006). This condition is unlikely to be encountered in regulatory toxicology of compounds not structurally related to glucocorticosteroids, although drugs, such as caffeine and other agents that amplify cAMP, can cause marked hypothalamo-pituitary-adrenal axis stimulation in rodents (Garside and Harvey, 1992; Hadley *et al*., 1990; Spindel *et al*., 1983) and elevated endogenous glucocorticoids may have moderate effects. Interestingly, while glucocorticoid excess in humans typically produces increased weight gain (Frank *et al*., 2004), studies of repeat dose administration of corticosterone in the rat typically show reductions in body weight gain (Harvey *et al*., 1992), illustrating species differences in response, although reduction of muscle mass and protein catabolism is consistent across many mammalian species.

ADRENAL INTERACTIONS: MODULATION OF THE RESPONSE TO TOXICITY

The natural stress response involving altered adrenocortical and adrenomedullary secretion profiles is a variable in toxicology (Harvey, 1994; Vogel, 1993) and alters the response to toxic insult. The glucocorticoids in particular are known to influence the tolerance of toxic insult (Harvey *et al*., 1994; Harvey, 1996b) and the coadministration of natural or synthetic glucocorticoids with toxic agents is known to modulate the target organ response to toxic challenge. In the brain, glucocorticoids exacerbate toxic insult (Brooke and Sapolsky, 2002; MacPherson *et al*., 2005; McIntosh and Sapolsky, 1996). In other organs, glucocorticoids appear to be protective and ameliorate toxic insult from a variety of compounds, for example, in liver [e.g., carbon tetrachloride (Lloyd and Franklin, 1991)], kidney [e.g., cisplatin, cephaloridine (Harvey *et al*., 1995; Koikawa *et al*., 1993)], heart/cardiomyocytes [e.g., cyclosporin A, doxorubicin, ischemia/oxidative damage (Chen *et al*., 2005; Florio *et al*., 2003; Valen *et al*., 2000)], and even auditory hair cells (Guzman *et al*., 2006) opening therapeutic amelioration possibilities for drugs whose clinical utility is curtailed by adverse reactions. The direction of effect of the glucocorticoid interaction with a toxicant generally depends on the target organ, the particular steroid, the properties/mechanisms of the toxicant, and

temporal administration relationships (e.g., Harvey *et al*., 1994; Harvey, 1996a; Harvey, 1996b; Brooke and Sapolsky, 2002). Other HPA hormones are reported to modulate the response to a toxic challenge, and interestingly CRH is reported to be protective in amyloid β-peptide toxicity in the brain (Pedersen *et al*., 2001).

There are several possible mechanisms by which the glucocorticoids may modulate toxicity and each organ or tissue may differ in its response to both glucocorticoid and toxicant. The most obvious is the primary pharmacological action of the glucocorticoids, including anti-inflammatory actions that may ameliorate tissue damage, and effects on blood glucose. As demonstrated from the above, the brain does not appear to respond in the same way as other organs, and it has recently been demonstrated that glucocorticoids actually exacerbate proinflammatory cytokine responses in hippocampal cells (MacPherson *et al*., 2005) rather than exerting classical anti-inflammatory actions. Further, hyperglycemia is also reported to worsen neurological injury particularly if mediated by ischemia or lactate acidosis (Wass and Lanier, 1996). Recent evidence suggests other molecular mechanisms by which glucocorticoids influence the response to toxicity; glucocorticoids can inhibit cell death (induced in cardiomyocytes by doxorubicin) and induce antiapoptosis, antioxidant, and detoxification genes (Chen *et al*., 2005), which may be beneficial in some tissues for the survival of acute insults. In contrast, the synthetic glucocorticoid dexamethasone has been shown to reduce doxorubicin P-glycoprotein efflux in rat hepatocytes (Fardel *et al*., 1993), and reduction of chemical efflux pumping of chemical toxicant out of cells would intuitively produce prolonged intracellular exposure and potentiate/exacerbate toxicity. Glucocorticoids, therefore, have different actions in different cells and tissues, and several mechanisms of glucocorticoid action may occur concurrently to variable degrees.

PRIORITIES OF ASSESSMENT IN ADRENAL TOXICOLOGY

Adrenal Hypertrophy: Differentiation of Functional Suppression Versus Stress

In the context of adrenal/endocrine toxicology, the major priority should be to identify compounds causing functional suppression of the adrenal, since there are clear examples of chemically induced adrenal suppression in human clinical toxicology and the effects are serious and rapid. In regulatory toxicology studies, adrenocortical inhibition/suppression may manifest as an enlarged adrenal gland particularly in rodents. In this case, the rodent adrenal may be enlarged because of continuous ACTH stimulation as the adrenal cortex is not producing corticosterone to provide negative feedback regulation of pituitary ACTH release (Harvey and Everett, 2006; Harvey *et al*., 2007). Unfortunately, enlarged adrenal and other adrenocortical findings are often incorrectly disregarded in regulatory toxicology studies as a stress-related finding without evidence of the actual mechanism of ACTH overstimulation. Establishing the cause of adrenal hypertrophy is therefore

important, and adrenocortical competence should be established before findings are attributed to stress.

As well as resulting from a loss of feedback inhibition of ACTH due to adrenocortical inhibition, adrenal hypertrophy may occur as a result of classical "stress" or pharmacological stimulation of the HPA axis, if these also cause persistent ACTH secretion. However, in the case of stress-induced adrenal hypertrophy, there are usually other histopathological indicators of adrenocortical competence and evidence of corticosteroid hypersecretion in other tissues such as atrophy or involution of the thymus (Harvey *et al*., 1992; Harvey *et al*., 2007). Enlarged adrenal in the absence of clear evidence of adrenal functional competency should not in isolation be considered to be a "simple" stress-related effect (stress effects are considered to be of limited toxicological significance) and possible functional suppression should be investigated further. Such functional suppression could involve inhibition of any of the steps in the steroidogenic pathway. For example, in an exemplary study, Shivanandappa et al. (1982) conducted a rat 90-day dietary toxicity study of benzene hexachloride and found marked adrenal hypertrophy with large vacuolated cells in the adrenal cortex. They conducted immunohistochemistry and reported accumulation of cholesterol-positive lipids, but of most importance, reduction of steroidogenic enzymes such as 3-hydroxysteroid dehydrogenase, Δ-4,5 isomerase,and 11β-hydroxysteroid dehydrogenase. Thus, the adrenal hypertrophy resulting from benzene hexachloride was due to adrenocortical steroidogenic enzyme inhibition and resultant unopposed ACTH drive of the adrenals due to a lack of corticosterone, which is of toxicological importance, rather than generalized stress which is of little concern. Measurement of adrenal steroids in blood has been recommended as a method of distinguishing the functional competence of the adrenal cortex (Harvey *et al*., 2007). Thus, adrenal hypertrophy resulting from inhibition of adrenal function is considered a potentially serious toxicological finding of direct relevance to humans.

Adrenal Atrophy: Loss of Trophic Support and Capacity

Conversely, small adrenals/adrenal atrophy is indicative of a loss of trophic support of the adrenal by ACTH, and this too may result in deficits in functional capability of the cortex to produce glucocorticoids. In this case, adrenal atrophy may result from inhibition of pituitary ACTH or hypothalamic function or, indeed, adrenal ACTH receptors and compounds such as valproic acid, bromocriptine, cyproheptadine, ketanserin, ritanserin, somatostatin analogues, glucocorticoids, 4-thio-β-D-arabinofuranosylcytosine, and hexachlorobenzene (Colagiovanni *et al*., 2006; Kasperlik-Zaluska *et al*., 2005; Lelli *et al*., 2007; Mercado–Asis *et al*., 1997; Sonino *et al*., 2005; Tringali *et al*., 2004) have previously been noted to impair hypothalamo-pituitary function (deficits in ACTH or CRH). In either case of adrenal hypertrophy or adrenal atrophy, measurement of pituitary-adrenocortical

function is warranted and mechanistic strategies are discussed later (Harvey *et al*., 2007).

Adrenocortical Hyperstimulation and Excess: Persistent Developmental Consequences

Although pituitary-adrenal hyperactivity through direct or indirect stress related mechanisms is considered a much less serious toxicological finding in regulatory toxicology studies compared with frank adrenal suppression, pituitary-adrenocortical overactivity nevertheless can also be detrimental (see discussion on Cushing's syndrome), particularly developmentally. It has long been known that ACTH stimulated adrenal steroids can cross the placenta and functionally suppress fetal HPA development in rodents, producing fetal adrenal atrophy (Milkovic *et al*., 1976; Skebelskaya, 1968). Recent studies of perinatal glucocorticoid exposure have also shown a variety of permanent effects in adulthood, for example, molecular (annexin-1), functional, and morphological changes in the anterior pituitary (Theogaraj *et al*., 2005) and in host defense cells in blood and lung (Theogaraj *et al*., 2006). These recent examples further illustrate that inappropriate glucocorticoid exposure during critical periods can have far-reaching and apparently irreversible adverse developmental effects, and alter physiological programing of glucocorticoid sensitive tissues in endocrine and immune systems (see also Spinedi *et al*., 2005 for neuroendocrine-immune interactions specifically involving the HPA axis in immunotoxicology). Baldwin (1996) reviews the role of natural and synthetic glucocorticosteroids in development and developmental toxicity in laboratory species and humans.

Investigative Strategy: ACTH Challenge Study and In Vitro Mechanism Elucidation

The need to develop a toxicology strategy for the assessment of adrenal function and the process of steroidogenesis as targets for toxicity has been pointed out (Harvey and Everett, 2003; Oskarsson *et al*., 2006; Sanderson, 2006), and a two phase strategy of studies considered relevant to regulatory evaluation of adrenocortical toxicity has been proposed (Harvey *et al*., 2007). The strategy is based on a short in vivo study where the competence of the adrenal in treated rodents is assessed by an ACTH challenge and resulting corticosterone secretion is measured in the blood as an endpoint marker of adrenocortical function. As adrenocortical impairment/suppression can be induced by inhibition of CRH or ACTH (Kasperlik-Zaluska *et al*., 2005; Mercado–Asis *et al*., 1997; Sonino *et al*., 2005; Tringali *et al*., 2004) as well as by inhibiting adrenocortical steroidogenic enzymes, an in vivo study in intact animals is required to test the integrity of the entire HPA axis by steroid secretion (Harvey *et al*., 2007). The ability of untreated rats to respond to an ACTH challenge by markedly increased corticosterone secretion, compared with test compound treated rats that cannot respond, would provide strong evidence of adrenocortical suppression. Basal

corticosterone measurements before ACTH challenge would provide information on hypothalmic-pituitary function (i.e., if basal corticosterone was low, but adequate upon ACTH challenge, it could be concluded that pituitary function was deficient rather than the adrenal). The use of an in vitro system, such as the H295R cell line, could then be employed as a second tier to elucidate mechanisms of action and provide evidence for, or exclude, direct adrenocortical effects such as altered StAR function or enzyme inhibition.

Comparative Adrenal Toxicology: Considerations and Limitations in Species Selection

In developing an adrenal toxicology assessment strategy, it is important to consider that species differences do exist, both in the sensitivity of activation of the HPA axis as discussed earlier, and in the direct response of the adrenal cortex to chemical toxicants. For example, *o*,*p*′-DDD[2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane; Mitotane] is a metabolite of DDT and an established adrenolytic compound used in human medicine for adrenocortical carcinoma, and is activated by CYPs in the human adrenal to exert its toxicity, but does not exert toxicity in the mouse adrenal (Hermansson *et al*., 2007). A closely related compound (also a metabolite of DDT) 3-MeS02-DDE [2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene] is an adrenal toxicant in mice, and studies have shown differences among rodent species in protein binding of 3-MeS02-DDE (irreversible protein binding is associated with the mechanism of toxicity); extensive binding was seen in hamster adrenal tissue, while guinea pig adrenals were devoid of binding, and although there was high binding in mouse adrenal tissue, there was only weak binding in the rat (Lindstrom *et al*., 2007). Thus, in the above example, if the mouse was used as the experimental model, it would only detect the adrenal toxicity of one of these structurally related analogues. Additionally, CYP17 is not well expressed in the rat adrenal cortex but is in the human (Hinson and Raven, 2006), and thus employing the rat only would probably miss compounds that are CYP17 inhibitors, although an effect could be detected in cortisol producing species such as dog or primate, or indeed in human cells. Thus, the choice of species is important and such mechanistic information can be useful in explaining species differences in adrenal toxicity between rat and mouse (and indeed between rat and nonrodents) that may be observed with some compounds for example, in regulatory toxicology studies.

Although animal models are accepted surrogates for human tissue responses, the use of human cells such as the H295R cell line in addition to rodent toxicology can increase confidence that adverse effects will not be undetected. Similarly, choice of in vitro model and human cell line is important. There are other human adrenocortical cell lines, such as H295A, but this line has been shown to differ in expression of 3β-hydroxysteroid dehydrogenase and 17,20-lyase activities, and has lower androgen production compared with H295R (Samandari *et al*., 2007), while the H295R cell line steroidogenic gene expression profile has been compared

with normal human adrenal (Oskarsson *et al*., 2006) and selected for validation in OECD toxicology programs for evaluation of endocrine disruption (Hecker and Giesy, 2007; Hecker *et al*., 2007). There are also rodent cell lines, but given the limitations in rodent tissue, particularly in comparison to human cells outlined above, these cannot be recommended when human cells are available.

CONCLUSION

The adrenal is an under-recognized target organ. It is vital for health and adrenal dysfunction leads to significant morbidity and even mortality. Numerous drugs have been documented to alter adrenal function as an unexpected side effect in humans (Mann, 1996), and adrenocortical suppression is considered the most common and serious of these adverse drug reactions. Further, an increasing number of chemicals have been reported to alter mammalian adrenocortical function in vivo, and in vitro systems have identified the molecular targets involved. The fact that free-living fish and birds show altered adrenal function indicates that environmental exposures are capable of inducing adrenal endocrine disruption, raising the question of whether the human population may also be vulnerable. Despite this, there are no recommendations to study adrenal dysfunction in regulatory endocrine disruption screening and testing strategies and this is considered a significant regulatory deficiency (Harvey and Everett, 2003; Harvey and Everett, 2006; Harvey *et al*., 2007).

A strategy has been proposed to evaluate adrenocortical toxicity relevant to mammalian regulatory toxicology assessment. The concept is to develop a standardized approach to assess the most important toxicological effect on the adrenal, which is considered to be functional adrenocortical suppression. The in vivo ACTH challenge study is designed to provide such information (and is based on a test in human and veterinary medicine) and considered particularly amenable to regulatory application. The H295R human cell line is also proposed as a method to elucidate mechanisms and is recommended as a research tool. Both methods require intra and interlaboratory validation [validation work using the H295R cell line is currently being conducted by OECD (Hecker and Giesy, 2007; Hecker *et al*., 2007)] with known positive and negative control standards, and standard operating procedure development. Both techniques provide hazard assessment data but it will be regulatory agencies that must consider the significance of such data in risk extrapolation models.

The extent of human subclinical adrenal effects from environmental chemical exposures is unknown. Although it is unlikely that acute, low-level human environmental chemical exposures would provoke adrenal suppression and Addisonian crisis, the extent to which environmental chemicals may act as a contributory or precipitating factor to human adrenal conditions following chronic low-level exposures will remain unknown unless purposefully studied.

REFERENCES

Ahlman H, Khorram-Manesh A, Jansson S, et al. Cytotoxic treatment of adrenocortical carcinoma. World J Surg 2001; 25:927–933.

Akingbemi BT, Youker RT, Sottas CM, et al. Modulation of rat Leydig cell steroidogenic function by di-(9,2-ethylhexyl)phthalate. Biol Reprod 2001; 65:1252–1259.

Albertson BD, Hill RB, Sprague KA, et al. Effects of the antiglucocorticoid RU486 on adrenal steroidogenic enzyme activity and steroidogenesis. Eur J Endocrinol 1994; 130:195–200.

Andersen HR, Vingaard AM, Rasmusse TH, et al. Effects of currently used pesticides in assays for estrogenicity and aromatase activity in vitro. Toxicol Appl Pharmacol 2000; 179:1–12.

Baldwin JA. Glucocorticosteroids, stress, and developmental toxicology. In: Harvey PW, ed. The adrenal in toxicology: Target organ and modulator of toxicity. London: Taylor & Francis; 1996:223—242.

Baos R, Blas J, Bortolotti GR, et al. Adrenocortical response to stress and thyroid status in free-living nestling white storks (Ciconia ciconia) exposed to heavy metal and arsenic contamination. Environ Health Perspect 2006; 114:1497–1501.

Barrett AM. Some factors affecting blood ACTH levels. Acta Endocrinol 1960; 51:421.

Billaudel B, Sutter BCJ. Immediate in vivo effects of corticosterone on glucose induced insulin secretion in rats. J Endocrinol 1982; 95:315–320.

Bisson M, Hontela A. Cytotoxic and endocrine disrupting potential of atrazine, diazinon, endosulfan, and mancozeb of adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicol Appl Pharmacol 2002; 180:110–117.

Blaha L, Hilscherova K, Mazurova E, et al. Alteration of steroidogenesis in H295R cells by organic sediment contaminants and relationships to other endocrine disrupting effects. Environ Int 2006; 32:749–757.

Bodart V, Rainey WE, Fournier A, et al. The H295R cell line contains functional atrial natriuretic peptide receptors that inhibit aldosterone biosynthesis. Mol Cell Endocrinol 1996; 118:137–144.

Bratusch-Marrain P, Vierhapper H, Waldhausl W, et al. Acute suppressive effects of ACTH induced cortisol secretion on serum prolactin levels in normal men. Acta Endocrinol 1982; 99:352–356.

Brooke SM, Sapolsky RM. Glucocorticoid exacerbation of gp120 neurotoxicity: Role of microglia. Exp Neurol 2002; 177:151–158.

Buckingham JC, Smith T, Loxley HD. The control of ACTH secretion. In: James VHT, ed. The Adrenal Gland. 2 nd ed. New York, NY: Academic Press, 1992:131–158.

Camacho AM, Cash R, Brough AJ, et al. Inhibition of adrenal steroidogenesis by aminoglutethimide and the mechanism of action. J Am Chem Soc 1967; 202:114–120.

Canton RF, Sanderson JT, Nijmeijer S, et al. In vitro effects of brominated flame retardents and metabolites on CYP17 catalytic activity: A novel mechanism of action. Toxicol Appl Pharmacol 2006; 216:274–281.

Champoux L, Rodrigue J, Trudeau S, et al. Contamination and biomarkers in the great blue heron, an indicator of the state of the St. Lawrence river. Ecotoxicology 2006; 15: 83–96.

Chandra AK, Chatterjee A, Ghosh R, et al. Chromium induced testicular impairment in relation to adrenocortical activities in adult albino rats. Reprod Toxicol 2007; 24:388–396.

Chang HW, Wu VC, Huang CY, et al. D4 Dopamine receptor enhances angiotensin-II stimulated aldosterone secretion through PKC and calcium signalling. Am J Physiol Endocrinol Metab 2008; 294:E622–629.

Chen QM, Alexander D, Sun H, et al. Corticosteroids inhibit cell death induced by doxorubicin in cardiomyocytes: Induction of antiapoptosis, antioxidant and detoxification genes. Mol Pharmacol 2005; 67:1861–1873.

Clark AJ, Metherell LAMechanisms of disease: The adrenocorticotropin receptor and disease. Nat Clin Pract Endocrinol Metab 2006; 2:282–290.

Colagiovanni DB, Drolet DW, Dihel L, et al. Safety assessment of 4-thio-beta-D-arabinofuranosylcytosine in the beagle dog suggests a drug-induced centrally mediated effect on the hypothalamic-pituitary-adrenal axis. Int J Toxicol 2006; 25:119–126.

Colby HD, Longhurst PA. Toxicology of the adrenal gland. In: Atterwill CK, Flack JD, eds. Endocrine Toxicology. Cambridge University Press, 1992:243—281.

Colby HD. The adrenal cortex as a toxicological target organ. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor &Francis, 1996:131–163.

Ding L, Murphy MB, He Y, et al. Effects of brominated flame retardants and brominated dioxins on steroidogenesis in H295R human adrenocortical carcinoma cell line. Environ Toxicol Chem 2007; 26:764–772.

Dorval J, Leblond V, Deblois C, et al. Oxidative stress and endocrine endpoints in white sucker (Catostomus commersoni) from a river impacted by agricultural chemicals. Environ Toxicol Chem 2005; 24:1273–1280.

Elkahloun AG, Powers JF, Nyska A, et al. Gene expression profiling of rat pheochromocytoma. Ann N Y Acad Sci 2006; 1073:290–299.

Fan W, Yanase T, Morinaga H, et al. Atrazine-induced aromatase expression is Sf-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans. Environ Health Perspect 2007; 115:720–727.

Fardel O, Lecureur V, Guillouzo A. Regulation of P-glycoprotein expression in cultured rat hepatocytes. FEBS Lett 1993; 327:189–193.

Fassnacht M, Beuschlein F, Vay S, et al. Aminoglutethimide suppresses adrenocorticotrophin receptor expression in the NCI-h295 adrenocortical tumor cell line. J Endocrionol 1998; 159:35–42.

Feltus FA, Kovacs WJ, Nicholoson W, et al. Epidermal growth factor increases cortisol production and type II 3 beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4)-isomerase expression in human adrenocortical carcinoma cells: evidence for a Stat5-dependent mechanism. Endocrinology 2003; 144:1847–1853.

Florio S, Ciarcia R, Crispino L, et al. Hydrocortisone has a protective effect on cyclosporin A-induced cardiotoxicity. J Cell Physiol 2003; 195:21–26.

Frank GR, Speiser PW, Griffin KJ, Safety of medications and hormones used in pediatric endocrinology: Adrenal. Pediatr Endocrinol Rev 2004; 2(Suppl 1):134–145.

Fu X, Latendresse JR, Muskhelishvili L, et al. Dietary modulation of 7, 12-dimethylbenz[a]anthracene (DMBA)-induced adrenal toxicity in female Sprague-Dawley rats. Food Chem. Toxicol 2005; 43:765–774.

Gala RR, Kothari LS, Haisenleder DJ. The influence of oral corticosterone replacement on plasma prolactin levels of adrenalectomized rats. Life Sci 1981; 29:2113–2117.

Garside DA, Harvey PW. Endocrine toxicology of the male reproductive system. In: Atterwill CK, Flack JD, eds. Endocrine Toxicology. Cambridge University Press, 1992:285–312.

Gassner HL, Toppari J, Quinteiro Gonzalez S, et al. Near-miss apparent SIDS from adrenal crisis. J Pediatr 2004; 145:178–183.

Gell JS, Oh J, Rainey WE, et al. Effect of estradiol on DHEAS production in the human adrenocortical cell line, H295R. J Soc Gynecol Invest 1998; 5:144–148.

Goldberg A. Etomidate. Lancet 1983; 1983 ii, 60.

Gracia T, Hilscherova K, Jones PD, et al. The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicol Environ Saf 2006; 63:293–305.

Gracia T, Hilscherova K, Jones PD, et al. Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds. Toxicol Appl Pharmacol 2007; 225:142–153.

Gumbleton M, Nicholls PJ. Molecular and systems pharmacology of glucocorticosteroids. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996:81–128.

Guzman J, Ruiz J, Eshraghi AA, et al. Triamcinolone acetonide protects auditory hair cells from 4-hydroxy-2,3-nonenal (HNE) ototoxicity in vitro. Acta Otolaryngol 2006; 126:685–690.

Hadley AJ, Flack JD, Buckingham JC. Modulation of corticotrophin release in vitro by methylxanthines and adenosine analogues. Br J Pharmacol 1990; 100(suppl): 337.

Hakki T, Bernhardt R. CYP17-and CYP11B-dependent steroid hydroxlases as drug development targets. Pharmacol Ther 2006; 111:27–52.

Harvey PW, Everett D. The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: Critical omissions from regulatory endocrine disrupter screening strategies for human health. J Appl Toxicol 2003; 23:81–87.

Harvey PW, Everett DJ. Regulation of endocrine-disrupting chemicals: Critical overview and deficiencies in toxicology and risk assessment for human health. Best Practice and Research Clinical Endocrinology and Metabolism 2006; 20:145–165.

Harvey PW, Johnson I. Approaches to the assessment of toxicity data with endpoints related to endocrine disruption. J Appl Toxicol 2002; 22:241–247.

Harvey PW, Rush KC, Cockburn A 1999. Endocrine and hormonal toxicology: An integrated mechanistic and target systems approach. In: Harvey PW, Rush KC, Cockburn A, eds. Endocrine and Hormonal Toxicology. Chichester: John Wiley, 1999: 3–11.

Harvey PW, Er J, Fernandes C, et al. Corticosterone does not cause testicular toxicopathology in the rat: Relevance to methylxanthines, ACTH and stress. Hum Exp Toxicol 1992; 11:505–509.

Harvey PW, Healing G, Rees SJ, et al. Glucocorticosteroid interactions with natural toxins: A mini review. Nat Toxins 1994; 2:341–346.

Harvey PW, Healing G, Major IR, et al. Glucocorticoid amelioration of nephrotoxicity: A study of cephaloridine-methylprednisolone interaction in the rat. Hum Exp Toxicol 1995; 14:554–561.

Harvey PW, Everett DJ, Springall CJ. Adrenal toxicology; a strategy for assessment of functional toxicity to the adrenal cortex and steroidogenesis. J Appl Toxicol 2007; 27:103–115.

Harvey PW. Stress and toxicity. Hum Exp Toxicol 1994; 13:275–276.

Harvey PW. An overview of adrenal gland involvement in toxicology. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996a:3–19.

Harvey PW. Glucocorticosteroid modulation of toxicity. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996b:185–204.

Healing G. Hormonal mechanisms in kidney toxicology. In: Harvey PW, Rush KC, Cockburn A, eds. Endocrine and Hormonal Toxicology. Chichester: John Wiley,; 1999:291–307.

Hecker M, Giesy JP. Novel trends in endocrine disruptor testing: The H295R steroidogenesis assay for identification of inducers and inhibitors of hormone production. Anal Bioanal Chem 2007.

Hecker M, Newsted JL, Murphy MB, et al. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: Hormone production. Toxicol Appl Pharmacol 2006; 217:114–124.

Hecker M, Hollert H, Cooper R, et al. The OECD validation program of the H295R steroidogenesis assay for the identification of in vitro inhibitors and inducers of testosterone and estradiol production. Phase 2: Inter-laboratory pre-validation studies. Env Sci Pollut Res 2007; 14(Special Issue 1):23–30.

Heneweer M, Van Den Berg M and Sanderson JT. A comparison of human H295R and rat R2 C cell lines as in vitro screening tools for effects on aromatase. Toxicol Lett 2004; 146:183–194.

Hermansson V, Asp V, Bergman A, et al. Comparative CYP-dependent binding of the adrenocortical toxicants 3-methylsulfonyl-DDE and *o,p′*-DDD in Y-1 adrenal cells. Arch Toxicol 2007; 81:793–801.

Hierlihy AM, Cooke GM, Curran IHA, et al. Effects of ciprofibrate on testicular and adrenal steroidogenic enzymes in the rat. Reprod Toxicol 2006; 22:37–43.

Hilscherova K, Jones PD, Gracia T, et al. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol Sci 2004; 81:78–89.

Hinson JP, Raven PW. Adrenal morphology and hormone synthesis and regulation. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996:23–52.

Hinson JP, Raven PW. Adrenal Toxicology. In: Harvey PW, Rush KC, Cockburn A, eds. Endocrine and Hormonal Toxicology. Chichester: John Wiley, 1999:67–89.

Hinson JP, Raven PW. Effects of endocrine-disrupting chemicals on adrenal function. Best Practice and Research Clinical Endocrinology and Metabolism 2006; 20:111–120.

Hinson JP, Kapas S, Smith DM. Adrenomedullin, a multifunctional regulatory peptide. Endocr Rev 2000; 21:138–167.

Hontela A, Rasmussen JB, Audet C, et al. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs and mercury. Arch Environ Contam Toxicol 1992; 22:278–283.

Hornsby PJ. Steroid and xenobiotic effects on the adrenal cortex: Mediation by oxidation and other mechanisms. Free Radical Biol Med 1989; 6:103–115.

Imagawa K, Okayama S, Takaoka M, et al. Inhibitory effect of efonidipine on aldosterone synthesis and secretion in human adrenocarcinoma (H295R) cells. J Cardiovasc Pharmacol 2006; 47:133–138.

Janossy A, Saez JM, Li JY. Carbachol induces homologus steroidogenic refractoriness of bovine fasciculata-reticularis cells. Mol Cell Endocrinol 2001; 172:147–155.

Johansson MK, Sanderson JT, Lund BO. Effects of 3-MeSO2-DDE and some CYP inhibitors on glucocorticoid steroidogenesis in the H295R human adrenocortical carcinoma cell line. Toxicol In Vitro 2002; 16:113–121.

Kaliner MA. Pharmacologic characteristics and adrenal suppression with newer inhaled corticosteroids: A comparison of ciclesonide and fluticasone propionate. Clin Ther 2006; 28:319–331.

Kamel F, Kubajak CL. Modulation of gonadotropin secretion by corticosterone: Interaction with gonadal steroids and mechanism of action. Endocrinology 1987; 121:561–568.

Kan SF, Kau MM, Low-Tone Ho L, et al. Inhibitory effects of bromocriptine on corticosterone secretion in male rats. Eur J Pharmacol 2003; 468:141–149.

Kapas S, Purbrick A, Hinson JP. Action of opioid peptides on the rat adrenal cortex: Stimulation of steroid secretion through specific mu opioid receptor. J Endocrinol 1995; 144:503–510.

Kasperlik-Zaluska AA, Zgliczynski W, Jeske W, et al. ACTH responses to somatostatin, valproic acid and dexamethasone in Nelson's syndrome. Neuro Endocrinol Lett 2005; 26:709–712.

Kau MM, Kan SF, Wang JR, et al. Inhibitory effects of digoxin and ouabain on aldosterone synthesis in human adrenocortical NCI-H295R cells. J Cell Physiol 2005; 205:393–401.

Kempna P, Hofer G, Mullis PE, et al. Pioglitazone inhibits androgen production in NCI-H295R cells by regulating gene expression of CYP17 and HSD3 B2. Mol Pharmacol 2007; 71:787–798.

Khisti RT, Kumar S, Morrow AL. Ethanol rapidly induces steroid acute regulatory protein expression and translocation in rat adrenal gland. Eur J Pharmacol 2003; 473:225–227.

Koikawa Y, Uozumi J, Ueda T, et al. Prophylactic effect of methylprednisolone against cisplatin-induced nephrotoxicity in rats. Toxicol Lett 1993; 66:281–285.

Kossor DC, Kominami S, Takemori S, et al. Role of the steroid 17 a-hydroxylase in spironolactone-mediated destruction of adrenal cytochrome P-450. Mol Pharmacol 1991; 40:321–325.

Kosti O, King PJ, Hinson JP. Tumour-derived human adrenocortical cells express beta adrenergic receptors: Steroidogenic effects of beta-adrenergic input. Endocr Res 2002; 28:363–367.

Leddingham IM, Watt I. Influence of sedation on mortality in multiple trauma patients. Lancet 1983; 1:1270.

Leddingham IM, Finlay WEI, Watt I, et al. Etomidate and adrenocortical function. Lancet 1983; 1:1434.

Lelli SM, Ceballos NR, Mazzetti MB, et al. Hexachlorobenzene as hormonal disruptor-studies about glucocorticoids: Their hepatic receptors, adrenal synthesis and plasma levels in relation to impaired gluconeogenesis. Biochem Pharmacol 2007; 73:873–879.

Letcher RJ, Sanderson JT, Bokkers A, et al. Effects of bisphenol A-related diphenylalkanes on vitellogenin production in male carp (Cyprinus carpio) hepatocytes and aromatase (CYP19) activity in human H295R adrenocortical carcinoma cells. Toxicol Appl Pharmacol 2005; 209:95–104.

Li LA, Lin TC. Interacting influence of potassium and polychlorinated biphenyl on cortisol and aldosterone biosynthesis. Toxicol Appl Pharmacol 2007; 220:252–261.

Li LA, Wang PW. PCB126 induces differential changes in androgen, cortisol and aldosterone biosynthesis in human adrenocortical H295R cells. Toxicol Sci 2005; 85:530–540.

Li LA, Wang PW, Chang LW. Polychlorinated biphenyl 126 stimulates basal and inducible aldosterone biosynthesis of human adrenocortical H295R cells. Toxicol Appl Pharmacol 2004; 195:92–102.

Li LA. Polychlorinated biphenyl exposure and CYP19 gene regulation in testicular and adrenocortical cell lines. Toxicol In Vitro 2007; 21:1087–1094.

Liakos P, Lenz D, Bernhardt R, et al. Transforming growth factor beta1 inhibits aldosterone and cortisol production in the human adrenocortical cell line NCI-H295R through inhibition of CYP11B1 and CYP11 B2 expression. J Endocrinol 2003; 176:69–82.

Liddle GW, Island DP, Lance EM, et al. Alterations in adrenal steroid patterns in man resulting from treatment with a chemical inhibitor of 11β-hydroxylase. J Clin Endocrinol 1958; 18:906–912.

Lin TCE, Chien SC, Hsu PC, et al. Mechanistic study of polychlorinated biphenyl 126-induced CYP11B1 and CYP11 B2 up-regulation. Endocrinology 2006; 147:1536–1544.

Lindhe O, Skoseid B, Brandt I. Cytochrome P450-catalysed binding of 3-methylsulfonyl-DDE and *o,p′*-DDD in human adrenal zona fasciculata/reticularis. J Clin Endocrinol Metab 2002; 87:1319–1326.

Lindstrom V, Brandt I, Lindhe O. Species differences in 3-methylsulphonyl-DDE bioactivation by adrenocortical tissue. Arch Toxicol 2007; 82:159–163.

Lloyd SA, Franklin MR. Modulation of carbon tetrachloride hepatotoxicity and xenobiotic metabolizing enzymes by corticosterone pre-treatment, adrenalectomy and sham surgery. Toxicol Lett 1991; 55:65–75.

Loose DS, Kan PB, Hirst MA, et al. Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450 dependent enzymes. J Clin Invest 1983; 71:1495–1499.

MacPherson A, Dinkel K, Sapolsky R. Glucocorticoids worsen excitiotoxin-induced expression of pro-inflammatory cytokines in hippocampal cultures. Exp Neurol 2005; 194:376–383.

Mann RD. Adverse drug reactions and the adrenal glands. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996:205–221.

McCarthy JL, Reitz CW, Wesson LK. Inhibition of adrenal corticosteroidogenesis in the rat by cyanotrimethylandrostenolone, a synthetic androstane. Endocrinology 1966; 79:1123–1139.

McIntosh LJ, Sapolsky RM. Glucocorticoids: Deleterious effects on neurons and potential exacerbation of environmental toxicity. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996:205–221.

Mercado–Asis LB, Yanovski JA, Tracer HL, et al. Acute effects of bromocriptine, cyproheptadine and valproic acid on plasma adrenocorticotropin secretion in Nelson's syndrome. J Clin Endocrinol Metab 1997; 82:514–517.

Metzeler K, Agonston A, Gratzl M. An intrinsic gamma-aminobutyric acid (GABA)ergic system in the adrenal cortex: Findings from human and rat adrenal glands and the NCI-H295R cell line. Endocrinology 2004; 145:2402–2411.

Mikhaylova IV, Kuulasmaa T, Jaaskelainen J, et al. Tumor necrosis factor-alpha regulates steroidogenesis, apoptosis and cell viability in the human adrenocortical cell line NCI-H295R. Endocrinology 2007; 148:386–392.

Milkovic K, Joffe J, Levine S. The effects of maternal and fetal corticosteroids on the development and function of the pituitary-adrenocortical system. Endokrinologie 1976; 76:60–65.

Miller DB. Caveats in hazard assessment- stress and neurotoxicity. In: Isaacson RL, Jensen KF, eds. The Vulnerable Brain and Environmental Risks, Vol 1, Malnutrition and Hazard Assessment. New York, NY: Plenum, 1992:239–266.

Montanaro D, Maggiolini M, Recchia AG, et al. Antiestrogens upregulate estrogen receptor beta expression and inhibit adrenocortical H295R cell proliferation. J Mol Endocrinol 2005; 35:245–256.

Muller-Vieira U, Angotti M, Hartman RW. The adrenocortical tumor cell line NCI-H295R as an in vitro screening system for the evaluation of CYP11 B2 (aldosterone synthase) and CYP11B1 (steroid-11beta-hydroxylase) inhibitors. J Steroid Biochem Mol Biol 2005; 96:259–270.

Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological action. Endocr Rev 1984; 5:25–44.

Newell-Price J, Bertagna X, Grossman AB, et al. Cushing's syndrome. Lancet 2006; 367(9522):1605–1617.

Nicol MR, Cobb VJ, Williams BC, et al. Vasoactive intestinal peptide (VIP) stimulates cortisol secretion from the H295R human adrenocortical tumor cell line via VPAC1 receptors. J Mol Endocrinol 2004; 32:869–877.

Noda M, Ohno S, Nakajin S. Mono-(2-ethylhexyl) phthalate (MEHP) induces nuclear receptor 4 A subfamily in NCI-H295R cells: A possible mechanism of aromatase suppression by MEHP. Mol Cell Endocrinol 2007; 274:8–18.

Nogueira EF, Vargas CA, Otis M, et al. Angiotensin-II acute regulation of rapid response genes in human, bovine and rat adrenocortical cells. J Mol Endocrinol 2007; 39:365–374.

Norris DO, Donahue S, Dores RM, et al. Impaired adrenocortical response to stress in brown trout, *Salmo trutta,* living in metal-contaminated waters of the Eagle River, Colorado. Gen Comp Endocrinol 1999; 113:1–8.

Ogle TF, Kitay JI. Ovarian and adrenal steroids during pregnancy and the oestrous cycle in the rat. J Endocrinol 1979; 74:89–98.

Ohno S, Shinoda S, Toyoshima S, et al. Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells. J Steroid Biochem Mol Biol 2002; 80:355–363.

Ohta S, Lai EW, Taniguchi S, et al. Animal models of pheochromocytoma including NIH initial experience. Ann N Y Acad Sci 2006; 1073:300–305.

Oskarsson A, Ulleras E, Plant KE, et al. Steroidogenic gene expression in H295R cells and the human adrenal gland: Adrenotoxic effects of lindane in vitro. J Appl Toxicol 2006; 26:484–492.

Pamenter RW, Hedge GA. Inhibition of thyrotropin secretion by physiological levels of corticosterone. Endocrinology 1980; 106:162–166.

Pedersen WA, McCullers D, Culmsee C, et al. Corticotrophin-releasing hormone protects neurons against insults relevant to the pathogenesis of Alzheimers disease. Neurobiol Dis 2001; 8:492–503.

Philip JG, John CD, Gillies GE, et al. Neuroendocrine and pituitary toxicity: Targets for drug action and potential consequences on immune function. In: Harvey PW, Rush KC, Cockburn A, eds. Endocrine and Hormonal Toxicology. Chichester: John Wiley, 1999:171–208.

Potts GO, Creange JE, Harding HR, et al. Trilostane, an orally active inhibitor of steroid biosynthesis. Steroids 1978; 32:257–267.

Quabius ES, Balm PH, Wendelaar Bongar SE. Inter-renal stress responsiveness of tilapia (Oreochromis mossambicus) is impaired by dietary exposure to PCB126. Gen Comp Endocrinol 1997; 108:472–482.

Rao ChV, Zhou XL, Lei, ZM. Functional luteinizing hormone/chorionic gonadotrophin receptors in human adrenal cortical H295R cells. Biol Reprod 2004; 71:579–587.

Raven PW, Hinson JP. Transport, actions and metabolism of adrenal hormones and pathology and pharmacology of the adrenal gland. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996:53–79.

Renshaw D, Hinson JP. Neuropeptide Y and the adrenal gland: A review. Peptides 2001; 22:429–438.

Ribelin WE. The effects of drugs and chemicals upon the structure of the adrenal gland. Fund Appl Toxicol 1984; 4:105–119.

Rodier W, Kitay JI. The influence of progesterone on adrenocortical function in the rat. Pro Soc Exp Biol Med 1974; 146:376–380.

Romero DG, Vergara GR, Zhu Z, et al. Interleukin-8 synthesis, regulation and steroidogenic role in H295R human adrenocortical cells. Endocrinology 2006; 147:891–898.

Rosol TJ, Yarrington JT, Latendresse J. Adrenal gland: Structure, function, and mechanisms of toxicity. Toxicol Pathol 2001; 29:41–48.

Samandari E, Kempna P, Nuoffer JM, et al. Human adrenal corticocarcinoma NCI-H295R cells produce more androgens than NCI-H295 A cells and differ in 3beta-hydroxysteroid dehydrogenase type 2 and 17, 20 lyase activities. J Endocrinol 2007; 195:459–472.

Sanderson JT, Slobbe L, Lansbergen GW, et al. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and diindolylmethanes differentially induce cytochrome P450 1A1, 1B1, and 19 in H295R human adrenocortical carcinoma cells. Toxicol Sci 2001a; 61:40–48.

Sanderson JT, Letcher RJ, Heneweer M, et al. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ Health Perspect 2001b; 109:1027–1031.

Sanderson JT, Boerma J, Lansbergen GW, et al. Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Toxicol Appl Pharmacol 2002; 82:44–54.

Sanderson JT, Hordijk J, Denison MS, et al. Induction and inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxicol Sci 2004; 82:70–79.

Sanderson JT. The steroid hormone biosynthetic pathway as a target for endocrine-disrupting chemicals. Toxicol Sci 2006; 94:3–21.

Sankar BR, Maran RR, Sudah S, et al. Chronic corticosterone treatment impairs Leydig cell 11beta-hydroxysteroid dehydrogenase activity and LH-stimulated testosterone production. Horm Metab Res 2000; 32:142–146.

Shivanandappa T, Krishnakumari MK, Majumder SK. Inhibition of steroidogenic activity in the adrenal cortex of rats fed benzene hexachloride (hexachlorocyclohexane). Experientia 1982; 38:1251–1253.

Skebelskaya YB. The effects of corticosteroid concentrations in the blood of gravid rats on the adrenocorticotrophic function of hypophysis of the fetus. Gen Comp Endocrinol 1968; 10:263–268.

Soll RM, Dollings PJ, Mitchell RD, et al. Guanabenz-related amidinohydrazones: Potent non-azole inhibitors of aldosterone biosynthesis. Eur J Medicinal Chem 1994; 29:223–232.

Sonino N, Boscaro M, Fallo F. Pharmacologic management of Cushing syndrome: New targets for therapy. Treat Endocrinol 2005; 4:87–94.

Spindel E, Griffiths L, Wurtman RJ. Neuroendocrine effects of caffeine. II. Effects on thyrotropin and corticosterone secretion. J Pharmacol Exp Ther 1983; 225:346–350.

Spinedi E, Perez NB, Gaillard RC. Neuroendocrine-immune interaction: Impact of gender, negative energetic balance condition, and developmental stage on the mechanisms of the acute phase response of the inflammatory process in mammals. In: Holladay SD, ed. Developmental Immunotoxicology. Boca Raton, FL: CRC Press, 2005:303–353.

Srinivas E, Murthy JN, Rao AR, et al. Recent advances in molecular modelling and medicinal chemistry aspects of phospho-glycoprotein. Curr Drug Metab 2006; 7:205–217.

Srivastava RK, Yaylor MF, Mann DR. Effect of immobilization stress on plasma luteinizing hormone, testosterone, and corticosterone concentrations and on 3 beta-hydroxysteroid dehydrogenase activity in the testes of adult rats. Proc Soc Exp Biol Med 1993; 204:231–235.

Sugawara T, Nomura E, Sakuragi N, et al. The effect of the arylhydrocarbon receptor on the human steroidogenic acute regulatory gene promoter activity. J Steroid Biochem Mol Biol 2001; 78:253–260.

Sugihara H, Miyabara S, Yun K, et al. The effect of a-melanocyte stimulating hormone on the adrenal cortex of the fetal rat. Endokrinologie 1982; 79:415–422.

Supornsilchai V, Soder O, Svechnikov K. Sesquiterpene lactone helenalin suppresses Leydig and adrenocortical cell steroidogenesis by inhibiting expression of the steroidogenic acute regulatory protein. Reprod Toxicol 2006; 22:631–635.

Supornsilchai V, Soder O, Svechnikov K. Stimulation of the pituitary-adrenal axis and of adrenocortical steroidogenesis ex vivo by administration of di-2-ethylhexyl phthalate to prepubertal male rats. J Endocrinol 2007; 192:33–39.

Szabo S, Lippe IT. Adrenal gland: Chemically induced structural and functional changes in the cortex. Toxicol Pathol 1989; 17:317–329.

Theogaraj E, John CD, Dewar A, et al. The long-term effects of perinatal glucocorticoid exposure on the host defence system of the respiratory tract. J Pathol 2005; 210:85–93.

Theogaraj E, John CD, Christian HC, et al. Perinatal glucocorticoid treatment produces molecular, functional, and morphological changes in the anterior pituitary gland of the adult male rat. Endocrinology 2006; 146:4804–4813.

Thomson LM, Kapas S, Carroll M, et al. Autocrine role of adrenomedullin in the human adrenal cortex. J Endocrinol 2001; 170:259–265.

Tischler AS, Powers JF, Alroy J. Animal models of pheochromocytoma. Histol Histopathol 2004; 19:883–895.

Toth IE, Hinson JP. Neuropeptides in the adrenal gland: Distribution, localization of receptors and effects on steroid hormone synthesis. Endocr Rev 1995; 21:39–51.

Tringali G, Aubry JM, Moscianese K, et al. Valproic acid inhibits corticotropin-releasing factor synthesis and release from the rat hypothalamus in vitro: Evidence for the involvement of GABAergic neurotransmission. J Psychiatry Neurosci 2004; 29:459–466.

Tucker MJ. The adrenal medulla as a toxicological target organ. In: Harvey PW, ed. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London: Taylor & Francis, 1996:165–182.

Vakharia K, Hinson JP. Lipopolysaccharide directly stimulates cortisol secretion by human adrenal cells by a cyclo-oxygenase-dependent mechanism. Endocrinology 2005; 146:1398–1402.

Vakharia K, Renshaw D, Hinson JP. Bacterial lipopolysaccharide directly stimulates cortisol secretion in human adrenal cells. Endocr Res 2002; 28:357–361.

Valen G, Kawakami T, Tahepold P, et al. Pretreatment with methylprednisolone protects the isolated rat heart against ischaemic and oxidative damage. Free Radic Res 2000; 33:31–43.

Vanttinen T, Liu J, Kuulasmaa T, et al. Expression of activin/inhibin signaling components in the human adrenal gland and the effects of activins and inhibins on adrenocortical steroidogenesis and apoptosis. J Endocrinol 2003; 178:479–489.

Vasquez SB, Kitay JI. Effects of prolactin on pituitary-adrenal function in intact and ovariectomized rats. Acta Endocrinol 1978; 88:745–758.

Vermeulen A, Paridaens R, Heuson JC. Effects of aminoglutethimide on adrenal steroid secretion. Clin Endocrinol 1983; 19:673–682.

Vinson GP, Bell JBG, Whitehouse BJ. Production of testosterone and corticosteroids by the rat adrenal gland incubated in vitro, and the effects of stimulation with ACTH, LH and FSH. J Steroid Biochem 1976; 7:407–411.

Voets M, Muller-Vieira U, Marchais-Oberwinkler S, et al. Synthesis of amidinohydrazones and evaluation of their inhibitory effect towards aldosterone synthase (CYP11 B2) and the formation of selected steroids. Arch Pharm 2004; 337:411– 416.

Vogel WH. The effect of stress on toxicological investigations. Hum Exp Toxicol 1993; 12:265–271.

Walsh LP, Stocco DM. Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. Biol Reprod 2000; 63:1024–1033.

Walsh LP, Kuratko CN, Stocco DM. Econazole and miconazole inhibit steroidogenesis and disrupt steroidogenic acute regulatory (StAR) protein expression post-transcriptionally. J Steroid Biochem Mol Biol 2000; 75:229–236.

Walsh LP, McCormick C, Martin C, et al. Roundup inhibits steroidogenic acute regulatory (StAR) protein expression. Environ Health Perspect 2000; 108:769–776.

Walsh LP, Webster DR, Stocco DM. Dimethoate inhibits steroidogenesis by disrupting transcription of the steroidogenic acute regulatory (StAR) gene. J Endocrinol 2000; 167:253–263.

Wass CT, Lanier WL. Glucose modulation of ischemic brain injury: Review and clinical recommendations. Mayo Clin Proc 1996; 71:801–812.

Watanabe M, Nakajin S. Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R. J Endocrinol 2004; 180:125–133.

Watanabe M, Noda M, Nakajin S. Effect of epidermal growth factor and prostaglandin on the expression of aromatase (CYP19) in human adrenocortical cell line NCI-H295R cells. J Endocrinol 2006; 188:59–68.

Whitworth EJ, Hinson JP, Vinson GP. Neuropeptides and adrenocortical proliferation in vitro. Endocr Res 2002; 28:677–681.

Whitworth EJ, Kosti O, Renshaw D, et al. Adrenal neuropeptides: Regulation and interaction with ACTH and other adrenal regulators. Microsc Res Tech 2003; 15:259–267.

Willenberg HS, Haase M, Papewalis C, et al. Corticotropin-releasing hormone receptor expression on normal and tumorous human adrenocortical cells. Neuroendocrinology 2005; 82:274–281.

Williams MT, Schaefer TL, Ehrman LA, et al. 3,4-methylenedioxymethamphetamine administration on postnatal day 11 in rats increases pituitary-adrenal output and

reduces striatal and hippocampal serotonin without altering SERT activity. Brain Res 2005; 1039:97–107.

Xu J, Lecanu L, Han Z, et al. Inhibition of adrenal cortical steroid formation by procaine is mediated by reduction of the cAMP-induced 3-hydroxy-3-methylglutaryl-coenzyme A reductase messenger ribonucleic acid levels. J Pharmacol Exp Ther 2003; 307:1148–1157.

Xu Y, Yu RM, Zhang X, et al. Effects of PCBs and MeSO2-PCBs on adrenocortical steroidogenesis in H295R human adrenocortical carcinoma cells. Chemosphere 2006; 63:772–784.

Zhang X, Yu RM, Jones PD, et al. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environ Sci Technol 2005; 39:2777–2785.

Zhao D, Xue H, Artemenko I, et al. Novel signaling stimulated by arsenite increases cholesterol metabolism through increases in unphosphorylated steroidogenic acute regulatory (StAR) protein. Mol Cell Endocrinol 2005; 231:95–107.

Part II

Hypothalamo-Pituitary-Adrenal Pathophysiology, Endocrinology, and Pharmacology

2

An Overview of Human Adrenal Dysfunction

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INTRODUCTION

The two adrenal glands are small endocrine glands weighing approximately 8 g and are situated in the retroperitoneum, immediately superior to the kidneys (hence the anatomical name, "suprarenal" gland) where they share, at least in part, the capsule of the kidney. The gland receives its blood supply through a number of small arteries which originate from the dorsal aorta. On the right, the adrenal vein drains directly into the inferior vena cava, whereas the left adrenal vein enters the left renal vein.

In mammals, the adrenal glands are divided into two main components, the outermost adrenal cortex and inner medulla, which have separate embryological derivations and function separately. For this reason, pathological processes affecting the cortex have little influence on the function of the medulla and vice versa. Therefore, the physiology and pathology of two parts of the adrenal can be considered separately.

THE NORMAL ADRENAL CORTEX

Anatomy

The adrenal cortex is divided into three distinct zones organized approximately as concentric shells, each with distinctive biochemistry. The outermost zone, occupying less than 5% of the total cortical volume and appearing as nests of closely packed small cells, is the *zona glomerulosa*. The largest layer is the *zona fasciculata* that contains larger cells arranged in columns making up three quarters of the adrenal cortex. The innermost part is the *zona reticularis*, the cells of which appear in "netlike" arrangements. In humans, this layer is formed between the age of 6 and 8 years at the poorly understood developmental landmark, "adrenarche."

Embryology

Epithelial cells that line the abdominal ("coelomic") cavity of the developing human embryo form the adrenal cortex in the 5th week of development. These cells proliferate to generate the outer definitive and the inner fetal zones of the adrenal cortex. This presence of the fetal zone is unique to certain primates. Only after birth does the gland reorganize itself into the more characteristic layers of the adult adrenal cortex.

The adrenal cortex is biochemically very active during fetal life and is correspondingly relatively larger than in postnatal life. This is largely attributable to the fetal zone, which, postnatally, is believed to involute or transform into other elements of the definitive cortex, when the relative size of the adrenal falls rapidly.

Biochemistry

The adrenal cortex secretes three main types of steroid hormones:

- Glucocorticoids—cortisol
- Mineralocorticoids—aldosterone
- Sex steroid precursors—dehydroepiandrosterone (DHEA) and androstenedione.

Synthesis of Steroid Hormones

Although the mechanisms underlying the determination and maintenance of the three adrenocortical zones remain incompletely understood, the three zones have very different biochemical activity. The zona glomerulosa secretes aldosterone, the zona fasciculata predominantly secretes cortisol but it also secretes sex steroid precursors while the zona reticularis secretes sex steroid precursors and to a lesser extent cortisol. This compartmentalized function is particularly interesting in light of the prevailing theory of adrenocortical aging, whereby cells migrate from outer glomerulosa to innermost reticularis prior to undergoing apoptosis with steroid secretion being modified along the way.

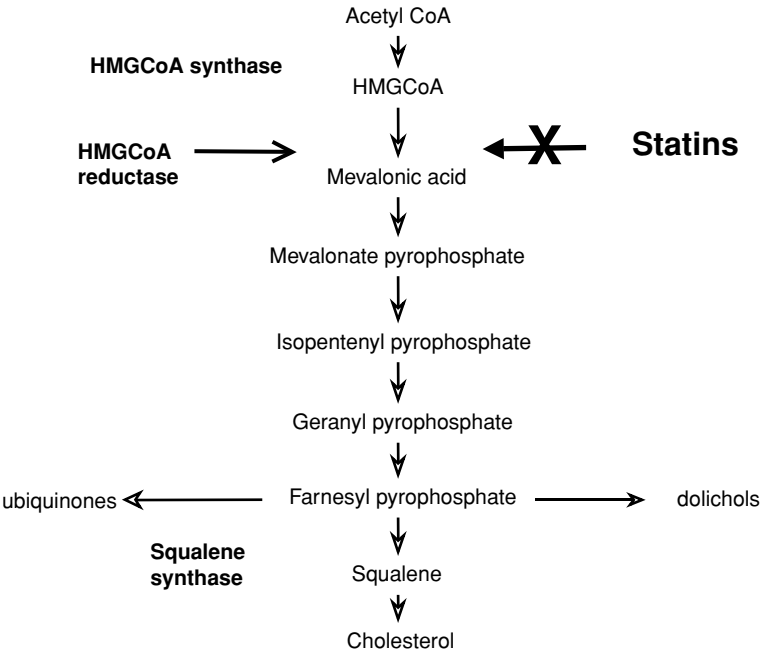

Figure 1 Biochemical pathway leading to the synthesis of cholesterol.

Steroidogenesis involves the enzymatic modification of the 4-carbon ring structure of cholesterol, and the final steroid product depends on the complement of enzymes that catalyze this process. Cholesterol is acquired in approximately equal amounts from the diet or de novo synthesis, mainly in the liver. Following ingestion, cholesterol is delivered to cells as a complex with low-density lipoprotein (LDL-cholesterol) and intracellular uptake is via the cell surface LDL-receptor. De novo biosynthesis commences with acetate and proceeds via hydroxymethylglutaryl coenzyme A (HMGCoA) and mevalonic acid. The rate-limiting step is the reduction of HMGCoA by the enzyme HMGCoA reductase (Fig. 1). The enzyme is inhibited by the "statin" class of drugs which are used to prevent cardiovascular disease.

In steroidogenic cells, cholesterol is stored as esters in large lipid-filled vesicles. Upon stimulation, cholesterol is transported into the mitochondrion under the control of the steroid acute regulatory (StAR) protein. The first and rate-limiting step in the synthesis of a steroid hormone is the conversion of cholesterol to pregnenolone by the removal of the cholesterol side chain by the enzyme CYP11A1 (Fig. 2).

Shuttling between the mitochondrion and endoplasmic reticulum allows further enzymatic modification of steroid intermediates. Many, but not all, of these

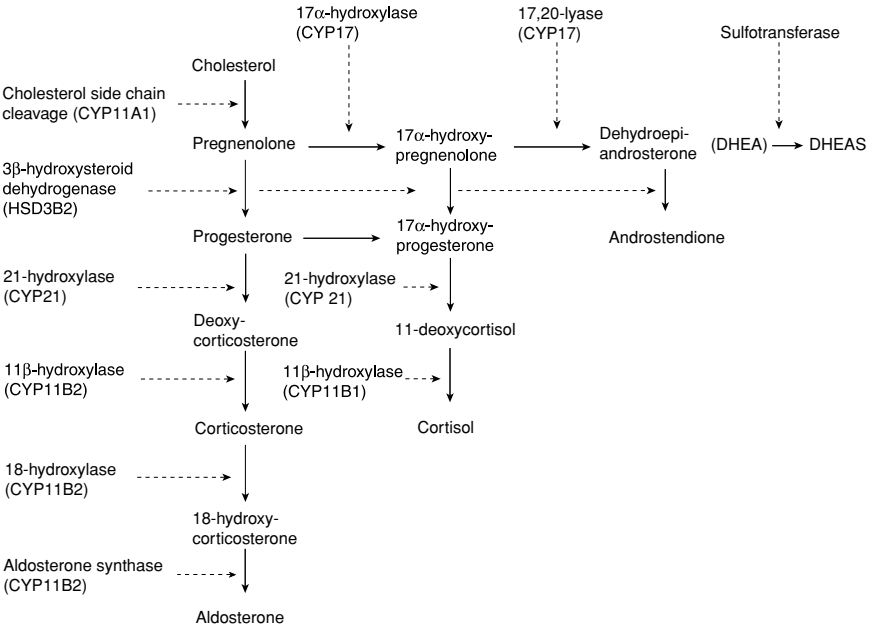

Figure 2 Biochemical pathway leading to the synthesis of adrenocortical steroid hormones.

enzymes are members of the cytochrome P450 superfamily. The two key enzymes are 3β-hydroxysteroid dehydrogenase (HSD3B2) and CYP17 (17α- hydroxylase); the former commits steroid precursors away from the sex steroid precursors toward aldosterone or cortisol, while CYP17 prevents the biosynthesis of aldosterone and provides substrate for further modifications to either cortisol (if HSD3B2 is active) or DHEA (if HSD3B2 is inactive).

Although the nomenclature for the corresponding genes has been unified, several other names remain in common usage (Table 1). Historically, the enzymes were labeled according to their enzymatic action at a specific carbon atom, with a Greek letter indicating orientation above or below the 4-carbon ring structure. For

Table 1 Alternative Names in Common Usage for Steroidogenic Enzymes

Gene name	Alternative common enzyme names and abbreviations
CYP11A1	Cholesterol side-chain cleavage enzyme (SCC) or desmolase
HSD3B2	Type 23β-hydroxyetcetc dehydrogenase (Type 2 3β-HSD)
CYP21	21-hydroxylase
CYP11B1	11β-hydroxylase
CYP11B2	Aldosterone synthase
CYP17	17α-hydroxylase/17,20-lyase

example, α-hydroxylase attaches a hydroxyl group in the alpha position to carbon 17. Awareness of these names is important, as several of the genes encoding these enzymes are mutated in congenital adrenal hyperplasia (CAH).

The common names used for steroids also adhere to a loose convention. The suffix "-ol" indicates an important hydroxyl group, as in cholester*ol* or cortis*ol*, whereas the suffix "-one" indicates an important ketone group (testoster*one*). The extra presence of "-di", as in diol (oestra*diol*) or dione (androstene*dione*), reflects two of these groups, respectively. "Ene" (androst*ene*dione) within the name indicates a significant double bond in the steroid nucleus.

The adrenal sex steroid precursors, DHEA and androstenedione, produced largely in the zona reticularis are converted into more potent sex hormones in peripheral target tissues.

Storage of Steroid Hormones

Steroid secreting cells do not store hormones but synthesize them as required. A consequence of this is a slow onset of action for steroid hormones following the initial stimulus to the relevant endocrine organ compared to hormones released from secretory granules by endocrine glands.

Mechanism of Hormone Action

Steroid hormones act by binding to steroid hormone receptors and functioning as transcription factors that influence target gene expression. While the major glucocorticoid, cortisol, and mineralocorticoid, aldosterone, have clearly defined functions, the role of DHEA remains unclear, other than serving as a precursor for extragonadal sex steroid hormone biosynthesis.

Steroid Hormone Receptors

Steroid hormone receptors are encoded by a single gene and belong to a larger family of nuclear receptors that are classified by their ligands (Fig. 3). Once in the nucleus, the receptors bind DNA and function as transcription factors; this need for transcription and translation to elicit an effect means that biological responses are relatively slow compared to peptide hormones that signal through cell surface receptors.

The nuclear import and export of the steroid hormone receptor appears to be an important regulatory mechanism in steroid action. A further characteristic of steroid hormones that affects their function is enzymatic modification within the target cell. For instance, in cells where mineralocorticoid action occurs, as in the kidney tubule, 11β-hydroxysteroid dehydrogenase (HSD11B2) inactivates cortisol to cortisone, a step which is thought to preserve aldosterone action at the mineralocorticoid receptor. Without this conversion, cortisol, which is present at much higher concentrations in the circulation than aldosterone, would be expected to swamp the mineralocorticoid receptor resulting in inappropriate overactivity. This is seen in the syndrome of apparent mineralocorticoid excess where

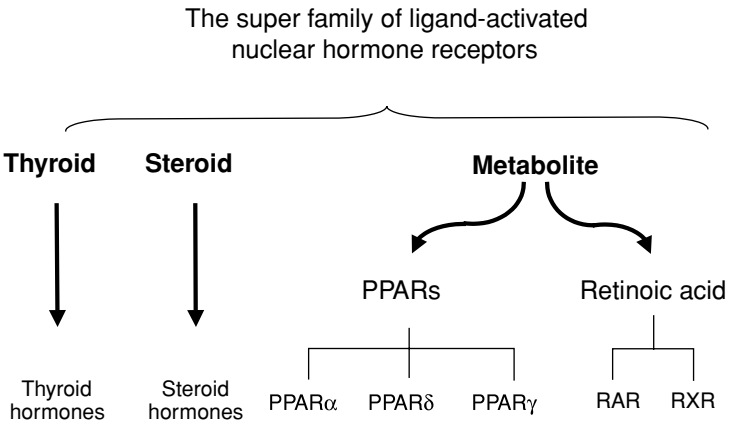

Figure 3 The superfamily of ligand-activated nuclear hormone receptors.

HSD11B2 does not function normally and patients present with hypertension and hypokalemia (Hammer and Stewart, 2006).

In their resting state, steroid hormone receptors without bound ligand are associated with heat-shock proteins that obscure the DNA-binding domain and prevent it from binding to DNA. Following binding of the steroid ligand, a conformational change occurs leading to dissociation of the heat-shock protein. This reveals two polypeptide loops stabilized by zinc ions known as zinc fingers. Once two steroid receptors are dimerized, these motifs bind to target DNA at the hormone response element.

Abnormalities of this process can lead to steroid hormone resistance syndromes. Mutations in the glucocorticoid receptor have been identified which lead to reduced hormone binding, reduced receptor number, and decreased DNA binding to the hormone response element (van Rossum and Lamberts, 2006).

Glucocorticoids

Secretion of Glucocorticoids

Glucocorticoids, predominantly cortisol in humans, are released from the adrenal cortex almost entirely under the control of the pituitary hormone, adrenocorticotrophic hormone (ACTH). The synthesis of ACTH from expression of the *pro-opiomelanocortin (POMC)* gene in anterior pituitary corticotrophs is regulated by the hypothalamic hormone, corticotropin-releasing hormone (CRH) in a classical endocrine negative feedback mechanism (Fig. 4).

Like other steroid hormones, glucocorticoids are not stored but synthesized according to acute changes in demand. Following the binding of ACTH to cell surface melanocortin type 2 receptors on the adrenal cortex, there is an increased flux within 5 minutes through the synthetic pathway from cholesterol to cortisol,

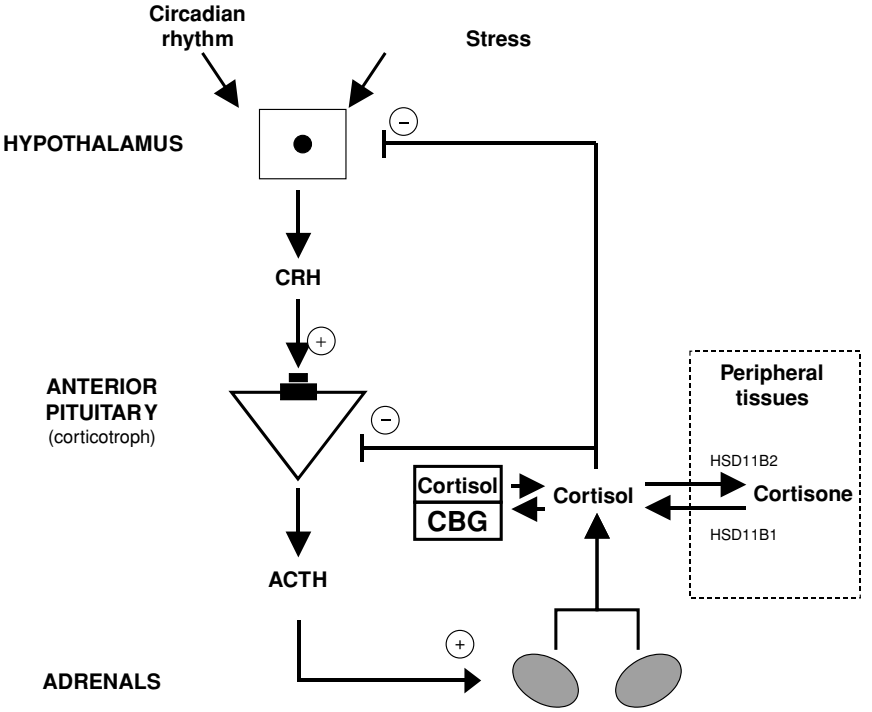

Figure 4 The hypothalamic-pituitary adrenal (HPA) axis. CRH release is influenced by higher brain function. ACTH secretion is increased by CRH, which in turn increases cortisol production by the adrenal gland. Both cortisol and ACTH feed back to reduce CRH secretion. Cortisol circulates largely bound to CBG. The concentration of cortisol is influenced by its conversion to cortisone by the activity of 11β-hydroxysteroid dehydrogenase.

particularly at the rate-limiting step catalyzed by CYP11A1, as well as an increase in blood flow through the adrenal gland (Fig. 2). Cortisol feeds back to the anterior pituitary and hypothalamus to inhibit CRH and ACTH production.

In addition to the acute regulation of cortisol secretion, peptides derived from the *POMC* gene, including ACTH, exert a trophic effect on the adrenal gland. The absence of anterior pituitary corticotrophs leads to atrophy of the fasciculata and reticularis zones of the adrenal cortex, while their overactivity induces a bilateral bulky increase in adrenocortical size. Contralateral growth occurs following unilateral adrenalectomy.

Superimposed on the acute feedback mechanism is a circadian rhythm. There is increased hypothalamic-pituitary-adrenal (HPA) axis activity prior to awakening in the morning, moderate activity during the day, with reduced functioning in the late evening and night time. Consequently, serum cortisol levels are highest in the morning and lowest around midnight. Plasma cortisol concentrations are

also highly variable, partly because of cortisol's relatively short half-life of 1 to 2 hours. In addition, cortisol secretion is stimulated by many physiological "stressors," including hypoglycemia, illness, fever, trauma, surgery, fear, pain, physical exertion, or extremes of temperature.

After secretion, cortisol is largely bound (>90%), within the circulation, to cortisol binding globulin (CBG), although only the free unbound component is able to enter cells. In peripheral tissues, predominantly the liver, cortisol is metabolized to inactive cortisone by the enzymatic action of type 2 11β-hydroxysteroid dehydrogenase (HSD11B2). Cortisone may be regenerated to the active hormone through the action of type 1 11β-hydroxysteroid dehydrogenase (HSD11B1), which is present in adipose tissue. One clinical correlation of this is that central obesity is frequently accompanied by hypercortisolemia, so-called Cushing syndrome of the omentum.

Function of Glucocorticoids

Glucocorticoid receptors occur in cells in virtually every organ of the body and so cortisol influences many body systems.

Intermediary Metabolism

As suggested by the name, glucocorticoids have a major role in intermediary metabolism where they act as insulin antagonists. Consequently, cortisol tends to increase blood glucose levels by promoting gluconeogenesis, inhibiting glucose uptake by fat and muscle and increasing hepatic glucose output. Lipolysis and protein catabolism are increased by cortisol. The antagonistic effect on insulin is enhanced by a permissive effect on adrenaline and glucagon. The net metabolic effect of cortisol action is to raise circulating free fatty acids and glucose, the latter stimulating glycogen synthesis. Excess cortisol also leads to an unfavorable serum lipid profile that is characterized by raised total cholesterol and triglyceride with decreased HDL cholesterol. In the long-term, cortisol stimulates adipocyte differentiation, particularly in a central or visceral distribution predisposing to centripetal obesity.

Skin, Muscle, and Bone

Glucocorticoids inhibit keratinocyte division and collagen synthesis in skin; while in muscle, the catabolic effects reduce protein synthesis resulting in atrophy. Similar catabolic effects in bone shift the balance of activity from osteoblast (the bone-forming cell-type) to osteoclast (the bone-resorbing cell-type), predisposing to osteoporosis and the net flow of amino acids towards the liver.

Salt and Water Homeostasis and Blood Pressure

Glucocorticoids possess some mineralocorticoid actions and may promote sodium resorption and potassium loss at the distal tubule and collecting ducts of the kidney. Unlike most of its actions, which are mediated through interaction with the glucocorticoid receptor, the effects on salt and water metabolism are through

the mineralocorticoid receptor, which cortisol binds with an equal affinity to aldosterone. The specificity for aldosterone is usually preserved by HSD11B2, which inactivates cortisol to cortisone at the major sites of mineralocorticoid action.

Cortisol increases glomerular filtration rate and inhibits arginine vasopressin to increase free water clearance. In addition to the mineralocorticoid effects, cortisol increases blood pressure by several other mechanisms including increased sensitivity of the vasculature to catecholamines.

Growth and Development

Cortisol is an important intrauterine hormone influencing fetal growth and development. It stimulates the differentiation of cell types to their mature phenotype. This is particularly important in the lung, where it stimulates the production of surfactant, which is necessary to reduce alveolar surface tension and the expansion of the fluid-filled fetal airways after birth. Too much glucocorticoid, however, inhibits growth which is in keeping with its largely catabolic effects on the musculoskeletal system.

Central Nervous System and Psyche

The role of glucocorticoids in the brain is highly complex, as illustrated by their potential to cause a range of emotional symptoms from euphoria to depression.

Anti-Inflammatory Effects

Glucocorticoids suppress circulating T lymphocytes and eosinophils but lead to a rise in neutrophil count. Within tissues, glucocorticoids rapidly suppress inflammation by inhibiting cytokine production and antagonizing macrophage action. Although these actions on inflammation and autoimmunity are less significant from a physiological perspective, they are important because of the therapeutic potential of potent synthetic steroids to treat a range of inflammatory and autoimmune disorders.

Mineralocorticoids and the Renin–Angiotensin System

Secretion of Aldosterone

Aldosterone is the most important mineralocorticoid in human. Its synthesis and secretion is regulated by the renin–angiotensin system in a negative feedback system (Fig. 5).

The juxtaglomerular apparatus of the kidney surrounds the afferent arteriole before it enters the glomerulus and forms a sensing mechanism for intravascular volume. In response to a fall in circulating volume, the enzyme, renin, is synthesized within and released from these cells. Renin acts upon its substrate, circulating angiotensinogen, to generate the decapeptide, angiotensin I, which is subsequently converted into angiotensin II. Angiotensin II binds to its type 2 receptor in the

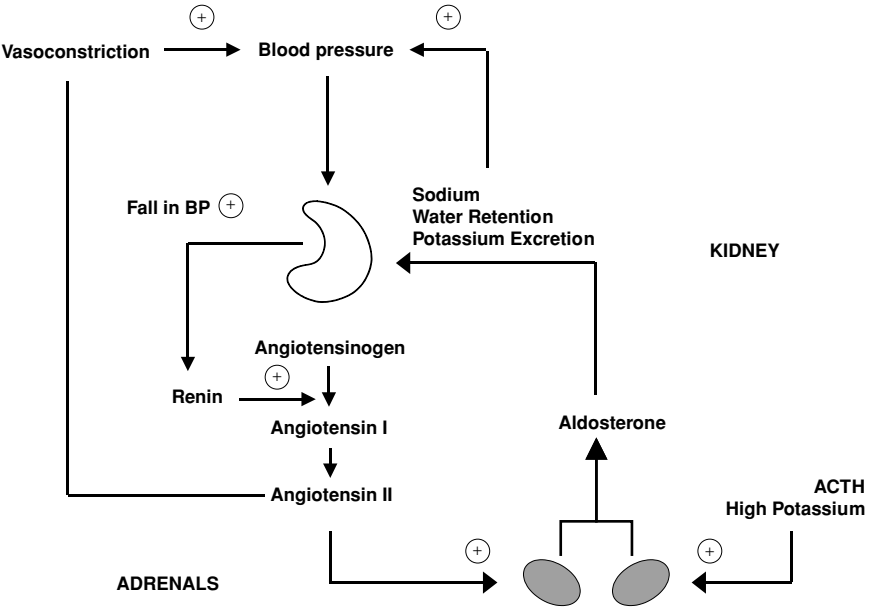

Figure 5 Regulation of salt, water, and blood pressure by aldosterone. Low blood pressure stimulates the release of renin from the juxta-glomerulat apparatus of the kidney. Renin converts angiotensinogen to angiotensin I, which is in turn converted to angiotensin II. Angiotensin stimulates production and release of aldosterone from the adrenal. Aldosterone promotes salt and water retention and increase in blood pressure. Angiotensin II has direct vasoconstrictive effects.

adrenal zona glomerulosa cells to stimulate aldosterone biosynthesis and secretion. Angiotensin II is also a potent arteriolar vasoconstrictor in its own right.

The volume of the zona glomerulosa layer correlates to its capacity to secrete mineralocorticoid. "Westernized" high salt diets, which expand the intravascular volume and raise blood pressure, suppress the renin–angiotensin system and lead to atrophy of the zona glomerulosa. Although the renin–angiotensin system is the major regulator of aldosterone, high potassium concentrations also stimulate aldosterone biosynthesis. ACTH also plays a minor role, although the relevance of this is uncertain as over- or undersecretion has no clinically significant impact on circulating aldosterone.

The circulating concentrations of aldosterone are approximately 1000-fold lower than cortisol. This is partly because of its lower affinity for serum carrier proteins and its shorter half life of 20 to 30 minutes.

Function of Aldosterone

Aldosterone acts through binding to the nuclear mineralocorticoid receptor to influence gene expression in target cells. Aldosterone acts on the Na–K-ATPase

transporter to increase sodium resorption in exchange for potassium excretion in the kidney and at other secretory epithelial sites, thus reducing the sodium content of urine, sweat, and saliva. The net effect is to increase osmotic potential within the circulation, causing expansion of circulating volume and a rise in blood pressure. There is also a direct effect of aldosterone to increase blood pressure through vasoconstriction.

Sex Steroid Precursors

Secretion of Sex Steroid Precursors

In the zona fasciculata and reticularis, the complement of steroidogenic enzymes determines whether the individual cells produce cortisol or the adrenal sex steroid precursors, DHEA and its downstream derivative androstenedione (Fig. 2). The regulation of this secretion is not fully understood. Although DHEA and androstenedione biosynthesis, like cortisol, appears to be primarily under the regulation of ACTH, the secretion of glucocorticoids and sex steroid precursors may be dissociated in some situations, for example, during puberty. During the 2nd and 3rd trimesters of fetal development, huge amounts of DHEA and its sulphated derivative, DHEAS, are generated. Postnatally, little sex steroid precursor is produced until adrenarche between 7 and 8 years, when the zona reticularis becomes functionally mature. Adrenal sex steroid precursor secretion peaks during puberty or early adulthood but remains high until 40 to 50 years, after which secretion falls.

Function of Sex Steroid Precursors

DHEA and androstenedione are not essential hormones and their potential direct action remains unclear. Although androstenedione possesses weak androgenic activity, it can be converted peripherally to the more potent androgen, testosterone, via 17β-hydroxysteroid dehydrogenase, or estradiol, via the subsequent action of aromatase (CYP19).

The role of sex steroid precursors seems important around the time of adrenarche. Peripheral metabolism of these hormones stimulates growth in middle childhood and is sometimes accompanied with pubic and axillary hair growth. Although this is a normal physiological process, it is important clinically because it can be mistaken for precocious puberty, the hallmarks of which include breast development in females and testicular enlargement in males.

CLINICAL DISORDERS OF THE ADRENAL CORTEX

The major clinical disorders affecting the adrenal cortex arise from either diminished or excess secretion of cortisol and aldosterone.

Hypoadrenalism

Hypoadrenalism refers to the clinical disorder where there is decreased secretion of adrenocortical hormones (Betterle *et al*., 2002). It may result from direct destruction of the adrenal gland, in which case it is known as primary hypoadrenalism, or diminished ACTH secretion that can result from pituitary or hypothalamic pathology, which is known as secondary or tertiary hypoadrenalism, respectively. Clinically, it is an important distinction because primary hypoadrenalism is associated with a deficiency in cortisol and aldosterone secretion, while in secondary hypoadrenalism aldosterone secretion is intact, reflecting the different regulation of secretion of glucocorticoids and mineralocorticoids.

Primary Hypoadrenalism

The clinical syndrome resulting from destruction of the adrenal cortex was first described by Thomas Addison in 1855 and now carries his eponymous title (Addison, 1868). The adjective "Addisonian" is used to refer to the clinical crisis, which results from acute, severe cortisol deficiency.

Etiology

There are many causes of primary adrenocortical destruction, but worldwide, the commonest cause is infection, particularly AIDS or tuberculosis (Table 2). In the western world, infection is rare and autoimmune destruction of the adrenal cortex is more common, accounting for more than 80% of the cases (Betterle *et al*., 2002). This may occur in isolation or as part of more widespread autoimmune destruction. The most commonly affected other tissue is the skin, resulting in vitiligo but other endocrine glands may be affected leading to pernicious anemia, hypo- or hyperthyroidism, alopecia, gonadal failure, type 1 diabetes, and myasthenia gravis.

Other causes are rare but in paediatric practice, inherited disorders need to be considered (Betterle *et al*., 2002). Adrenoleukodystrophy is a rare inherited disorder of very long chain fatty acid metabolism that results in progressive demyelination of the central nervous system and hypoadrenalism, particularly affecting glucocorticoid secretion (Moser, 1997). Congenital adrenal hypoplasia occurs in 1 in 12,500 live births (Vaidya *et al*., 2000). Familial X-linked and autosomal recessive inheritance forms as well as sporadic cases have been identified. Specific mutations, for example, in the *AHC* gene, encoding the orphan nuclear receptor, NROB1 (also known as DAX1), have been identified (Vaidya *et al*., 2000).

Clinical Features

The clinical manifestations of the combined glucocorticoid and mineralocorticoid deficiency become apparent when more than 90% of the adrenal gland is destroyed (Oelkers, 1996; Ten *et al*., 2001). The features relate to reduced vascular volume and tone, renal sodium loss, bowel water and electrolyte loss, the loss of the cortisol action on hepatic and peripheral metabolism, and removal of negative feedback

Table 2 Causes of Primary Adrenal Failure

Autoimmune
Infection
Tuberculosis
Fungal infection
Histoplasmosis
Blastomycosis
Coccidiomycosis
Cryptococcosis
Paracoccidiomycosis
Acquired immunodeficiency syndrome (AIDS)
Cytomegalovirus
Congenital or hereditary
Adrenal hypoplasia
Adrenal cysts
ACTH receptor mutations
Allgrove syndrome
Adrenoleucodystrophy
Adrenomyeloneuropathy
Drugs
Inhibitors of cortisol synthesis
Aminoglutethamide
Metyrapone
Mitotane
Ketoconazole
Increased cortisol clearance
Barbiturates
Phenytoin
Rifampicin
Hemorrhage
Infection (Waterhouse–Friderichsen syndrome)
Anticoagulation
Trauma
Thrombosis
Systemic lupus erythematosis
Polyarteritis nodosa
Neoplastic
Metastatic tumor
Adrenal Carcinoma
Infiltrative
Amyloidosis
Hemochromatosis
Sarcoidosis
Bilateral adrenalectomy
Neonatal
Maternal Cushing

Table 3 Signs and Symptoms of Hypoadrenalism

Weight loss and anorexia
Fatigue and weakness
Nausea, vomiting, abdominal pain, and diarrhea
Generalized wasting and muscle cramps
Hypoglycemia (especially in children)
Dizziness and postural hypotension
Loss of body hair
Pigmentation of light-exposed areas, pressure points, scars, and buccal mucosa (primary disease only)
Vitiligo (associated with autoimmune adrenalitis)
Circulatory shock (in acute circumstances)

(Table 3). The latter leads to a classical pigmentation that is seen in skin creases, scars, and other unusual places, like the buccal mucosa. The reduced cortisol feedback leads to increased ACTH synthesis. ACTH is a cleavage product derived from expression of the *POMC* gene, which also gives rise to melanocyte stimulating hormone (MSH). Accordingly, increased ACTH synthesis inevitably increases MSH production and secretion, and this in turn increases skin pigmentation. This only occurs in primary hypoadrenalism as MSH production is a consequence of increased *POMC* expression from diminished negative feedback from cortisol.

Diagnosis

The diagnosis is often suggested by the presence of hyponatremia and hyperkalemia. Aldosterone deficiency leads to a failure of sodium reabsorption and potassium excretion in the distal tubules of the kidney. The glucocorticoid deficiency leads to loss of vascular tone and hypovolemia, which in turn causes release of vasopressin with subsequent water retention and hemodilution. Hyponatremia ensues as a result of excessive renal loss and the vasopressin response. Although potassium concentrations are elevated, they may be normal if there has been excessive concomitant potassium loss as a result of vomiting or diarrhea. In children and neonates, hypoglycemia is present in more than 90% of cases because of impaired gluconeogenesis and, indeed, hypoglycemic fitting may be the initial presentation.

Specific testing of the HPA axis is required to confirm the diagnosis (Betterle *et al*., 2002; Oelkers, 1996). A serum cortisol in excess of 550 nmol/L safely excludes Addisonian crisis in most situations; conversely, in emergency situations, the finding of a plasma cortisol less than 200 nmol/L in combination with an elevated ACTH in excess of 80 ng/L is highly supportive of primary adrenal failure.

Relying on a single measurement to make the diagnosis, however, is generally ill-advised because of the highly variable secretion of cortisol. The "gold standard" investigation for diagnosis of primary hypoadrenalism is dynamic testing. Serum cortisol is measured in the morning, before and 30 minutes after an intramuscular or intravenous injection of 250 μg tetracosactide, a soluble synthetic

ACTH product, containing the first 24 amino acids of biological ACTH. This test, which is designed to stimulate maximal cortisol secretion, is commonly known as the "short Synacthen test" and "Cortrosyn stimulation test" after the trade names for tetracosactide in the United Kingdom and United States, respectively. There is a lack of consensus about the definition for the cutoff for a normal response, but cortisol values post-ACTH administration in excess of 525 nmol/L identifies those above the 5th centile; thus, 95% of the adult population will achieve serum cortisol levels above this value. In primary adrenocortical disease, plasma renin concentration and activity will be increased and this may be accompanied by reduced plasma aldosterone concentrations.

Treatment

Oral hydrocortisone, the pharmacological term for cortisol, is the mainstay of replacement therapy in hypoadrenalism (Betterle *et al*., 2002; Oelkers, 1996). Alternative glucocorticoids are available but rarely indicated. Replacement with the synthetic mineralocorticoid, fludrocortisone is also needed in primary hypoadrenalism. In emergency situations, hydrocortisone may be given parenterally.

Historically, endocrinologists have tended to over-replace cortisol, leading to iatrogenic hyperadrenalism. A better understanding of adrenal physiology has indicated that normal adrenal cortices produce the equivalent of approximately 10 to 15 mg of oral ingested hydrocortisone daily, but detrimental effects are only seen when more than 20 mg of hydrocortisone is ingested daily. The modern adult replacement dose is usually 15 to 20 mg hydrocortisone daily; 10 mg on awakening and the remainder either as a single dose mid-afternoon (5 or 10 mg) or in equally divided 5 mg doses at midday and mid-to-late afternoon, in an attempt to mirror the normal circadian variation in cortisol secretion of high levels in the morning and low levels by bedtime. Disturbance to this profile can present as either difficulty or tiredness executing daily tasks (inadequate cortisol) and inability to sleep at night (too much cortisol).

Although some endocrinologists advocate an intermittent series of measurements during the day, a "cortisol day curve," little evidence supports its use in demonstrating clinical benefit.

As the patients are entirely dependent on tablets, the normal ability of the adrenal cortex to increase cortisol output during illness or stress is lost. It is vital to ensure that patients receiving glucocorticoid replacement understand that this treatment is essential and noncompliance can lead to a potentially fatal Addisonian crisis, which presents as circulatory collapse, hyponatremia, hyperkalemia, and hypoglycemia. This medical emergency demands immediate treatment with intravenous hydrocortisone. Patients should be advised to double replacement doses during intercurrent illness and to carry a "steroid card" and alert bracelet to identify to healthcare professionals in an emergency that the person is dependent on exogenous glucocorticoids.

Fludrocortisone is much longer acting than hydrocortisone and therefore can be taken once daily, commonly 100 μg in adults. The efficacy of mineralocorticoid

replacement can be guided by measurements of renin concentration or plasma renin activity and blood pressure. Inadequate replacement causes raised renin levels whereas, over-replacement tends to generate hypokalemia and hypertension.

Secondary Hypoadrenalism

Secondary hypoadrenalism occurs if the anterior pituitary corticotrophs secrete insufficient ACTH as a result of pituitary or hypothalamic pathology. ACTH deficiency leads to cortisol deficiency and loss of adrenal sex steroid precursors but aldosterone biosynthesis is maintained, as this is largely ACTH independent. In adult endocrinology, hypopituitarism is most commonly caused by compression from nonfunctioning pituitary adenomas or their treatment by surgery or radiotherapy. In pediatric practice, congenital absence or malformation of the pituitary gland are other important causes.

The clinical features are similar to primary hypoadrenalism and the principles of glucocorticoid-replacement therapy are the same. The diagnosis is suggested by the presence of low 0900-hour plasma ACTH and cortisol ($<$100 nmol/L) and confirmed by dynamic testing. Traditionally, the investigation of choice was an insulin stress test. The hypoglycemia leads to a brisk rise in ACTH and cortisol in normal individuals. This investigation is contraindicated in those with cardiac disease, epilepsy, and blackouts, and is potentially hazardous because of the hypoglycemia. More recently, the ACTH stimulation test has been increasingly advocated as the first line investigation because of its simplicity and safety. The main concern with this test is that it is not testing pituitary function and may miss ACTH insufficiency of recent onset within the preceding three months.

Hyperadrenalism

Hyperadrenalism refers to the clinical disorder where there is excess secretion of adrenocortical hormones. Despite advances in our basic research and clinical understanding of hyperadrenalism, it remains one of the most challenging problems in clinical endocrinology.

Glucocorticoid Excess

Etiology

The constellation of clinical features of glucocorticoid excess is known as "Cushing syndrome" after Harvey Cushing, the American neurosurgeon who first described the condition in 1912, some 20 years before its hormonal basis was discovered (Cushing, 1932; Cushing, 1912). The commonest cause of glucocorticoid excess is iatrogenic resulting from the use of exogenous steroid drugs to treat inflammatory conditions such as asthma. Pathological causes are rare but occur more frequently in women (Newell-Price *et al.*, 1998). The incidence appears to be rising, as the diagnosis is being increasingly considered by clinicians working in settings outside endocrinology.

Table 4 Etiology of Pathological Causes of Cushing Syndrome

	Proportion of Cases
ACTH dependent	84%
Pituitary Cushing	68%
Ectopic ACTH	10.5%
ACTH Source unknown	5.5%
ACTH Independent	16%
Adrenal Adenoma	8%
Adrenal Carcinoma	6.5%
Nodular Hyperplasia	1.5%

Source: Newell-Price *et al*., 1998

The etiological cause of endogenous Cushing syndrome can be divided into three main categories (Newell-Price *et al*., 1998):

- ACTH independent, resulting from excessive autonomous glucocorticoid secretion from adrenocortical abnormalities.
- ACTH dependent, resulting from excessive ACTH secretion. This group can be further divided into:
  - Excess ACTH production from the pituitary known as Cushing disease.
  - Ectopic ACTH secretion by nonpituitary sources.
- Ectopic sources include malignant tumors, such as small cell carcinoma of the lung, and carcinoid tumors. Rarely these tumors can secrete ectopic corticotropin-releasing hormone.

The commonest cause of Cushing syndrome is a benign pituitary tumor (Cushing disease) accounting for 66% of all cases and 79% of ACTH-dependent cases (Newell-Price *et al*., 1998). Approximately one-fifth of the cases result from adrenocortical tumors (Table 4) (Newell-Price *et al*., 1998).

Pseudo-Cushing Syndrome

A careful history is needed to exclude conditions, such as depression, obesity, and alcoholism, which can increase serum cortisol and complicate the differential diagnosis of Cushing syndrome (Groote and Meinders, 1996; Koelz and Girard, 1976; Stokes, 1995). This is called "pseudo-Cushing syndrome". This condition has many of the clinical features of Cushing syndrome together with evidence of cortisol hypersecretion. Differentiating the conditions can often be challenging; for example, depression can cause pseudo-Cushing syndrome yet also be a manifestation of Cushing syndrome. Some features such as proximal myopathy, easy bruising, and osteoporosis are usually absent in pseudo-Cushing syndrome, and other clinical features resolve once the underlying condition is treated. Similarly, subtle differences exist in investigation; diurnal variation in serum cortisol is usually retained in pseudo-Cushing syndrome, while in depression the cortisol will

Table 5 Symptoms and Signs of Cushing Syndrome

Symptoms and signs	Frequency
Central obesity or weight gain	78–97%
Hypertension	47–90%
Plethora	78–94%
Moon face	88–92%
Hirsutism	58–84%
Thin skin	84%
Poor wound healing	42%
Pigmentation	8–14%
Abnormal glucose tolerance	39–94%
Dyslipidemia	39%
Easy bruising	17–77%
Weakness	45–90%
Osteopenia ± fracture	48–83%
Menstrual changes	20–86%
Decreased libido	33–100%
Mood disturbance or psychosis	25–67%
Headache	47–58%
Striae	50–64%
Edema	48–66%
Acne	21–82%
Buffalo hump	34–67%
Male pattern hair loss	13–51%
Children	
Diminished growth	80–83%
Altered bone age	8–11%

rise with insulin-induced hypoglycemia (Butler and Besser, 1968; Newell-Price *et al*., 1998).

Clinical Features

The widespread action of glucocorticoids means that their excess causes many clinical features (Table 5) (Newell-Price *et al*., 1998). The symptoms and signs are frequently missed, however, because of insidious onset and because they are extremely common within the population. For example, it is estimated that only approximately 3% to 4% of people with type 2 diabetes have glucocorticoid excess (Leibowitz *et al*., 1996). Some signs, such as proximal myopathy and violaceous striae, are more discriminating but occur less frequently (Ross and Linch, 1982; Ross, Marshall-Jones,, Friedman, 1966). It is therefore incumbent on the clinician to have a high index of suspicion for potential patients with Cushing syndrome. Some ACTH-secreting tumors only release ACTH intermittently and therefore, some clinical features, such as depression, may occur in a cyclical manner (Atkinson *et al*., 1985).

The presentation of ectopic ACTH secretion is sometimes markedly different from other causes of Cushing syndrome and so a careful history and examination can help differentiate the etiology of the glucocorticoid excess (Meador *et al*., 1962). The excessively high cortisol concentrations may lead to profound weakness and hypokalemia together with marked pigmentation resulting from high ACTH production. These features point toward ectopic ACTH secretion but the lack of these features does not exclude the diagnosis.

Diagnosis

Diagnosing Cushing syndrome can be challenging but often the most important step is to consider the diagnosis (Findling and Raff, 2006). The cardinal biochemical features of Cushing syndrome are:

- Loss of the normal diurnal rhythm of glucocorticoid secretion
- Excess total daily glucocorticoid secretion (Trainer and Grossman, 1991)

Given the prevalence of relatively nonspecific symptoms and signs, high-sensitivity screening tests are used in assessing patients suspected of having Cushing syndrome. Random plasma cortisol estimations are not useful in diagnosing glucocorticoid excess.

Out-Patient Diagnosis of Cushing Syndrome

1. *Loss of diurnal variation*
   One of the earliest signs of glucocorticoid excess in Cushing syndrome is persistently high secretion at normal restful bedtime. The normal diurnal variation can be detected in the serum and a cortisol less than 50 nmol/L effectively excludes Cushing syndrome (Newell-Price *et al*., 1995). Free cortisol also passes into the saliva. With the advent of highly sensitive assays and expert centers with robustly validated normal ranges, salivary cortisol estimation can become an attractive way of detecting loss of diurnal variation (Raff *et al*., 1998). With patients being able to post samples from home, this assay has become an effective way of detecting cyclical Cushing syndrome.
2. *24-hour urinary free cortisol*
   Under normal conditions, unbound free cortisol is filtered by the kidney. Although the majority is reabsorbed, approximately 1% of total adrenocortical cortisol production is excreted in the urine, where it provides an integrated measure of glucocorticoid secretion over a specific time period (Trainer and Grossman, 1991). A 24-hour collection of urinary free cortisol therefore provides an easy noninvasive means of assessing potential glucocorticoid excess. It is usual to perform 2 to 3 collections to increase the chance of making a positive diagnosis. The main disadvantage of this screening test is the inconvenience to the patient and potential inadequacy of collection; an incomplete collection will under-represent true glucocorticoid status and risk a false-negative result (Newell-Price *et al*., 1998). Overall the urinary free cortisol performs well as a screening test because of its high sensitivity despite

relatively low specificity (Newell-Price *et al*., 1998). Thus, if several urinary free cortisol collections are normal, Cushing syndrome is unlikely.

3. *Low dose dexamethasone suppression test*
   The principle underlying the low dose dexamethasone suppression test is that the administration of exogenous glucocorticoids leads to the inhibition of ACTH and cortisol secretion in healthy individuals (Liddle, 1960). Dexamethasone, a potent synthetic steroid, is administered by one of the following two methods: Either 1mg at 2300 hrs to midnight with serum cortisol measured between 0800 and 0900 hours the following morning (Nugent *et al*., 1965); or for eight doses of 0.5 mg every 6 h, ending at midnight with serum cortisol similarly measured between 0800 and 0900 hrs, the following morning (Kennedy *et al*., 1984). The latter test is claimed to be more specific but is more complicated to administer (Newell-Price *et al*., 1995).

   Dexamethasone is used because it does not cross-react with cortisol in cortisol immunoassays. Suppression of cortisol to less 50 nmol/L effectively excludes Cushing syndrome (Newell-Price *et al*., 1995).

   False-negative results may occur in chronic renal impairment and hypothyroidism (Ramirez *et al*., 1982). The commonest reason for a false-positive result is not taking the tablet or doing so at an incorrect time. Malabsorption or hepatic enzyme inducing drugs, such as phenytoin and rifampicin, may also reduce dexamethasone concentrations to less than the level needed to suppress pituitary ACTH secretion (Jubiz *et al*., 1970; Putignano *et al*., 1998). Failure to suppress the cortisol may also occur in situations where there is an increase in cortisol binding globulin, such as pregnancy or estrogen therapy. False-positive results may occur in as many as 50% of women taking the oral contraceptive pill, therefore, either this should be discontinued for 6 weeks to allow the CBG to return to normal before any investigation or an alternative screening test should be performed (Tiller *et al*., 1988).
4. *Other investigations*
   In addition to cortisol, sex steroid precursor (DHEA, androstenedione) production may be elevated (Barbetta *et al*., 2001). These hormones may be converted elsewhere to potent androgens that cause hirsutism and, along with elevated cortisol, menstrual irregularities in women.

Etiology of Glucocorticoid Excess

The final stage of the diagnostic process is to determine the site of pathology leading to glucocorticoid excess.

1. *ACTH dependent vs. ACTH independent*
   Having diagnosed glucocorticoid excess, a primary adrenocortical cause of Cushing syndrome is indicated by suppressed levels of ACTH (Newell-Price *et al*., 1998; Raff and Findling, 1989). The precise cause can then be defined by imaging the adrenal gland with a fine-cut CT scan.

2. *Differential diagnosis of ACTH-dependent Cushing syndrome*
   Where ACTH remains inappropriately within the normal range or frankly elevated in the presence of excessive cortisol secretion, it is necessary to distinguish between pituitary and ectopic sources of ACTH secretion. ACTH is usually higher when secreted ectopically, although there is a significant overlap with a pituitary source (Howlett *et al*., 1986). Ectopic tumors also often cosecrete several other peptide hormones, which are amenable to assay (Howlett *et al*., 1985). The mainstay of discriminating these two possibilities is the high dose dexamethasone suppression test.

   a. *High dose dexamethasone suppression test*
      The principle underlying the high dose dexamethasone suppression test is that tumorous anterior pituitary corticotrophs remain partially sensitive to the negative feedback of glucocorticoids while ectopic sources do not (Newell-Price *et al*., 1998). The test is performed in a similar manner to the low dose test except that 2 mg of dexamethasone is given every 6 hours instead of 0.5 mg, or 8 mg is administered in a single dose rather than 1 mg (Tyrrell *et al*., 1986). For pituitary sources, the post-dexamethasone cortisol is usually less than half of the baseline cortisol; less than 50% fall supports an ectopic source (Newell-Price *et al*., 1998).
   b. *Corticotropin-releasing hormone test*
      The principle of this test is that the intravenous administration of CRH leads to a rise in ACTH and cortisol in pituitary disease but only rarely in ectopic causes (Grossman *et al*., 1987), consistent with greater expression of CRH receptors in pituitary tumors than in ectopic ones (Newell-Price *et al*., 1998).

      The test is performed by administering 1 μg/ kg body weight of CRH with measurement of ACTH and cortisol before and up to 2 hours after the injection. Overall, the sensitivity and specificity of the test is 86% to 93% and 88% to 100%, respectively, with 7% to 14% of those with pituitary disease failing to respond to CRH (Newell-Price *et al*., 1998).
   c. *Inferior petrosal sinus sampling*
      Venous sampling via catheters from the inferior petrosal sinuses, which drain venous blood from the anterior pituitary, provides further information regarding the source of ACTH (Corrigan *et al*., 1977). The presence of a significant concentration gradient of ACTH between this site and the periphery provides evidence of a pituitary rather than an ectopic source (Newell-Price *et al*., 1998; Trainer and Grossman, 1991). The administration of CRH to stimulate pituitary production of ACTH increases this gradient in pituitary disease and therefore improves sensitivity of the test. As samples are taken from both sides, this test may help lateralize the anatomical location of a pituitary tumor and assist the surgeon planning conservative hypophysectomy (Newell-Price *et al*., 1998; Oldfield *et al*., 1985).

Although this test is usually well tolerated, rare serious side effects such as brain stem infarction may occur. Thus, depending on local access to this invasive radiology and on individual endocrinologist preferences, some advocate inferior petrosal sinus sampling only when diagnostic doubt remains after high dose dexamethasone suppression test and pituitary imaging, whereas others regard it as an important component in the analysis of all suspected pituitary-dependent Cushing syndrome.

d. *Imaging*
The modality of choice for pituitary disease is magnetic resonance imaging (MRI), but where this is not available or contraindicated, CT scanning is also helpful. These tests should be reserved until after a biochemical diagnosis has been made to prevent several "pitfalls". For example, ACTH-secreting tumors may be small and not seen on scanning while coexisting, yet unrelated nonfunctioning pituitary tumors are relatively common. High-resolution CT scanning may identify an ectopic tumor but, as these may also be small, nuclear medicine scintigraphy may help localize the tumor.

Treatment of Cushing Syndrome

As glucocorticoid excess causes considerable morbidity and excess mortality, predominantly from cardiovascular disease, it is important to normalize glucocorticoid production and diurnal rhythm.

The treatment of choice is surgery, as this offers the best chance of long-term cure. For adrenal adenomas, unilateral adrenalectomy is undertaken frequently via a laparoscopic approach. This procedure is well tolerated and carries a low incidence of complications. Transsphenoidal surgery to remove pituitary adenomas is less effective leading to remission in 42% to 86% of cases, depending on how accessible and easily visualized the pituitary tumor is at operation (Atkinson *et al*., 2005). The success of surgery can be evaluated by the immediate postoperative assessment of serum cortisol (Atkinson *et al*., 2005; Trainer *et al*., 1993). Once the cause of excess glucocorticoid is removed, the underlying HPA axis is so suppressed that endogenous cortisol may not return to normal for several months. Consequently undetectable cortisol in the immediate postoperative period demonstrates curative surgery. Furthermore hydrocortisone therapy is needed until the HPA axis recovers to prevent symptoms and signs of adrenal insufficiency. Conversely, lack of adrenal insufficiency 4 to 6 weeks postoperatively is evidence of persistent hypercortisolemia and noncurative surgery (Pereira *et al*., 2003). In this scenario or with recurrent Cushing syndrome, there are several treatment options. In pituitary disease, complete hypophysectomy is an option but pituitary radiotherapy is more commonly used where surgery has failed or the tumor is inoperable. Conventional radiotherapy leads to remission in 53% to 100% of cases, with more focused application of gamma irradiation (gamma knife) being a useful modality in some patients (Vance, 2005). Following conventional radiotherapy, normalization of cortisol secretion may take up to 10 years and so medical therapy is also

needed during this period. It also commonly causes panhypopituitarism in adults and so lifelong hormone replacement of other hormone axes is needed (Vance, 2005). Gamma knife irradiation can act quicker and also spare normal pituitary tissue. Rarely radiotherapy may induce the development of a second tumor or temporal lobe epilepsy as a result of temporal lobe infarction and it also appears to increase subsequent risk of cerebrovascular disease (Findling and Raff, 2006).

The aim of medical therapies is to inhibit glucocorticoid secretion. Ketoconazole, aminoglutethimide and metyrapone all inhibit cortisol biosynthesis, and doses are commonly titrated to lower cortisol secretion into the normal range (Sonino *et al*., 2005). With time, these drugs lose their effectiveness as the increased ACTH secretion leads to escape of the competitive inhibition of steroidogenesis (Findling and Raff, 2006). Mitotane may be helpful in this situation as it reduces steroid production through a number of mechanisms (Sonino *et al*., 2005). There are no effective drugs that act on the hypothalamus and pituitary (Findling and Raff, 2006). Mifepristone is a glucocorticoid receptor antagonist and has been used in rare circumstances to block cortisol action (Chu *et al*., 2001).

As the morbidity and mortality of Cushing syndrome reflects the increased cortisol production rather than the underlying tumor, bilateral adrenalectomy is an option where other measures have failed (Young and Thompson, 2007). The cure rate for this procedure is nearly 100% but patients are rendered hypoadrenal and, therefore, need lifelong steroid replacement. Reduced feedback on the pituitary can lead to uncontrollable enlargement of the pituitary corticotroph tumor, so-called Nelson syndrome (Findling and Raff, 2006).

Mineralocorticoid Excess or Primary Hyperaldosteronism

Etiology

Primary hyperaldosteronism, first described by Conn in 1955 (Conn, 1995), is defined as the presence of increased aldosterone concentrations in the presence of low renin, and usually causes hypertension with a classical association of hypokalemia (Mattsson and Young, 2006). It is the commonest cause of secondary hypertension affecting up to 5% to 13% of hypertensive patients or approximately 8.5 million people in the United States. The prevalence increases with the severity of hypertension and is responsible for 17% to 20% of "resistant" hypertension (Calhoun *et al*., 2002). There are no consistent differences found in prevalence with age, gender, or ethnicity (Calhoun *et al*., 2002; Mosso *et al*., 2003). Primary hyperaldosteronism is caused by adenomas of the zona glomerulosa in approximately 30% of cases while nearly two thirds result from bilateral idiopathic hypersecretion, commonly from bulky hyperplastic glands (Mattsson and Young, 2006). Approximately 1% result from adrenal carcinoma.

There are rare familial forms of primary hyperaldosteronism. Type 1 familial hyperaldosteronism is inherited in an autosomal dominant manner and is caused by recombination between CYP11B1 and CYP11B2 (Mulatero *et al*., 2004). The abnormal chimeric gene places aldosterone synthase activity in the adrenal cortex

under the control of ACTH. Type 2 familial hyperaldosteronism is more common but the causative gene defect has not been identified, although it has been linked to the 7p22 region (So *et al*., 2005).

Clinical Features

Classically, Conn syndrome presents with hypokalemic hypertension but any symptoms tend to be vague. Hypertension may present with headaches and visual disturbances; hypokalemia may cause muscle fatigue or tiredness. In addition to the effect of hypertension, increased aldosterone concentrations have an adverse effect on the heart, causing increased left ventricular wall thickness and impaired diastolic function.

The clinical features are usually milder in bilateral idiopathic hypersecretion than for adrenal adenomas.

Diagnosis

The diagnosis is suggested by the presence of hypokalemia (Mattsson and Young, 2006). It is important that agents such as diuretics, β blockers and ACE inhibitors are stopped for at least 2 weeks before the investigation because of their effect on the renin–angiotensin system. Serum potassium should be restored to the normal range with oral supplementation in the days prior to testing and the patient should be sodium replete. The diagnosis is confirmed by the measurement of aldosterone and renin. Paired samples are taken while supine, on wakening, and then after 2 hours in the erect posture. Alternatively, a single measurement in a semi-recumbent position has proven as effective and is easier.

An abnormality is suggested when the serum aldosterone is inappropriately high in the presence of a suppressed plasma renin concentration or activity. This combination generates a high aldosterone to renin ratio. Challenge tests with fludrocortisone or salt loading, together with a finding of high urinary potassium excretion offer confirmatory evidence of hyperaldosteronism. Imaging with MRI or CT and, if needed, adrenal vein sampling helps to localize the potential source of mineralocorticoid excess.

Treatment

The treatment of choice for an isolated adrenal tumor is laparoscopic unilateral adrenalectomy (Mattsson and Young, 2006). Postoperatively, potassium supplementation should be withdrawn and antihypertensive doses reduced. Depending on the duration of the hyperaldosteronism, approximately one-third of patients will be able to stop antihypertensive treatment altogether (Sawka *et al*., 2001). A sodium-rich diet is temporarily recommended because adrenal production of aldosterone in the contralateral adrenal may be suppressed. Serum potassium should be monitored for 4 weeks and occasionally fludrocortisone supplementation is required.

Medical therapy is preferred for those with bilateral idiopathic hypersecretion. Spironolactone has been used for many years as a mineralocorticoid antagonist. Indeed, a clue to diagnosis can come with its use followed by a rapid fall in

previously refractory high blood pressure (Mattsson and Young, 2006). Spironolactone also antagonizes the androgen receptor, necessitating contraceptive advice in fertile women to guard against feminization of a male fetus. In men, spironolactone can induce sexual dysfunction and gynecomastia, which occurs in up to half of men in a dose-dependent fashion (Jeunemaitre *et al*., 1987). The affinity of spironolactone for the progesterone receptor may also lead to menstrual irregularities.

Eplerenone, which has been developed by replacing the 17α thioacetyl group of spironolactone with a carbomethoxy group, has a much lower affinity for the androgen and progesterone receptors but retains its affinity for the mineralocorticoid receptor (Sica, 2005). Consequently this drug is an effective treatment for bilateral idiopathic hypersecretion or for those with tumors that are unfit for surgery.

Amiloride, a potassium sparing diuretic, has been used as an alternative to mineralocorticoid receptor antagonists (Mattsson and Young, 2006).

Type 1 familial hyperaldosteronism, which is also known as glucocorticoid responsive aldosteronism, is treated with glucocorticoid replacement to suppress pituitary ACTH production (Mattsson and Young, 2006).

Sex Steroid Precursor Tumors

Tumors from the fasciculata and reticularis zones can secrete excess sex steroid precursors, either alone or as part of Cushing syndrome. These steroids are converted in the periphery to androgens and, potentially, estrogens that cause feminization in men leading to gynecomastia. The tumors can be diagnosed by serum measurement of DHEA (or DHEAS), androstenedione, testosterone, and assessment of glucocorticoid status, accompanied by appropriate imaging with CT or MRI. The main differential diagnosis is a gonadal tumor and where it is difficult to differentiate between the two, catheterization and sampling of the adrenal veins can be helpful. The treatment of choice is adrenalectomy.

Other Tumors of the Adrenal Cortex

Adrenocortical Carcinoma

The commonest malignant tumor of the adrenal cortex is metastatic, as primary adrenal carcinoma is rare (Allolio and Fassnacht, 2006). Overall, the incidence of primary adrenal carcinoma is 1 to 2 per million population, with a peak occurring in childhood and a second higher peak in the 4th and 5th decade. There are some geographical variations; in Brazilian children, the incidence in 10 times higher than elsewhere and is related to a *P53* tumor suppressor gene mutation (Ribeiro *et al*., 2001). The incidence is around 50% higher in women than men (Allolio and Fassnacht, 2006).

The vast majority are functional (60% to 80%), secreting either glucocorticoids alone or with sex steroid precursors (Allolio and Fassnacht, 2006). The

clinical picture tends to be one of rapidly progressive Cushing syndrome and virilization accompanied by the more general effects of an aggressive tumor.

A careful endocrine assessment is needed prior to surgical treatment. Preoperative imaging by fine-cut CT or MRI is needed to characterize and stage the lesion. Biopsy of the lesion should only be attempted when a surgical diagnosis is impossible.

The primary treatment is usually surgery, but this is rarely curative because most tumors have metastasized at the time of presentation. Radiotherapy may have modest benefits but is often ineffective.

Mitotane (*o*,*p*′-DDD) is the only specific medical treatment for adrenal carcinoma. It exerts a specific cytotoxic effect on adrenocortical cells causing focal degeneration in the zona fasciculata and zona reticulosa. Mitotane has a narrow therapeutic window and adverse effects often limit its use. Complete or partial response occurs in 13% to 50% of patients leading to palliation of symptoms of hormone excess and reduced tumor growth.

Glucocorticoid replacement is needed because mitotane induces adrenal insufficiency through its adrenolytic action. Experience with cytotoxic chemotherapy remains limited but several combinations, including cisplatin, have been tried.

The prognosis of adrenal carcinoma is poor but depends on tumor stage. Recurrence rates are of up to 85%. The overall 5 year survival is between 16% and 38%, varying from 60% for stage 1 disease to no survivors in stage 4 disease (Allolio and Fassnacht, 2006).

Incidental Adrenal Tumors

An increasing problem is the management of asymptomatic adrenal tumors identified on imaging for other reasons. It is estimated that between 1% to 4% of all abdominal imaging studies will reveal an unexpected adrenal tumor (Shen *et al*., 2005). After 40 years of age, these "incidentalomas" are common, potentially affecting 1 in 20 individuals.

90% of the incidentalomas are less than 2 cm and the vast majority are benign nonfunctioning cortical adenomas Table 6) (Shen *et al*., 2005; Sturgeon and Kebebew, 2004).

Table 6 Pathology of Adrenal Incidentalomas

Benign nonfunctioning adenoma	Approximately 80%
Adrenal carcinoma	5%
Phaeochromocytoma	5–10%
Cortisol secreting tumor	5%
Adrenal metastasis	2.5%
Aldosteronoma	1%
Cysts	<1%
Myelolipoma	<1%

When incidentalomas are found, it is important to exclude inappropriate secretion of glucocorticoids, mineralocorticoids, sex steroid precursors, and catecholamines by endocrine investigation.

The overall risk of malignancy is approximately 5% and this increases with the size of the tumor (Sturgeon and Kebebew, 2004). The risk is less than 2% for tumors less than 4 cm and 6% for tumors between 4 and 6 cm, while 25% of tumors larger than 6cm are malignant (Shen *et al*., 2005). The 2002 National Institutes of Health consensus statement recommended adrenalectomy if the tumor is larger than 6 cm, but other guidelines have recommended lower thresholds between 3 and 6 cm (NIH., 2004; Sturgeon and Kebebew, 2004). Other features that are indicative of malignancy include the radiographic appearance, lymphadenopathy, and the presence of metastases. Smaller nonsuspicious tumors can be managed conservatively with monitoring every 6 to 12 months.

Congenital Adrenal Hyperplasia

Congenital adrenal hyperplasia (CAH) comprises a group of inherited autosomal recessive disorders that are caused by mutations in the genes that encode enzymes involved in adrenocortical steroidogenesis (Fig. 2) (Krone *et al*., 2007).

Etiology of CAH

Inactivating mutations in CYP21 (encoding 21-hydroxylase) account for 90% of cases of CAH. The incidence of the classical form of CYP21 deficiency is 1 in 7,000 to 15,000 live births, making it one of the most common inherited metabolic disorders (Speiser *et al*., 1985). The nonclassical form is much more prevalent affecting 1 per 500 to 1,000 individuals in various Caucasian populations (Speiser *et al*., 1985).

Clinical Features of CAH

The clinical consequences of CAH are significant, and include adrenal insufficiency, ambiguous genitalia in females, short stature, hirsutism, and infertility. Precocious puberty may occur in boys. Glucocorticoid replacement to control these features is complex and necessarily unphysiological, leading to the signs and symptoms of Cushing syndrome.

CYP21 deficiency may present in either the "simple virilizing" form or "salt wasting" form, characterized by hypotension and hyperkalemia in the neonatal period. CYP21 catalyzes a key step in glucocorticoid production and therefore mutations in its encoding gene are invariably associated with glucocorticoid deficiency (White and Speiser, 2000). This leads to reduced negative feedback at the anterior pituitary and increased ACTH secretion. The high ACTH stimulates the remaining intact adrenocortical steroidogenic pathways. In the simple virilizing form, in female neonates, this results in high levels of potent androgens, which virilize the external genitalia and, later in life, cause isosexual precocious puberty.

The excessive sex steroids also cause accelerated early growth followed by growth arrest and short stature. These features are also present in the salt-wasting form but, in addition, there is severe renal sodium loss as a consequence of concomitant aldosterone deficiency, presenting as a life-threatening adrenal crisis between 7 and 21 days of life.

In adolescence and adulthood, women may present with anovulatory irregular menstrual cycles while men may develop azo- or oligospermia. Sexual function may be affected in women, not least because of the long lasting psychosocial and physical effects of genital ambiguity and its surgical treatment.

In the nonclassical form, individuals may present in childhood with isosexual precocious puberty or in early adulthood with hirsutism, oligomenorrhea, and anovulation in a pattern that resembles polycystic ovarian syndrome (White and Speiser, 2000).

Diagnosis

The position of CYP21 in the biosynthetic pathway means that 17α-hydroxyprogesterone (17OHP), the enzyme's substrate, is markedly increased (White and Speiser, 2000). As newborn screening has been shown to reduce the morbidity and mortality associated with CAH (Therrell *et al*., 1998), measurement of 17OHP in the early neonatal period has been advocated to identify affected children with CYP21 mutations, and prevent incorrect gender assignment and salt-wasting crises. If levels of basal 17OHP are inadequately raised to make the diagnosis, it can be confirmed by ACTH stimulation testing (New *et al*., 1983). Basal ACTH concentrations are elevated because of diminished negative feedback by cortisol, and the administration of further synthetic ACTH causes a rise in 17-hydroxyprogesterone but a blunted response in cortisol.

The diversity in the clinical presentation has led to studies examining the relationship between genotype and phenotype. Different mutations have been identified and, broadly, less severe phenotypes are associated with greater residual CYP21 activity.

Treatment

Glucocorticoid is replaced orally to restore the negative feedback on ACTH secretion and, thus, minimize androgen overproduction (White and Speiser, 2000). Glucocortioids with a longer half-life than hydrocortisone may need to be given in the evening or at bedtime to suppress ACTH secretion in the early hours of the morning (Shimon *et al*., 1995). These steroids are more potent than hydrocortisone and frequently lead to signs of excessive replacement including features of Cushing syndrome and growth restriction in children (Jaaskelainen and Voutilainen, 1997). Balancing this treatment can be complex and in some situations, bilateral adrenalectomy to remove the source of sex steroid precursors is needed (Van Wyk *et al*., 1996). In this scenario, life-long hydrocortisone replacement (as for Addison syndrome) is needed. Where renin levels or activity are raised in CAH, mineralocorticoid is also given.

THE NORMAL ADRENAL MEDULLA

The adrenal medulla functions alongside the sympathetic nervous system and together these can be regarded as forming a single sympatho-adrenomedullary system.

Anatomy

The adrenal medulla, which is surrounded by the cortex, comprises approximately 10% of the total weight of the adrenal gland. It is formed of chromaffin cells that are the equivalent of postganglionic neurones. Much of the chromaffin cell is made up of secretory granules, which contain the stored catecholamines, adrenaline and noradrenaline, and proteins called chromogranins.

Embryology

In contrast to the outer cortex, the adrenal medulla is derived from the neuroectoderm (neural crest) cells that migrate in a forward direction from the vertebral column to the periaortic region. These cells give rise to the sympathetic chain of ganglia that innervate much of the gut and blood vessels. Some specialize by invading the adrenal cortex to form the chromaffin cells of the adrenal medulla and innervated by preganglionic sympathetic neurones that emanate from T7-L3.

The clinical importance of this is that adrenomedullary disorders can occasionally occur in unexpected places because of embryological "rests" of cells.

Catecholamine Biosynthesis

Catecholamines have a 3,4-dihydroxyphenyl core which is derived from a tyrosine precursor. The biosynthesis of catecholamines occurs in three or four steps (Fig. 6). The hydroxylation of tyrosine is rate-limiting and subject to negative feedback by the downstream hormone products, noradrenaline and dopamine. The last step, converting noradrenaline to adrenaline, reflects the unusual embryological development of the adrenal medulla in mammals. Expression of phenylethanolamine *N*-methyltransferase (PNMT), the enzyme required for adrenaline production, depends upon high concentrations of glucocorticoid that are only present in the adrenal medulla because of the centripetal drainage of blood from the outer adrenal cortex.

Release of Catecholamines

After biosynthesis, catecholamines are stored within the cell in vesicles. Following nervous nicotinic stimulation, the chromaffin cell surface is depolarized. This increases the sodium permeability and stimulates an influx of calcium ions and release of the catecholamine.

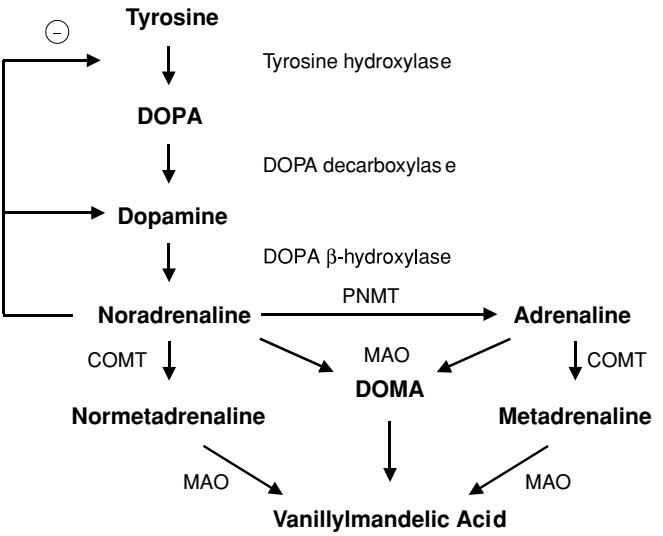

Figure 6 Synthesis and metabolism of catecholamines. *Abbreviations*: PNMT, phenylethanolamine *N*-methyltransferase; MAO, mono-amine oxidase; COMT, catechol-*O*-methyltransferase; DOMA, 3,4, dihydroxmandelic acid; DOPA, 3,4, dihydroxphenylalanine.

Adrenaline and noradrenaline are major stress hormones responsible for the body's "fright, fight, and flight" responses following stressful stimulation. There are multiple physiological stimuli that influence catecholamine secretion (Table 7).

Table 7 Physiological Stimuli Affecting Catecholamine Release

Emotion	Anxiety
	Pain
	Anger
	Venepuncture
Standing	Two- to threefold rise within 2–5 min of standing
Circadian variation	Most peaks occur between 0600 and1800 hrs.
	Lowest during sleep
Age	Increase with age
Gender	Variation during menstrual cycle
Exercise	Rapid increase with exercise
Obesity	Increase with obesity
Salt intake	Increase with low salt diet
Cardiac status	Increase with cardiac disease
Thyroid status	Increase with hypothyroidism

Table 8 The Effects of Catecholamines

Adrenaline	Noradrenaline
Increases systolic blood pressure and heart rate	Increases systolic and diastolic blood pressure rise leading to increased mean arterial pressure
Diverts blood supply to limb muscle beds and away from the gut	Decreases heart rate
Decreases gut motility	
Bronchodilation and reduces mucus secretion	
Piloerection	
Mydriasis (pupil dilation)	

Secreted catecholamines are rapidly cleared from the circulation within 1 to 2 minutes, by metabolism to inactive forms and uptake (Fig. 6). The rapid release and clearance provides a quick means of responding to a stressful stimulus.

Actions of Catecholamines

The subtle differences in the actions of adrenaline and noradrenaline reflect their relative affinities for the different adrenoreceptors, predominantly α and β subtypes 1 and 2 (Table 8). Noradrenaline stimulates α and $\beta 1$ receptors and so does not cause bronchodilation, which is a $\beta 2$ response. The distribution of α and $\beta 2$ receptors is such that catecholamines lead to vasodilatation in skeletal muscle beds while causing vasoconstriction in the gut.

The combined effects of both catecholamines raise blood pressure, divert nutrients away from nonessential organs, and promote delivery to the muscles that are active in the fight or flight responses to danger. The hormones also play important metabolic roles; both hormones act as insulin antagonists and raise fatty acids and blood glucose by stimulating glycogenolysis in liver and muscle, and hepatic gluconeogenesis.

CLINICAL DISORDERS OF THE ADRENAL MEDULLA

Catecholamines from the adrenal medulla are not essential for health and deficiency of adrenal catecholamines produces no clinical condition. In contrast to the need for glucocorticoid replacement following bilateral adrenalectomy, catecholamine replacement is not required. All clinical disorders of the adrenal medulla relate to excess catecholamine production.

Phaeochromocytoma

Phaeochromocytoma, a tumor of the chromaffin cells that secretes excess catecholamines, is the most important clinical disease of the adrenal medulla (Bravo and Tagle, 2003).

Etiology

These rare tumors can occur sporadically or as part of familial syndromes, such as multiple endocrine neoplasia. It is often said that these are the "10% tumor" because 10% are bilateral, 10% are malignant, and 10% are extra-adrenal. Although only a rule of thumb, this serves as a useful reminder that the vast majority of phaeochromocytomas are benign but may occur outside the adrenal along the sympathetic chain. Previously it was also thought that 10% were inherited. With greater molecular genetic investigation and greater knowledge of causative mutations, however, it appears that this is an underestimation with the true value being more than 20% (Mannelli *et al*., 2007).

Familial Syndromes Including Phaeochromocytoma

Phaeochromocytomas arise from genetic abnormalities of the relevant gene in the affected cell-type. In approximately 25% of phaeochromocytomas, a mutation is inherited in one copy of a genome via the germline, such that every cell in the body is affected and vulnerable to a second "hit" that results in tumor formation.

Although phaeochromocytomas may arise in isolation, they may be part of a syndrome involving multiple endocrine neoplastic abnormalities.

Multiple Endocrine Neoplasia (MEN) syndromes are rare genetic conditions but may occur in a familial inherited fashion or as a result of new mutations, in which case they present sporadically (de Groot *et al*., 2006; Doherty, 2005). Two MEN syndromes have been described. MEN type 1 includes tumors of the parathyroid, pancreatic tumors and pituitary glands. Phaeochromocytomas are classically a part of MEN type 2 (MEN-2) alongside medullary carcinoma of the thyroid (MTC) and parathyroid adenomas (MEN-2a) or in combination with neurofibromata and Marfanoid habitus (MEN-2b). MEN-2 is inherited as an autosomal dominant trait with a high degree of penetrance and is caused by mutations in the *RET* proto-oncogene, which encodes a cell surface receptor with tyrosine kinase activity (de Groot *et al*., 2006). Loss of *RET* function leads to potent growth stimulation, hyperplasia, and tumor formation. The MEN syndromes are rare, but bilateral or extramedullary tumors, or phaeochromocytomas at a young age should raise suspicion and instigate a thorough family history, additional examination, and biochemical testing. Phaeochromocytoma may also be a part of Von Hippel Lindau syndrome and Von Recklinghausen neurofibromatosis syndrome (Mannelli *et al*., 2007).

Clinical Features

The classical triad of symptoms in phaeochromocytoma are hypertension, throbbing bilateral headache, and palpitation. These and other distinctive symptoms of phaeochromocytoma relate to the excessive, unregulated, and episodic catecholamine release. Constant or episodic hypertension is the most common finding, occurring in 90% to 100% of cases. The symptoms are often episodic and the frequency can vary from daily to monthly. Less commonly, tremor, angina, or nausea can be apparent.

Diagnosis

The episodic nature of the excess catecholamine secretion can make the diagnosis challenging and in cases where there is a high index of suspicion, investigations should be repeated over time.

A widely available screening test to detect excess catecholamines has been the measurement of metabolites in urine over 24 hours which provides an integrated, representative assessment of catecholamine secretion (Pacak *et al*., 2007). This can be done randomly or, in cases with infrequent symptoms, immediately after an attack. Most laboratories will measure the metabolites, metadrenaline and normetadrenaline, together with the parent hormones, adrenaline and noradrenaline, and possibly their precursor, dopamine (Fig. 6). This combined approach is important. Although very large or extraadrenal phaeochromocytomas escape the normal influence of cortisol on PNMT and secrete an increased proportion of noradrenaline, it is rare for patients to present with oversecretion of one hormone in the absence of its metabolites. Most phaeochromocytomas secrete a mixed profile of catecholamines. Of note, vanillylmandelic acid is inadequate as a screening metabolite in the urine as its estimation misses approximately one-third of the cases (Mannelli *et al*., 2007). Increasingly, serum measurements of metadrenaline and normetadrenaline are being recognized as the optimal tool which excludes phaeochromocytoma because of their low false-negative rate (Mannelli *et al*., 2007; Pacak *et al*., 2007). They are especially useful in high-risk populations such as those with familial neoplasia syndromes.

Once a biochemical diagnosis has been made, imaging, ideally by MRI, aids localization for surgical intervention. In specialized centers, nuclear medicine uptake scans with mIBG may be possible.

Treatment

Managing phaeochromocytoma focuses on two aspects: Blocking the effects of catecholamine excess using α and β adrenoreceptor blockers, and then surgical removal of the offending tumor or adrenal gland. It is important to provide α blockade first to reduce the risk of a hypertensive crisis from unopposed α adrenoreceptor stimulation. Adequate α and β adrenoreceptor blockade is

essential prior to surgery, as manipulation of the tumor intraoperatively can result in catastrophic release of stored catecholamines.

BIBLIOGRAPHY

The following major international endocrinology textbooks make an excellent source of additional reading: Kronenberg H, Melmed S, Polonsky KS, Larsen R. Williams Textbook of Endocrinology. 11th ed. Saunders 2007; Grossman A. Clinical Endocrinology. 2nd ed. Blackwell Science Ltd. 1997.

REFERENCES

Addison T. On the constitutional and local effects of disease of the suprarenal capsules. In: A Collection of the Published Writing of the Late Thomas Addison, M.D., Physician to Guy's Hospital. London: New Sydenham Society; 1868. [Reprinted in Medical Classics 2:244–293].

Allolio B, Fassnacht M. Clinical review: Adrenocortical carcinoma: Clinical update. J Clin Endocrinol Metab 2006; 91:2027–2037.

Atkinson AB, Kennedy AL, Carson DJ, et al. Five cases of cyclical Cushing's syndrome. BMJ 1985; 291:1453–1457.

Atkinson AB, Kennedy A, Wiggam MI, et al. Long-term remission rates after pituitary surgery for Cushing's disease: The need for long-term surveillance. Clin Endocrinol 2005; 63:549–559.

Barbetta L, Dall'Asta C, Re T, et al. Androgen secretion in ectopic ACTH syndrome and in Cushing's disease: Modifications before and after surgery. Horm Metab Res 2001; 33:596–601.

Betterle C, Dal Pra C, Mantero F, et al. Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: Autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. Endocr Rev 2002; 23:327–364.

Bravo EL, Tagle R. Pheochromocytoma: state-of-the-art and future prospects. Endocr Rev 2003; 24:539–553.

Butler PW, Besser GM. Pituitary-adrenal function in severe depressive illness. Lancet 1968; 1:1234–1236.

Calhoun DA, Nishizaka MK, Zaman MA, et al. Hyperaldosteronism among black and white subjects with resistant hypertension. Hypertension 2002; 40:892–896.

Chu JW, Matthias DF, Belanoff J, et al. Successful long-term treatment of refractory Cushing's disease with high-dose mifepristone (RU 486). J Clin Endocrinol Metab 2001; 86:3568–3573.

Conn JW. Presidential address: Part I painting background; Part II primary aldosteronism, a new clinical syndrome. J Lab Clini Med 1995; 45:3–17.

Corrigan DF, Schaaf M, Whaley RA, et al. Selective venous sampling to differentiate ectopic ACTH secretion from pituitary Cushing's syndrome. N Engl J Med 1977; 296:861–862.

Cushing HW. The basophil adenomas of the pituitary body and their clinical manifestations (pituitary basophilism). Bull John Hopkins Hosp 1932; 50:137–195.

Cushing HW. The pituitary body and its disorders. Philadelphia: JB Lippincott. 1912.

de Groot JW, Links TP, Plukker JT, et al. RET as a diagnostic and therapeutic target in sporadic and hereditary endocrine tumors. Endocr Rev 2006; 27:535–560.

Doherty GM. Multiple endocrine neoplasia type 1. J Surg Oncol 2005; 89:143–150.
Findling JW, Raff H. Cushing's Syndrome: Important issues in diagnosis and management. J Clin Endocrinol Metab 2006; 91:3746–3753.
Groote VR, Meinders AE. On the mechanism of alcohol-induced pseudo-Cushing's syndrome. Endocr Rev 1996; 17:262–268.
Grossman A, Howlett TA, Kopelman PG. The use of CRF-41 in the differential diagnosis of Cushing's syndrome and obesity. Horm Metab Res Suppl 1987; 16:62–64.
Hammer F, Stewart PM. Cortisol metabolism in hypertension. Best Pract Res Clin Endocrinol Metab 2006; 20:337–353
Howlett TA, Price J, Hale AC, et al. Pituitary ACTH dependent Cushing's syndrome due to ectopic production of a bombesin-like peptide by a medullary carcinoma of the thyroid. Clin Endocrinol 1985; 22:91–101.
Howlett TA, Drury PL, Perry L, et al. Diagnosis and management of ACTH-dependent Cushing's syndrome: Comparison of the features in ectopic and pituitary ACTH production. Clin Endocrinol 1986; 24:699–713.
Jaaskelainen J, Voutilainen R. Growth of patients with 21-hydroxylase deficiency: an analysis of the factors influencing adult height. Pediatr Res 1997; 4130–33.
Jeunemaitre X, Chatellier G, Kreft-Jais C, et al. Efficacy and tolerance of spironolactone in essential hypertension. Am J Cardiol 1987; 60:820–825.
Jubiz W, Meikle AW, Levinson RA, et al. Effect of diphenylhydantoin on the metabolism of dexamethasone. N Engl J Med 1970; 283;11–14.
Kennedy L, Atkinson AB, Johnston H, et al. Serum cortisol concentrations during low dose dexamethasone suppression test to screen for Cushing's syndrome. Br Med J (Clin Res Ed) 1984; 289:1188–1191.
Koelz A, Girard J. Cushing's syndrome or obesity. Bilateral adrenal hyperplasia in a boy 10 years of age. Eur J Pediatr 1976; 121237–246.
Krone N, Dhir V, Ivison HE, et al. Congenital adrenal hyperplasia and P450 oxidoreductase deficiency. Clin Endocrinol 2007; 66:162–172.
Leibowitz G, Tsur A, Chayen SD, et al. Pre-clinical Cushing's syndrome: An unexpected frequent cause of poor glycaemic control in obese diabetic patients. Clin Endocrinol 1996; 44:717–722.
Liddle GW. Tests of pituitary-adrenal suppressibility in the diagnosis of Cushing's syndrome. J Clin Endocrinol Metab 1960; 20:1539–1560.
Mannelli M, Simi L, Gagliano MS, et al. Genetics and biology of pheochromocytoma. Exp Clin Endocrinol Diabetes 2007; 115:160–165.
Mattsson C, Young WF, Jr. Primary aldosteronism: Diagnostic and treatment strategies. Nat Clin Pract Nephrol 2006; 2:198–208.
Meador CK, Liddle GW, Island DP, et al. Cause of Cushing's syndrome in patients with tumors arising from "nonendocrine" tissue. J Clin Endocrinol Metab 1962; 22:693–703.
Moser HW. Adrenoleukodystrophy: Phenotype, genetics, pathogenesis and therapy. Brain 1997; 120(Pt 8):1485–1508.
Mosso L, Carvajal C, Gonzalez A, et al. Primary aldosteronism and hypertensive disease. Hypertension 2003; 42:161–165.
Mulatero P, Morello F, Veglio F. Genetics of primary aldosteronism. J Hypertens 2004; 22:663–670.
New MI, Lorenzen F, Lerner AJ, et al. Genotyping steroid 21-hydroxylase deficiency: Hormonal reference data. J Clin Endocrinol Metab 1983; 57:320–326.

Newell-Price J, Trainer P, Perry L, et al. A single sleeping midnight cortisol has 100% sensitivity for the diagnosis of Cushing's syndrome. Clin Endocrinol 1995; 43:545–550.

Newell-Price J, Trainer P, Besser M, et al. The diagnosis and differential diagnosis of Cushing's syndrome and pseudo-Cushing's states. EndocrRev 1998; 19:647–672.

NIH. State of the Science statement on management of the clinical inapparent adrenal mass ("incidentaloma"). NIH Consensus State Sci Statements 2004; 19:1–23.

Nugent CA, Nichols T, Tyler FH. Diagnosis of Cushing's Syndrome; single dose dexamethasone suppression test. Arch Intern Med 1965; 116:172–176.

Oelkers W. Adrenal insufficiency. N Engl J Med 1996; 335:1206–1212.

Oldfield EH, Chrousos GP, Schulte HM, et al. Preoperative lateralization of ACTH-secreting pituitary microadenomas by bilateral and simultaneous inferior petrosal venous sinus sampling. N Engl J Med 1985; 312:100–103.

Pacak K, Eisenhofer G, Ahlman H, et al. Pheochromocytoma: Recommendations for clinical practice from the First International Symposium. October 2005. Nat Clin Pract Endocrinol Metab 2007; 3:92–102.

Pereira AM, van Aken MO, van DH,et al. Long-term predictive value of postsurgical cortisol concentrations for cure and risk of recurrence in Cushing's disease. J Clin Endocrinol Metab 2003; 88:5858–5864.

Putignano P, Kaltsas GA, Satta MA, et al. The effects of anti-convulsant drugs on adrenal function. Horm Metab Res 1998; 30:389–397.

Raff H, Findling JW. A new immunoradiometric assay for corticotropin evaluated in normal subjects and patients with Cushing's syndrome. Clin Chem 1989; 35:596–600.

Raff H, Raff JL, Findling JW. Late-night salivary cortisol as a screening test for Cushing's syndrome. J Clin Endocrinol Metab 1998; 83:2681–2686.

Ramirez G, Gomez-Sanchez C, Meikle WA, et al. Evaluation of the hypothalamic hypophyseal adrenal axis in patients receiving long-term hemodialysis. Arch Intern Med 1982; 142:1448–1452.

Ribeiro RC, Sandrini F, Figueiredo B, et al. An inherited p53 mutation that contributes in a tissue-specific manner to pediatric adrenal cortical carcinoma. Proc Natl Acad Sci U S A 2001; 98:9330–9335.

Ross EJ, Linch DC. Cushing's syndrome–killing disease: discriminatory value of signs and symptoms aiding early diagnosis. Lancet 1982; 2:646–649.

Ross EJ, Marshall-Jones, P, Friedman M. Cushing's syndrome: diagnostic criteria. Q J Med 1966; 35:149–192.

Sawka AM, Young WF, Thompson GB, et al. Primary aldosteronism: Factors associated with normalization of blood pressure after surgery. Ann Intern Med 2001; 135:258–261.

Shen WT, Sturgeon C, Duh QY. From incidentaloma to adrenocortical carcinoma: the surgical management of adrenal tumors. J Surg Oncol 2005; 89:186–192.

Shimon I, Kaiserman I, Sack J. Home monitoring of 17 alpha-hydroxyprogesterone levels by filter paper blood spots in patients with 21-hydroxylase deficiency. Horm Res 1995; 44:247–252.

Sica DA. Pharmacokinetics and pharmacodynamics of mineralocorticoid blocking agents and their effects on potassium homeostasis. Heart Fail Rev 2005; 10:23–29.

So A, Duffy DL, Gordon RD, et al. Familial hyperaldosteronism type II is linked to the chromosome 7p22 region but also shows predicted heterogeneity. J Hypertens 2005; 23:1477–1484.

Sonino N, Boscaro M, Fallo F. Pharmacologic management of Cushing syndrome: new targets for therapy. Treat Endocrinol 2005; 4:87–94.
Speiser PW, Dupont B, Rubinstein P, et al. High frequency of nonclassical steroid 21-hydroxylase deficiency. Am J Hum Genet 1985; 37:650–667.
Stokes PE. The potential role of excessive cortisol induced by HPA hyperfunction in the pathogenesis of depression. Eur Neuropsychopharmacol 1995; 5(Suppl):77–82.
Sturgeon C, Kebebew E. Laparoscopic adrenalectomy for malignancy. Surg Clin North Am 2004; 84:755–774.
Ten S, New M, Maclaren N. Clinical review 130: Addison's disease 2001. J Clin Endocrinol Metab 2001; 86:2909–2922.
Therrell BL, Jr, Berenbaum SA, Manter-Kapanke V, et al. Results of screening 1.9 million Texas newborns for 21-hydroxylase-deficient congenital adrenal hyperplasia. Pediatrics 1998; 101:583–590.
Tiller JW, Maguire KP, Schweitzer I, et al. The dexamethasone suppression test: a study in a normal population. Psychoneuroendocrinology 1988; 13:377–384.
Trainer PJ, Grossman A. The diagnosis and differential diagnosis of Cushing's syndrome. Clin Endocrinol 1991; 34:317–330.
Trainer PJ, Lawrie HS, Verhelst J, et al. Transsphenoidal resection in Cushing's disease: undetectable serum cortisol as the definition of successful treatment. Clin Endocrinol 1993; 38:73–78.
Tyrrell JB, Findling JW, Aron DC, et al. An overnight high-dose dexamethasone suppression test for rapid differential diagnosis of Cushing's syndrome. Ann Intern Med 1986; 104:180–186.
Vaidya B, Pearce S, Kendall-Taylor P. Recent advances in the molecular genetics of congenital and acquired primary adrenocortical failure. Clin Endocrinol 2000; 53403–418.
van Rossum EF, Lamberts SW. Glucocorticoid resistance syndrome: A diagnostic and therapeutic approach. Best Pract Res Clin Endocrinol Metab 2006; 20611–626.
Van Wyk JJ, Gunther DF, Ritzen EM, et al. The use of adrenalectomy as a treatment for congenital adrenal hyperplasia. J Clin Endocrinol Metab 1996; 81:3180–3190.
Vance ML. Pituitary radiotherapy. Endocrinol Metab Clin North Am 2005; 34:479–487, xi.
White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev 2000; 21:245–291.
Young WF, Jr, Thompson GB. Role for laparoscopic adrenalectomy in patients with Cushing's syndrome. Arq Bras Endocrinol Metabol 2007; 51:1349–1354.

3

The Hypothalamo-Pituitary-Adrenocortical Axis: Endocrinology, Pharmacology, Pathophysiology, and Developmental Effects

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INTRODUCTION

Glucocorticoids exert wide ranging effects in the body which, collectively, are essential for normal growth and development and for the maintenance of homeostasis. Chronic disturbances in the circulating levels of glucocorticoids, induced pathologically (e.g., Addison's disease and Cushing's syndrome), surgically (adrenal resection) or pharmacologically (e.g., administration of anti-inflammatory steroids), are well known to induce a plethora of unwanted effects. However, growing body of data suggests that more subtle, long-term changes in the secretion and/or activity of glucocorticoids may also have serious deleterious effects. In particular, they may predispose the organism to a variety of diseases which are endemic in the western world and also emerging in developing countries, including type 2 diabetes, hypertension, and depression. This review will consider (a) the physiological and pharmacological actions of the glucocorticoids, (b) the mechanisms that control the secretion of these steroid hormones and enable them to produce tissue-specific actions and (c) the potential role of the glucocortiocoids in the etiology of disease.

Glucocorticoids are unusual hormones. They influence the activity of almost every cell in the body; they modulate the expression of approximately 10% of the

genome; they are essential for life but are also increasingly implicated in the pathogenesis of disease; they produce many unwanted effects when given therapeutically in supraphysiological doses; yet, over 50 years after their introduction to the clinic as anti-inflammatory drugs, glucocorticoids still have an important place in the treatment of disease. Understanding the biology of these hormones and their synthetic analogues remains a major challenge for biologists in the 21st century.

FUNCTIONS OF GLUCOCORTICOIDS

Glucocorticoids, cortisol in man and its rodent counterpart corticosterone, exert widespread actions in the body which are essential for the maintenance of homeostasis and for the organism to prepare for, respond to, and cope with physical and emotional stress (Sapolsky *et al.*, 2000). They are important regulators of immune and inflammatory processes and are required for numerous processes associated with host defence. These properties confer many of the stress protective actions of the steroids, as they quench the pathophysiological responses to tissue injury and inflammation and, thereby, prevent them proceeding to a point where they threaten the survival of the host (Munck and Naray-Fejes-Toth, 1992). In addition, they form the basis of their use as anti-inflammatory and immunosuppressive drugs. Glucocorticoids also exert a spectrum of other important physiological actions. They promote the breakdown of carbohydrate and protein and exert complex effects on lipid deposition and breakdown. They raise blood pressure, in part through their ability to alter the sensitivity of tissues to catecholamines, and exert "aldosterone-like" actions which modulate electrolyte and water balance. They have complex effects on bone, modulate cellular differentiation, exert both positive and negative effects on cell growth, and are proapoptotic. Within the central nervous system, glucocorticoids target both neurones and glial cells. During development these actions underpin important organizational events in the brain, while in adulthood, they contribute to neuronal plasticity and are implicated in the processes of neurodegeneration. Other central effects include complex changes in mood and behavior and modulation of food intake, body temperature, pain perception, and neuroendocrine function (Pearson Murphy, 2002).

In conditions in which sustained, pronounced elevations in circulating glucocorticoids occur, due either to hypersecretion of endogenous steroids (Cushing's syndrome/disease) or prolonged administration of exogenous steroids, the above effects become exaggerated and a plethora of unwanted pathologies emerge. These include a significant redistribution of fat and a positive effect on adipocyte differentiation, giving rise to centripetal obesity together with a characteristic moon face and buffalo hump; protein wasting and associated muscle weakness; hyperglycemia and insulin-resistant diabetes mellitus (steroid diabetes); hypertension, raised cholesterol, altered serum lipids, and salt and water retention; immunodeficiency, poor wound healing, and loss of connective tissue leading to easy bruising; impaired growth and development; osteoporosis; menstrual irregularities, infertility, and other endocrine-related changes; depression and, sometimes, impaired

cognitive function. Conversely, insufficient glucocorticoid secretion, which may arise from Addison's disease (an autoimmune disorder causing degeneration of the adrenal cortex), the adrenogenital syndrome (an inherited disorder of glucocorticoid synthesis) or pituitary disease, is characterized by a vulnerability to stress, white blood cell excess, lymphoid tissue hypertrophy, hypotension, mood disturbances, weakness/lethargy, weight loss, and hypoglycemia. Adrenal insufficiency is often insidious in onset and may go undetected until stress or illness precipitates a crisis. By contrast, acute adrenal insufficiency usually manifests as shock in previously undiagnosed patients (Buckingham, 2006).

For many years glucocorticoid-associated pathologies were associated solely with substantive long-term changes in the glucocorticoid *milieu*. More recent findings, however, indicate that quite subtle but sustained changes in glucocorticoid secretion and/or activity are potentially hazardous and that they may contribute to the pathogenesis of a number of common diseases including the following: hypertension and other cardiovascular disease; insulin resistance, obesity, and type 2 diabetes; depression; autoimmune-inflammatory disease; and reproductive dysfunction (De Kloet *et al*., 1998; Gold *et al*., 2002; Seckl, 2004).

MECHANISMS OF GLUCOCORTICOID ACTION

Receptors

Glucocorticoids exert their actions, principally, via intracellular receptors that belong to the nuclear receptor superfamily and regulate the transcription of target genes. The biological actions of the steroids are thus generally slow in onset and persist for some time after the steroid has been cleared from the circulation. Two main types of glucocorticoid receptors have been identified, the mineralocorticoid receptor (MR, sometimes called the type 1 glucocorticoid receptor) and the glucocorticoid receptor (GR, also known as the type 2 glucocorticoid receptor) (De Kloet *et al*., 1998).

The MR has a high and approximately equal affinity for cortisol, corticosterone, and the mineralocorticoid, aldosterone (K_d approximately 0.5 to 2 nM). It shows a discrete distribution, being localized mainly to the distal renal tubule and other cells/tissues concerned with Na^+/K^+ balance (e.g., sweat glands, parotid glands, and colon), but is also found in specific brain regions, notably in neurones within the limbic system, entorhinal cortex and, to a lesser extent, the hypothalamus. The GR, by contrast, is of lower affinity (K_d cortisol/corticosterone approximately 10 to 20 nM), is glucocorticoid-selective, and does not bind aldosterone readily. GRs are widely distributed in the body; cell responsiveness depends not only on the presence of GR, but also receptor concentration which is known to fluctuate, for example, during development, during the cell cycle, and following disturbances in endocrine status (De Kloet *et al*., 1998). Measures of GR expression are thus sometimes taken as a measure of tissue sensitivity to the steroids.

Approximately 95% of cortisol/corticosterone in the plasma is bound to plasma protein; the unbound portion is free to diffuse from the circulation to bathe the tissues. The plasma concentration of unbound steroid at the nadir of the circadian rhythm is in the region of 0.5- to 1 nM, well below the levels found at the peak of the circadian rhythm, in stress or following administration of exogenous steroids. From this, it was reasoned that the MR (K_d 0.5–2.0 nM) is responsible for mediating the effects of very low concentrations of glucocorticoids. However, when glucocorticoids levels are raised, for example, in stress, when the free cortisol/corticosterone level may exceed 300 nM, the MRs are saturated and the GRs effect the observed biological actions. From these findings, it was easy to understand why hydrocortisone (cortisol) shows mineralocorticoid as well as glucocorticoid activity when used clinically in high doses, and how small modifications of the cortisol molecule alter markedly the ratio of mineralocorticoid to glucocorticoid activity. It was not, however, obvious how MRs in tissues such as the kidney are preferentially regulated in vivo by aldosterone, the principal endogenous mineralocorticoid, despite their high affinity for cortisol/corticosterone. This conundrum was resolved by studies which revealed tissue-specific mechanisms for the delivery of glucocorticoids to their intracellular receptors (De Kloet *et al*., 1998).

Delivery of Endogenous Glucocorticoids to Their Receptors

Access to Target Cells

While measurements of cortisol/corticosterone in the circulation provide a reasonable index of the activity of the HPA axis, they provide a surprisingly poor marker of delivery of the steroids to receptors in their target cells. As indicated above, approximately 95% of cortisol/corticosterone in the circulation is bound to a carrier protein (corticosteroid binding globulin, CBG) and, in principle, only the free steroid has ready access to target cells. However, in some cases (e.g., in inflamed tissues), local serine proteases facilitate delivery by liberating free steroid from its binding globulin, whereas in others (e.g., pituitary gland), locally expressed CBG may limit access by absorbing free steroid. The ability of glucocorticoids in the systemic circulation to reach target cells is also compromised by transporter proteins which belong to the ATP-binding cassette family and serve as a barrier by actively extruding steroids from cells. These proteins, which are also called multidrug resistant P-glycoproteins (MDRs), are expressed in a tissue-specific manner and, like CBG, show substrate specificity. They thus provide a mechanism for tissue- and steroid-specific delivery of glucocorticoids to target cells, a phenomenon which may contribute to the subtle differences in pharmacological profile of various corticosteroids. In addition, dysregulation of these proteins may have a role in the development of glucocorticoid resistance. Particular interest has focused on the expression of MDRs in blood barrier, as these appear to limit the access of steroids such as dexamethasone and, to a lesser extent, cortisol

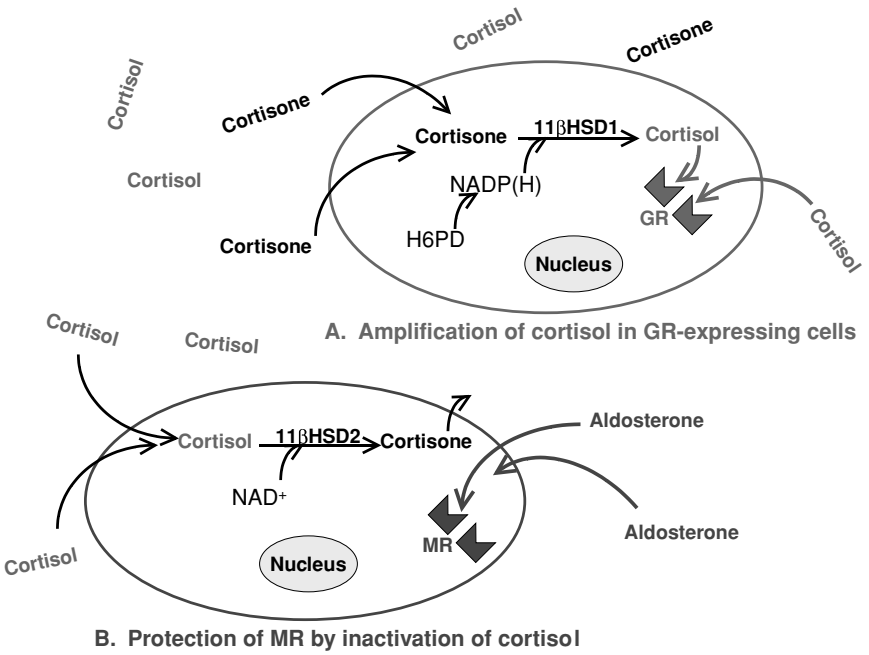

Figure 1 Schematic diagram illustrating the (**A**) amplification of glucocorticoid action in GR-expressing cells by type 1 11β-hydroxysteroid dehydrogenase (11β-HSD1), which acts as a reductase and reactivates cortisone and **(B)** protection of mineralocorticoid receptors (MRs) by type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) which inactivates cortisol. The reductase activity of 11β-HSD1 is dependent upon the local generation of NADP(H) by hexose-6-phosphate dehydrogenase (H6PD). In rodents, 11β-HSD1 and 11β-HSD2 catalyze the interconversion of corticosterone and 11-deoxycorticosterone.
Source: Reproduced from Buckingham (2006) with permission.

and corticosterone to the brain (Meijer *et al*., 2003; Webster and Carlstedt–Duke, 2002).

Pre-receptor Metabolism

Probably the most important factor regulating the access of endogenous glucocorticoids to their receptors (GR or MR) is local metabolism of the steroids within the target cells themselves by 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes, a phenomenon sometimes termed pre-receptor metabolism. 11β-HSD catalyzes the interconversion of cortisol and its inert metabolite cortisone (or corticosterone and 11-deoxycorticosterone in rodents, Fig. 1) (Buckingham, 2006). Its existence was first recognized in 1953, but it was not until the late 1980s, due largely to the seminal experiments of groups in Edinburgh and Melbourne, that the pivotal role of this enzyme family began to become apparent.

Stewart and colleagues (Stewart *et al*., 1988) were the first to recognize that the kidney is a major site for the conversion of cortisol to cortisone and that in the normal state intrarenal 11β-HSD inactivates cortisol and, thus, allows preferential access and binding of aldosterone to MR. Other findings confirmed this view and revealed that MR-expressing tissues which are normally highly sensitive to aldosterone (kidney, parotid sweat glands, and colon) show high levels of 11β-HSD activity. However, some tissues that express MRs in abundance (e.g., hippocampus) are not recognized targets for aldosterone and show little, if any, 11β-HSD activity, suggesting that that cortisol (or corticosterone) is the primary MR ligand at these sites (Edwards *et al*., 1988; Funder *et al*., 1988). These landmark studies opened a new chapter in the biology of glucocorticoids and, as described briefly below, it is now apparent that "pre-receptor" interconversion of active and inactive glucocorticoids is a critical determinant of the specificity of the MR and also of the degree of GR activation in a number of tissues (Fig. 1).

Two distinct species of 11β-HSD have been cloned and characterized, type 1 and type 2 (i.e., 11β-HSD1 and 11β-HSD2) (Edwards *et al*., 1996). 11β-HSD1 is expressed mainly in liver, adipose tissue (particularly omental), and brain, but also found in other tissues, and is subject to regulation by a variety of factors including glucocorticoids, stress, sex steroids, cytokines and peroxisome proliferator–activator receptors ligands. 11β-HSD1 is a low-affinity, NADP(H)-dependent enzyme and in in vitro systems shows bidirectional activity (i.e., both dehydrogenase and reductase). However, in vivo it appears to function solely as a reductase, relying on hexose-6-phosphate dehydrogenase (with which it is colocalized in the endoplasmic reticulum) to generate NADP(H) (Draper *et al*., 2003). 11β-HSD1 thus serves to regenerate biologically active cortisol/corticosterone from inert cortisone/11-dehydrocorticosterone.

As 11β-HSD1 is found mainly in tissues in which the high-affinity MR is sparse but the low-affinity GR is abundant, it has been argued that its principal role is to amplify the local concentration of active glucocorticoids in those tissues in which the steroids have a key regulatory role, for example, the liver. Support for this hypothesis emerged from a number of studies, including phenotypic analysis of 11β-HSD1–null mice (Seckl, 2004) and has led to the view that 11β-HSD1 may be an important factor in the development of insulin resistance, obesity, and other metabolic disturbances. Consequently, drugs which block 11β-HSD1 are now important targets for the pharmaceutical industry. There is also a growing interest in the role of 11β-HSD1 in the brain, particularly in the hypothalamus, hippocampus, cortex, and cerebellum where the enzyme is abundant. Of particular note is a recent study showing improved cognitive function in elderly subjects treated with an inhibitor of 11β-HSD1 (Sandeep *et al*., 2004) and evidence for an association between of 11β-HSD1 haplotypes and susceptibility to Alzheimer disease (De Quervain *et al*., 2004).

11β-HSD2 is a high-affinity, NAD^+-dependent, constitutive enzyme which acts exclusively as a dehydrogenase. Analysis by in situ hybridization and immunohistochemistry has confirmed data based on measures of enzyme activity that

11β-HSD2 is colocalized with the MR in tissues, such as the kidney, parotid gland, sweat glands, colon, and vascular smooth muscle cells. 11β-HSD2–null mice show a high degree of mortality; those that survive, develop severe corticosterone-dependent hypertension and other features of apparent mineralocorticoid excess (Kotelevtsev *et al*., 1999) as also do patients with specific mutations of the 11β-HSD2 gene (Edwards *et al*., 1996), and animals or humans in which 11β-HSD2 activity is blocked with glycyrrhetinic acid (the active component of liquorice) or its derivative carbenoxolone. 11β-HSD2 is also expressed in some tissues which lack MR, e.g., placenta and developing brain, where it appears to provide protection from the potentially harmful effects of excess cortisol/corticosterone (Brown, 1996). Conversely, at loci in which 11β-HSD2 is not expressed (for example, adult brain and cardiac myocytes) or is inactivated by a change in redox state (for example, in damaged tissue), cortisol/corticosterone may act as the primary ligand of the MR (Funder, 2005).

Signal Transduction at Glucocorticoid Receptors

Genomic Actions

Glucocorticoid receptors regulate, directly or indirectly, specific changes in DNA transcription and, hence, protein generation. They are located mainly in the cytoplasm as multiprotein complexes which include various heat shock proteins (e.g., hsp90). The GR shares a high degree of sequence homology with other members of the nuclear receptor family and comprises five distinct domains (Vinson, 1997). The N-terminal A/B domains include the activational function domain 1 (AF1) which facilitates transcriptional activity. The C-domain includes two cysteine-rich Zn^{2+} fingers and is responsible for receptor dimerization and DNA-binding. The D-domain, or hinge region, aids nuclear translocation as also does the C-terminal E-domain. The E-domain is also responsible for ligand binding, includes a second activational function domain 2 (AF2), and may also have the ability to silence basal promoter activity.

Early studies showed that activated GRs act as transcription factors which induce or repress the expression of target genes by direct interaction with specific glucocorticoid response elements in the promoter region. Access of the ligand–receptor complex to the glucocorticoid response elements is effected via a process involving dissociation of the chaperone proteins and sequential phosphorylation, dimerization and nuclear translocation of the receptor (Kawata *et al*., 2008). It is also subject to regulation by intracellular proteins called coactivators and corepressors. These proteins, which may be expressed in a cell- or tissue-specific manner, are recruited by the activated ligand–receptor complex. They act, at least in part, as histone acetylases/deacetylases that modulate the structure of the core histone proteins that support the helical DNA structure, a process called chromatin remodeling. Coactivator proteins may thus facilitate access of RNA II polymerase and associated transcriptional complexes to target DNA sequences by acetylating key residues in the histone core and causing the DNA to unwind. Conversely,

corepressor modules limit the access of these regulatory molecules by histone deacetylation which winds the DNA tightly around the histone core (Beato *et al*., 1995; Goulding, 2004).

In addition to acting as positive, or in some cases negative, transcription factors, activated GRs may also modulate gene transcription indirectly through protein–protein interactions. Initial evidence to this effect emerged from studies in cell lines which revealed (a) that activated GRs repress activator protein 1 (AP-1, c-fos/c-jun) induced transcription of the collagenase gene, (b) that c-fos and c-jun effectively oppose the suppressive influence of glucocorticoid receptors on collagenase expression, and (c) that AP-1 and glucocorticoid receptor can be coprecipitated. Subsequent studies confirmed that GRs bind to AP-1 and thereby prevent its transcriptional activity. They also demonstrated that GR suppress transcription evoked by nuclear factor kappa B (NF-κB), but augment the responses to Stat-5 (Barnes and Adcock, 2003; Reichardt *et al*., 1998; Wintermantel, 2005; Yang–Yen *et al*., 1990). Glucocorticoid receptors may thus act as corepressors or coactivators of other transcription factors, as well as acting as transcription factors in their own right.

Further advances in our understanding of the significance of protein–protein interactions in effecting the actions of glucocorticoids came from analyses involving point mutations of the GR. In particular, key mutations in the Zn^{2+} fingers demonstrated clearly that the transrepression effected through interactions with other transcription factors (e.g., AP-1 or NF-κB) does not require either dimerization or DNA binding of the receptor (Reichardt *et al*., 1998). Conversely, the majority of endocrine and metabolic actions of the steroids appear to be mediated by direct binding of glucocorticoid receptor dimers to DNA (i.e. transactivation).

These findings have led to a search for compounds that possess strong transrepressional activity via NF-κB or AP-1 (and hence ability to suppress the expression of proinflammatory genes) but have little direct transactivational activity, as such compounds should reduce the risk of systemic side effects when used as anti-inflammatory drugs. Several such compounds (termed dissociated steroids) appear promising in in vitro models (e.g., RU 24858, and RU 40066), but the separation of anti-inflammatory and transactivational properties in vivo is disappointing, although a nonsteroidal GR agonist (ZK 216348) shows an improved therapeutic index versus prednisolone.

Nongenomic Actions

Although glucocorticoid actions are effected mainly by changes in gene transcription, some occur too rapidly to be explained in this way. For example, cortisol hyperpolarizes hippocampal neurones within 1 to 2 minutes contact and depresses the release of ACTH from the anterior pituitary gland over a similar time-frame (See also section, "Feedback Regulation of the HPA Axis" later in this chapter). The mechanisms responsible for these rapid effects are unknown, although there is evidence that they may reflect membrane perturbations which compromise energy-dependent functions, alterations in Ca^{2+} flux, or interactions with specific

membrane bound G-protein–coupled receptors (De Kloet *et al*., 2008; Kawata *et al*., 2008). Other data indicate that the nongenomic actions of glucocorticoids are effected via the intracellular glucocorticoid receptors and that postreceptor signaling involves a complex kinase cascade (Solito *et al*., 2003). A role for the intracellular receptors is further supported by evidence that the activated glucocorticoid–receptor complex may regulate posttranscriptional events including mRNA transcript stability, mRNA translation, and posttranslational processing. Such actions may be particularly relevant to steroid effects manifest before the full transcriptional effects of the genomic actions are apparent (Buckingham, 2002).

Splice Variants of the Glucocorticoid Receptor

The responses to glucocorticoids often vary quite considerably among individuals. This may be explained, in part, by GR polymorphisms or haplotypes. However, increasing evidence suggests that differential expression of splice variants of the GR gene is important in this regard. Two splice variants, GR-β and GR-γ, are well characterized. GR-β is a C-terminally truncated GR variant that is unable either to bind ligand or to induce gene transcription. Its expression is augmented by proinflammatory cytokines (e.g., tumor necrosis factor-α), and molecular studies suggest that it may act as a dominant negative inhibitor of GR and, thereby, contribute to the phenomenon of glucocorticoid resistance (Goulding, 2004). GR-γ is the most widely expressed variant identified to date. Its amino acid sequence includes an additional residue (arginine) between the two Zn^{2+} fingers of the DNA-binding domain. This renders the receptor less transcriptionally active than the native GR and its relative expression may thus affect tissue sensitivity to glucocorticoids (Stevens *et al*., 2004).

GLUCOCORTICOID-REGULATED GENES

The advent of microarray technology has facilitated the identification of numerous glucocorticoid-regulated genes. However, the picture is far from clear. Predictably, these studies have identified genes concerned with inflammation and immunity, cell growth, differentiation, and apoptosis; endocrine function and metabolism; signal transduction and membrane transport; neurotransmission; and bone turnover. However, they have also identified a large number of genes whose functions are either unknown or fall outside these categories. In addition, they have revealed a highly complex scenario in which the profile of differentially expressed genes differs according to the cell type(s) studied, the steroid contact time, and the nature of the steroid itself. Transferred to the in vivo situation, the expression profile will inevitably be modified further by the local milieu, which will also include many other factors that contribute to the regulation of these target genes.

While difficult to interpret, the data from microarray illustrate admirably the amazing complexity of glucocorticoid action and the resultant capacity of the steroids to exert very fine control over a broad range of physiological and

pathophysiological functions. This is amply illustrated by consideration of the plethora of glucocorticoid-regulated genes concerned with the processes of inflammation (Barnes and Adcock, 2003; Goulding, 2004).

CONTROL OF GLUCOCORTICOID SECRETION

Cortisol (the principal glucocorticoid in man) and its rodent counterpart corticosterone are synthesized from cholesterol in cells of the zona fasciculata of the adrenal cortex. Their release into the systemic circulation is pulsatile or "ultradian" (Droste *et al*., 2008; Windle *et al*., 2001), and pulse amplitude varies according to a distinct circadian pattern which is co-ordinated by the hypothalamic suprachiasmatic nucleus. Serum glucocorticoid concentrations are thus maximal just before waking, and decline thereafter reaching a nadir some 6 to 8 hours later. Pulse frequency may alter in certain pathological conditions and this may have important consequences for the kinetics of the dimerized steroid-bound receptor in the nucleus and, hence, for the processes of gene transcription (Droste *et al*., 2008). Glucocorticoids are also released in response to physical and/or emotional trauma. The "stress response" is superimposed upon the existing circadian tone and varies in magnitude according to the nature, intensity, and duration of the stimulus and the individual's previous experience (Buckingham, 2002; Sapolsky *et al*., 2000).

The circadian and stress-induced secretion of the glucocorticoids is governed by the hypothalamo-pituitary axis (Fig. 2) (Buckingham, 2006). The hypothalamus receives, monitors, and integrates neural and humoral information from many sources. It thus acts as a sensor of changes in the external and internal environment and of the body clock. Using this information, the hypothalamus responds to adverse circumstances, be they physical or emotional, and circadian factors by activating the final common pathway to glucocorticoid synthesis. This pathway involves the release of two hypothalamic neurohormones, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which drive the release of a pituitary hormone, the adrenocortocotrophic hormone (ACTH), which, in turns promotes the synthesis and release of cortisol/corticosterone by the adenal cortex. The sensitivity of the hypothalamo-pituitary-adrenocortical (HPA) axis to incoming stimuli is modulated by a servo system through which the sequential release of CRH/AVP and ACTH from the hypothalamus and anterior pituitary gland is negatively regulated by the glucocorticoids themselves. The magnitude of the HPA response to stress thus depends upon the preexisting glucocorticoid tone (Buckingham *et al*., 1996).

ACTH and Its Precursor, Pro-opiomelanocortin

ACTH is a polypeptide hormone comprising 39 amino acid residues. Its first 24 amino acids are highly conserved and confer the steroidogenic activity of the

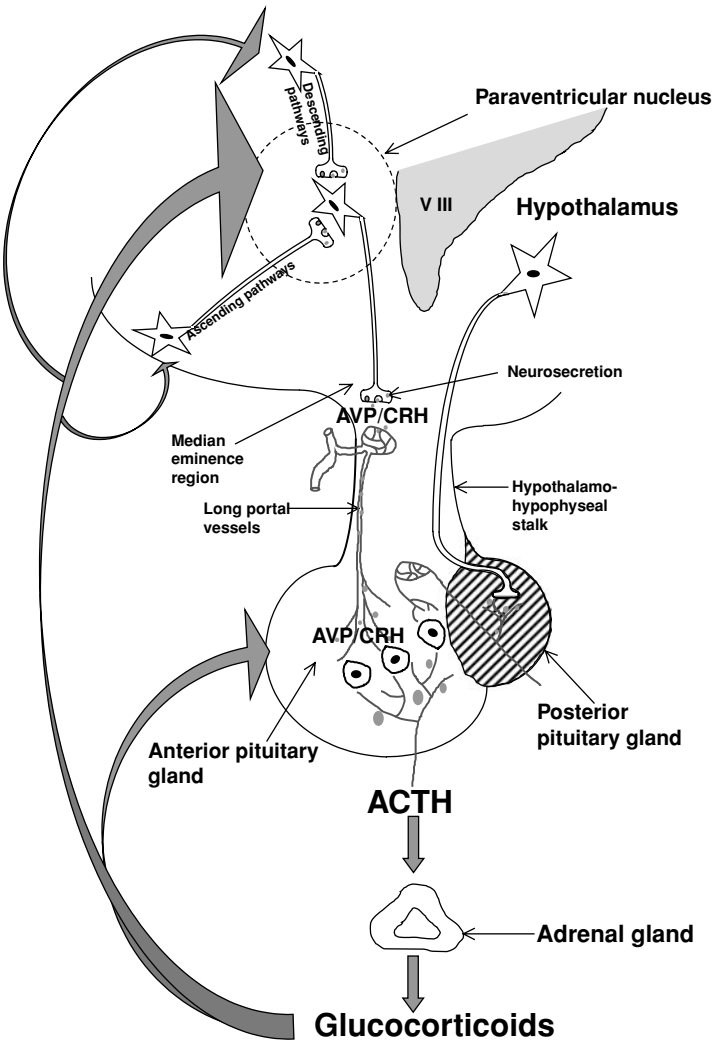

Figure 2 Schematic diagram illustrating the hypothalamo-pituitary-adrenocortical axis and principal loci of glucocorticoid feedback control. Note that the CRH/AVP secreting neurones are innervated by ascending nervous pathways from the brain-stem nuclei and by descending pathways from the limbic system and other centers (e.g., cortex). Circadian regulation is effected mainly via pathways from the suprachiasmatic nucleus (not shown). Local factors derived from glial cells (e.g., cytokines) and humoral factors (e.g., glucose) may also modulate the secretion of CRH/AVP. Similarly, locally produced substances also modulate the secretion of ACTH and glucocorticoids. *Abbreviations*: CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; ACTH, adrenocorticotrophin hormone. *Source*: Reproduced from Buckingham (2006) with permission.

molecule while amino acids 25 to 39 show considerable species variation. ACTH is synthesized in specialized cells (called corticotrophs) in the anterior pituitary gland by posttranslational processing of a precursor protein, pro-opiomelanocortin (POMC) (Bicknell, 2008). POMC processing takes place after the protein has been packaged into secretory vesicles and results in the formation of several other proteins which are coreleased with ACTH; these include β-lipotrophin (which may be further processed to β-endorphin) and the N-terminal peptide, N-POC$^{1-74}$, also known as pro-γ-melanocyte stimulating hormone (pro-γ-MSH). The POMC gene is also expressed at a number of other sites in the body (e.g., the hypothalamic arcuate nucleus, placenta, and peripheral blood leukocytes), where its protein product is differentially processed to yield tissue-specific arrays of peptides which fulfil very different functions. For example, in the brain, the principal products of POMC are β-endorphin, which play an important role in the regulation of pain perception, and α-melanocyte stimulating hormone (α-MSH) whose functions include the regulation of food intake (Bicknell, 2008).

ACTH acts via type 2 melanocortin receptors (MC-R2, a G-protein–coupled seven transmembrane domain receptor) on its target cells in the zona fasciculata of the adrenal cortex to activate the rate limiting enzyme in the steroidogenic pathway (cholesterol side chain cleavage enzyme, CYP11A1, which catalyses the conversion of cholesterol to prenenolone) and, hence, the synthesis of the glucocorticoids, cortisol/corticosterone (Chan *et al*., 2008). The newly synthesized steroids are then released by diffusion across the plasma membrane into the systemic circulation. This process is assisted by ACTH which also acts via type 3 melanocortin receptors (MC-R3) in the adrenal cortex to cause a local vasodilation; this increases local blood flow and thereby facilitates steroid release by increasing the concentration gradient across the plasma membrane. ACTH also promotes adrenal androgen synthesis by zona reticularis cells and, in some conditions, may also promote aldosterone synthesis by the zona glomerulosa cells.

Two further peptides derived from the N-terminus of POMC also have important roles in the adrenal cortex. Pro-γ-MSH and its C-terminal peptide (N-POC$_{50-70}$ or γ-3-MSH) potentiate steroidogenesis by liberating free cholesterol from its stored ester and, thereby, increasing the availability of free mitochondrial cholesterol, the precursor for steroidogenesis (Al-Dujaili *et al*., 1981). N-POC$_{1-48/49}$ induces mitogenesis of the steroidogenic cells. Interestingly, in normal conditions the principal N-terminal product of POMC is N-POC$_{1-74}$. However, in conditions of adrenal insufficiency, when glucocorticoids levels are low, adrenal growth is promoted by increased production of N-POC$_{1-48/49}$. This is effected by increased N-terminal processing of POMC in the pituitary corticotrophs (Estivariz *et al*., 1988). In addition, circulating pro-γ-MSH is converted in the adrenal cortex to N-POC$_{1-48/49}$ and γ-3-MSH by a serine protease produced locally in the capsular region (Bicknell *et al*., 2001). This mechanism is believed to be critically important to the process of compensatory adrenal growth in conditions of adrenal insufficiency.

Corticotropin-Releasing Hormone and Vasopressin

CRH, a 41-amino acid residue peptide, was first described by Vale and colleagues (1981) as the principal hypothalamic factor driving the secretion of ACTH (Vale *et al*., 1981). Subsequent studies revealed that CRH is expressed by hypothalamic parvocellular neurones, which originate in the paraventricular nucleus (PVN) and terminate in the external layer of the median eminence in close apposition with vessels of the hypothalamo-hypophyseal vessels. CRH is released from these neurones in response to stress and in accord with the circadian rhythm and acts on specific receptors, type 1 CRH receptors (CRH-R1) (Aguilera *et al*., 2004), on the corticotrophs (a) to evoke the release of preformed ACTH from secretory vesicles in the corticotrophs, (b) to increase pituitary POMC gene expression (and thereby replace the ACTH released) and (c) in the longer term, to promote corticotroph mitogenesis. CRH-R1 is a Gs-protein–coupled 7-transmembrane domain receptor, which is positively coupled to adenylyl cyclase. Activation of this receptor thus results in increased intracellular accumulation of 3',5' adenosine cyclic monophosphate (cAMP) (Labrie *et al*., 1987). The CRH gene is also expressed at a number of other sites in the brain, in particular, the limbic system and the brain stem nuclei where it has important roles in mediating the autonomic and behavioral responses to stress and may also contribute to the pathogenesis of depression and other psychiatric disorders (De Kloet, 2008). In addition, CRH is expressed in a number of peripheral cells and tissues (e.g., placenta, leukocytes) and is increasingly associated with the pathogenesis of inflammation (Kalantaridou *et al*., 2007).

Although principally associated with the regulation of osmotic balance, vasopressin has been implicated in the regulation of HPA activity for well more than 50 years. McCann and colleagues were the first to show that vasopressin possesses corticotropin-releasing activity in a variety of model systems. The significance of this finding was disputed on the grounds that concentrations of vasopressin required to induce ACTH release were several orders of magnitude higher than those in the systemic circulation. However, reports that (a) rats which congenitally lack vasopressin (Brattleboro strain) show impaired HPA activity that is corrected by vasopressin-replacement therapy (Buckingham, 1981), (b) vasopressin is present in the hypophyseal portal vessels in concentrations well above than those in the systemic circulation (Plotsky and Sawchenko, 1987), and (c) vasopression potentiates the corticotropin-releasing activity of CRH (Buckingham, 1981; Gillies *et al*., 1982) reopened the debate on the role of this neuropeptide in the regulation of ACTH release. It is now established that vasopressin is coexpressed with CRH in some of the parvocellular neurones which project from the PVN to the median eminence; the proportion of neurones expressing both peptides varies but increases markedly in conditions of chronic stress and adrenal insufficiency (Fig. 3) (Shibata *et al*., 2007).

Vasopressin acts via type 1b, phospholipase C–coupled vasopressin receptors on the corticotrophs to induce ACTH release directly (Antoni, 1993). Alone,

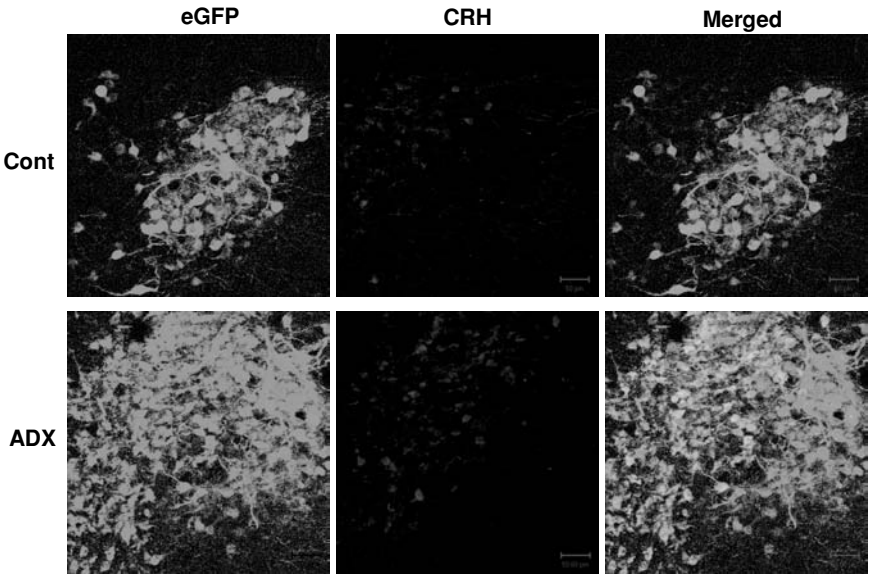

Figure 3 (*See color insert*) Micrographs of the mouse PVN of transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of the vasopressin promoter. These data illustrate the effects of adrenalectomy on the expression of eGFP (a surrogate for vasopressin, AVP) and CRH in the parvocellular and magnocellular regions of PVN. Note that in the controls, AVP (green) is localized mainly to the magnocellular region (left panel, top), while CRH (red) is found mainly in the parvocellular region (middle panel, top); there is negligible colocalization of the two peptides (right panel, top). Adrenalectomy causes a profound increase in AVP in the parvocellular region of the PVN (left panel, bottom) together with a more modest increase in parvocellular CRH (middle panel, bottom); there is a significant degree of colocalization of the peptides (orange cells, right panel, bottom). *Source*: Adapted from Shibata et al. (2007) and reproduced with permission.

its effects are modest, as also are those of CRH; however, it potentiates very markedly the ACTH-releasing activity of CRH, and the synergistic actions of the two peptides are now recognized to be critical to the regulation of ACTH release. The mechanism by which the synergy occurs has not been fully explained but appears to involve potentiation of the CRH-induced accumulation of cAMP and hence, the process of exocytosis. Interestingly however, while the CRH-induced increases in POMC expression and corticotroph mitogenesis are also cAMP-dependent, vasopressin has no measurable effect on either parameter in either the presence or absence of CRH.

The relative importance of CRH and vasopressin in effecting the ACTH responses to stress has been difficult to ascertain, largely because of the complexities of measuring the release of one or both hormones into the portal vessels, or effectively quenching their activities by genetic, immunological, or

pharmacological methods without inducing compensatory effects. Nonetheless, a substantive body of data now indicates that CRH is the primary driver of the HPA response to acute stress, be it physical or emotional, but that vasopressin assumes an increasingly important role in conditions of chronic stress. This view has been reinforced by findings that vasopressin 1b-receptor null mice show marked deficiencies in their pituitary–adrenocortical responses to chronic stress (Lolait, 2007). In this context, it is interesting to note that vasopressin is now implicated in the overactivity of the HPA axis observed in subjects with depression.

Stress-Induced Activation of the HPA Axis

The HPA axis plays a critical role in enabling the organism to prepare for, respond to, and cope with physical or psychological stress. The mechanisms by which different stresses promote the release of CRH and AVP are a subject of much current research as too are the processes which underlie the adaptive responses to repeated or chronic stress.

Acute Stress

It is now broadly established that stresses which have a psychological component use cortico-limbic pathways to drive the HPA response while those that are "physical" in nature, e.g., hypotension, use the ascending noradrenergic pathways which project from the brain stem nuclei (A1 in the ventrolateral medulla and A2 in the nucleus tractus solitarius) to the PVN. This dogma is well supported by data from studies involving, for example, measurement of the stress-induced expression of the immediate early gene, c-*fos*, in discrete brain regions or of c-*fos* and CRH mRNAs in the PVN of animals in which the relevant ascending or descending neuronal inputs to the PVN have been lesioned surgically or pharmacologically. These studies have emphasized repeatedly the critical role of the amygdala, hippocampus, and bed nucleus stria terminalis in mediating the responses to acute psychogenic stressors (e.g., restraint), and the supporting roles of cortical regions, most notably the prefrontal and cingulate cortices.

Among the most potent activators of the HPA axis are insults to the host defence system (e.g., infections and other immunological insults), which threaten the well-being of the organism. The mechanisms by which such assaults trigger the release of glucocorticoids have been hotly debated. Early studies exploring the responses to bacterial (injection of lipopolysaccharide, LPS) or viral (e.g., Newcastle disease) toxins pointed to cytokine-dependent mechanisms which trigger the release of CRH and, possibly, AVP from the hypothalamus (Sapolsky *et al*., 1987). This view was supported by evidence that CRH antisera block the ACTH response to a peripheral injection of IL-1β (Sapolsky *et al*., 1987). Furthermore, TNF-α, IL-1β, and IL-6 increase the release of (a) ACTH in vivo when injected into the third ventricle (Loxley *et al*., 1993) and (b) CRH and AVP from isolated hypothalami in vitro (Loxley *et al*., 1993). However, questions emerged about the ability of the cytokines, which are proteins of molecular

weight 17 to 26 kDa, to cross the blood barrier to target the CRH/AVP neurones directly. The concept that transport across the barrier may be effected via a specific active pump or by diapedis of cytokine-producing leukocytes received scant experimental support. Observations that peripheral injections of LPS and proinflammatory cytokines (e.g., IL-1β) induce proinflammatory cytokine expression in discrete regions of the brain (e.g., hypothalamus) pointed to an alternative mechanism(s). Considerable attention focused on the potential role of vagal afferents. Importantly, intraperitoneal injection of IL1β was shown to release eicosanoids from activated peritoneal macrophages; the eicosanoids target specific receptors on sensory neurones and thus activate vagal afferents that project to the brain stem (nucleus tractus solitarius, NTS); here they activate fibers in the ventral noradrenergic bundle which lead to the parvocellular PVN to activate CRH/AVP secreting neurones and also induce local cytokine synthesis (Laye *et al*., 1995). Other data advocate a role for the blood–brain barrier in mediating the central responses to systemic immune insults. In particular, they indicate that LPS and proinflammatory cytokines in the blood stream target receptors on the endothelial cells of the blood–brain barrier, most notably in the brain stem (pons region); this results in the local release of eicosanoids which diffuse to receptors in the NTS, causing activation of the ventral noradrenergic bundle, central cytokine generation, and the release of CRH/AVP (Sapolsky *et al*., 2000).

While there can be little doubt that the hypothalamus plays a critical role in driving the pituitary adrenocortical responses to immune/inflammatory stress, a further body of data suggests that direct actions of bacterial/viral proteins and cytokines on the pituitary gland and adrenal cortex serve to enhance and sustain the rise in glucocorticoids secretion. At the pituitary level glial-like cells, termed folliculostellate (FS) cells, express LPS receptors (toll-like 2 receptors, tlr-2 receptors) which provoke the release of local cytokines, e.g., IL-6 and leukocyte inhibitory factor, which act on the corticotrophs to augment CRH-driven ACTH release (Giacomini *et al*., 2007). Similarly, at the adrenal level proinflammatory cytokines, e.g., IL-6, increase ACTH-driven corticosterone release. In addition, our recent studies have identified mRNAs for members of the formyl peptide receptor family (which are targets for formylated bacterial peptides and also for certain viral proteins), in both the anterior pituitary gland and adrenal cortex (John *et al*., 2007). These receptors influence ACTH release (John *et al*., 2007) and are hugely upregulated by treatment with LPS (Buss *et al*., 2008).

Repeated and Chronic Stress

Until comparatively recently, studies on the stress-induced activation of the HPA axis centered mainly on acute stresses. However, acute stress does not model the chronic stress which is implicated in the pathogenesis of "stress-related" disease. To address this problem, various models of repeated (intermittent) or chronic (sustained) stress have been developed. In most instances, tolerance develops to repeated exposure to homotypic stress, and the amount of ACTH and corticosterone released in response to successive events is thus reduced. Cross tolerance

may develop to stressors that use similar afferent inputs to the hypothalamus, but exposure to a novel (heterotypic) stress may precipitate a normal or even exaggerated HPA response.

Several groups have used models in which an inflammatory response develops over a period of days and then resolves as models of chronic stress (e.g., experimentally induced arthritis or allergic encephalomyelitis). In these models, increases in pituitary POMC expression and plasma ACTH and corticosterone emerge just before or at the time of onset of the clinical symptoms of disease and continue to rise as the disease progresses, correlating with the severity of disease; in addition, these parameters decline as the disease regresses and return to normal on recovery. Paradoxically, however, while CRH mRNA expression in the parvocellular PVN increases before disease symptoms emerge, it declines as the disease develops as also does the release of CRH into the hypophophyseal portal blood (Aguilera *et al*., 1997; Harbuz *et al*., 1992; Harbuz *et al*., 1993). In contrast, the release of vasopressin into the portal vessels is increased at this time as also is the expression of AVP mRNA in the parvocellular PVN. In addition, AVP binding in the anterior pituitary gland increases, whereas CRH binding decreases when inflammation is maximal. Taken together, these data suggest a critical role for AVP in effecting the pituitary adrenocortical response to chronic inflammatory stress (Chowdrey *et al*., 1995). The underlying neural mechanisms appear to involve both noradrenergic (Harbuz *et al*., 1994) and serotonergic (Harbuz *et al*., 1998) pathways. A further important finding was that the hypersecretion of corticosterone induced by chronic inflammation is characterized by an increase in frequency but not the amplitude of secretory events, and that this profile compromises the response to acute noise stress (10 min) but not to an inflammatory stress, e.g., injection of LPS (Windle *et al*., 2001). Indeed, the peripheral and central immunological responses to LPS during the chronic inflammatory phase of experimentally induced arthritis are significantly increased (Grinevich *et al*., 2002). Conversely, when given prior to the induction of inflammatory disease, LPS protects the organism from the development of the disease. This protective effect is associated with reduced production of proinflammatory cytokines and increased production of the anti-inflammatory cytokine IL-10 (Richards *et al*., 2006). It is also dependent on the interval between the injection of LPS and the induction of inflammation, may also involve increased activation of the HPA axis (Richards *et al*., 2006).

An alternative model of chronic stress which is attracting increasing attention is chronic mild variable stress (CMVS). In CMVS paradigms, animals (normally rodents) are exposed to successive periods of mild but unpredictable stress designed to mimic the everyday hassles of life. Typically, the stressors used may include cage tilt, wet bedding, water deprivation, noise, light, restraint, novel environment for variable periods of several hours with occasional periods of calm. The order in which the stressors are applied is randomized, as too are the intervening stress-free periods. The test period may persist for periods of 7 days to 4 weeks. At the end of the test period, HPA activity is typically enhanced, as evidenced

by thymic atrophy, adrenal hypertrophy, raised plasma ACTH and corticosterone, and increases in CRH mRNA and, to a lesser extent, AVP mRNA in the parvocellular PVN. In addition, MR expression is reduced in the hippocampus while GR expression is reduced in both the hippocampus and the prefrontal cortex. Taken together, these data suggest that CMVS increases HPA activity and that this may be due in part to impairment on the negative feedback actions of glucocorticoids in the brain (Herman *et al*., 1995). There is evidence that rats subjected to CMVS not only respond to a novel stress (restraint) with a normal rise in plasma ACTH and corticosterone, but are tolerant to stresses used in the CMVS paradigm (Simpkiss and Devine, 2003). Others, however, have reported that CVMS-treated rats take several days to regain their responsiveness to psychogenic stressors, but that the response to physical stress (hypoxia) is intact (Ostrander *et al*., 2006). The processes responsible for the adaptive responses of the HPA axis to CMVS warrant further investigation but may include recruitment of cell groups within the bed nucleus stria terminalis or functional reorganization of stress-integrative circuits (Choi *et al*., 2008).

Feedback Regulation of the HPA Axis

In all species studied, the functional activity of the HPA axis is tightly regulated by the negative feedback effects of the glucocorticoids. The actions of the steroids are exerted at several levels in the axis, involve both MR and GR, and are effected via several molecular mechanisms which operate in different time spans. It is well established that sustained alterations in circulating glucocorticoids brought about by e.g., administration of glucocorticoids or surgical removal of the adrenal gland have a profound effect on the expression of the gene encoding CRH and AVP in the parvocellular PVN (Fig. 3), and POMC in the pituitary gland. These actions, which are slow in onset, are consequent in part on direct actions of the steroids on GR in the PVN and pituitary gland, respectively, causing transrepression of the target genes. In addition, the steroids act at other loci in the brain, notably GR and also MR in the hippocampus and GR elsewhere in the limbic system, the prefrontal cortex, and the brain stem nuclei to modulate the neural drive to the hypothalamus and, thereby, down-regulate the expression of CRH, AVP, and POMC in, for example, conditions of stress or in accord with the circadian rhythm.

In normal circumstances, CRH/AVP and ACTH are stored in appreciable amounts in secretory vesicles in the median eminence and anterior pituitary gland where they are available for immediate release by exocytosis. A second important mechanism of glucocorticoid feedback is to prevent the release of the preformed hormones. This process enables the steroids to exert more immediate effects on the resting and stress-induced activity of the axis. Two molecular mechanisms have been shown to contribute to these rapid actions. The first emerges in seconds/minutes and may involve an action of the steroid on the cell membrane which alters the Ca^{2+} flux and thereby prevents exocytosis (De Kloet *et al*., 2008).

This action appears to be important in terminating the release of CRH/AVP and ACTH in conditions of stress (De Kloet *et al*., 2008). The second involves a glucocorticoid-regulated protein, annexin 1 (ANXA1) (John *et al*., 2004).

ANXA1 was originally identified by Flower and colleagues (Flower and Blackwell, 1979) as a glucocorticoid-inducible protein that inhibits the activity of phospholipase A*2* and, hence, the generation of proinflammatory eicosanoids. Subsequent studies using immunoneutralization and antisense strategies, ANXA1 peptides and, most recently, ANXA1-null mice have revealed a role for ANXA1 in the regulation of neuroendocrine function. In particular, ANXA1 serves as a mediator of the early inhibitory effects of glucocorticoids on the basal and stress-induced secretion of CRH/AVP and ACTH (John *et al*., 2004). The mode of action of ANXA1 is unusual in that it appears to act in a paracrine or juxtacrine manner. This is perhaps best exemplified in the anterior pituitary gland. Here, ANXA1 is expressed by the nonsecretory FS cells, where it is found mainly in the projections that make contact with the endocrine cells (Fig. 4) (Chapman *et al*., 2002). Glucocorticoids act via a nongenomic mechanism to cause rapid (within 15 min) serine-phosphorylation and translocation of the protein to the cell surface. The exported protein acts via membrane-bound receptors on adjacent corticotrophs to suppress ACTH release. Our recent studies suggest that these receptors are members of the formyl peptide receptor family (John *et al*., 2007). ANXA1 may thus target receptors recognized by bacterial and viral peptides and also by endogenous anti-inflammatory eicosanoids (e.g., lipoxin A4).

AGE- AND SEX-RELATED CHANGES IN HPA FUNCTION

Development

The functional development of the HPA axis begins in midfetal life. In the later stages of gestation, the fetus produces ACTH and glucocorticoids; it also responds to stress with an increase in pituitary-adrenocortical activity which is driven by CRH released from the hypothalamus and is sensitive to the feedback action of the glucocorticoids (Buckingham *et al*., 1997). The fetus does not, however, produce CBG and, hence, only "free" glucocorticoid is found in the plasma; in addition, circadian activity does not develop until the postnatal period. Paradoxically, in the rat and several other species, HPA activity regresses postnatally and a period ensues in which the neonate produces at most only modest amounts of corticosterone when exposed to stress (e.g. endotoxemia, foot shock). This period of relative adrenal insufficiency, termed the stress-hyporesponsive phase , persists for some 14 to 21 days in the rat and, thus, coincides with an important stage of glucocorticoid-sensitive growth and development (e.g. of the immune system). It may thus serve to protect the organism from the potentially harmful effects of the steroids in this critical phase of development (See also section on the Fetal Origin of Disease). The etiology of the stress-hyporesponsive phase is poorly defined but appears to be associated with immaturity of the neuronal circuitry driving the secretion of

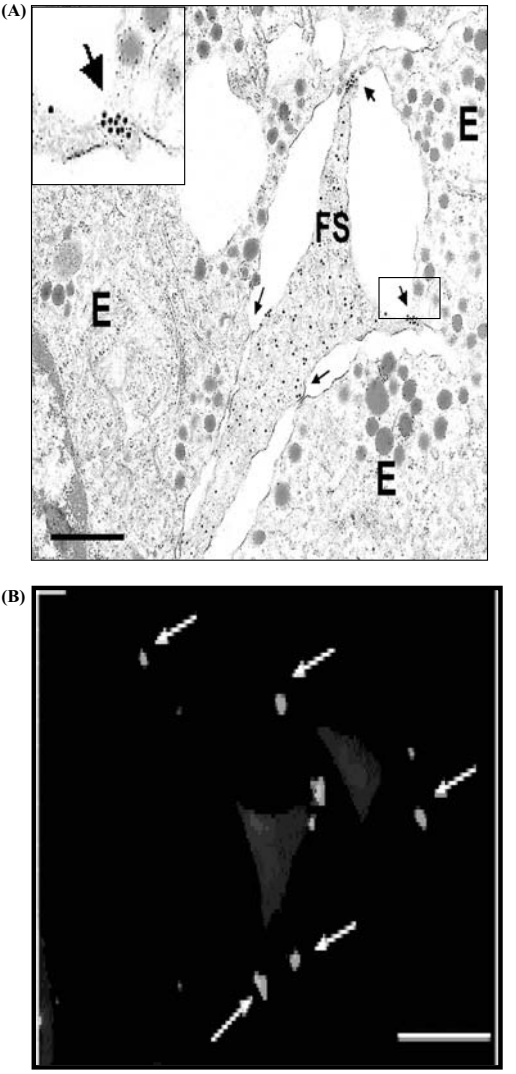

Figure 4 (*See color insert*) Expression of annexin 1 in FS cells. **(A)** Electron micrograph from a freeze substituted mouse anterior pituitary section showing immunogold detection of annexin 1 in an FS cell adjacent to three endocrine **(E)** cells. Gold particles (15 nm) are scattered over the cytoplasm and adjacent to the plasma membrane of the cell; they are also localized on the FS cell surface at the ends of processes contacting endocrine cells (see also enlarged inset). Arrows indicate intercellular junctions. Scale bar: 1 μm. **(B)** Immunofluorescence staining of annexin 1 in a murine folliculostellate (TtT/GF) cell line. Surface annexin 1 fluorescence (green) is evident on processes of TtT/GF cell (nucleic acids stained red with propidium iodide). Arrows indicate bright patches of cell surface annexin I. Scale bars: 20 μm. *Source*: Adapted from Chapman *et al*. (2002) and reproduced with permission.

CRH/AVP neurones and to increased sensitivity of the axis to the feedback actions of the glucocorticoids (Buckingham *et al*., 1997).

Adulthood

In the adult, distinct sexually dimorphic patterns of glucocorticoid secretion emerge. Basal and stress-induced glucocorticoid concentrations are consistently higher in the female than the male with further increases occurring towards the middle of the menstrual/estrous cycle, just prior to ovulation. These changes are due in part to the positive effects of estrogen on the production of CBG. In addition, there is growing evidence that the sex steroids also modify the secretion of glucocorticoids, with androgens and estrogens producing opposing actions. In support of this argument ovariectomy reduces and castration increases the frequency of corticosterone pulses without affecting pulse amplitude. Similarly, ovariectomy attenuates and castration exaggerates the stress-induced expression of hypothalamic CRH- and AVP mRNAs and pituitary POMC mRNA, and the associated increase in glucocorticoid secretion (Seale *et al*., 2004). In the female, the effects of ovariectomy are reversed by estradiol and, thus, have been attributed to estrogen deficiency (Seale *et al*., 2004), a view which is supported by earlier evidence that estradiol acts directly on the hypothalamus to augment both the expression (Vamvakopoulos and Chrousos, 1994) and the release (Buckingham, 1981) of CRH. However, other data suggest that progesterone may also have a role as, when present in substantive amounts (e.g., in the midluteal phase), it binds readily to but is only weakly active at MR in the hippocampus and may therefore serve as an antagonist of the feedback actions of glucocorticoids. Conversely, in the male, the effects of castration on basal and stress-induced HPA activity are reversed by testosterone replacement therapy, suggesting that androgens exert a negative influence on the functional activity of the system (Seale *et al*., 2004).

In both sexes, the serum glucocorticoid concentrations tend to increase in aging individuals. The reasons for this are unknown but this phenomenon may be an important contributory factor to the age-related decline in immunocompetence and the emergence of degenerative diseases of the brain and bone.

Adaptive Responses in Pregnancy and Lactation

The maternal HPA axis undergoes significant adaptive change during pregnancy and lactation (Brunton and Russell, 2008), which may serve to protect the mother and her progeny from the potentially adverse effects of stress at this critical time (See also the section, "Organizational Actions of Glucocorticoids" later in the chapter).

In the rat, basal HPA activity is unchanged at the nadir of the circadian cycle in early pregnancy, as indexed by measure of circulating ACTH and corticosterone and expression of mRNAs for POMC in the pituitary gland, CRH and AVP in the PVN, and GR in the PVN and hippocampus. However, the diurnal rise in HPA activity is quenched, a phenomenon that may favor implantation, and remains so

until midprenancy when the circadian pattern of corticosterone, but not ACTH secretion, is reinstated. In late pregnancy, basal HPA activity is reduced in the rat as evidenced by reductions in hypothalamic CRH and AVP pituitary POMC mRNAs, and in pituitary CRH-R1 and V1b receptor binding. These changes have been attributed to increased glucocorticoid feedback effected both by upregulation of hippocampal GR and by increased local generation of corticosterone in the PVN and pituitary gland, due to increased activity of 11βHSD1.

Stress-induced HPA activity is largely unchanged in the early stages of pregnancy. However, from midpregnancy onwards, the HPA responses to a wide range of psychological and physical stresses are severely blunted. This is associated with a diminution of the effectiveness of afferent stimuli passing from the limbic system (psychogenic stress) or brain stem (physical stress) nuclei. With respect to the latter, the activity of the noradrenergic neurones projecting from the brain stem to the PVN is attenuated by upregulation of the inhibitory opioidergic mechanisms, apparently as a result of generation of the neurosteroid, allopregnanalone, from progesterone (Brunton and Russell, 2008).

During the first week of lactation in rodents, basal HPA activity increases progressively, and a modest circadian profile is reinstated. Similarly, basal salivary cortisol levels are increased in lactating women. Suckling provides a potent stimulus to the HPA axis, with consequent rises in plasma ACTH and corticosterone. However, as in late pregnancy, the axis remains refractory to stress.

ORGANIZATIONAL ACTIONS OF GLUCOCORTICOIDS

Glucocorticoids and Development

Glucocorticoids play an important role in the regulation of growth and development. For example, they are required for the normal maturation of the lung and the central nervous system and they play a critical role in the control of postnatal growth. Many of these actions are "activational", i.e., they are reversed in the absence of steroid. However, some actions of the steroids in development are irreversible or "organizational" and effectively "programme" adult physiology. Disturbances in the glucocorticoid milieu at critical stages of development may thus have long-term and potentially harmful effects on physiology. Perhaps not surprisingly, glucocorticoid levels are normally maintained within very narrow levels during development. Furthermore, in rodents at least, the neonate is refractory to stress and is thus protected from the potentially harmful effects of raised corticosteroid levels (Buckingham *et al*., 1997).

The Fetal Origin of Disease

Evidence that adverse events in early life may increase disease susceptibility in adulthood first emerged from the work of Barker's group, which noted the correlation between low birth weight and increased risk of cardiovascular and metabolic disorders in later life (Barker *et al*., 1989). Subsequent studies in

rodents, pigs, sheep, and humans linked malnutrition and stress in early life to an array of common adult pathologies, including hypertension, coronary heart disease, impaired glucose tolerance, hyperlipidemia, type 2 diabetes mellitus and CNS disturbances including anxiety and locomotor dysfunction. The molecular mechanisms underpinning these events are under extensive investigation, with evidence pointing to roles for genetic factors, nutrients, and mediators such as growth factors, cytokines, and hormones. Support for a role for glucocorticoids emerged from several sources. Firstly, malnutrition and early life stress produce permanent tissue-specific changes in GR expression, in particular, causing downregulation of GR within the HPA axis and thereby impairing the negative feedback regulation of glucocorticoid secretion in adulthood. Secondly, many of the pathological consequences of early life stress or malnutrition are similar to those of Cushing's syndrome, i.e., hypertension, insulin resistance, osteoporosis, and behavioral changes. Thirdly, the developing fetus is protected from fluctuations in maternal cortisol/corticosterone by the abundance of 11βHSD-2 in the placenta, which effectively captures and inactivates the steroid before it reaches the fetus. Correlations between low birth weight and placental 11βHSD-2 have been described. Moreover, rats exposed in utero to the nonselective 11βHSD-2 inhibitor, carbenoxolone, develop hypertension in adult life (Seckl, 2004).

More direct evidence of a role for glucocorticoids has emerged from studies in experimental animals exposed to dexamethasone during perinatal life. Unlike cortisol and corticosterone, dexamethasone is a poor substrate for 11βHSD-2 and, thus, passes readily from the mother to the developing fetus. Furthermore in the neonate, dexamethasone has ready access to brain tissues due to the immaturity of the ATP-binding–cassette transporter, P-glycoprotein in the blood–brain barrier.

Consequences of Perinatal Glucocorticoid Treatment

Early studies indicated that adult rats treated with glucocorticoids in the first seven days of postnatal life show significant changes in CNS function including loss of GRs, altered monoamine turnover, deficits in motor coordination, hyperreactivity with stereotypy, impaired conditioned avoidance, and disturbances in reproductive function (Benesova and Pavlik, 1989; Olton *et al*., 1975). Subsequent studies have focused mainly on the impact of glucocorticoid treatment during gestation.

In rats, prenatal dexamethasone treatment given either throughout gestation or during the week before parturition, produces tissue-specific changes in glucocorticoid receptor expression together with an array of metabolic, cardiovascular, neuroendocrine, and behavioral pathologies in adulthood (Kofman, 2002; Seckl, 2004). The exact profile is dependent on the time and duration of glucocorticoid exposure and the sex of the individual. Similar, but less extensive, findings have been reported in the sheep and the guinea pig. In the rat, prenatal dexamethasone treatment augments the basal and stress-induced activity of the adult HPA axis. These changes have been attributed in part to downregulation of the GRs within the HPA axis, in particular the hippocampus, and consequent diminution of the negative feedback effects of glucocorticoids on the secretion of ACTH, although

other adaptive responses may contribute to the overall effect (Theogaraj *et al*., 2005). The hyperactivity of the HPA axis is accompanied by hypertension in the adult rat. This may be partly explained by the raised corticosteroid levels, but the "programed" rats also exhibit altered responses to vasoactive substances such as endothelin and increased expression of type 1 and type 2 angiotensin receptors. Moreover, in the sheep the baroreceptor reflexes are impaired by antenatal glucocorticoid treatment (O'Regan *et al*., 2004; Seckl, 2004).

Prenatal dexamethasone treatment in both the rat and the sheep induces hyperglycemia and hyperinsulinemia in adulthood. These changes are accompanied not only by raised serum glucocorticoids, but also by increased GR expression in the liver and visceral fat pads and, hence, increased tissue sensitivity to the diabetogenic properties of the steroid. A consequence of this is the upregulation of hepatic phosphoenolpyruvate carboxykinase, an enzyme which is rate limiting in gluconeogenesis and which, when overexpressed, impairs glucose tolerance. The consequences of raised GR expression in adipose tissue require further study but may contribute to the manifestation of insulin resistance (Seckl, 2004; Seckl, 2004).

Diverse behavioral changes occur in adult rats subjected to antenatal glucocorticoid treatment, with increased fear and anxiety-type behaviors being reported by several groups (Kofman, 2002). These have been associated with changes in the amygdala where expression of the anxiogenic peptide, CRH, is increased. As glucocorticoids increase CRH expression in the central nucleus of the amygdala and basal corticosterone levels and GR expression in the amygdala are both augmented by antenatal glucocorticoid treatment, it is plausible that the glucocorticoids have a causal role. In addition, there is evidence that prenatal glucocorticoid treatment produces long-term changes in rodent central dopaminergic systems (Diaz *et al*., 1997; McArthur *et al*., 2005) and impairments of myelination in sheep.

Clinical Implications

To what extent the observations made in experimental animals can be translated to man is unclear. However, there is cause for concern as synthetic glucocorticoids, for example, dexamethasone, are used in perinatal medicine to mature the lung in conditions of threatened or actual preterm birth. A limited number of cohort studies have provided evidence of impairments in cognitive function and a higher incidence of cerebral palsy in children aged 4 years and of raised blood pressure and increased frequency of infectious disease. However, such data need to be verified by randomized controlled trials which take into account differences in the dosing regime and type of corticosteroid employed (Kofman, 2002; Newnham, 2001; Seckl, 2004).

GLUCOCORTICOIDS AND DISEASE

As indicated elsewhere in this review, glucocorticoids are increasingly implicated in the pathogenesis of common diseases, e.g. hypertension, obesity, type 2

diabetes, and immune/inflammatory disorders. In many cases, their pathological effects appear to be primarily related to dysregulation of the processes regulating the delivery of the steroids to their receptors, receptor dysfunction, or the potential consequences of inappropriate early life-programing, rather than alterations in cortisol secretion itself. However, abnormalities in HPA function have been described in a number of psychiatric conditions. For example, increased HPA activity is common in major depression, anorexia nervosa, obsessive compulsive disorders, and panic states, whereas HPA function is generally reduced in post-traumatic stress and seasonal affective disorders. Particular interest has centered on the role of cortisol in severe depression (Juruena *et al*., 2004).

Hyperactivity of the HPA axis in major depression is one of the most consistent findings in biological psychiatry. A substantial proportion of patients show raised concentrations of cortisol in the plasma, urine, and cerebrospinal fluid, exaggerated adrenocortical responses to exogenous ACTH and enlarged pituitary and adrenal glands. In addition, CRH levels in the hypothalamus and cerebrospinal fluid are increased and CRH is now strongly implicated in the manifestation of the disease symptoms (e.g., decreased appetite, psychomotor disturbances, and altered sleep patterns) (Juruena *et al*., 2004). These changes have been attributed in part to failure of the negative feedback effects of cortisol in the brain and pituitary gland, a hypothesis that is supported by evidence that depressed patients are relatively unresponsive to the suppressive effects of dexamethasone on basal and CRH-stimulated ACTH release and on CRH levels in the cerebrospinal fluid. These and other data have led to suggestions that cortisol resistance may have a causal role in the pathogenesis of depression (Pariante *et al*., 2004; Ridders *et al*., 2005). In support of this premise, a number of structurally unrelated antidepressant drugs augment brain GR expression and enhance the sensitivity of the HPA axis to glucocorticoid feedback inhibition when given long-term in both normal animals and in animal models of disease (Lopez *et al*., 1998; Pariante *et al*., 2004). These data are consistent with clinical reports that the beneficial effects of antidepressant drugs are associated with normalization of HPA function as also do data suggesting that antidepressants augment GR signaling by inhibiting the membrane steroid transporters and, thereby, increasing the intracellular concentrations of the steroid (Muller *et al*., 2004; Pariante *et al*., 2004). These and other data support the premise that defective cortisol signaling plays an important part in the pathogenesis of depression and that characterization of the underlying molecular defects may identify new targets for therapeutic intervention.

CONCLUSIONS

A short review cannot possibly do justice to the literature surrounding the HPA axis. Suffice it to say that, in recent years our understanding of the biology of the glucocorticoids and the mechanisms which control their secretion has increased exponentially as increasing layers of complexity have been uncovered and explored. These findings have given significant insight to the critical role of these steroid

hormones in the maintenance of homeostasis and, when dysregulated, the pathogenesis of disease. They have also made major inroads to our understanding of the molecular basis of the advantageous and unwanted effects of the steroids as drugs, and paved the way for the development of more selective compounds.

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REFERENCES

Aguilera G, Jessop DS, Harbuz MS, et al. Differential regulation of hypothalamic pituitary corticotropin releasing hormone receptors during development of adjuvant-induced arthritis in the rat. J Endocrinol 1997; 153:185–191.

Aguilera G, Nikodemova M, Wynn PC, et al. Corticotropin releasing hormone receptors: Two decades later. Peptides 2004; 25:319–329.

Al-Dujaili EA, Hope J, Estivariz FE, et al. Circulating human pituitary pro-gamma-melanotropin enhances the adrenal response to ACTH. Nature 1981; 291:156–159.

Antoni FA. Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. Front Neuroendocrinol 1993; 14:76–122.

Barker DJ, Osmond C, Golding J, et al. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. Bmj 1989; 298:564–567.

Barnes PJ, Adcock IM. How do corticosteroids work in asthma? Ann Intern Med 2003; 139:359–370.

Beato M, Herrlich P, Schutz G. Steroid hormone receptors: Many actors in search of a plot. Cell 1995; 83:851–857.

Benesova O, Pavlik A. Perinatal treatment with glucocorticoids and the risk of maldevelopment of the brain. Neuropharmacology 1989; 28:89–97.

Bicknell AB, Lomthaisong K, Woods RJ, et al. Characterization of a serine protease that cleaves pro-gamma-melanotropin at the adrenal to stimulate growth. Cell 2001; 105;903–912.

Bicknell AB. The tissue-specific processing of pro-opiomelanocortin. J Neuroendocrinol 2008; 20:692–699.

Brown RW, Diaz R, Robson AC, et al. The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. Endocrinology 1996; 137:794–797.

Brunton PJ, Russell JA. The expectant brain: Adapting for motherhood. Nat Rev Neurosci 2008; 9:11–25.

Buckingham JC, Christian HC, Gillies GE, et al. The hypothalmo-pituitary-adrenocortical immune axis. In: Kendall MD, Marsh JA, eds. The Physiology of Immunity. Florida, USA: CRC Press Inc; 1996:331–354.

Buckingham JC, Flower RJ, Kikkert R, et al. Secretion of corticotrophin-releasing hormone and arginine vasopressin by the neonatal rat hypothalamus in vitro: Age-dependent

maturation of the responses to cytokines and modulation by glucocorticoids and lipocortin 1. Dev Brain Dys 1997; 10:482–502.
Buckingham JC. The influence of vasopressin on hypothalamic corticotrophin releasing activity in rats with inherited diabetes insipidus. J Physiol 1981; 312:9–16.
Buckingham JC. Glucocorticoids, role in stress. In: Fink G, ed. Encyclopaedia of Stress. San Diego: Academic Press; 2002:261–270.
Buckingham JC. Glucocorticoids: Exemplars of multi-tasking. Br J Pharmacol 2006; 147(Suppl 1):S258–S268.
Buss NAPS, John CD, Cover PO, et al. Modulation of the expression of mRNAs for annexin 1 and formyl peptide receptors following inflammatory stress. PA2 Online 2008; 119.
Chan LF, Clark AJ, Metherell LA. Familial glucocorticoid deficiency: Advances in the molecular understanding of ACTH action. Horm Res 2008; 69:75–82.
Chapman L, Nishimura A, Buckingham JC, et al. Externalization of annexin I from a folliculo-stellate-like cell line. Endocrinology 2002; 143:4330–4338.
Choi DC, Evanson NK, Furay AR, et al. The anteroventral bed nucleus of the stria terminalis differentially regulates hypothalamic-pituitary-adrenocortical axis responses to acute and chronic stress. Endocrinology 2008; 149:818–826.
Chowdrey HS, Larsen PJ, Harbuz MS, et al. Evidence for arginine vasopressin as the primary activator of the HPA axis during adjuvant-induced arthritis. Br J Pharmacol 1995; 116:2417–2424.
De Kloet ER, Vreugdenhil E, Oitzl MS, et al. Brain corticosteroid receptor balance in health and disease. Endocr Rev 1998; 19:269–301.
De Kloet ER, Karst H, Joels M. Corticosteroid hormones in the central stress response: Quick-and-slow. Front Neuroendocrinol 2008; 29:268–272.
De Kloet ER. About stress hormones and resilience to psychopathology. J Neuroendocrinol 2008; 20:885–892.
De Quervain DJ, Poirier R, Wollmer MA, et al. Glucocorticoid-related genetic susceptibility for Alzheimer's disease. Hum Mol Genet 2004; 13:47–52.
Diaz R, Fuxe K, Ogren SO. Prenatal corticosterone treatment induces long-term changes in spontaneous and apomorphine-mediated motor activity in male and female rats. Neuroscience 1997; 81:129–140.
Draper N, Walker EA, Bujalska IJ, et al. Mutations in the genes encoding 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. Nat Genet 2003; 34:434–439.
Droste SK, De Groote L, Atkinson HC, et al. Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to forced swim stress. Endocrinology 2008.
Edwards CR, Stewart PM, Burt D, et al. Localisation of 11 beta-hydroxysteroid dehydrogenase–tissue specific protector of the mineralocorticoid receptor. Lancet 1988; 2:986–989.
Edwards CR, Benediktsson R, Lindsay RS, et al. 11 beta-Hydroxysteroid dehydrogenases: Key enzymes in determining tissue-specific glucocorticoid effects. Steroids 1996; 61:263–269.
Estivariz FE, Morano MI, Carino M, et al. Adrenal regeneration in the rat is mediated by mitogenic N-terminal pro-opiomelanocortin peptides generated by changes in precursor processing in the anterior pituitary. J Endocrinol 1988; 116:207–216.
Flower RJ, Blackwell GJ. Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. Nature 1979; 278:456–459.

Funder JW, Pearce PT, Smith R, et al. Mineralocorticoid action: Target tissue specificity is enzyme, not receptor, mediated. Science 1988; 242:583–585.
Funder JW. Mineralocorticoid receptors: Distribution and activation. Heart Fail Rev 2005; 10:15–22.
Giacomini D, Acuna M, Gerez J, et al. Pituitary action of cytokines: Focus on BMP-4 and gp130 family. Neuroendocrinology 2007; 85:94–100.
Gillies GE, Linton EA, Lowry PJ. Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. Nature 1982; 299:355–357.
Gold PW, Drevets WC, Charney DS. New insights into the role of cortisol and the glucocorticoid receptor in severe depression. Biol Psychiatry 2002; 52:381–385.
Goulding NJ. The molecular complexity of glucocorticoid actions in inflammation: A four-ring circus. Curr Opin Pharmacol 2004; 4:629–636.
Grinevich V, Harbuz M, Ma XM, et al. Hypothalamic pituitary adrenal axis and immune responses to endotoxin in rats with chronic adjuvant-induced arthritis. Exp Neurol 2002; 178:112–123.
Harbuz MS, Rees RG, Eckland D, et al. Paradoxical responses of hypothalamic corticotropin-releasing factor (CRF) messenger ribonucleic acid (mRNA) and CRF-41 peptide and adenohypophysial proopiomelanocortin mRNA during chronic inflammatory stress. Endocrinology 1992; 130:1394–1400.
Harbuz MS, Leonard JP, Lightman SL, et al. Changes in hypothalamic corticotrophin-releasing factor and anterior pituitary pro-opiomelanocortin mRNA during the course of experimental allergic encephalomyelitis. J Neuroimmunol 1993; 45:127–132.
Harbuz MS, Chover-Gonzalez AJ, Biswas S, et al. Role of central catecholamines in the modulation of corticotrophin-releasing factor mRNA during adjuvant-induced arthritis in the rat. Br J Rheumatol 1994; 33:205–209.
Harbuz MS, Marti O, Lightman SL, et al. Alteration of central serotonin modifies onset and severity of adjuvant-induced arthritis in the rat. Br J Rheumatol 1998; 37:1077–1083.
Herman JP, Adams D, Prewitt C. Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Neuroendocrinology 1995; 61:180–190.
John CD, Christian HC, Morris JF, et al. Annexin 1 and the regulation of endocrine function. Trends Endocrinol Metab 2004; 15:103–109.
John CD, Sahni V, Mehet D, et al. Formyl peptide receptors and the regulation of ACTH secretion: Targets for annexin A1, lipoxins, and bacterial peptides. Faseb J 2007; 21:1037–1046.
Juruena MF, Cleare AJ, Pariante CM. The hypothalamic pituitary adrenal axis, glucocorticoid receptor function and relevance to depression. Rev Bras Psiquiatr 2004; 26:189–201.
Kalantaridou S, Makrigiannakis A, Zoumakis E, et al. Peripheral corticotropin-releasing hormone is produced in the immune and reproductive systems: Actions, potential roles and clinical implications. Front Biosci 2007; 12:572–580.
Kawata M, Nishi M, Matsuda K, et al. Steroid receptor signalling in the brain: Lessons learned from molecular imaging. J Neuroendocrinol 2008; 20:673–676.
Kofman O. The role of prenatal stress in the etiology of developmental behavioral disorders. Neurosci Biobehav Rev 2002; 26:457–470.
Kotelevtsev Y, Brown RW, Fleming S, et al. Hypertension in mice lacking 11beta-hydroxysteroid dehydrogenase type 2. J Clin Invest 1999; 103:683–689.
Labrie F, Giguere V, Meunier H, et al. Multiple factors controlling ACTH secretion at the anterior pituitary level. Ann N Y Acad Sci 1987; 512:97–114.

Laye S, Bluthe RM, Kent S, et al. Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS. Am J Physiol 1995; 268:R1327–R1331.

Lolait SJ, Stewart LQ, Roper JA, et al. Attenuated stress response to acute lipopolysaccharide challenge and ethanol administration in vasopressin V1b receptor knockout mice. J Neuroendocrinol 2007; 19:543–551.

Lopez JF, Chalmers DT, Little KY, et al. A.E. Bennett Research Award. Regulation of serotonin1 A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. Biol Psychiatry 1998; 43:547–573.

Loxley HD, Cowell AM, Flower RJ, et al. Effects of lipocortin 1 and dexamethasone on the secretion of corticotrophin-releasing factors in the rat: in vitro and in vivo studies. J Neuroendocrinol 1993; 5:51–61.

McArthur S, McHale E, Dalley JW, et al. Altered mesencephalic dopaminergic populations in adulthood as a consequence of brief perinatal glucocorticoid exposure. J Neuroendocrinol 2005; 17:475–482.

Meijer OC, Karssen AM, De Kloet ER. Cell- and tIssue-specific effects of corticosteroids in relation to glucocorticoid resistance: Examples from the brain. J Endocrinol 2003; 178:13–18.

Muller MB, Uhr M, Holsboer F, et al. Hypothalamic-pituitary-adrenocortical system and mood disorders highlights from mutant mice. Neuroendocrinology 2004; 79:1–12.

Munck A, Naray-Fejes-Toth A. The ups and downs of glucocorticoid physiology. Permissive and suppressive effects revisited. Mol Cell Endocrinol 1992; 90:C1–C4.

Newnham JP. Is prenatal glucocorticoid administration another origin of adult disease? Clin Exp Pharmacol Physiol 2001; 28:957–961.

O'Regan D, Kenyon CJ, Seckl JR, et al. Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology. Am J Physiol Endocrinol Metab 2004; 287:E863–E870.

Olton DS, Johnson CT, Howard E. Impairment of conditioned active avoidance in adult rats given corticosterone in infancy. Dev Psychobiol 1975; 8:55–61.

Ostrander MM, Ulrich-Lai YM, Choi DC, et al. Hypoactivity of the hypothalamo-pituitary-adrenocortical axis during recovery from chronic variable stress. Endocrinology 2006; 147:2008–2017.

Pariante CM, Thomas SA, Lovestone S, et al. Do antidepressants regulate how cortisol affects the brain? Psychoneuroendocrinology 2004; 29:423–447.

Pearson Murphy BE. Glucocorticoids overview. In: Kink G, eds. Encyclopaedia of Stress. San Diego: Academic Press, 2002:244–260.

Plotsky PM, Sawchenko PE. Hypophysial-portal plasma levels, median eminence content, and immunohistochemical staining of corticotropin-releasing factor, arginine vasopressin, and oxytocin after pharmacological adrenalectomy. Endocrinology 1987; 120:1361–1369.

Reichardt HM, Kaestner KH, Tuckermann J, et al. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 1998; 93:531–541.

Richards LJ, Chover-Gonzalez A, Harbuz MS, et al. Protective effects of endotoxin in a rat model of chronic inflammation are accompanied by suppressed secretion of pro-inflammatory cytokines and biphasic alteration in hypothalamo-pituitary-adrenal axis activity. J Neuroendocrinol 2006; 18:875–882.

Ridders S, Chourbaji S, Hellweg R, et al. Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. J Neurosci 2005; 25:6243–6250.

Sandeep TC, Yau JL, MacLullich AM, et al. 11 beta-hydroxysteroid dehydrogenase inhibition improves cognitive function in healthy elderly men and type 2 diabetics. Proc Natl Acad Sci U S A 2004; 101:6734–6739.

Sapolsky R, Rivier C, Yamamoto G, et al. Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. Science 1987; 238:522–524.

Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 2000; 21:55–89.

Seale JV, Wood SA, Atkinson HC, et al. Gonadectomy reverses the sexually diergic patterns of circadian and stress-induced hypothalamic-pituitary-adrenal axis activity in male and female rats. J Neuroendocrinol 2004; 16:516–524.

Seale JV, Wood SA, Atkinson HC, et al. Gonadal steroid replacement reverses gonadectomy-induced changes in the corticosterone pulse profile and stress-induced hypothalamic-pituitary-adrenal axis activity of male and female rats. J Neuroendocrinol 2004; 16:989–998.

Seckl JR. 11 beta-hydroxysteroid dehydrogenases: changing glucocorticoid action. Curr Opin Pharmacol 2004; 4:597–602.

Seckl JR. Prenatal glucocorticoids and long-term programming. Eur J Endocrinol 2004; 151(Suppl 3):U49–U62.

Shibata M, Fujihara H, Suzuki H, et al. Physiological studies of stress responses in the hypothalamus of vasopressin-enhanced green fluorescent protein transgenic rat. J Neuroendocrinol 2007; 19:285–292.

Simpkiss JL, Devine DP. Responses of the HPA axis after chronic variable stress: Effects of novel and familiar stressors. Neuro Endocrinol Lett 2003; 24:97–103.

Solito E, Mulla A, Morris JF, et al. Dexamethasone induces rapid serine-phosphorylation and membrane translocation of annexin 1 in a human folliculostellate cell line via a novel nongenomic mechanism involving the glucocorticoid receptor, protein kinase C, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase. Endocrinology 2003; 144:1164–1174.

Stevens A, Donn R, Ray D. Regulation of glucocorticoid receptor gamma (GR gamma) by glucocorticoid receptor haplotype and glucocorticoid. Clin Endocrinol (Oxf) 2004; 61:327–331.

Stewart PM, Corrie JE, Shackleton CH, et al. Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. J Clin Invest 1988; 82:340–349.

Theogaraj E, John CD, Christian HC, et al. Perinatal glucocorticoid treatment produces molecular, functional, and morphological changes in the anterior pituitary gland of the adult male rat. Endocrinology 2005; 146:4804–4813.

Vale W, Speiss J, Rivier C, et al. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 1981; 213:1394–1397.

Vamvakopoulos NC, Chrousos GP. Hormonal regulation of human corticotropin-releasing hormone gene expression: Implications for the stress response and immune/inflammatory reaction. Endocr Rev 1994; 15:409–220.

Vinson GP. Hormone receptors and signal transduction. In: Buckingham JC, Gillies G.E, Cowell AM, eds. Stress, Stress Hormones and the Immune System. Chichester, UK: J Wiley & Sons Ltd; 1997:95–126.

Webster JI, Carlstedt–Duke J. Involvement of multidrug resistance proteins (MDR) in the modulation of glucocorticoid response. J Steroid Biochem Mol Biol 2002; 82:277–288.

Windle RJ, Wood SA, Kershaw YM, et al. Increased corticosterone pulse frequency during adjuvant-induced arthritis and its relationship to alterations in stress responsiveness. J Neuroendocrinol 2001; 13:905–911.

Wintermantel TM, Berger S, Greiner EF, et al. Evaluation of steroid receptor function by gene targeting in mice. J Steroid Biochem Mol Biol 2005; 93:107–112.

Yang–Yen HF, Chambard JC, Sun YL, et al. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 1990; 62:1205–1215.

Part III

Adrenal Toxicology In Vivo and In Vitro

4

The Adrenal Medulla as a Target Organ in Toxicologic Studies of Rats and Mice

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INTRODUCTION

Among the endocrine organs, the adrenal gland is reportedly the most susceptible to compound-induced lesions (Ribelin, 1984). The cells of the adrenal medulla are derived from neural crest ectoderm in contrast to the mesodermal origin of the adrenal cortex. The different origins and functions of the two components of the adrenal make them susceptible to different toxicologic insults and evoke different types of responses. In the medulla, proliferative lesions are the most common pathological finding. There can also be degenerative changes, although these are less common. The proliferative lesions include diffuse or nodular hyperplasia; benign, malignant, or complex pheochromocytoma; ganglioneuroma; and neuroblastoma. Proliferative lesions can be spontaneous or can be induced by chemicals

and dietary modifications. In general, both spontaneous and xenobiotic-induced medullary proliferative lesions tend to be much more frequent in rats than in mice and, in rats, most studies report a higher incidence in males than in females (Tischler *et al*., 1997; Tischler, 1994).

NORMAL STRUCTURE AND FUNCTION OF THE ADRENAL MEDULLA

The structure and anatomic organization of the adrenal medulla were extensively and meticulously studied by RE Coupland, whose classic book *The Natural History of the Chromaffin Cell* (Coupland, 1965) began the modern era of chromaffin cell biology (reviewed in detail in Ref. Tischler, 1994). The medulla constitutes only about 10% of total adrenal weight, and is surrounded by cortical tissue except at the hilus where blood vessels enter the gland. Medullary cells can also penetrate to the capsule at the hilar region. The boundary between the cortex and medulla is typically well-defined, but irregular. Branches of the renal and suprarenal arteries penetrate the adrenal capsule at the hilus, and supply numerous capillaries to the cortical regions and primary arterioles to the medullary regions of the left and right adrenal glands, respectively. The cortical capillaries also supply blood to the medulla via cortical-medullary sinusoids, thereby exposing medullary cells to blood rich in glucocorticoids. Blood leaves the adrenal gland via the medullary veins.

The medulla is composed principally of neuroendocrine cells known as chromaffin cells, a term applied more than a century ago based on the "chromaffin reaction"—a brown coloration observed after the tissue was immersed in chromate salts. The reaction was originally believed to indicate a specific affinity for chromium, but subsequently proved to be due principally to oxidation of stored catecholamines. Chromaffin cells are polyhedral neuroendocrine cells arranged in small packets or short branching cords separated by an extensive vascular network. They contain pale basophilic finely granular cytoplasm and vesicular nuclei. A few neurons of at least two types (Holgert *et al*., 1996) and both myelinated and unmyelinated nerve fibers are scattered throughout the medulla, although they are not always easily observed without the aid of special stains or immunohistochemistry.

The adrenal medulla is an important neuroendocrine organ that secretes the catecholamines epinephrine (E), also known as adrenaline, and norepinephrine (NE), also known as noradrenaline, in response to stressful conditions. Other constituents of neuroendocrine secretory granules are cosecreted. Chromaffin cells originate from the neural crest during embryogenesis and are regulated by the sympathetic nervous system for the life of the animal. The active secretion of the adrenal medulla contains approximately 80% epinephrine and 20% norepinephrine. Correspondingly, the rodent adrenal medulla contains a separate population of NE-cells that comprise approximately 20% of the total chromaffin cell population.

The production and secretion of epinephrine and norepinephrine are regulated by both neural and hormonal signals. The central nervous system input to the

adrenal medulla is from the medulla oblongata, pons, and hypothalamus via the thoracolumbar spinal cord. Preganglionic sympathetic nerve fibers from the spinal cord directly synapse on chromaffin cells and catecholamines are released by stimulation of cholinergic and peptidergic receptors. A small amount of innervation from sensory ganglia and intrinsic neurons is also present. Different neuropeptide content of nerve endings, innervating E-cells and NE-cells suggests that innervation of the two chromaffin cell populations is derived from partly different sources and that the cells respond to partly different stimuli (Holgert *et al*., 1996). Transsynaptic signals and glucocorticoids from adrenal cortical blood induce the expression of catecholamine biosynthetic enzymes, thereby maintaining catecholamine stores. To a lesser extent, released catecholamines reciprocally influence adrenal cortical steroidogenesis (Schinner and Bornstein, 2005).

The adrenal medulla is essentially a modified sympathetic ganglion. The synthesis and storage of catecholamines is qualitatively similar in chromaffin cells and sympathetic neurons, with one major exception. Only adrenergic chromaffin cells express the enzyme phenylethanolamine *N*-methyltransferase (PNMT), which methylates norepinephrine to produce epinephrine, while both neurons and noradrenergic chromaffin cells lack PNMT. Although all three of these closely related cell types produce norepinephine, in adrenergic chromaffin cells the majority of norepinephrine is converted to epinephrine by this additional enzyme (Wong, 2003).

Epinephrine is the "fight or flight" hormone, resulting in short-term stress reactions which include increased heart rate, stroke volume, and cardiac contraction strength, dilated pupils, bronchial dilatation, and constriction of arterioles in the skin and gut while dilating arterioles in skeletal muscles and liver. It elevates blood sugar level by increasing catalysis of glycogen to glucose in the liver, and at the same time begins the breakdown of lipids in fat cells. Epinephrine also exerts a suppressive effect on the immune system. Norepinephrine exerts effects similar to those of epinephrine, although it will constrict almost all blood vessels. Norepinephrine also increases the rate and force of contraction of the heart and increases glycogenolysis, thus increasing the level of circulating free fatty acids.

In addition to the classic hormones epinephrine and norepinephrine, chromaffin cells contain a complex mixture of proteins and peptides that are also stored in secretory granules and released by cholinergic stimulation. These include granin proteins, neuropeptides such as neuropeptide Y, Met-enkephalin, adrenomedullin, and calcitonin gene-related peptide, and several enzymes that are involved in hormone synthesis or processing but may also serve other purposes. The major constituents of chromaffin cell secretory granules by weight are the granin proteins (Feldman and Eiden, 2003; Helle, 2004), chromogranins A and B and, to a lesser extent, secretogranin II (chromogranin C). In addition to proposed roles in secretory granule biogenesis, these proteins serve as multifunctional prohormones, differentially expressed and processed in different tissues to give rise to biologically active fragments with autocrine, paracrine, or systemic effects (Feldman and Eiden, 2003; Helle, 2004). The latter include an antimicrobial effect that could

boost the immune system, when it is suppressed by epinephrine during times of stress. Fragments of chromogranin A also stimulate insulin release and relax blood vessels—functions similarly reduced by epinephrine during stress. Neuropeptide Y, which is present in nerve fibers within the adrenal cortex and medulla as well as in the medullary chromaffin cells, (Majane *et al*., 1985) exhibits potent stimulatory actions on vascular smooth muscle (Hexum *et al*., 1987). Adrenomedullin (ADM) is a potent hypotensive peptide that modulates adrenal gland secretion, inhibits agonist-stimulated aldosterone secretion, is an indirect suppressor of the immune system, a potent angiogenic factor, and a survival factor for cancer cells (Mazzocchi *et al*., 1999; Nakamura *et al*., 2006). Calcitonin gene-related peptide is also a potent hypotensive peptide that belongs to the peptide family that includes ADM. Calcitonin gene-related peptide has similar functions to ADM such as inhibition of agonist-stimulated aldosterone secretion and stimulation of basal catecholamine release (Tortorella *et al*., 2001).

CHARACTERIZATION OF THE ADRENAL MEDULLA BY IMMUNOHISTOCHEMISTRY IN NORMAL AND PATHOLOGICAL STATES

The chromaffin reaction is obsolete. Immunohistochemical staining procedures applicable to paraffin sections and routinely available in most pathology laboratories can be used to identify subsets of normal adrenal medullary cells and to diagnose and functionally characterize adrenal medullary proliferative lesions (Hill *et al*., 2003; Tischler *et al*., 1990). Although numerous neural and endocrine markers are potentially applicable, only a few are required for the practical purposes of differential diagnosis and functional assessment. Immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, will readily distinguish all chromaffin cells and their proliferative lesions from normal or proliferative adrenal cortex. Proliferative chromaffin cell lesions also usually show extensive staining for chromogranin A (CgA), which is the single most specific and reliable generic neuroendocrine marker currently utilized in pathology practice. However, in contrast to TH, expression of CgA will not discriminate adrenal medullary tumors from other neuroendocrine neoplasms, e.g., from the lung or Gastrointestinal tract, which may be a concern in determining the origin of metastases to the lung or liver. In addition, poorly differentiated tumors may contain sparse secretory granules and therefore stain only weakly for CgA, while TH which is a cytosolic enzyme, is not dependent on granule content. Adrenergic or noradrenergic function can be inferred, respectively, by the presence or absence of immunoreactive PNMT (Fig. 1).

Although immunohistochemistry is an extremely valuable and relatively straightforward technique, a caveat is that it must be applied judiciously and with appreciation of potential artefacts. The latter include technical artefacts such as enhancement of endogenous biotin staining by heat-based antigen retrieval methods in staining protocols that employ a biotin bridge (Srivastava *et al*., 2004),

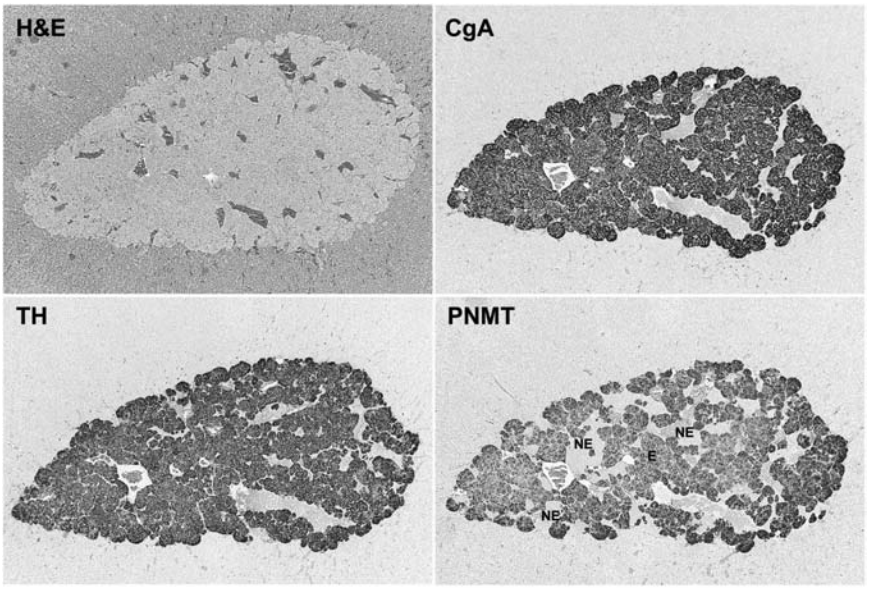

Figure 1 (*See color insert*) Immunohistochemical characterization of adrenal medullary cells. Immunoreactivity for tyrosine hydroxylase (TH), which is an enzyme required for all catecholamine synthesis, will distinguish chromaffin cells and their proliferative lesions from normal or proliferative adrenal cortical cells. Phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that synthesizes epinephrine (E) from norepinephrine (NE), can be used to infer the expression of an adrenergic phenotype. The islands of chromaffin cells that lack PNMT are noradrenergic Chromogranin A (CgA), which is a major constituent of the matrices of neuroendocrine secretory granules, is another reliable marker of chromaffin cells. However, unlike TH, it will not discriminate adrenal medullary tumors from other neuroendocrine tumors.

nonspecific staining of E-cells by serum (Tischler *et al*., 1998), and cross-reactivities of commercially available antibodies that are often of dubious quality (Rhodes and Trimmer, 2006). Mitochondria often exhibit nonspecific interactions with antibodies, as does lipofuscin in the adrenal cortex. Nonspecific staining by any given antibody is not predictable. Moreover, no controls available in routine diagnostic immunohistochemistry are wholly adequate, including substitution of normal serum or IgG in place of primary antibody. Excellent monoclonal antibodies are widely available for TH. Most antibodies that will recognize rodent CgA or PNMT are polyclonal and must be carefully validated.

SPONTANEOUS (AGING) LESIONS

The spectrum of reported spontaneous age-related lesions in the adrenal medulla includes hyperplasia, benign and malignant pheochromocytoma, complex

Table 1 Criteria for Proliferative Lesions of the Rodent Adrenal Medulla

Focal Hyperplasia
• Circumscribed focus of medullary cells that blends with surrounding normal parenchyma • No or minimal compression • Minimal alteration in architecture with cells arranged in packets or solid clusters slightly larger than normal • Minimal to mild alteration in size, shape, and staining qualities of affected cells (nuclei and cytoplasm)
Pheochromocytoma, benign
• Well-delineated mass of medullary cells • Minimal to marked compression of surrounding parenchyma • Altered architecture with cells arranged in large solid clusters or thick trabeculae; growth pattern may be variable • Mild to marked alteration in size, shape, and staining qualities of affected cells; cellular atypia and pleomorphism may be marked
Pheochromocytoma, malignant
• Invasion of capsule and periadrenal soft tissue • Metastasis

Source: Adapted from Hamlin and Banas, 1990

pheochromocytoma, ganglioneuroma, and neuroblastoma. The incidences of these lesions vary among rodent species and strains.

The incidences of both medullary hyperplasia and neoplasia increase with age and the histological differences represent a morphological continuum, with "gray areas" that can sometimes present a diagnostic dilemma. Hamlin and Banas (Hamlin and Banas, 1990) have suggested useful criteria for proliferative lesions of the adrenal medulla in rats (Table 1) (Hamlin and Banas, 1990). In both rats and mice, chromaffin cell hyperplasia can be either focal or diffuse. When focal, the lesions may be unilateral or bilateral and are often multiple. Although these hyperplastic foci may occur anywhere in the medulla, they appear to most often arise near the corticomedullary junction (Fig. 2), suggesting that cortical steroids might contribute to cell growth or survival in these lesions. The hyperplastic cells blend with the surrounding normal parenchyma with little to no compression of the surrounding tissue or cortical intrusion. The architecture is minimally altered, with cells typically arranged in packets or clusters. These cells are usually more basophilic and smaller than adjacent chromaffin cells (Fig. 2). Less frequently, these cells may be the same size as, or larger than, the surrounding chromaffin cells, with abundant cytoplasm, vesicular nuclei, and prominent nucleoli (Fig. 3). A similar morphological spectrum is seen in pheochromocytomas. Diffuse

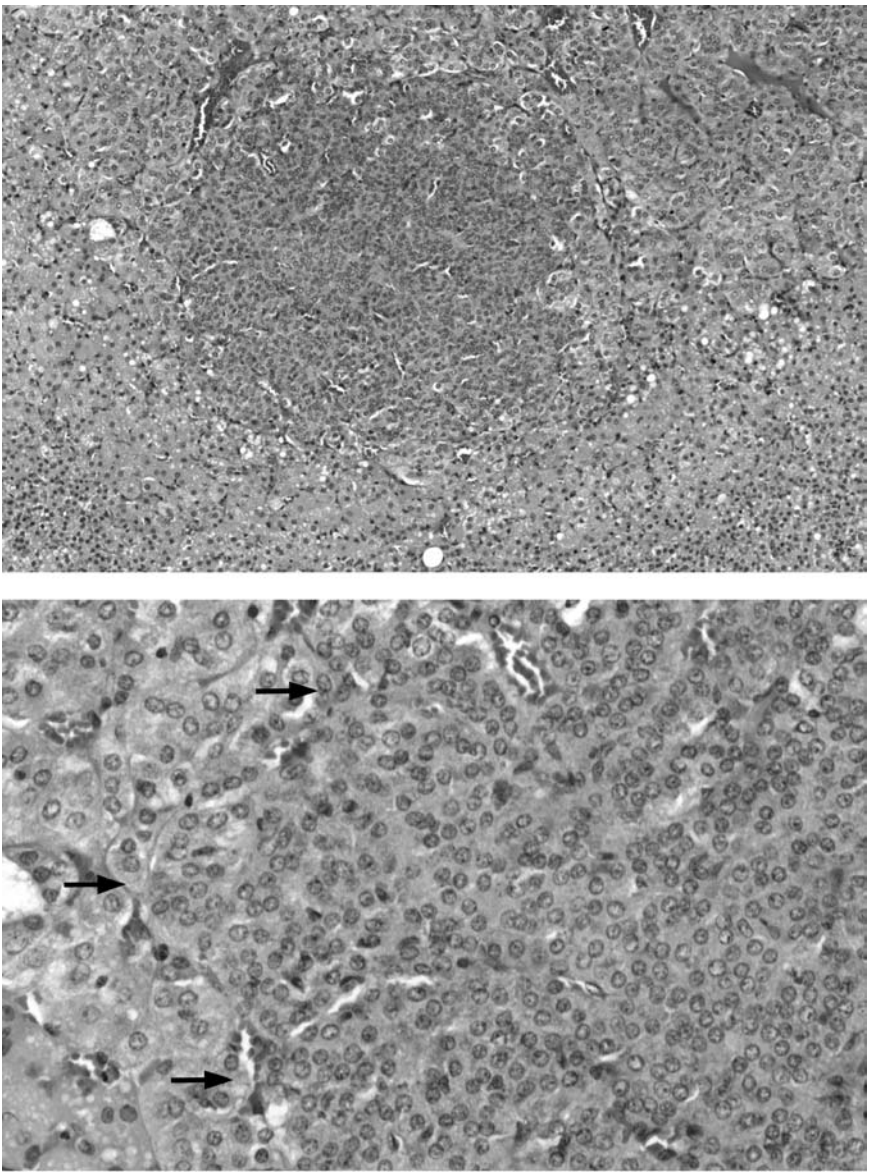

Figure 2 (*See color insert*) Focal chromaffin cell hyperplasia in a male F344 rat that is located at the corticomedullary junction. At low magnification, this focal lesion is hypercellular, slightly more basophilic than the adjacent medulla, with no significant compression of the surrounding cortical or medullary cells. At higher magnification (lower panel), the arrows indicate the junction with the larger normal chromaffin cells to the left and the smaller hyperplastic chromaffin cells to the right. The hyperplastic cells are uniform in appearance and arranged in clusters or packets.

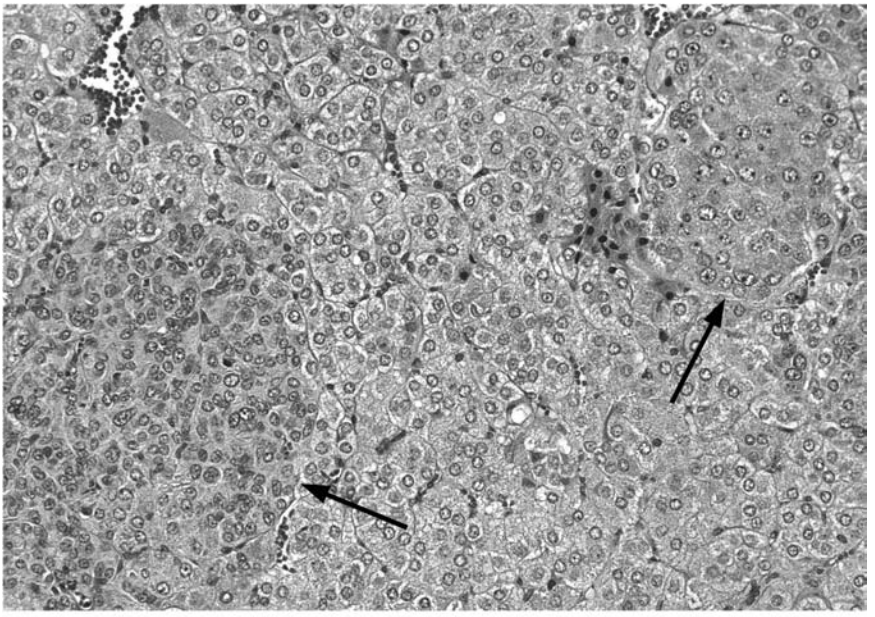

Figure 3 (*See color insert*) This figure contrasts the usual morphology of chromaffin cell hyperplasia (*small cells, left arrow*) with a less common lesion (*right arrow*) in which the hyperplastic cells are larger than the surrounding normal chromaffin cells and have abundant cytoplasm, vesicular nuclei, and prominent nucleoli.

hyperplasia is usually bilateral. The medullary region may be larger than normal and may extend into the cortex without compression of cortical tissue. In mice, the spontaneous incidence of medullary hyperplasia is strain-dependent, but it is not known if a higher incidence of hyperplasia correlates with a higher incidence of neoplasia. There is a higher spontaneous incidence of medullary hyperplasia in rats compared to mice but the incidences are also strain-dependent and vary with sex, diet, endocrine conditions, and environmental factors. In the rat, medullary hyperplastic lesions are clinically nonfunctional. However, they are capable of catecholamine biosynthesis as shown by immunohistochemical staining for TH. They are usually noradrenergic, as indicated by the absence of immunoreactive PNMT (Fig. 4) and, in contrast to the normal adrenal medulla, contain little or no innervation (Tischler *et al*., 1999). In the rat, these lesions may sometimes be seen in conjunction with hyperplastic lesions of other endocrine glands such as the pancreatic islets, thyroid C cells, and pituitary pars distalis, thereby resembling unusual mixed multiple endocrine neoplasia syndromes that occasionally occur in humans (Lee *et al*., 1982; Pellegata *et al*., 2006).

Pheochromocytomas are catecholamine-producing tumors of the adrenal medulla. The term (derived from the Greek *phaios*, i.e., dusky, and *chroma*, i.e., color) was intended to allude to the color change imparted by the chromaffin

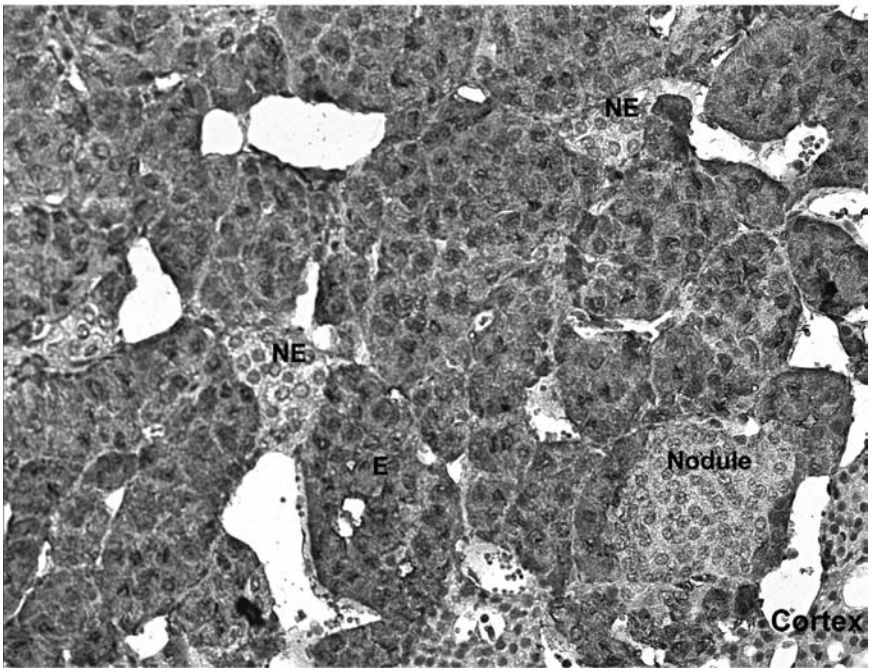

Figure 4 (*See color insert*) In the rat, medullary hyperplastic lesions are usually noradrenergic. Note the absence of immunoreactive PNMT within the hyperplastic nodule compared to the positive reaction within the surrounding normal medullary adrenergic chromaffin cells. *Abbreviations*: E, Epinephrine cells; NE, Norepinephrine cells.

reaction without denoting a mechanism for it. Spontaneous pheochromocytomas are common in rats but rare in mice, except in recently developed genetically engineered mouse strains. Their incidence varies among rat strains (Tischler *et al*., 1989; Tischler *et al*., 1997), with high incidences in strains including F344, Wistar, Long-Evans, and Sprague Dawley. Rat strains with low incidences of pheochromocytomas include Osborne-Mendel, Charles River, Holtzman, and WAG/Rij. Other factors related to the incidence of pheochromocytomas include chronic elevated levels of growth hormone or prolactin associated with pituitary tumors, stimulation of the autonomic nervous system and dietary factors. Food restriction and modifications of nutrient content were shown to markedly decrease the frequency of adrenal medullary hyperplasia and pheochromocytomas in control animals in toxicologic studies conducted after the 1990s (Haseman *et al*., 2003). In most rat studies, the spontaneous lesions are more common in males than females. NTP historical control data for spontaneous pheochromocytomas (2007) show the male predisposition in F344 rats. This sex predilection is not seen in mice (Table 2).

Pheochromocytomas may be single or multiple and unilateral or bilateral. In the rat, they are often bilateral and multicentric whereas in the mouse they

Table 2 Incidence of Adrenal Medullary Tumors in Control Animals from NTP Studies, all Routes, all Vehicles, October, 2007[a]

	Male B6C3F1 mice	Female B6C3F1 mice	Male F344 rats	Female F344 rats
Pheochromocytoma, Benign	4/1135	14/1239	208/1443	32/1339
Pheochromocytoma, Malignant	4/1135	7/1239	34/1443	11/1339
Pheochromocytoma, Complex	0/1135	1/1239	5/1443	2/1339
Ganglioneuroma	0/1135	0/1239	2/1443	0/1339
Neuroblastoma	0/1135	0/1239	1/1443	0/1339

[a]http://ntp.niehs.nih.gov.

are more frequently unilateral. They are typically well-delineated aggregates of neoplastic medullary cells, which may protrude into the cortex with variable compression of the surrounding parenchyma. The neoplastic cells may be arranged in clusters, sheets, or trabecular cords several layers thick (Fig. 5). The sinusoids may be ectatic and blood-filled. Cellular pleomorphism is common and may occur within different areas of the same tumor. The individual cells may vary from large polygonal cells with prominent cytoplasm to small fusiform cells with scant basophilic cytoplasm. The nuclear size and chromatin features may vary as well, ranging from large vesicular nuclei with prominent nucleoli to small hyperchromatic nuclei. Mouse pheochromocytomas can be either adrenergic or noradrenergic (Hill *et al*., 2003). Focal PNMT expression is sometimes seen in tumors that, overall, are PNMT-negative. Until recently, rat pheochromocytomas were believed to be almost always noradrenergic (Tischler *et al*., 1990). However, recent reassessment of archived NTP pheochromocytomas has revealed a number of tumors that did express PNMT (Powers *et al*., 2008). A possible association of PNMT expression with tumors in female rats requires confirmation in further studies.

Diagnosis of malignant pheochromocytomas may be challenging due to the morphologic continuum that exists between benign and malignant tumors. By convention, malignancy is diagnosed if there is penetration of the adrenal gland capsule, invasion of periadrenal tissue or blood vessels, or distant metastases (Fig. 6). Metastases are rare, but when present, tend to occur in the regional lymph nodes, lung, or liver. It is important to note that the convention applied to diagnosis of malignancy is a potential source of confusion in assessing the relevance of rodent pheochromocytomas to their human counterparts. According to the current WHO classification, malignancy of human pheochromocytomas is defined by the presence of metastases (DeLellis *et al*., 0000), not local invasion. The best argument for this restriction is based on tumor biology. Despite its

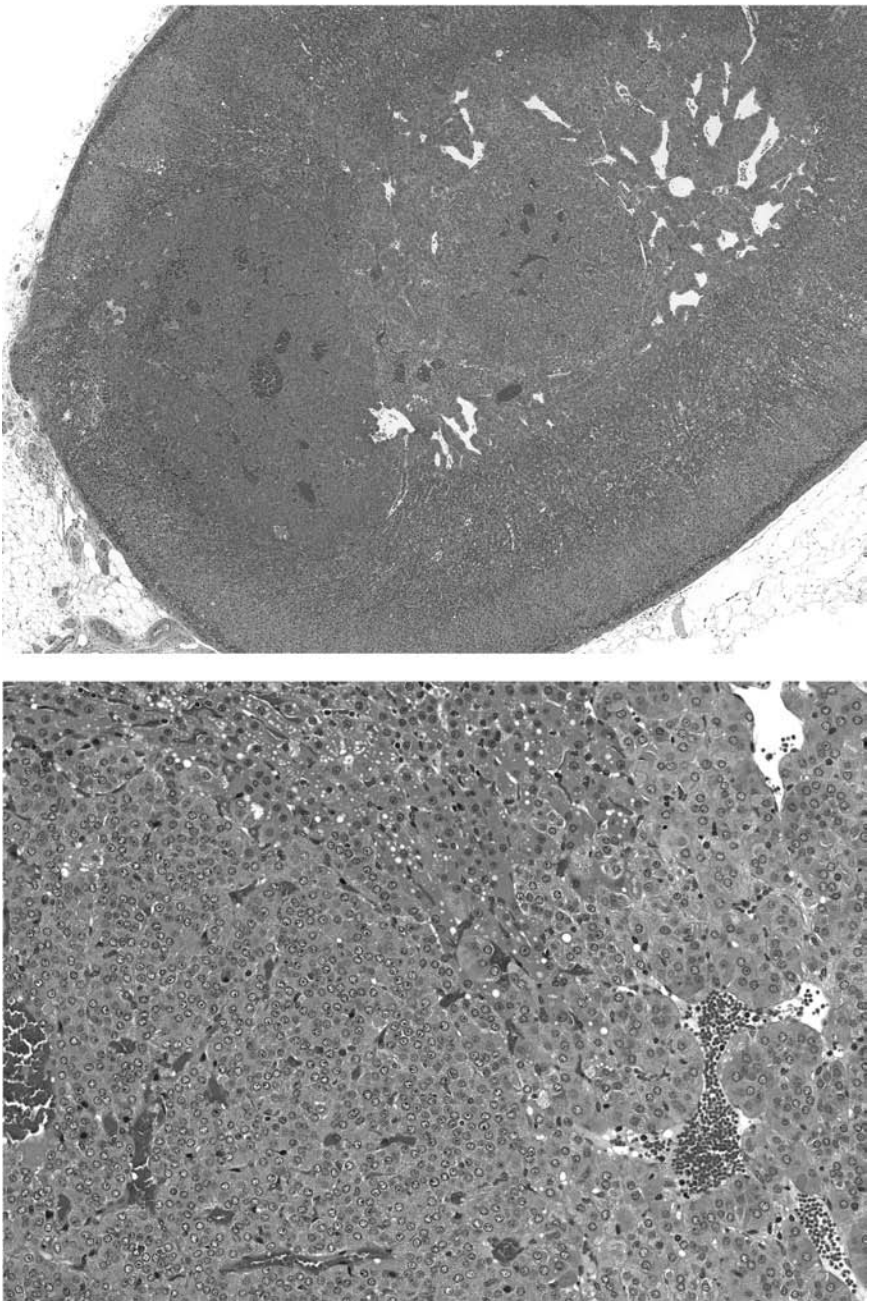

Figure 5 (*See color insert*) Pheochromocytoma in a male F344 rat with extension into the cortex. The neoplastic chromaffin cells are arranged in irregular clusters with ectatic and blood-filled sinusoids.

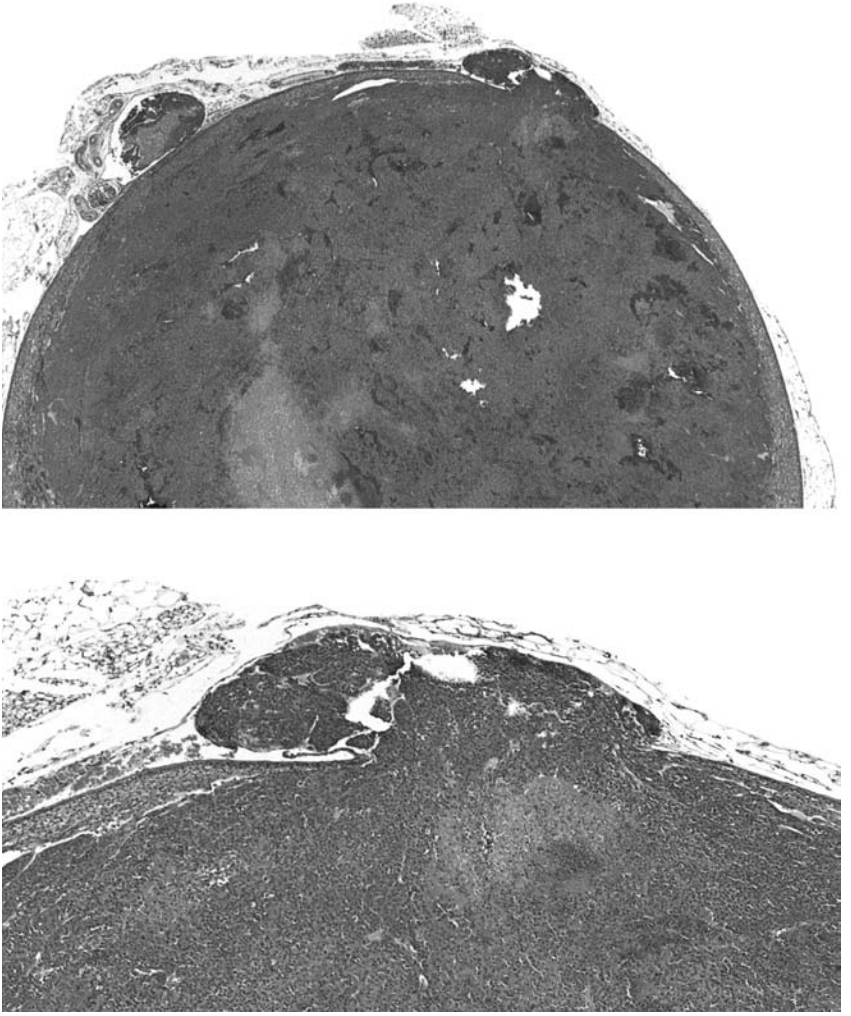

Figure 6 (*See color insert*) Malignant pheochromocytoma in a male F344 rat with complete effacement of normal adrenal gland architecture and invasion of capsule and periglandular tissue. Malignancy is not always this straightforward. By convention, malignancy is diagnosed if there is penetration of the adrenal gland capsule, invasion of periadrenal tissue or blood vessels, or distant metastases.

potential lethality, local invasion alone is a poor predictor of metastases, and the absence of apparent invasion does not preclude development of metastases. The two types of aggressive behavior may therefore have different biological underpinnings, i.e., some tumors capable of invasion may lack properties required for subsequent dissemination, similar to basal cell carcinomas of the skin.

The term "complex pheochromocytoma" is used to describe neoplasms with a mixture of neoplastic chromaffin cells and neoplastic neuronal cells, but with the neural component comprising less than 80% of the tumor mass (Fig. 7) (DeLellis *et al*., 0000; Longeart, 1996). In human pathology, the corresponding term is "composite pheochromocytoma," a subtype of ganglioneuroblastoma (Shimada and Roald, 2000). These tumors contain a mixture of neoplastic chromaffin cells, moderately to well-differentiated neurons (ganglion cells), satellite cells, Schwann cells, and neurofibrils. These tumors may also contain prominent areas of neuroblast-like cells. The ganglion cells are typically large and polyhedral with abundant finely granular eosinophilic or slightly basophilic cytoplasm (Fig. 7). The nuclei contain finely stippled chromatin with variably prominent nucleoli. Electron microscopy reveals a well-developed rough endoplasmic reticulum (Nissl substance) and neurosecretory granules that are usually sparser than the granules in chromaffin cells and tend to accumulate in cell processes. Immunohistochemically, the ganglion cells are usually TH-positive but PNMT-negative and their cell bodies tend to stain only weakly for CgA because of the sparse granule content (Tischler, 2000). This mixed phenotype tumor is probably derived from a progenitor with the ability to develop into either neurons or chromaffin cells. However, the nature of such a progenitor is unclear. The unitary hypothesis of a single pluripotent sympathoadrenal progenitor (primitive sympathoblast), favored in the 1980s and 1990s, is no longer tenable. Recent studies show that the adrenal medulla is initially populated by several types of apparently different progenitors, which may have different fates (Unsicker *et al*., 2005).

Ganglioneuromas and neuroblastomas are medullary neoplasms composed predominantly of neuronal cells. Ganglioneuromas consist of greater than 80% mature ganglion cells and Schwann cells in a matrix of nerve fibers (Fig. 8). Neuroblasts and immature cells are not a feature of ganglioneuromas. Neuroblastomas are immature neuronal neoplasms defined by a predominance (>80%) of neoplastic neuroblast cells (Fig. 9) (Longeart, 1996). The neoplastic cells are arranged in sheets or thick trabeculae separated by a fine eosinophilic fibrillary network with indistinct cell borders. The cells are closely packed with hyperchromatic round or oval to triangular or carrot-shaped nuclei and scant fibrillary pale cytoplasm with indistinct cell borders (Fig. 9). Rosette formation is occasionally observed.

In the adrenal medulla, complex pheochromocytoma, ganglioneuroma, and neuroblastoma are extremely rare. None have been reported to be induced by xenobiotics. Interestingly, tumors of the neuroblastoma group (neuroblastoma, ganglioneuroblastoma, ganglioneuroma) in humans almost always occur in children, but mixed lineage tumors more often occur in adults (Shimada and Roald, 2000). There is no reported age-specificity of rodent tumors in the neuroblastoma group.

TOXICOLOGICALLY INDUCED MEDULLARY LESIONS

In general, the adrenal medulla is a less common site for chemically induced lesions than the adrenal cortex and a much less common site than other organs such as the

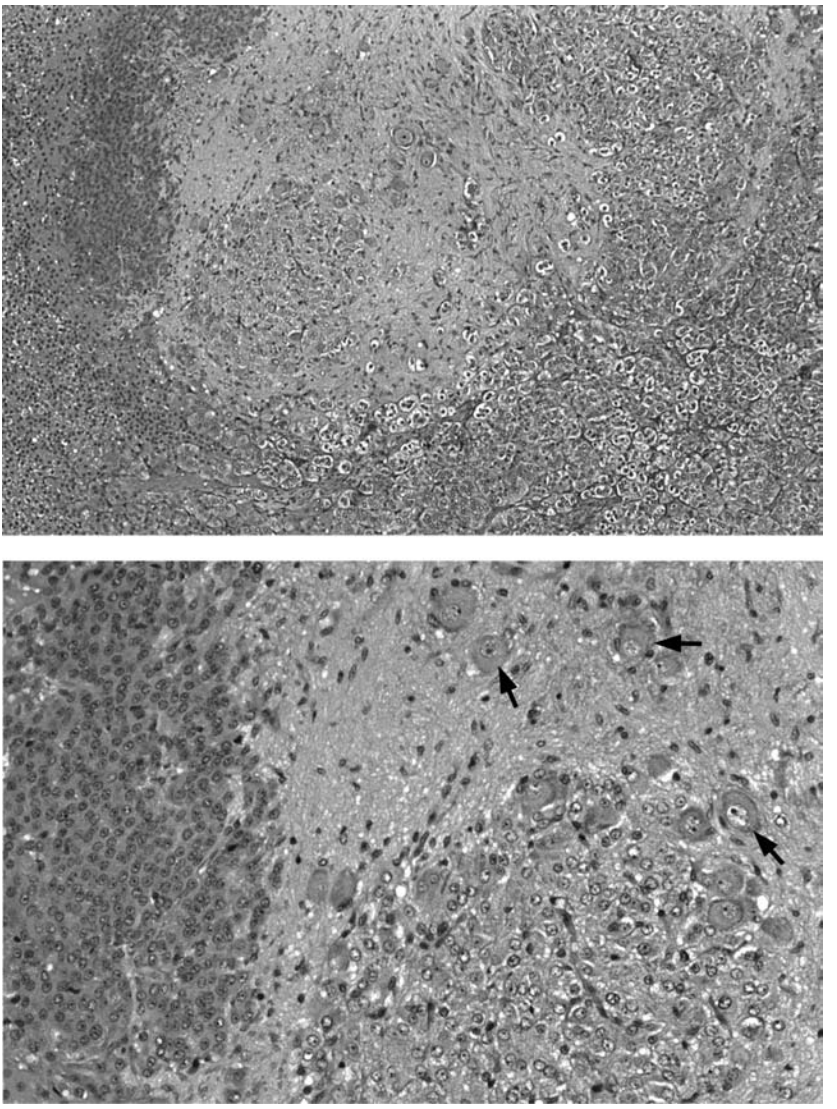

Figure 7 (*See color insert*) The term "complex pheochromocytoma" is reserved for neoplasms that have a neural component that comprises less than 80% of the tumor mass. They may contain a mixture of neoplastic chromaffin cells, neuroblasts, mature neurons (ganglion cells), Schwann cells, and neurofibrils. These are low- and high-magnification images from a male F344 rat treated with a high dose of hexachloroethane. In the upper panel, there are multifocal regions of neoplastic chromaffin cells surrounded by an eosinophilic matrix. There is a more basophilic aggregate of hyperplastic chromaffin cells in the upper left-hand corner. The lower panel shows that at higher magnification there are well-differentiated ganglion cells (arrows) amid an eosinophilic neurofibrillary matrix.

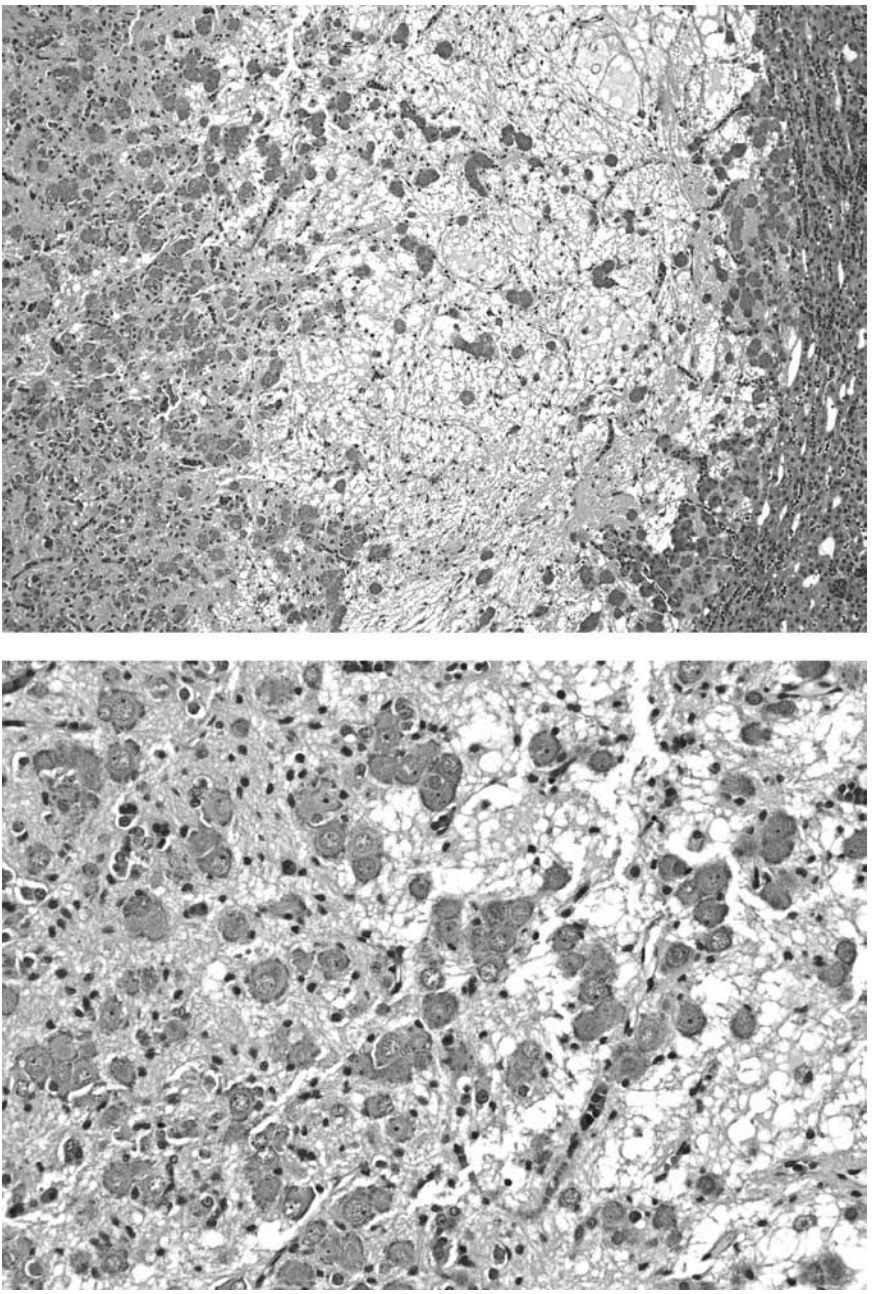

Figure 8 (*See color insert*) Ganglioneuroma is a term reserved for a rare medullary neoplasm that consists of mostly (>80%) ganglion cells and Schwannian stroma. These low- and high-magnification images of a ganglioneuroma from a male F344 rat illustrate scattered mature ganglion cells amid mature stroma.

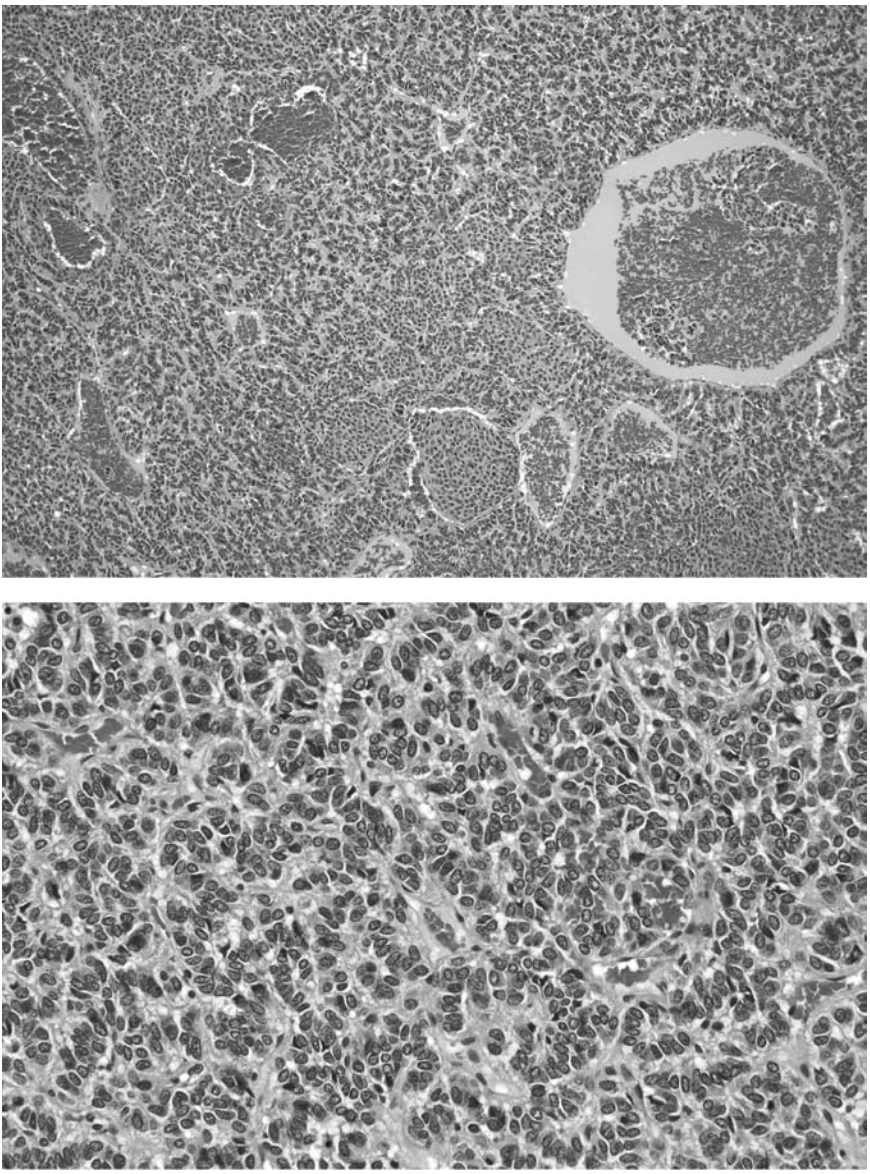

Figure 9 (*See color insert*) In rodents, the term "neuroblastoma" is reserved for medullary neoplasms with a predominance (approximately 80%) of neoplastic neuroblast cells. In these images from a male F344 rat, there are sheets and irregular aggregates of tightly packed small hyperchromatic neoplastic neuroblast cells separated by a fine fibrillary network. The cells have oval to triangular or carrot-shaped nuclei with scant pale cytoplasm and indistinct cell borders. Occasional rosette formation can be seen.

liver or lung. The incidence of toxicologically-induced adrenal medullary lesions is highly dependent on the species, strain, and sex of the animals. Hyperplasia and pheochromocytoma are the most common chemically induced adrenal medullary proliferative lesions observed in both rats and mice. These lesions can result from the administration of exogenous compounds or from dietary modifications, and these tend to occur more frequently in the rat than the mouse. In rats, increased frequencies of hyperplasias and pheochromocytomas have been associated with a variety of genotoxic and nongenotoxic agents. These include, but are not limited to, exposure to growth hormone, reserpine, estrogens, radiation, retinol acetate, nicotine, and diets high in complex carbohydrates (Cheng, 1980; Kurokawa *et al*., 1985; Lynch *et al*., 1996; Saiko *et al*., 1998; Tischler *et al*., 1995). Degenerative medullary lesions may also occur but, due to the rapid repopulation of these damaged cells in as little time as 24 hours, chemically induced degeneration or necrosis may be overlooked if tissues taken at short time points are not examined (Chen-Pan *et al*., 1999). The mechanism of chromaffin cell repopulation is not well understood (Rosol, 2001). Adult chromaffin cells in both rats and mice have a low rate of basal proliferation demonstrable by bromodeoxyuridine incorporation or mitotic counts after administration of colcemid. Administration of reserpine, which causes a decrease in blood pressure and a reflex increase in transsynaptic stimulation of chromaffin cells, also causes a marked increase in chromaffin cell proliferation, suggesting that physiological signals that regulate catecholamine secretion can also alter the size of the chromaffin cell population in response to increased functional demand. Reserpine has been proposed as a model system for studying the association of signal transduction with chromaffin cell proliferation (Tischler *et al*., 1996; Tischler *et al*., 1997). However, mitogenic effects of reserpine typically require approxiamtely 48 hours to become apparent. Reserpine causes pheochromocytomas in rats after long-term administration, suggesting that its effect on chromaffin cell proliferation might provide a setting in which oncogenic mutations can occur. Short-term administration of reserpine is mitogenic for both E-cells and NE-cells in rats. It therefore remains unclear why adrenal medullary hyperplasia and pheochromocytomas in rats are usually noradrenergic.

As of 2008, there are 22 chemicals studied by the NTP that are associated with a response of "clear" evidence of pheochromocytoma in mice and/or rats. The sex/species association is as follows; male rats: 14 , female rats: 8, male mice: 6, female mice: 4. Table 3 indicates those chemicals associated with "clear evidence" of pheochromocytoma. In many studies that show a treatment-related increase in the incidence of pheochromocytoma, it is common to see an increase in the incidence of focal hyperplasia also. This finding is not surprising because hyperplasia is considered to be a part of the continuum of the development of adrenal medullary tumors. Hypertrophy of the medullary cells in B6C3F1 mice has been reported as a treatment-related lesion in only one NTP study (Pentachloroanisole, Technical Report 414, available at http://ntp.niehs.nih.gov).

Table 3 NTP Chemicals with Clear Evidence[a] of Treatment-Related Pheochromocytoma in 2-Year Studies in Mice and Rats

Chemical	NTP TR	Route of exposure	Sex and Species
Bromoethane (ethyl bromide)	TR 363	Inhalation	Female mice
C.I Basic Red 9 monohydrochloride	TR 285	Dosed-feed	Male and female rats and mice
Chlorinated Paraffins: C23, 43% Chlorine	TR 305	Gavage	Male mice
P-Chloroaniline Hydrochloride	TR 351	Gavage	Male rats
4-Chloro-M-Phenylenediamine	TR 085	Dosed-feed	Male rats and female mice
C.I. Acid Red 114	TR 405	Water	Male and female rats
Cobalt Sulfate Heptahydrate	TR 471	Inhalation	Female rats; male and female mice
Decalin	TR 513	Inhalation	Male rats
1,4-Dichlorobenzene (P-Dichlorobenzene)	TR 319	Gavage	Male rats, male and female mice
Furan	TR 402	Gavage	Male and female rats and mice
Gallium Arsenide	TR 492	Inhalation	Female rats
Hexachloroethane	TR 361	Gavage	Male rats
Indium Phosphide	TR 499	Inhalation	Male and female rats and mice
4,4'-Methylenedianiline Dihydrochloride	TR 248	Water	Male and female rats and mice
Mirex	TR 313	Dosed-feed	Male and female rats
Nickel Subsulfide	TR 453	Inhalation	Male and female rats
Oxymetholone	TR 485	Gavage	Female rats
Pentachlorophenol, Dowicide EC-7	TR 349	Dosed-feed	Male and female mice
Phenolphthalein	TR 465	Dosed-feed	Male rats; male and female mice
Reserpine	TR 193	Dosed-feed	Male rats; male and female mice
Talc	TR 421	Inhalation	Female rats
1,1,2-Trichloroethane	TR 074	Gavage	Male and female mice

Abbreviation: TR, technical report.

[a]Clear Evidence of Carcinogenic Activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.

Source: http://ntp.niehs.nih.gov, Summaries & Associations of Study Results.

SUGGESTED NONGENOTOXIC MECHANISMS IN THE PROMOTION OF ADRENAL PHEOCHROMOCYTOMA IN THE MALE F344 RAT—THE NTP STUDIES

Because exogenous agents that induce adrenal medullary neoplasia frequently lack the ability to cause DNA damage, investigators have suggested that many of these influence the carcinogenic response through an indirect mechanism (Tischler *et al*., 1996). We reviewed the literature regarding the potential association between adrenal pheochromocytoma and disease in the kidney and lung. Herein we overview those studies and their conclusions.

Potential Association Between Severe Nephropathy and Pheochromocytoma

The rationale for this investigation was previous work demonstrating that marked stimulation of chromaffin-cell proliferation occurs following oral administration of vitamin D to the rat in vivo (Tischler *et al*., 1996). Vitamin D is known to stimulate Ca^{2+} absorption. In contrast, use of vitamin D, its active metabolite calcitriol, lactose, or xylitol in adrenal medullary cell culture does not result in a mitogenic effect. It was therefore hypothesized that the mitogenic effects of altered Ca^{2+} homeostasis may be related to changes in the nature or intensity of neurally derived signals that stimulate chromaffin-cell proliferation. A direct effect of Ca^{2+} on the nicotinic or muscarinic acetylcholine receptors of the chromaffin cell may also be involved.

Chronic progressive nephropathy (CPN) is a commonly occurring spontaneous disease in aging F344/N rats. Severity of CPN is greater in males than in females. Chronic renal failure is known to be associated with the inability to secrete phosphate, resulting in hyperphosphatemia. Reduced production of the active metabolite of vitamin D, due to decreased numbers of nephrons, and hypocalcemia, due to decreased calcium intestinal absorption, also occur (Woodard and Jones, 1997). Low serum calcium levels stimulate parathyroid hormone secretion. In severe cases of CPN in rats, associated with disturbed calcium/phosphorous homeostasis, chronic stimulation of the chromaffin cells to proliferate may result, which may eventually lead to hyperplasia and neoplasia. Thus, possible associations between the severity of CPN, alterations related to secondary hyperparathyroidism, and pheochromocytoma were examined in selected studies conducted at the NTP (Nyska and Haseman, 1999). Since higher spontaneous incidences of both severe CPN and pheochromocytoma occur in males, only data derived from this gender were used in the investigation.

The NTP historical control database was first scrutinized to determine whether any association could be documented between the severity of CPN and the occurrence of adrenal pheochromocytoma in unexposed animals. Following this analysis, the 125 most recent NTP studies conducted in F344 rats were examined to determine how frequently chemicals that caused increased severity of CPN were associated with an increased incidence of pheochromocytoma. Finally, the

Table 4 Incidence of Adrenal Pheochromocytoma (%) vs Chronic Progressive Nephropathy Severity at Different Survival Times in Control Male F344/N Rats from National Toxicology Program 2-Year Studies

Nephropathy Severity	Survival time (months)				
	0–15	15–21	21–24	24	Total
Inhalation study controls					
0–2	0/39	34/212 (16)	30/84 (36)	20/53 (38)	84/388 (22)
3	0/1	12/81 (15)	36/93 (39)	37/100 (37)	85/275 (31)
4	0/0	4/33 (12)	59/103 (57)	62/101 (61)	125/237(53)
Total	0/40	50/326 (15)	125/280 (45)	119/254 (47)	294/900 (33)
Feeding study controls					
0–2	2/38 (5)	20/185 (11)	48/146 (33)	60/209 (29)	130/578 (22)
3	0/1 (0)	3/28 (11)	32/73 (44)	59/141 (42)	94/243 (39)
4	0/1 (0)	1/7 (14)	11/25 (44)	15/40 (38)	27/73 (37)
Total	2/40 (5)	24/220 (11)	91/244 (37)	134/390 (34)	251/894 (28)

Source: Adapted from Nyska and Haseman, 1999.

association between the incidence of pheochromocytoma and severity of CPN in those NTP studies with chemically related increased rates of pheochromocytoma was examined.

In control male F344 rats surviving beyond 21 months, the incidence of pheochromocytoma was consistently higher in animals with more severe CPN (Table 4) (Nyska and Haseman, 1999). This association was significant ($p < 0.05$) for NTP controls of both the inhalation study ($n = 900$) and the feeding study ($n = 900$).

An association was not consistently observed when dosed groups were considered. Most NTP studies with increased severity of CPN in male F344 rats did not show a corresponding increase in the incidence of pheochromocytoma. Although 22% (28/125) of NTP studies revealed a chemically related increased severity of CPN, only three of these showed a corresponding significant increase in the incidence of pheochromocytoma. Of six NTP studies with increased incidence of pheochromocytoma, animals with pheochromocytoma from five of those exhibited some degree of increased severity of CPN. The estimated strength of the correlation with the severity of CPN varied from study to study and, however, was often quite different from that indicated by an analysis of the more extensive NTP control databases. Additionally, this association could not always account for the increased incidence of pheochromocytoma observed in dosed animals. As the existence of an association between CPN severity and pheochromocytoma incidence did not definitively establish a cause-and-effect relationship, the suggestion was made that more work is required to better understand this correlation and what impact, if any, CPN may have on the interpretation of experimental results.

In another retrospective investigation applied to the NTP database, the potential effect of quality of diet on the incidence of tumors, including pheochromocytomas, and severity of nephropathy in untreated control groups of F344 rats was tested (Haseman *et al*., 2003). The NIH-07 open-formula diet was used in NTP rodent carcinogenicity studies from 1980 to 1994. A new diet designated the NTP-2000 diet was begun in 1994, containing different contents of protein, fat, fiber, vitamins, and minerals. The retrospective investigation compared body weight, survival, tumor incidence, and severity of nephropathy in untreated control groups of F344 rats fed with NTP-2000 or NIH-07 diet, using data from 22 separate 2-year feed and inhalation studies. The results indicated, among other findings, that use of the NTP-2000 diet was associated with decreased incidences of pheochromocytoma and decreased severity of nephropathy, especially in males.

The potential association of severe CPN and the incidence of pheochromocytoma was considered in the case of Stoddard solvent IIC, widely used in the manufacture of paints and varnishes, tested by the NTP for potential carcinogenicity by inhalation in F344/N rats (Doi *et al*., 2004). After 2 years, dose-related increased incidences of adrenal medullary hyperplasia and benign and malignant pheochromocytomas, as well as increased severity of nephropathy, were noted, among other lesions. Statistical testing for potential correlation between increases in both nephropathy severity and incidences of pheochromocytoma indicated a weak and statistically insignificant association, suggesting that the exposure-related increases in pheochromocytomas in male rats were unrelated to the severity of the nephropathy. Since the compound is nongenotoxic, the mechanism by which Stoddard solvent IIC induces adrenal medullary neoplasms remains unclear.

In view of the findings to date, we suggest that investigators evaluating the potential carcinogenic effect of a chemical on the adrenal medulla in male F344 rats be alert to the possible correlation between the severe CPN and pheochromocytoma, and the impact that this association may have in some instances on the interpretation of carcinogenic effects observed at this site (Doi *et al*., 2004). We believe that this association should be considered and tested in every relevant case where chemically related increased severity of nephropathy and pheochromocytoma are noted.

Potential Association Between Severe Lung Pathology and Pheochromocytoma

The rationale for this examination was the concept that systemic hypoxemia is one of the stimuli that augments the secretion of catecholamines and that prolonged functional stimulation of E-cells may lead to their hypertrophy followed by hyperplasia and, finally, neoplasia (Capen, 1996; Myles and Ducker, 1971; Steinsland *et al*., 1970). The suggestion was thus proffered that systemic hypoxemia, occurring in space-occupying lung disease, such as inflammation or neoplasms, reduces the gas exchange area and stimulates secretion of catecholamines from the adrenal

medulla where chronic endocrine hyperactivity may lead to hyperplasia and neoplasia (Ozaki and Haseman, 2002).

In recent years, the NTP performed several 2-year inhalation studies in F344 rats to evaluate the effects of particulate compounds. The results demonstrated variably extensive pulmonary inflammatory lesions and/or lung tumors, and significantly increased incidences of adrenal medullary hyperplasias and pheochromocytomas induced by several compounds in males and females. Retrospective evaluation of 9 of these recent studies in male F344 rats revealed significant ($p < 0.01$) associations of pheochromocytoma with the severity of inflammation and fibrosis in the cases of nickel oxide, cobalt sulfate, indium phosphide, talc, and nickel subsulfide (Baysal, 2006). Studies of gallium arsenide, vanadium pentoxide, molybdenum trioxide, and nickel sulfate hexahydrate revealed an increased incidence and/or severity of nonneoplastic lung lesions, but no increased incidence of pheochromocytoma. However, pheochromocytoma was significantly correlated ($p < 0.01$) with the severity of pulmonary fibrosis and inflammation per se in the gallium arsenide and molybdenum trioxide studies. In the studies of vanadium pentoxide and nickel sulfate hexahydrate, no relationship between nonneoplastic lung lesions and pheochromocytoma was manifested. Retrospective investigation, therefore, supported possible roles of pulmonary fibrosis and inflammation in the induction of pheochromocytoma in some, but not all, studies of F344 male rats. In those studies in which no association between the severity of lung pathology and pheochromocytoma was noted, hypoxemia may not have been sufficiently severe and/or prolonged to promote the proliferation of adrenal medullary cells. The existence of a hypoxemic threshold, defined as a factor of severity of alveolar space occupation multiplied by the duration of reduced normal oxygenation, has been suggested (Ozaki and Haseman, 2002).

MECHANISTIC CONSIDERATIONS AND RELEVANCE TO HUMAN DISEASE

In contrast to xenobiotic-induced changes in many other target organs, there is currently no evidence that the proliferative lesions seen as the major adrenal medullary pathology in toxicologic studies of rats or mice have relevance to human risk assessment. Similar lesions are very rare in humans, except in a few hereditary tumor syndromes; and there is no epidemiologic evidence that the lesions can be induced in humans under any circumstances. Nonetheless, the rodent tumors express many of the same genes as their human counterparts and are potentially valuable for mechanistic studies of roles of those genes in tumor biology.

Hereditary disorders associated with development of human pheochromocytomas are multiple endocrine neoplasia (MEN) 2a and 2b, von Hippel-Lindau disease (VHL), and neurofibromatosis type 1 (NFI), due, respectively, to mutations of the *RET* protooncogene and the *VHL* and *NFl* tumor-suppressor genes. The list of hereditary susceptibility disorders was recently expanded to include

familial syndromes caused by mutations of succinate dehydrogenase (*SDH*) genes that also appear to function as tumor-suppressors (Baysal, 2006). The lesson from genetic studies of human pheochromocytomas is that multiple routes can lead to development of the same type of tumor, presumably as a result of cross-talk or functional overlap between signaling pathways, but the pathological cellular consequences of the signaling events are largely unknown. Somatic mutations of the genes responsible for hereditary pheochromocytomas are uncommon in sporadic tumors, suggesting that the mutations may act during specific developmental windows to prevent apoptotic culling of tumor progenitors (Lee *et al*., 2005).

A genetic basis for the high frequency and ready inducibility of rat adrenal medullary lesions in toxicologic studies has not been identified, and spontaneous mutations of rat or mouse orthologs of the human susceptibility genes have thus far not been found. A hereditary predisposition to develop pheochromocytomas was recently discovered as part of a mixed MEN syndrome in Sprague-Dawley rats with a germline mutation of the *Cdkn1b* gene, which encodes the cell cycle checkpoint protein p27kip (Pellegata *et al*., 2006). The mutation was found to prevent expression of *p27* in the rat tumor tissues through a post-transcriptional mechanism. A similar syndrome caused by a nonsense mutation was subsequently identified in a human kindred (Pellegata *et al*., 2006). In rats, a partially overlapping tumor syndrome had been described in the 1980s in Long-Evans hooded rats (Lee *et al*., 1982), but the genetic defect was never identified. Unfortunately, analysis of archived pheochromocytomas that arose both in NTP toxicological studies and in the Long-Evans model do not show apparent loss of p27 (Powers *et al*., 2008). A loss of function mutation that still permits protein expression has not been ruled out.

In contrast to their wild type counterparts, genetically engineered mice harboring a variety of mutations frequently develop pheochromocytomas, and the number of altered genes shown to cause pheochromocytomas in mouse models increases (Tischler *et al*., 2004). In some cases, orthologous genes are responsible for transmission of human pheochromocytoma syndromes [*Ret* (51), *Nf1* (52)], while others have secondary involvement in some cases or minimal direct relevance [*Pten* (53), *Ink4a/Arf* (You *et al*., 2002), *Kip1* (King *et al*., 2005), *Cx32* (King *et al*., 2005), *Rb* (Williams *et al*., 1994), *c-Mos* (Schulz *et al*., 1992), *ErbB-2* (Lai *et al*., 2007)]. As with human pheochromocytomas, the overriding lesson is that different pathways can lead to the same tumor type. The mouse models have been particularly important in showing how combinations of defects contribute to tumor development, for example, loss of the gap junction protein connexin32 together with loss of the cell cycle checkpoint protein p27kip1 (King *et al*., 2005). A potential toxicological application of the mouse models is to study the effects of xenobiotic agents and diet on tumor development, which could help to explain variable penetrance of pheochromocytoma susceptibility syndromes. To our knowledge, the only such study to date is one in which chronic administration of reserpine failed to induce pheochromocytomas in *Nf1* knockout mice (Powers *et al*., 2000).

CELL CULTURE MODELS

Pheochromocytomas exhibit many characteristics of both chromaffin cells and neurons. In addition, as first reported more than 30 years ago, rat (Tischler and Greene, 1975) and human (Tischler *et al*., 1980) pheochromocytoma cells undergo dramatic transdifferentiation into cells that resemble neurons in response to nerve growth factor. The PC12 pheochromocytoma cell line, developed from a rat pheochromocytoma in 1976 (Greene and Tischler, 1976), is widely used for studying many aspects of neurobiology, including the mechanisms of action of neurotoxins. Mouse pheochromocytoma (MPC) cell lines (Powers *et al*., 2000), recently developed from *Nf1* knockout mice (Jacks *et al*., 1994), supplement PC12 cells and differ from them in a number of respects, including expression of *PNMT* and high levels of *Ret* (Powers *et al*., 2002). MPC cells have not yet been used for toxicology. Pheochromocytoma cell lines are difficult to establish from any species and no validated human lines currently exist. Interestingly, both PC12 and MPC cells arose in irradiated animals, suggesting that radiation-induced changes may have permitted propagation of the cells in culture. The nature of those changes remains to be determined.

CONCLUSION

Among the endocrine organs, the adrenal gland is reportedly the most susceptible to compound-induced lesions. In the medulla, the proliferative lesions include diffuse or nodular hyperplasia, benign, malignant, or complex pheochromocytoma, ganglioneuroma, and neuroblastoma. Proliferative lesions can be spontaneous or can be induced by chemicals and dietary modifications. In general, both spontaneous and xenobiotic-induced medullary proliferative lesions tend to be much more frequent in rats than in mice and, in rats, most studies report a higher incidence in males than in females. Degenerative medullary lesions may also occur but, due to the rapid repopulation of these damaged cells in as little time as 24 hours, chemically induced degeneration or necrosis may be overlooked if tissues taken at short-time points are not examined.

Immunohistochemistry can be used to identify subsets of normal adrenal medullary cells and to diagnose and functionally characterize adrenal medullary proliferative lesions. However it must be applied judiciously and with appreciation of potential artefacts, such as nonspecific staining and cross-reactivity. Immunoreactivity for TH will distinguish all chromaffin cells and their proliferative lesions from normal or proliferative adrenal cortex. Proliferative chromaffin cell lesions also usually show extensive staining for CgA; however, unlike TH, antibodies to CgA will also stain other types of neuroendocrine cells.

As of 2008, there are 22 chemicals studied by the NTP that are associated with a response of clear or "some" evidence of pheochromocytoma in mice and/or rats. The sex/species association is as follows; male rats: 14, female rats: 8, male mice: 6, female mice: 4. In many studies that show a treatment-related increase

in the incidence of pheochromocytoma, it is common to see an increase in the incidence of focal hyperplasia also.

Retrospective evaluations of the NTP database concerning potential involvement of indirect mechanisms in the adrenal medullary response indicated that there was no definitive cause-and-effect relationship between pheochromocytoma incidence and severity of nephropathy, and a suggestion was made that more work is required to better understand this correlation and what impact, if any, CPN may have on the interpretation of experimental results. On the other hand, the retrospective investigation supported possible roles of pulmonary fibrosis and inflammation in the induction of pheochromocytoma in some, but not all, studies of F344 male rats.

REFERENCES

Baysal BE. Role of mitochondrial mutations in cancer. Endocr Pathol 2006; 17(3):203–212.

Capen CC. Functional and pathologic interrelationships of the pituitary gland and hypothalamus in animals. In: Jones TC, Capen CC, Mohr U, eds. 2nd ed. Endocrine System. Berlin: Springer-Verlag, 1996:3–33.

Chen-Pan C, Pan IJ, Yamamoto Y, et al. Prompt recovery of damaged adrenal medullae induced by salinomycin. Toxicol Pathol 1999; 27(5):563–572.

Cheng L. Pheochromocytoma in rats: Incidence, etiology, morphology and functional activity. J Environ Pathol Toxicol 1980;4(5–6):219–228.

Coupland RE. The Natural History of the Chromaffin Cell. London: Longmans Green, 1965.

DeLellis RA, Lloyd RV, Heitz PU, et al. Tumours of Endocrine Organs. In: World Health Organization Classification of Tumors. Lyon, IARC Press.

Doi A, Peckham J, Chou B, et al. Development of alpha2u-globulin nephropathy and adrenal medullary pheochromocytomas in male rats following exposure to Stoddard solvent IIC. Inhal Toxicol 2004; 16(5):247–257.

Feldman SA, Eiden LE. The chromogranins: Their roles in secretion from neuroendocrine cells and as markers for neuroendocrine neoplasia. Endocr Pathol 2003; 14(1):3–23.

Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A 1976; 73(7):2424–2428.

Hamlin MH, Banas DA. Adrenal Gland. In: Boorman GA, Eustis SL, Elwell MR, eds. Pathology of the Fischer Rat, Reference and Atlas. San Diego, CA, Academic Press, Inc, 1990:511.

Haseman JK, Ney E, Nyska A, et al. Effect of diet and animal care/housing protocols on body weight, survival, tumor incidences, and nephropathy severity of F344 rats in chronic studies. Toxicol Pathol 2003; 31(6):674–681.

Helle KB. The granin family of uniquely acidic proteins of the diffuse neuroendocrine system: Comparative and functional aspects. Biol Rev Camb Philos Soc 2004; 79(4):769–794.

Hexum TD, Majane EA, Russett LR, et al. Neuropeptide Y release from the adrenal medulla after cholinergic receptor stimulation. J Pharmacol Exp Ther 1987; 243(3):927–930.

Hill GD, Pace V, Persohn E, et al. Comparative immunohistochemical study of spontaneous and chemically induced pheochromocytomas in B6C3F1 mice. Endocr Pathol 2003; 14(1):81–91.

Holgert H, Dagerlind A, Hokfelt T. Phenotype of intraadrenal ganglion neurons during postnatal development in rat. J Comp Neurol 1996; 371(4):603–620.

Holgert H, Holmberg K, Hannibal J, et al. PACAP in the adrenal gland–relationship with choline acetyltransferase, enkephalin and chromaffin cells and effects of immunological sympathectomy. Neuroreport 1996; 8(1):297–301.

Jacks T, Shih TS, Schmitt EM, et al. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet 1994; 7(3):353–361.

King TJ, Gurley KE, Prunty J, et al. Deficiency in the gap junction protein connexin32 alters p27Kip1 tumor suppression and MAPK activation in a tissue-specific manner. Oncogene 2005; 24(10):1718–1726.

Kurokawa Y, Hayashi Y, Maekawa A, et al. High incidences of pheocfhromocytomas after long-term administration of retinol acetate to F344/DuCrj rats. J Natl Cancer Inst 1985; 74(3):715–723.

Lai EW, Rodriguez OC, Aventian M, et al. ErbB-2 induces bilateral adrenal pheochromocytoma formation in mice. Cell Cycle 2007; 6(15):1946–1950.

Lee AK, DeLellis RA, Blount M, et al. Pituitary proliferative lesions in aging male Long-Evans rats. A model of mixed multiple endocrine neoplasia syndrome. Lab Invest 1982; 47(6):595–602.

Lee S, Nakamura E, Yang H, et al. Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: Developmental culling and cancer. Cancer Cell 2005; 8(2):155–167.

Longeart LE. Adrenal medullary tumors, mouse. In: Jones TC, Capen CC, Mohr U, eds. Indocrine System. Berlin: Springer-Verlag, 1996:421–427.

Lynch BS, Tischler AS, Capen C, et al. Low digestible carbohydrates (polyols and lactose): Significance of adrenal medullary proliferative lesions in the rat. Regul Toxicol Pharmacol 1996; 23(3):256–297.

Majane EA, Alho H, Kataoka Y, et al. Neuropeptide Y in bovine adrenal glands: Distribution and characterization. Endocrinology 1985; 117(3):1162–1168.

Mazzocchi G, Albertin G, Andreis PG, et al. Distribution, functional role, and signaling mechanism of adrenomedullin receptors in the rat adrenal gland. Peptides 1999; 20(12):1479–1487.

Myles WS, Ducker AJ. The excretion of catecholamines in rats during acute and chronic exposure to altitude. Can J Physiol Pharmacol 1971; 49(8):721–726.

Nakamura M, Han B, Nunobiki O, et al. Adrenomedullin: A tumor progression factor via angiogenic control. Curr Cancer Drug Targets 2006; 6(7):635–643.

Nyska A, Haseman JK, Hailey JR, et al. The association between severe nephropathy and pheochromocytoma in the male F344 rat–the National Toxicology Program experience. Toxicol Pathol 1999; 27(4):456–462.

Ozaki K, Haseman JK, HaileyJR, et al. Association of adrenal pheochromocytoma and lung pathology in inhalation studies with particulate compounds in the male F344 rat—The National Toxicology Program experience. Toxicol Pathol 2002; 30(2):263–270.

Pellegata NS, Quintanilla-Martinez L, Siggelkow H, et al. Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. Proc Natl Acad Sci U S A 2006; 103(42):15558–15563.

Powers JF, Schelling K, Brachold JM, et al. High-level expression of receptor tyrosine kinase Ret and responsiveness to Ret-activating ligands in pheochromocytoma cell lines from neurofibromatosis knockout mice. Mol Cell Neurosci 2002; 20(3):382–389.

Powers JF, Evinger MJ, Tsokas P, et al. Pheochromocytoma cell lines from heterozygous neurofibromatosis knockout mice. Cell Tissue Res 2000; 302(3):309–320.

Powers J, Picard K, Nyska A, Tischler A. Adrenergic Differentiation and Ret Expression in Rat Pheochromocytomas. Endocrine Pathology 2008; 19(1): 9–16.

Rhodes KJ, Trimmer JS. Antibodies as valuable neuroscience research tools versus reagents of mass distraction. J Neurosci 2006; 26(31):8017–8020.

Ribelin WE. The effects of drugs and chemicals upon the structure of the adrenal gland. Fundam Appl Toxicol 1984; 4(1):105–119.

Rosol TJ, Yarrington JT, Latendresse J,et al. Adrenal gland: Structure, function, and mechanisms of toxicity. Toxicol Pathol 2001; 29(1):41–48.

Saiko Y, Homma Y, Kawabe K, et al. Optimal conditions to induce rat adrenal medullary changes, such as pheochromocytoma, by nicotine. Int J Urol 1998; 5(6):575–581.

Schinner S, Bornstein SR. Cortical-chromaffin cell interactions in the adrenal gland. Endocr Pathol 2005; 16(2):91–98.

Schulz N, Propst F, Rosenberg MM, et al. Patterns of neoplasia in c-mos transgenic mice and their relevance to multiple endocrine neoplasia. Henry Ford Hosp Med J 1992; 40(3–4):307–311.

Shimada H, Roald B. Histology: Tumors of the Neuroblastoma. In: Brodeur GM, Sawada T, Tsuchida Y, Voute PA, ed. Neuroblastoma. Elsevier Science, 2000:341–354.

Smith-Hicks CL, Sizer KC, Powers JF, et al. C-cell hyperplasia, pheochromocytoma and sympathoadrenal malformation in a mouse model of multiple endocrine neoplasia type 2B. Embo J 2000; 19(4):612–622.

Srivastava A, Tischler AS, Delellis RA. Endogenous biotin staining as an artifact of antigen retrieval with automated immunostaining. Endocr Pathol 2004; 15(2):175–178.

Steinsland OS, Passo SS, Nahas GG. Biphasic effect of hypoxia on adrenal catecholamine content. Am J Physiol 1970; 218(4):995–998.

Tischler AS, Greene LA. Nerve growth factor-induced process formation by cultured rat pheochromocytoma cells. Nature 1975; 258(5533):341–342.

Tischler AS, DeLellis RA, Biales B, et al. Nerve growth factor-induced neurite outgrowth from normal human chromaffin cells. Lab Invest 1980; 43(5):399–409.

Tischler AS, Ruzicka LA, Donahue SR ,et al. Chromaffin cell proliferation in the adult rat adrenal medulla. Int J Dev Neurosci 1989; 7(5):439–448.

Tischler AS, Ruzicka LA, Van Pelt CS, et al. Catecholamine-synthesizing enzymes and chromogranin proteins in drug-induced proliferative lesions of the rat adrenal medulla. Lab Invest 1990; 63(1):44–51.

Tischler AS, Ziar J, Downing JC, et al. Sustained stimulation of rat adrenal chromaffin cell proliferation by reserpine. Toxicol Appl Pharmacol 1995; 135(2):254–257.

Tischler AS, Powers JF, Downing JC, et al. Vitamin D3, lactose, and xylitol stimulate chromaffin cell proliferation in the rat adrenal medulla. Toxicol Appl Pharmacol 1996; 140(1):115–123.

Tischler AS, Powers JF, Shahsavari M, et al. Comparative studies of chromaffin cell proliferation in the adrenal medulla of rats and mice. Fundam Appl Toxicol 1997; 35(2):216–220.

Tischler AS, Tsokas P, Shahsavari M, et al. Immunoreactivity of normal rabbit serum with epinephrine (E) cells of the rat adrenal medulla after microwave antigen retrieval. Cell Tissue Res 1998; 293(3):563–566.

Tischler AS, Powers JF, Pignatello M, et al. Vitamin D3-induced proliferative lesions in the rat adrenal medulla. Toxicol Sci 1999; 51(1):9–18.

Tischler AS, Powers JF, Alroy J. Animal models of pheochromocytoma. Histol Histopathol 2004; 19(3):883–895.

Tischler AS, DeLellis RA, Nunnemacher G, et al. Acute stimulation of chromaffin cell proliferation in the adult rat adrenal medulla. Lab Invest 1988; 58(6):733–735.

Tischler AS. Coupland R. E. Changes in structure and function of the adrenal medulla. In: Mohr U, Dungworth CC, Capen CC, eds. Pathobiology of the Aging Rat. Vol 2. Washington, DC: ILSI Press, 1994:244–268.

Tischler AS. Divergent differentiation in neuroendocrine tumors of the adrenal gland. Semin Diagn Pathol 2000; 17(2):120–126.

Tortorella C, Macchi C, Forneris M, et al. Calcitonin gene-related peptide (CGRP), acting via CGRP type 1 receptors, inhibits potassium-stimulated aldosterone secretion and enhances basal catecholamine secretion from rat adrenal gland. Int J Mol Med 2001; 8(3):261–264.

Unsicker K, Huber K, Schutz G, et al. The chromaffin cell and its development. Neurochem Res 2005; 30(6–7):921–925.

Williams BO, Schmitt EM, Remington L, et al. Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. Embo J 1994; 13(18):4251–4259.

Wong DL. Why is the adrenal adrenergic? Endocr Pathol 2003; 14(1):25–36.

Woodard JC. Skeletal system. In: Jones TC, Hunt RD, King NW, eds. Veterinary Pathology. 6th ed. Baltimore, MD: Williams & Wilkins, 1997:899–946.

You MJ, Castrillon DH, Bastian BC, et al. Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. Proc Natl Acad Sci U S A 2002; 99(3):1455–1460.

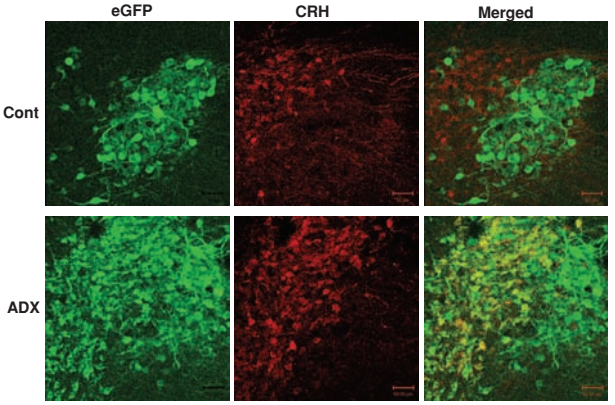

Figure 3.3 Micrographs of the mouse PVN of transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of the vasopressin promoter. (See page 90 for complete legend.)

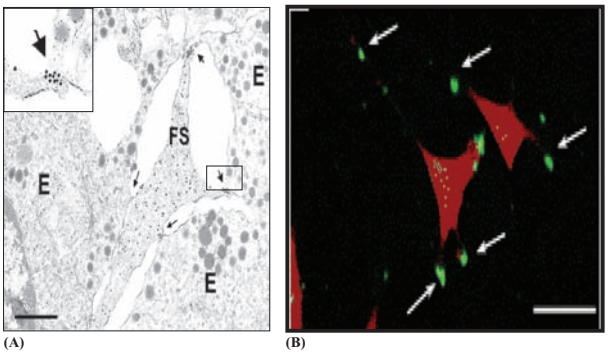

Figure 3.4 Expression of annexin 1 in FS cells. (See page 96 for complete legend.)

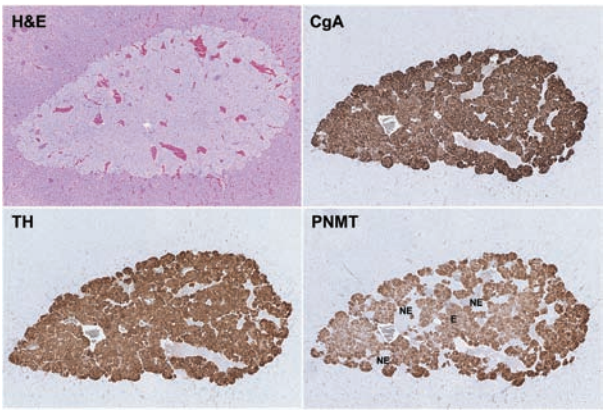

Figure 4.1 Immunohistochemical characterization of adrenal medullary cells. (See page 115 for complete legend.)

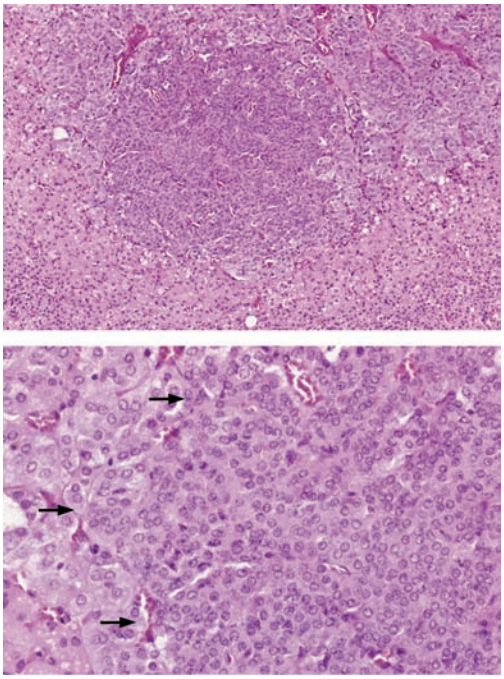

Figure 4.2 Focal chromaffin cell hyperplasia in a male F344 rat that is located at the corticomedullary junction. (See page 117 for complete legend.)

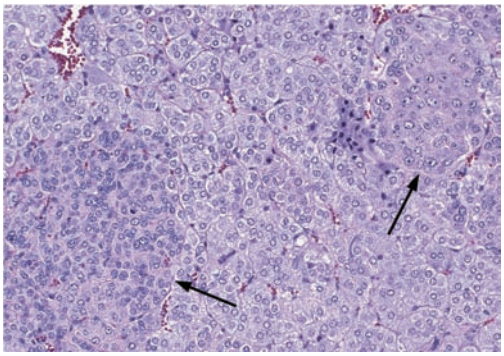

Figure 4.3 This figure contrasts the usual morphology of chromaffin cell hyperplasia (*small cells, left arrow*) with a less common lesion (*right arrow*) in which the hyperplastic cells are larger than the surrounding normal chromaffin cells and have abundant cytoplasm, vesicular nuclei, and prominent nucleoli.

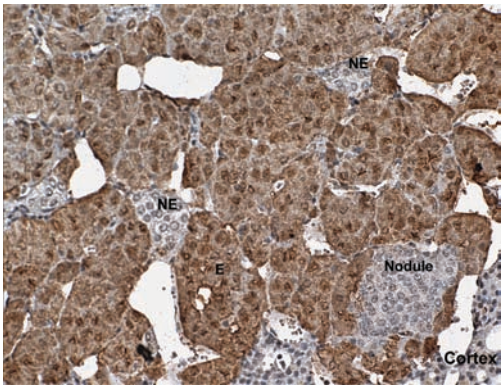

Figure 4.4 In the rat, medullary hyperplastic lesions are usually noradrenergic. Note the absence of immunoreactive PNMT within the hyperplastic nodule compared to the positive reaction within the surrounding normal medullary adrenergic chromaffin cells. *Abbreviations*: E, Epinephrine cells; NE, Norepinephrine cells.

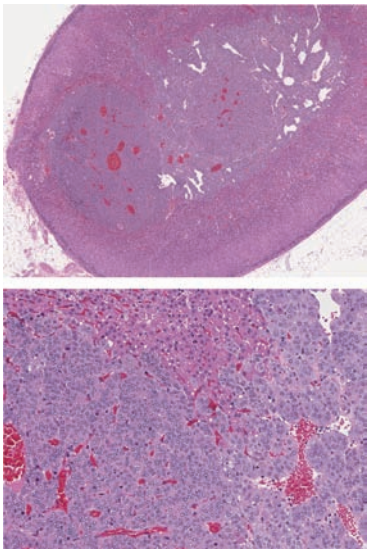

Figure 4.5 Pheochromocytoma in a male F344 rat with extension into the cortex. The neoplastic chromaffin cells are arranged in irregular clusters with ectatic and blood-filled sinusoids.

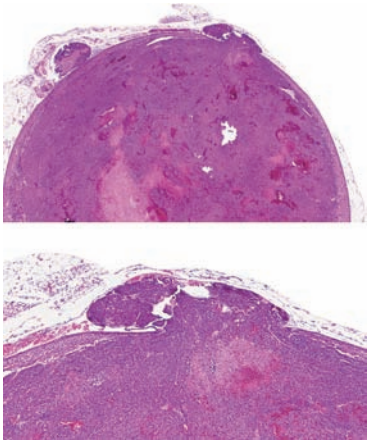

Figure 4.6 Malignant pheochromocytoma in a male F344 rat with complete effacement of normal adrenal gland architecture and invasion of capsule and periglandular tissue. (See page 122 for complete legend.)

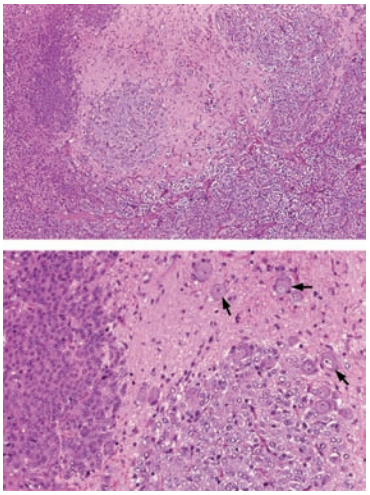

Figure 4.7 The term "complex pheochromocytoma" is reserved for neoplasms that have a neural component that comprises less than 80% of the tumor mass. (See page 124 for complete legend.)

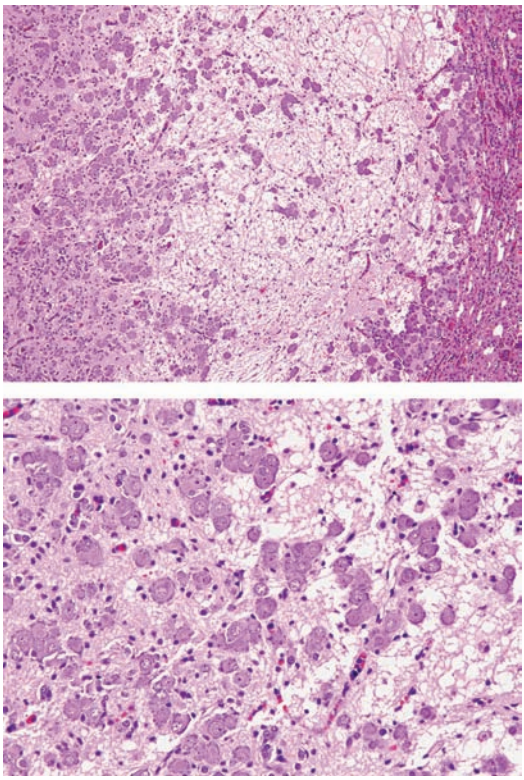

Figure 4.8 Ganglioneuroma is a term reserved for a rare medullary neoplasm that consists of mostly (>80%) ganglion cells and Schwannian stroma. These low- and high-magnification images of a ganglioneuroma from a male F344 rat illustrate scattered mature ganglion cells amid mature stroma.

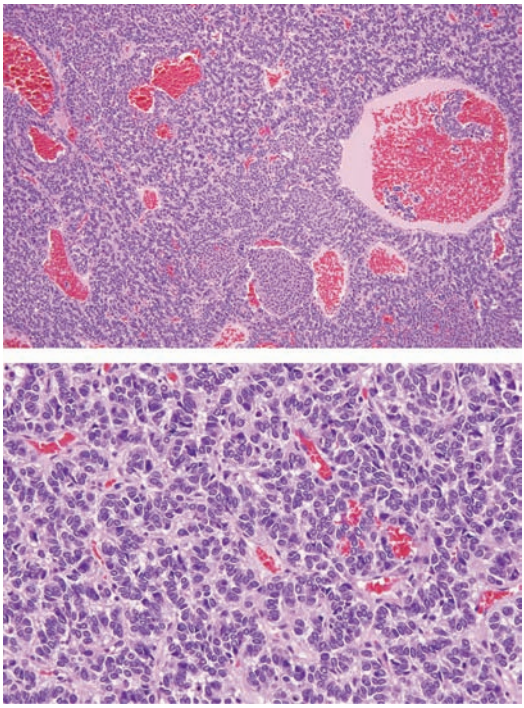

Figure 4.9 In rodents, the term "neuroblastoma" is reserved for medullary neoplasms with a predominance (approximately 80%) of neoplastic neuroblast cells. (See page 126 for complete legend.)

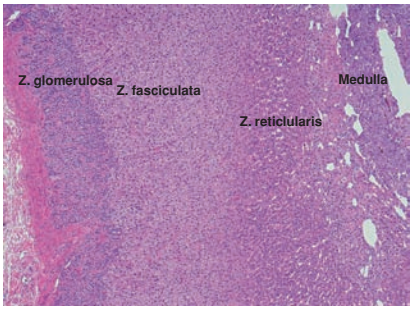

Figure 5.1 Normal structure of the adrenal cortex in *M. fascicularis*.

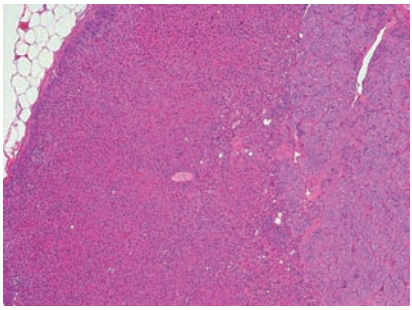

Figure 5.2 Normal structure of the adrenal cortex in *C. jacchus*. Note lack of zona reticularis.

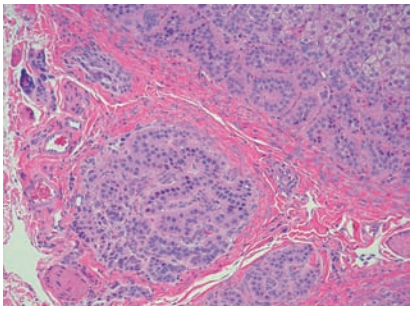

Figure 5.3 Capsular extrusion of adrenal cortex in *M. fascicularis*.

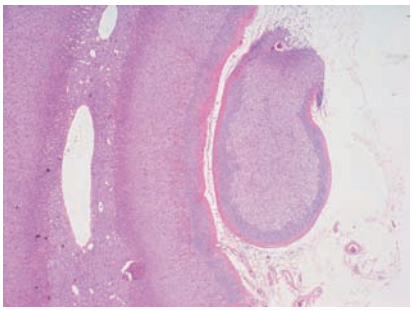

Figure 5.4 Ectopic adrenal cortical tissue on the surface of an adrenal gland in *M. fascicularis*. Note the presence of zona glomerulosa and fasciculata.

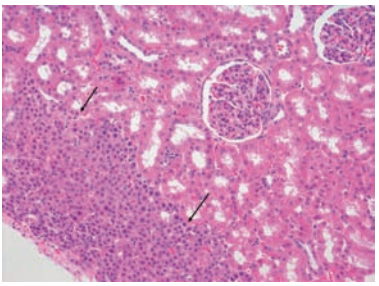

Figure 5.5 Ectopic adrenal cortical tissue under the capsule of a kidney (*arrows*) in *M. fascicularis*.

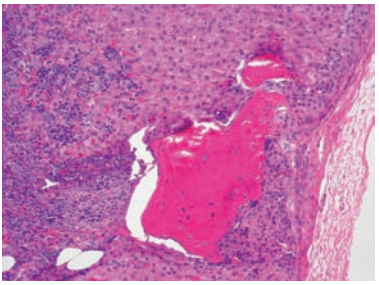

Figure 5.6 Ectopic osseous tissue in the cortex of *C. jacchus*.

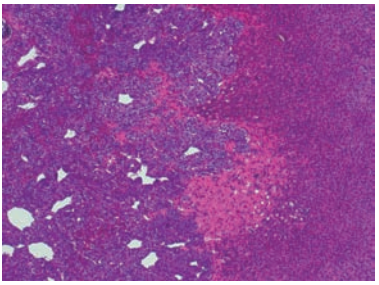

Figure 5.7 Amyloid deposit at the corticomedullary junction of *M. fascicularis*.

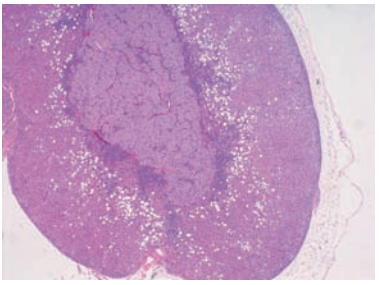

Figure 5.8 Moderate cortical fatty vacuolation in the adrenal cortex of *C. jacchus*.

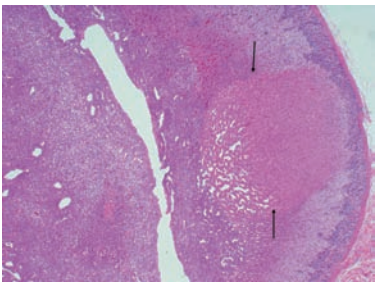

Figure 5.9 Large eosinophilic focus (*arrows*) in the adrenal cortex of *M. fascicularis*.

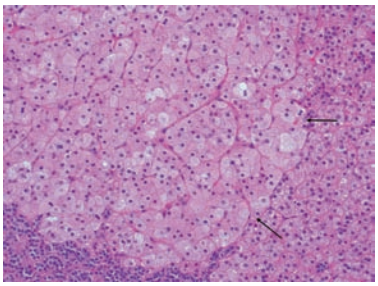

Figure 5.10 Focus of hypertrophic cells (*arrows*) in the adrenal cortex of *M. fascicularis*.

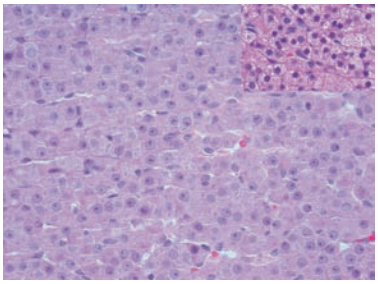

Figure 5.11 Diffuse hypertrophy of cotical cells in *M. fascicularis*. (*Inset*) Normal sized cortical cells. Note also increased eosinophilia and basophilic stippling of hypertrophied cells.

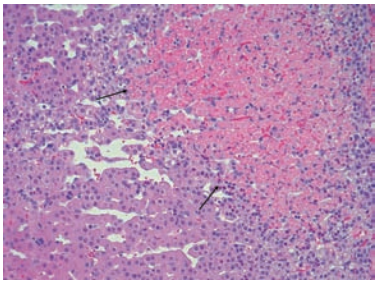

Figure 5.12 Focus of necrotic cells (*arrows*) in the adrenal cortex of *M. fascicularis*.

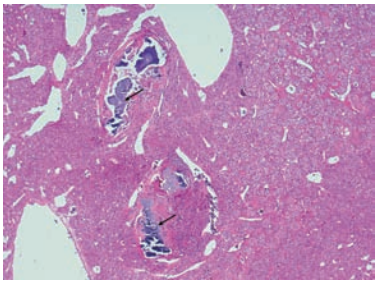

Figure 5.13 Foci of mineralization (*arrows*) in the cortex of *M. fascicularis*.

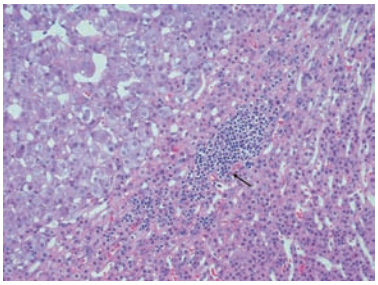

Figure 5.14 Inflammatory cell focus (*arrow*) in the cortex of *M. fascicularis*.

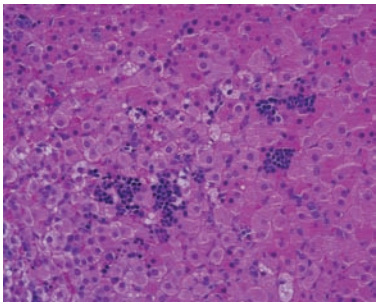

Figure 5.15 Erythropoiesis in the adrenal cortex of *C. jacchus*.

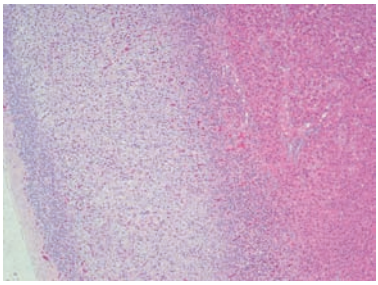

Figure 5.16 Tight adhesion between adrenal cortex (*left*) and liver (*right*) in *M. fascicularis*.

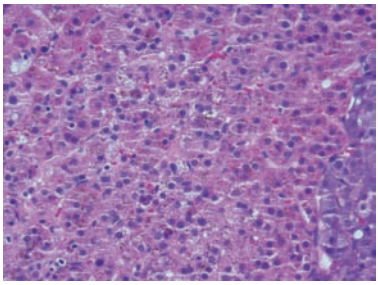

Figure 5.17 Dark-brown lipofuscin granules in cortical cells of an aged *M. fascicularis*.

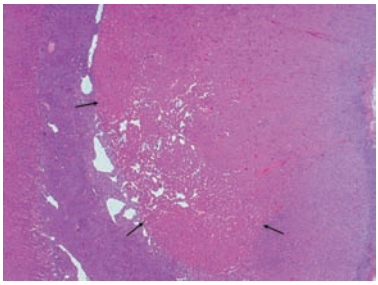

Figure 5.18 Adrenal cortical adenoma (*arrows*) in *M. fascicularis*.

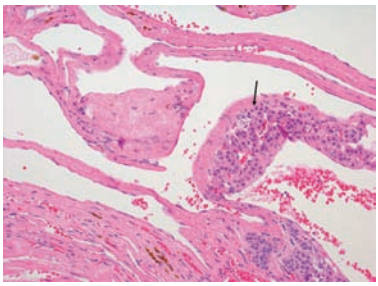

Figure 5.19 Cavernous hemangioma in *M. fascicularis*. Note also island of cortical cells within the tumor *(arrow)*.

5

Adrenal Gland Background Pathology of Primates in Toxicological Studies

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INTRODUCTION

Nonhuman primates represent unique animal models for human diseases because of their phylogenetic relationship to man. Especially, the cynomolgus monkey (*Macaca fascicularis*) is now commonly used for experimentation due to its convenient size, rather omnivorous natural diet, and ability to stay healthy in captivity. It is the animal of choice in toxicological studies on drugs or chemicals (as second, nonrodent species), as well as in AIDS research and studies on vaccine development and safety. Also, the common marmoset (*Callithrix jacchus*) is currently increasingly selected as laboratory primate in many fields of biomedical research, including toxicology. Whenever only small amounts of a drug are available to run a preclinical toxicological study, required by regulatory authorities before the marketing of a new drug, the marmoset may be the appropriate species due to its small size. In contrast, rhesus monkeys (*Macaca mulatta*) that were formerly more frequently used in toxicological testing now only rank third in use behind the two other species. Other primate species, e.g., African green monkeys (*Chlorocebus aethiops*) or pigtailed macaques (*Macaca nemestrina*) may be used for specific scientific questions. This chapter will primarily focus on the three types of primates most widely used in toxicology.

Generally, there are adrenal gland lesions in primates, which on one hand can occur spontaneously but, on the other hand, can also be induced by a chemical substance or a drug in toxicological testing. Therefore, a precise knowledge of the

Table 1 Adrenal Weights (gram) of Different Primate Species (adult animals, historical data, unpublished)

	Absolute weight				Range			
	Males		Females		Males		Females	
Species	*x*	SD	*x*	SD	Min	Max	Min	Max
Cynomolgus monkey	0.58	0.13	0.53	0.12	0.31	1.04	0.32	0.83
Marmoset	0.09	0.02	0.13	0.03	0.05	0.17	0.09	0.19
Rhesus monkey	0.86	0.22	0.89	0.29	0.53	1.43	0.54	1.83

possible naturally occurring changes (as well as their spontaneous frequencies) is necessary when making a judgement as toxicologist/toxicological pathologist on the significance of a finding in a toxicological study. This chapter is intended to assist and support those who are involved in the interpretation of these studies in primates.

ANATOMY, HISTOLOGY, AND EMBRYOLOGY

The adrenal glands are paired organs, each located on either side of the anterior poles of the kidneys. They are supplied with blood from branches of the aorta, and from the phrenic, renal, and lumbar arteries, which form a vascular plexus. The glands are divided into two distinct areas, the cortex, which occupies two-thirds of the gland, and the medulla, which comprises the remaining one-third. Organ weights of different laboratory primate species are presented in Table 1. In man, the adrenal is divided into head, body, and tail and the medulla is lacking in the tail of the gland.

The cortex is characterized histologically by three separate regions in most primate species (Fig. 1). The gland is enclosed by a thin fibrous capsule, and immediately below this lies the zona glomerulosa that is composed of small cells with round nuclei arranged in twisted cords or oval groups. This zone comprises approximately 15% of the cortex. Below this is the largest zone, the zona fasciculata, which comprises 70% of the cortex. Here the cells are arranged in cords, two or more cells wide, and separated by small capillaries. These cells quite frequently contain numerous small lipid droplets that give their cytoplasm a foamy appearance. The inner part of the cortex, the zona reticularis, contributes the remaining 15% of the cortex, and is composed of similar cells to the zona fasciculata but arranged in more narrow cords. However, a zona reticularis is missing (Fig. 2) in the common marmoset (Levine *et al.*, 1982; Pattisson, 2005). The adrenal medulla is composed of homogenous, basophilic, polyhedral cells arranged in small irregular packets separated by sinusoids. Numerous large central veins are characteristic of the medulla. A few ganglion cells are found scattered among these chromaffin medullary cells (Takayama *et al.*, 1999). Ultrastructurally, cortical cells show many lipid droplets which contain

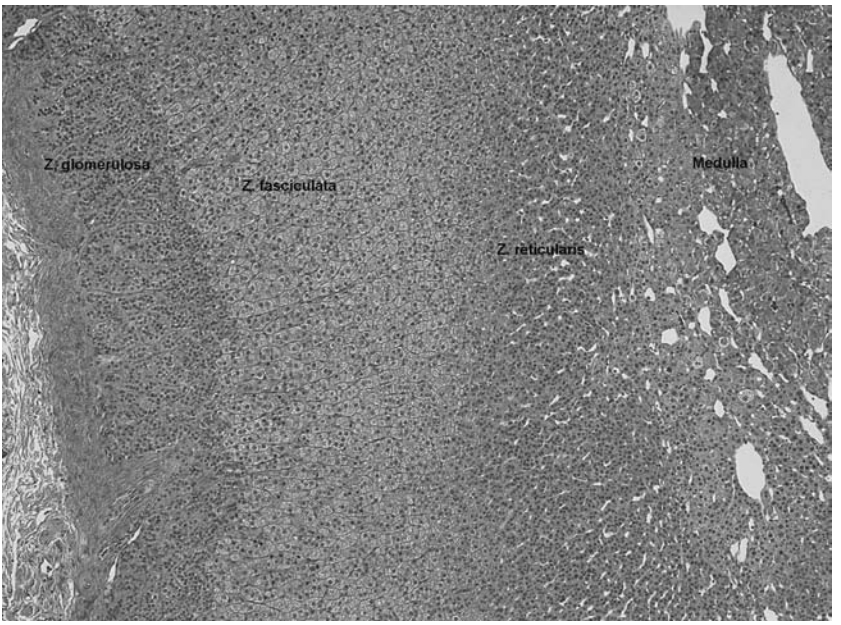

Figure 1 (*See color insert*) Normal structure of the adrenal cortex in *M. fascicularis*.

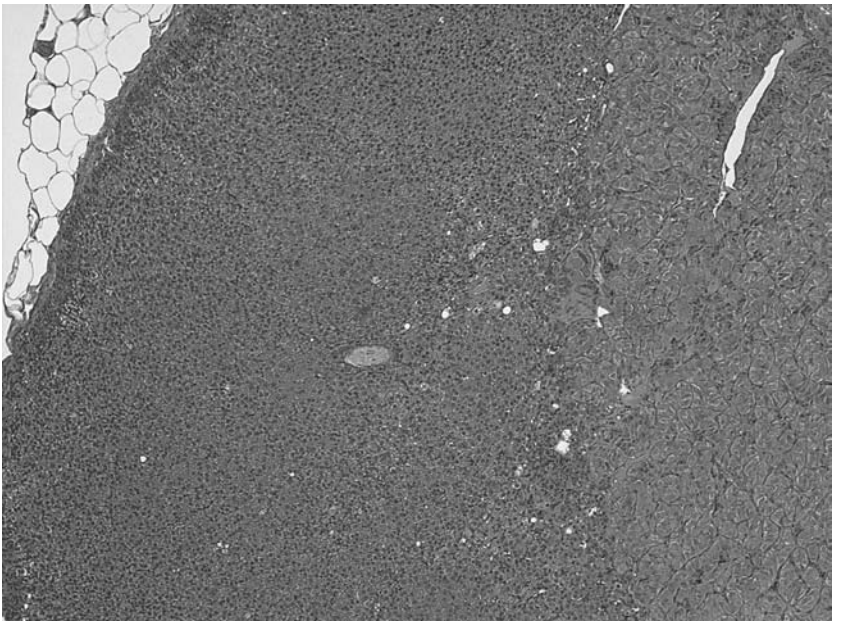

Figure 2 (*See color insert*) Normal structure of the adrenal cortex in *C. jacchus*. Note lack of zona reticularis.

cholesterol, the basic steroid precursor. The droplets are close to the smooth endoplasmic reticulum and the mitochondria; all the cells of the zona glomerulosa show less smooth endoplasmic reticulum than those of the other two zones, and the cells of zona reticularis have fewer lipid droplets. The Golgi apparatus is prominent in the cells of the zona glomerulosa but poorly developed in the cells of the inner cortical zones. The cells of the medulla show rough endoplasmic reticulum, a Golgi apparatus, mitochondria and, unlike the cells of the cortex, scattered secretory granules (Brenner, 1966; Capen *et al*., 1991).

During fetal life, all primates have a provisional or fetal adrenal cortex. It is replaced postnatally by the definitive or adult cortex with its familiar three zones. Involution of the fetal cortex is accompanied by necrosis and hemorrhage. Involution prior to parturition is a sign of fetal distress. Mineralization of the adrenal, usually at the corticomedullary junction and often visible grossly, is an incidental finding in macaques of all ages, and possibly a sequel to destruction of the fetal cortex (Lowenstine, 2003).

NON-NEOPLASTIC LESIONS

Most lesions in cynomolgus monkeys, rhesus monkeys, and marmosets occur with very low frequency and must therefore be considered as rather sporadic findings. Only extramedullary hematopoiesis, cortical vacuolation, and inflammatory cell foci in marmosets, as well as capsular extrusion of adrenal cortex in cynomolgus monkeys and cortical mineralization in rhesus monkeys do occur in a greater portion of animals. Lesions of all three primate species are summarized in Tables 2–4.

Capsular Extrusion of Adrenal Cortex

Capsular extrusion of adrenal cortex (also called accessory cortical nodule or extracortical nodules) is quite common in *M. fascicularis*. The accessory nodules of cortical tissue are mostly located within the glands capsule (Fig. 3), surrounded by an own thin capsule and composed of normal appearing cortical cells, resembling those of the zona glomerulosa or zone fasciculata. Because the great majority of cynomolgus monkeys of our colony reveals this lesion, it is not recorded normally.

Ectopic Cortical Tissue

Ectopic adrenal cortical tissue can be infrequently seen within the periadrenal or perirenal fat. It consists of cortical cells which might in very rare cases even show a zonation like the adrenal gland (Fig. 4). Medullary cells are always lacking within these structures. Even more rare is a displacement of cortical cells into another organ, primarily the kidney. We have observed a focus of sharply demarcated cortical tissue under the renal capsule of a cynomolgus monkey (Fig. 5). Prentice and Jorgeson (Prentice and Jorgeson, 1979) even reported five cases of ectopic adrenal tissue in the kidney of rhesus monkeys. A further case of this lesion affecting the

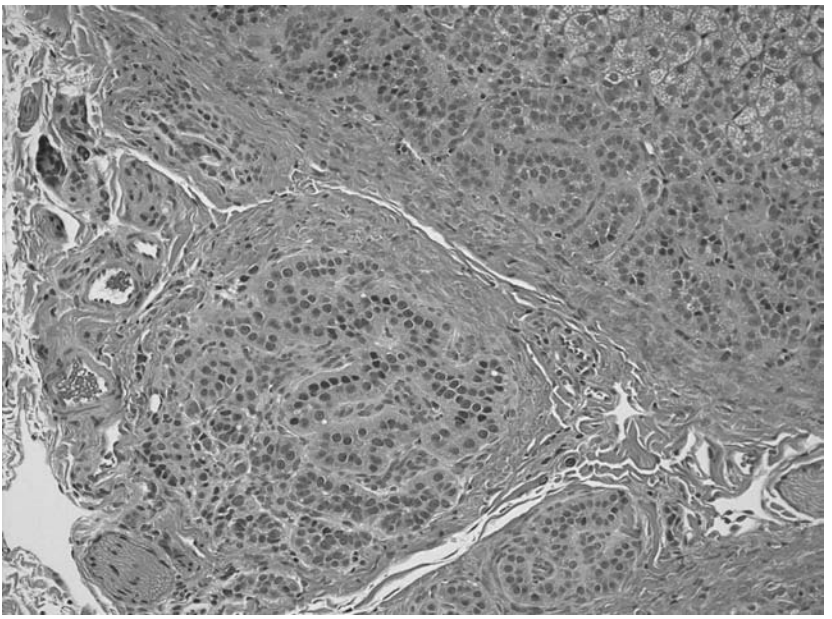

Figure 3 (*See color insert*) Capsular extrusion of adrenal cortex in *M. fascicularis*.

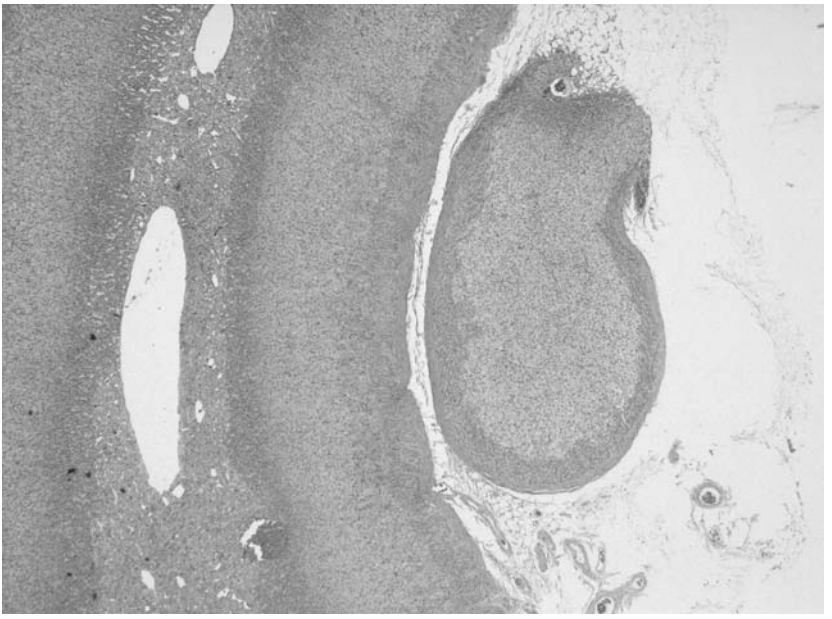

Figure 4 (*See color insert*) Ectopic adrenal cortical tissue on the surface of an adrenal gland in *M. fascicularis*. Note the presence of zona glomerulosa and fasciculata.

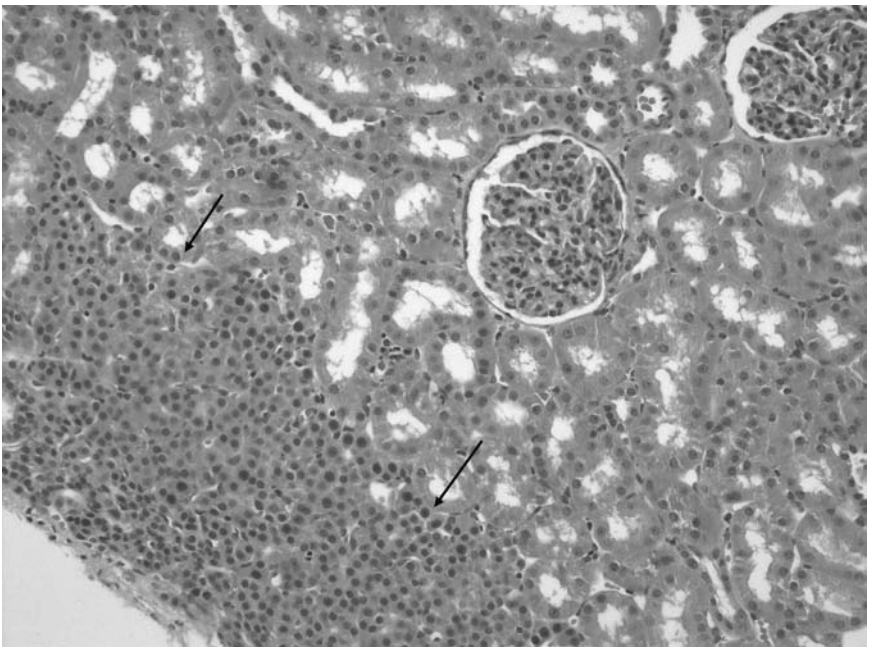

Figure 5 (*See color insert*) Ectopic adrenal cortical tissue under the capsule of a kidney (*arrows*) in *M. fascicularis*.

kidney of a rhesus monkey was documented by Schmidt (Schmidt, 1956). Conaway (Conaway, 1969) found adrenal cortical tissue at the ovarian hilus of patas monkeys (*Erythrocebus patas*).

Ectopic Bone

Ectopic bone (Fig. 6) was observed in the cortex of the adrenal gland of *C. jacchus*. It must be regarded as part of a broader spectrum of organs with infrequent osseous metaplasia, including the lung, the aorta, and the testes in marmosets of our colony. Bone formation in the adrenal gland was also described before in a rhesus monkey (Schmidt, 1957).

Amyloidosis

Amyloidosis comprises a heterogeneous group of diseases having in common the extracellular deposition of amyloid protein fibrils. All types of amyloid bind Congo red, which exhibits green birefringence when examined microscopically under polarized light. Amyloidosis is classified based on the type of amyloid proteins. Primary amyloidosis contains fibrillar proteins with light chain regions of immunoglobulins and is associated with plasma cell dyscrasias, myeloma, and reticulum cell sarcoma. Secondary amyloidosis contains proteins which are amino terminal fragments of serum amyloid A. Serum amyloid A is produced in the liver

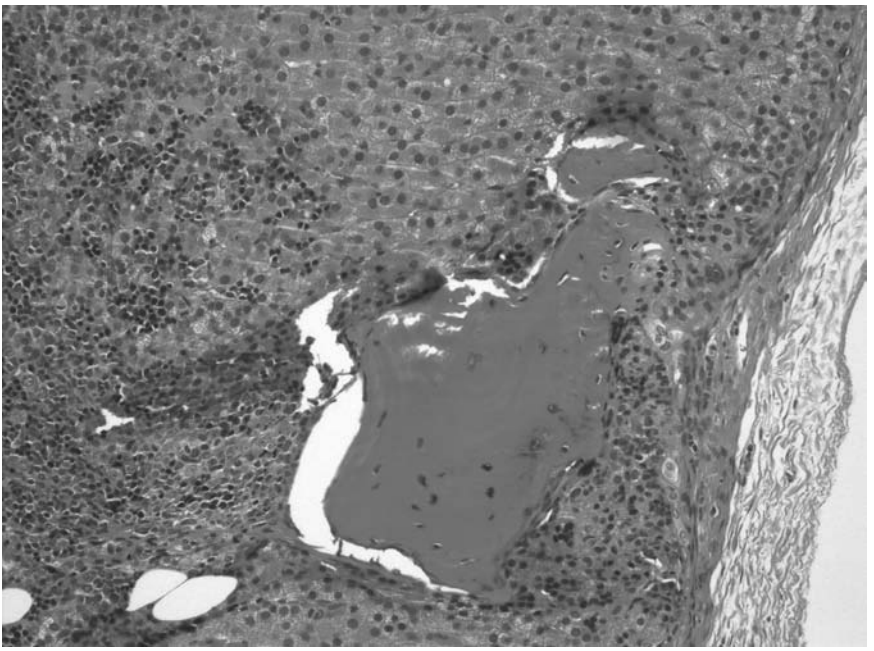

Figure 6 (*See color insert*) Ectopic osseous tissue in the cortex of *C. jacchus*.

and released into the bloodstream during the acute phase of infectious and non-infectious inflammation, various neoplasms, and in familial Mediterranean fever.

Adrenal amyloidosis was not observed in the adrenal gland of control marmosets, cynomolgus monkeys, and rhesus monkeys that belonged to toxicological studies, probably due to the young age of these animals. However, adrenal amyloidosis occurred in seven out of 32 cynomolgus monkeys (together with amyloidosis of other organs) of our geriatric colony ($\geq$ 12 years) that was kept for studies on age-related diseases like Alzheimer disease. It was located in the cortex with preference to the cortico-adrenal junction (Fig. 7). Generalized amyloidosis (including adrenal gland) was also found in 10.4% of animals of a colony of *M. mulatta*, pigtailed macaques (*M. nemestrina*), and *M. fascicularis* of the Adler Primatological Centre (Naumenko and Krylova, 2003), and in 57 rhesus monkeys out of 128 examined histologically by Blanchard et al. (Blanchard *et al*., 1986). *C. jacchus* and *M. nemestrina* were found to have amyloid deposits in one or more organs, including adrenal gland, in studies of Lundlage et al. (Lundlage *et al*., 2005) and Slattum et al. (Slattum *et al*., 1989).

Cortical Fatty Vacuolation

Fatty vacuolization is sporadically observed in the cortex of *M. fascicularis* but more frequently in the *C. jacchus*. While in cynomolgus monkeys few clusters of

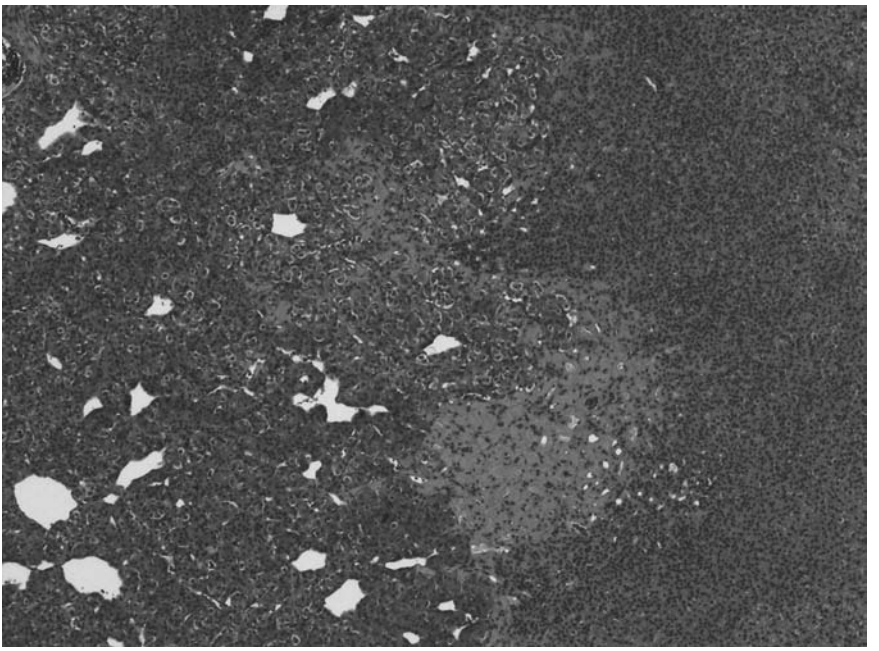

Figure 7 (*See color insert*) Amyloid deposit at the corticomedullary junction of *M. fascicularis*.

vacuoles are present in the cortex, a higher degree of cortical vacuolization can be seen in marmosets, occasionally affecting numerous cells of the cortex (Fig. 8). Vacuoles stain positive in Susan black and oil-red O stain.

Eosinophilic Focus

Eosinophilic foci are more or less spherical accumulations of eosinophilic cells within the zona fasciculata (Fig. 9). They are seen infrequently in *M. fascicularis* and *C. jacchus*. Larger foci even may show minimal compression at one side.

Focal Cortical Cell Hypertrophy

This change is comprised of enlarged cortical cells that often reveal a coarser vacuolation than normal zona fasciculata cells and have lost their cord-like orientation. Typically, the hypertrophic focus consists of these cells, but intermingled eosinophilic cells may also be present (Fig. 10). Low-grade compression of surrounding tissue can occasionally occur. Hyperplastic cortical nodules with a different staining feature were observed in two out of eight woolly monkeys

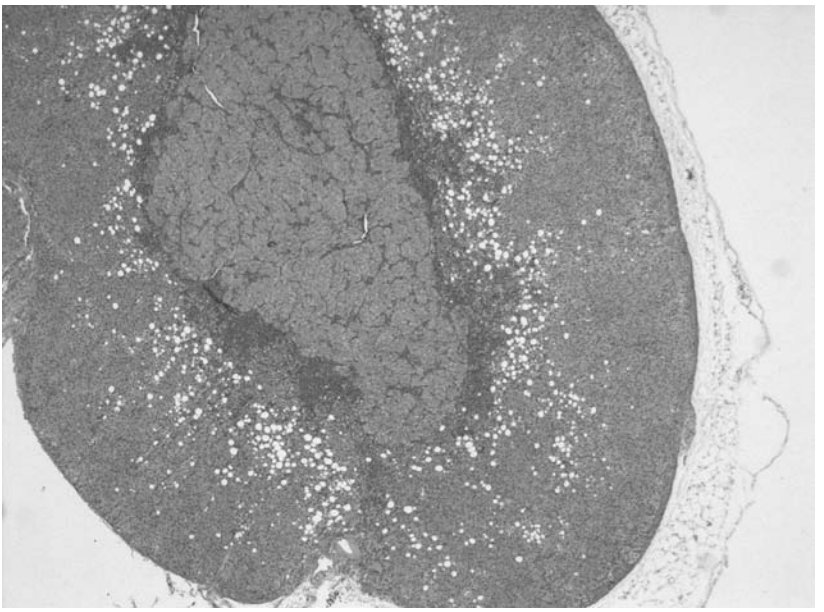

Figure 8 (*See color insert*) Moderate cortical fatty vacuolation in the adrenal cortex of *C. jacchus*.

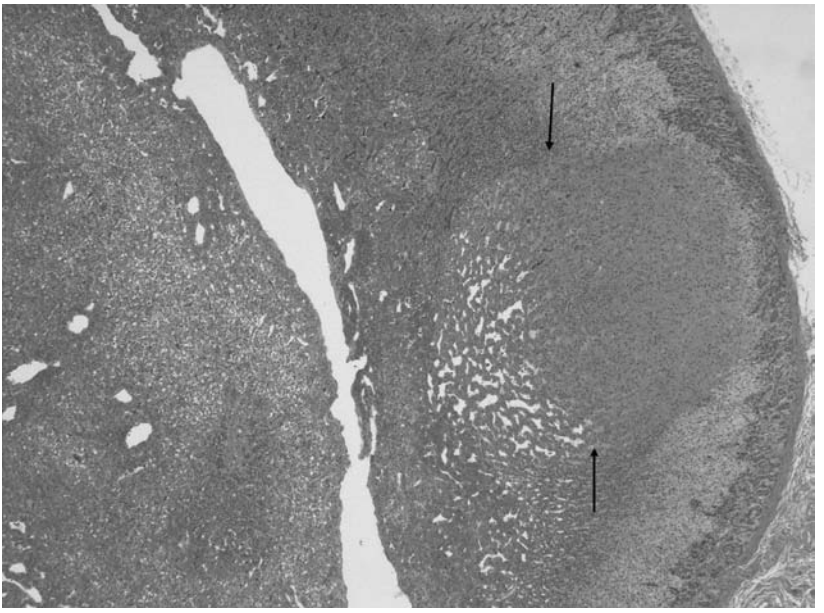

Figure 9 (*See color insert*) Large eosinophilic focus (*arrows*) in the adrenal cortex of *M. fascicularis*.

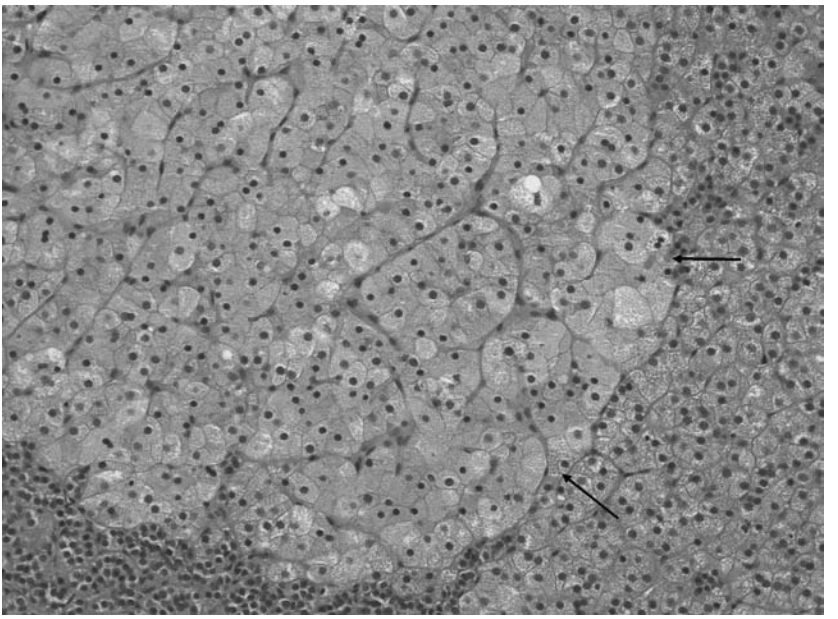

Figure 10 (*See color insert*) Focus of hypertrophic cells (*arrows*) in the adrenal cortex of *M. fascicularis*.

(*Lagothrix lagothricha*) by Henderson (Henderson, 1970). These nodules had a deeply basophilic staining cytoplasm and hyperchromatic nuclei.

Diffuse Cortical Cell Hypertrophy

Diffuse cortical cell hypertrophy (Fig. 11) is a condition that can be seen in cynomolgus monkeys and other primates suffering from various diseases or chronic stress for other reasons. Since only healthy terminal killed control animals are included in our historical data files, the incidence is very low in these data. Macroscopically, adrenal glands may show a brown discoloration and enlargement. Occasionally (when the adrenal is exactly trimmed in a plane through the middle of the medulla), an increased width of the cortex is discernible. Histologically, it is characterized by increased size of cortical cells, with obviously bigger nuclei, affecting large parts or the complete cortex. Diffuse cell hypertrophy is almost always accompanied by increased cytoplasmic eosinophilia (depletion of vacuoles). Occasionally, a fine basophilic stippling can additionally be seen in the cytoplasm. Stress-related loss of intracellular lipid associated with increased eosinophilia was already observed in the zona fasciculata and reticularis of squirrel monkeys (*Saimirii sciureus*) after 1-hour fixation in a primate restraining chair compared to animals killed (decapitated) immediately after removal from their cage (Penny and Brown, 1971).

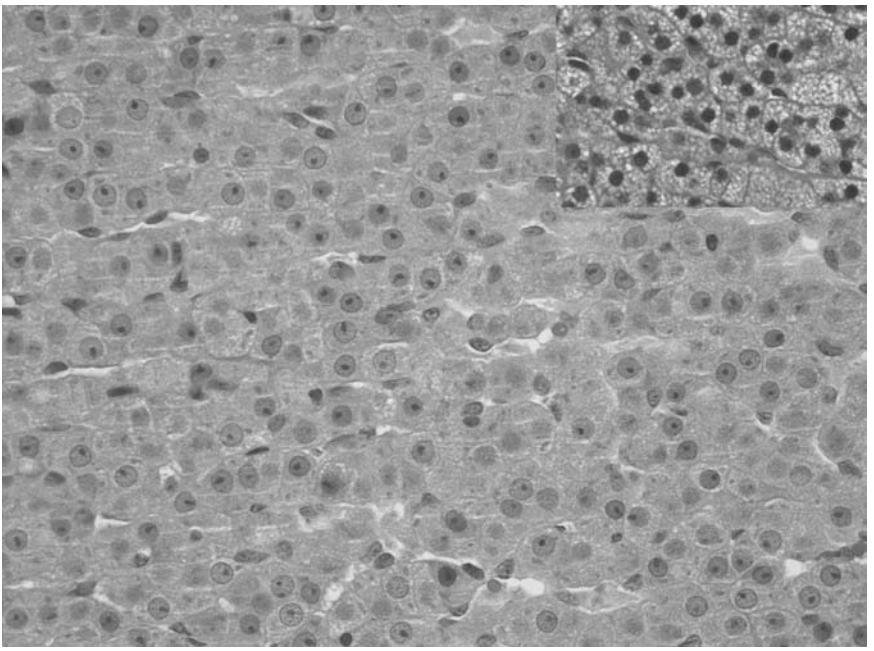

Figure 11 (*See color insert*) Diffuse hypertrophy of cotical cells in *M. fascicularis*. *Inset:* Normal sized cortical cells. Note also increased eosinophilia and basophilic stippling of hypertrophied cells.

Cortical Necrosis

Cortical necrosis is occasionally found in the cortex of *M. fascicularis* and *C. jacchus*. Changes represent sharply demarcated coagulative necrosis (Fig. 12) and are characterized by karyolytic or pyknotic nuclei and a strongly eosinophilic condensed cytoplasm. In a series of more than 3000 necropsies on chacma baboons (*Papio ursinus*), adrenal cortical necrosis was observed in 50% of the baboons that revealed cardiomyopathy, and in 63% with diarrhea (Weber and Greeff, 1973).

Cortical/Corticomedullary Mineralization

Foci of mineralization do occur, focal or multifocal, and are located either at the corticomedullary junction or within the zona fasciculata as bluish/purple (HE stain) or black (Kossa stain) deposits (Fig. 13). Some of them reveal a laminated arrangement of the calcium deposit and an inflammatory reaction is generally absent. Occasionally, also the medulla can be affected. Adrenal mineralization is rare in *M. fascicularis*. In historical data of our colony only 11 cases could be identified among 449 control animals of both sexes. In contrast, a much higher incidence can be observed in rhesus monkeys. With eight cases out of 83 rhesus control animals, cortical mineralization was present in about 10% of animals of

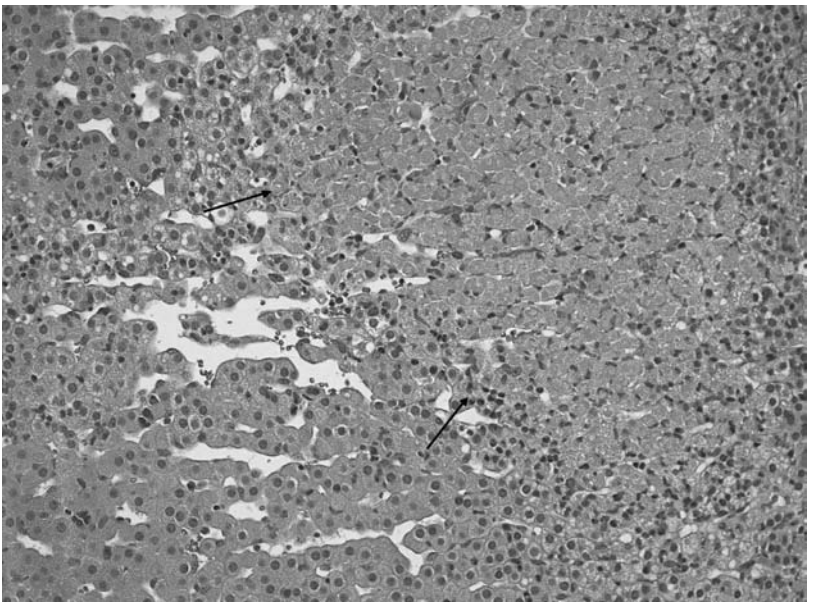

Figure 12 (*See color insert*) Focus of necrotic cells (*arrows*) in the adrenal cortex of *M. fascicularis*.

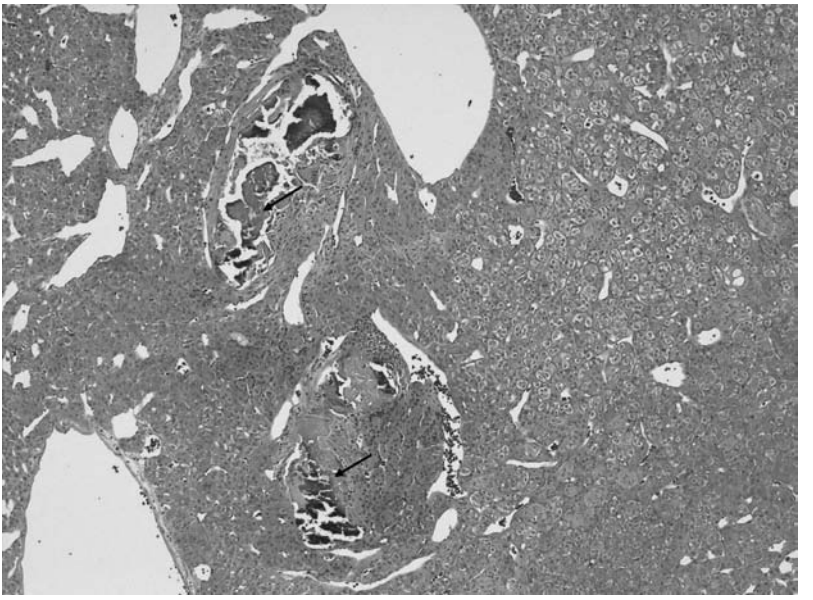

Figure 13 (*See color insert*) Foci of mineralization (*arrows*) in the cortex of *M. fascicularis*.

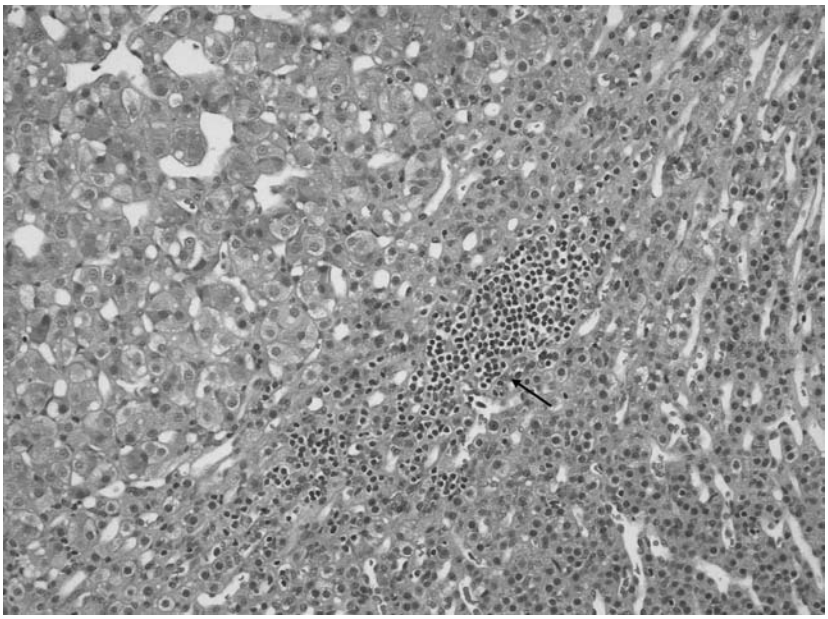

Figure 14 (*See color insert*) Inflammatory cell focus (*arrow*) in the cortex of *M. fascicularis.*

this species. Findings in our colony confirm previous data on cortical mineralization in primates. Kast et al. (Kast *et al*., 1994) examined this lesion in *M. mulatta* and *M. fascicularis*. Cortical mineralization (mainly affecting the corticomedullary junction) was present in 45% of male rhesus monkeys, 45% of female rhesus monkeys, but only in 6% of male cynomolgus monkeys. A study on adrenal calcification by Majeed and Gopinath (Majeed and Gopinath, 1980) on *M. mulatta*, *M. fascicularis,* and baboons (*Pabio spp*.) reported this change in 52.6%, 4.7% and 2.4%, respectively. Examination of adrenal glands of 604 baboons (Skelton-Stroud and Ishmael, 1985) showed calcification in 20 primates with a much higher prevalence in males. Mineralization of the adrenal capsule was seen by Henderson (Henderson, 1970) in three out of eight woolly monkeys.

Inflammatory Cell Foci

Inflammatory cell foci may be seen in any of the three primate species used in toxicological testing. Infiltrates are predominantly composed of lymphocytes and macrophages (Fig. 14), but polymorphonuclear granulocytes may also be present in low number. Together with inflammatory cell foci in the adrenal gland control laboratory primates show comparable infiltrates with varying incidence and generally low severity in a great number of organs. Accumulations of lymphoid cells at the corticomedullary junction were seen in a woolly monkey by Henderson (Henderson, 1970).

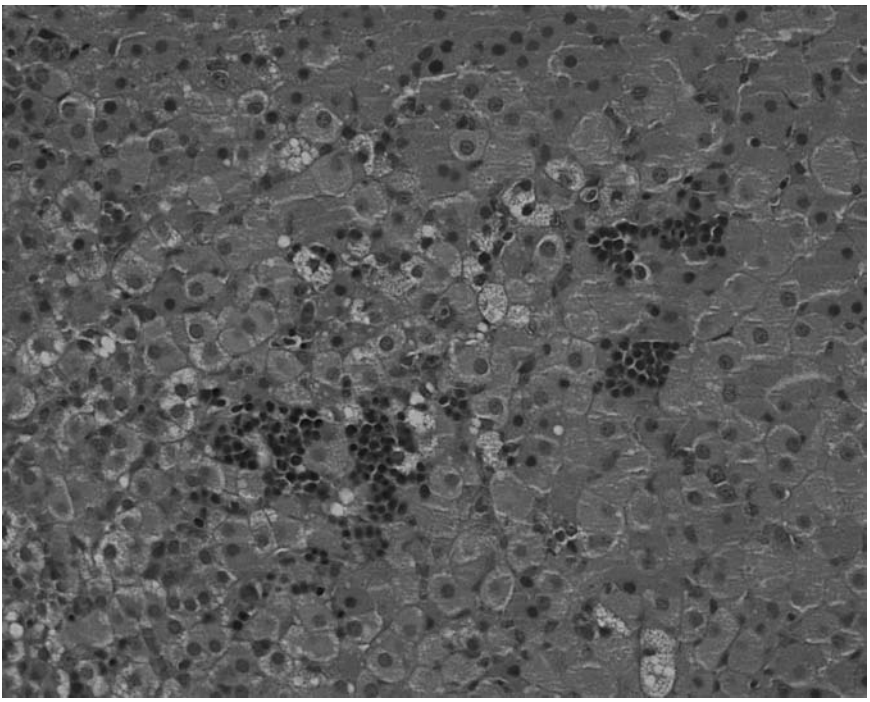

Figure 15 (*See color insert*) Erythropoiesis in the adrenal cortex of *C. jacchus*.

Extramedullary Hematopoiesis

One of the most common findings in *C. jacchus*, not seen in other laboratory primates like rhesus and cynomolgus monkeys (Ito *et al*., 1992), is extramedullary hematopoiesis. Foci of hematopoiesis show erythroblastic, myeloblastic, and megacaryocytic cell elements and are located in the cortex (Fig. 15). While in rodents the spleen is the most important site of extramedullary hematopoiesis (Andrews, 1998), in marmosets, a spectrum of organs including even pulmonary alveolar walls and the choroid plexus, is involved. Especially in the adrenal glands of marmosets, extramedullary hematopoiesis may be so severe that hematopoietic cells obscure the normal tissue architecture. In other studies on marmoset background pathology, the lesion was observed in 79% (Okazaki *et al*., 1996) and 25% (Tucker, 1984) of animals, respectively. In a study on spontaneous lesions of marmosets, 54% of animals were involved (Kaspareit *et al*., 2006). According to Tucker (Tucker, 1984), repeated bleeding in toxicological studies can increase the degree of hematopoiesis.

Capsular Adhesion

Sporadically, there can occur tight adhesion of the adrenal capsule to adjacent organs in *M. fascicularis*. Organs involved mostly include the liver (Fig. 16) or

Table 2 Incidence of Spontaneous Adrenal Gland Lesions in Control Cynomolgus monkeys (historical data, unpublished)

Adrenal gland lesion	Males ($n = 228$)	Females ($n = 221$)	Total ($n = 449$)
Cortical pigmentation	5	7	12
Cortical mineralization	8	3	11
Inflammatory cell foci	9	5	14
Focal hypertrophy	7	6	13
Eosinophilic focus	9	8	17
Cortical vacuolation	2	4	6
Fibrosis/adhesion	4	3	7
Ectopic adrenal cortex	2	3	5

the kidney. The connection normally is so tight that a separation of both tissues at necropsy can only be achieved with a loss of tissue of either organ. A higher degree of attachment to the liver has been observed by Mousa and van Esch (Mousa and van Esch, 2004) in two cynomolgus monkeys. In most fused areas, the margins between hepatic and adrenocortical or adrenomedullary parenchymal cells were indistinct and hepatocytes could be found in close contact with those cell types. In slides stained for reticulin-fibers, the reticular network of the liver and adrenal sinusoids clearly could be seen merging together. Hepatoadrenal adhesion/fusion also was observed in *Papio spp*. by Skelton-Stroud and Ishmael (Skelton-Stroud and Ishmael, 1985), with real fusion represented by several small nests of hepatocytes located within the zona fasciculata in one female control baboon.

Lipofuscin Pigmentation

This change is a microscopically detected, age-related pigment accumulation. Lipofuscin pigmentation is characterized by the deposition of yellow to brown

Table 3 Incidence of Spontaneous Adrenal Gland Lesions in Control Marmosets

Adrenal gland lesion	Males ($n = 102$)	Females ($n = 103$)	Total ($n = 205$)
Cortical atrophy	–	1	1
Cortical vacuolation	20	12	32
Cortical necrosis	1	1	2
Hematopoiesis	64	45	109
Inflammatory cell foci	34	21	55
Diffuse cortical cell hyperplasia	1	1	2
Focal hypertrophy	2	1	3
Eosinophilic focus	4	–	4

Source: Kaspareit *et al*., 2006.

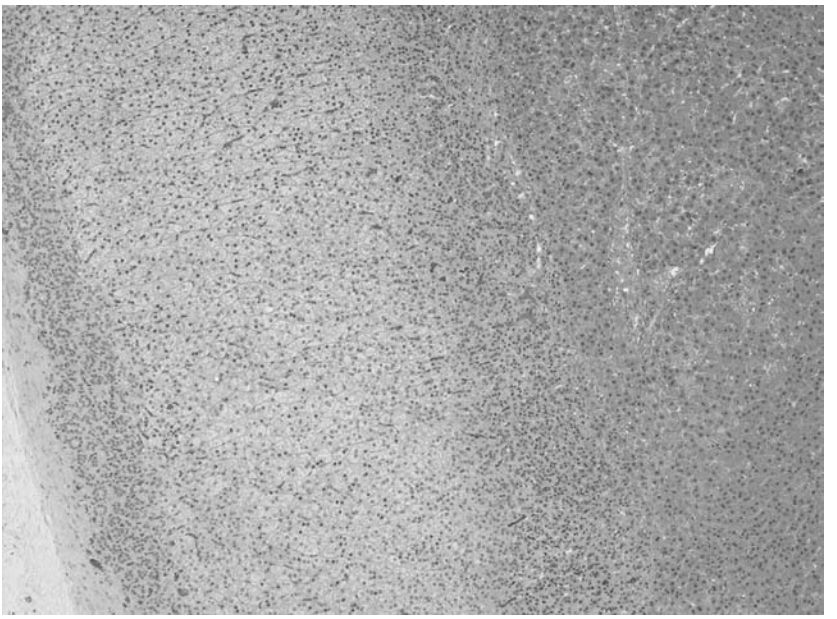

Figure 16 (*See color insert*) Tight adhesion between adrenal cortex (*left*) and liver (*right*) in *M. fascicularis*.

granular pigment in the cells of the zona reticularis and fasciculata. Initially, the pigment contained in the cytoplasm has a relatively uniform distribution, giving the cytoplasm a faintly brown discoloration. Finally, dark-brown granules fill the cells, (Fig. 17) which might be slightly distended. It must be distinguished from hemosiderin-laden macrophages, which also might occur in the same region. Special stains may be required to distinguish between hemosiderin and lipofuscin. Iron stains, such as prussian blue, will confirm hemosiderin. Lipofuscin may exhibit autofluorescence when unstained sections are irradiated with ultraviolet light. Lipofuscin pigmentation is rather infrequent in mature control cynomolgus monkeys aged 4 to 6 years of our toxicological studies. It was observed only in 12 of 449 control animals. In contrast, marked pigmentation was seen in 5 out of 32 cynomolgus monkeys of our geriatric colony (age $\geq$ 12 years). Ito et al. (Ito *et al.*,

Table 4 Incidence of Spontaneous Adrenal Gland Lesions in Control Rhesus monkeys (historical data, unpublished)

Adrenal gland lesion	Males ($n = 46$)	Females ($n = 37$)	Total ($n = 83$)
Cortical mineralization	3	5	8
Inflammatory cell foci	2	–	2
Ectopic adrenal cortex	1	–	1
Eosinophilic focus	1	1	2

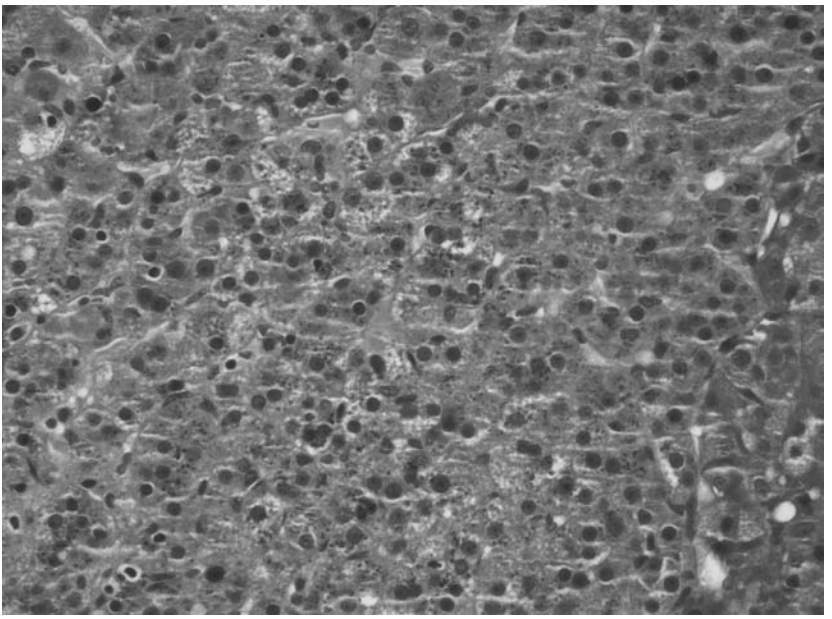

Figure 17 (*See color insert*) Dark-brown lipofuscin granules in cortical cells of an aged *M. fascicularis*.

1992) observed lipofuscin pigmentation in 10.9% of males and 7.7% of females of wild-caught *M. fascicularis* of indonesian and philipinian origin.

Adrenal Cysts

Adrenal cysts of epithelial origin were observed as incidental findings in two female saddleback tamarins (*Saguinus fuscicollis*) by Brack (Brack, 1998). In the first animal, a small cluster of cysts was observed at the corticomedullary border with PAS-positive contents and a lining of cuboidal cells. The second one had two large cysts in the cortex with PAS-negative contents and a bi- to multilayered epithelium.

NEOPLASMS

While a lot of information is available on tumor types and incidences that occur in the common laboratory rodents (mice, rats, hamsters), little is known about neoplasms in primates. Part of the information is derived from necropsies of zoo primates. These animals normally live till the end of their natural life span and, therefore, have a higher risk to develop tumors with increased age than laboratory primates. In contrast, primates that are kept in toxicological laboratories are regularly sacrificed in studies before they reach an age where neoplasms

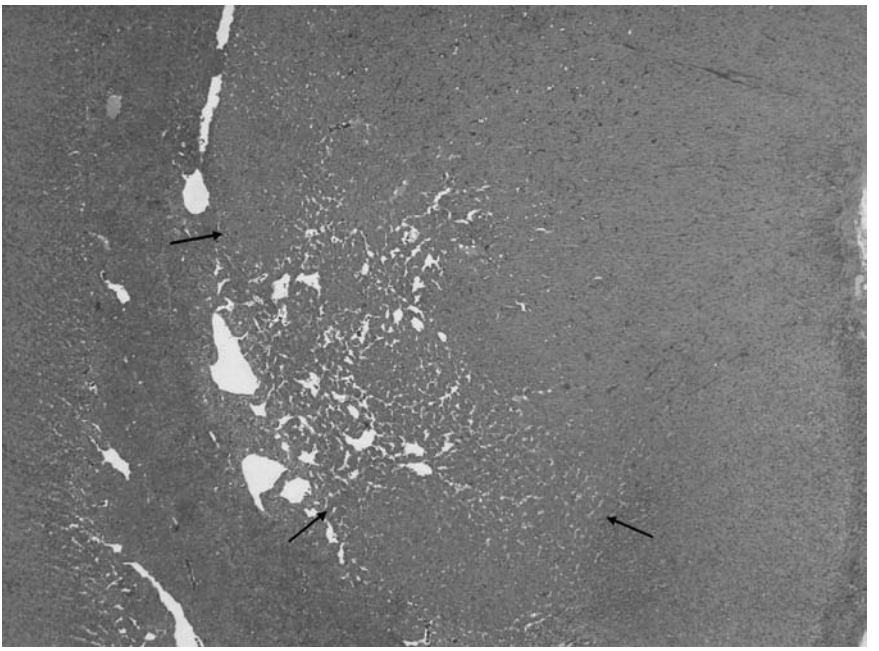

Figure 18 (*See color insert*) Adrenal cortical adenoma (*arrows*) in *M. fascicularis*.

are more frequent and, therefore, show tumors only sporadically. However, for the interpretation of the occurrence of preneoplastic or neoplastic findings as spontaneous or treatment-related in toxicological studies, information from any source can be helpful.

In our colony of cynomolgus monkeys, a total of 5 adrenal gland tumors were observed (Kaspareit *et al*., 2007). All these tumors were benign neoplasms. Four of them were cortical adenomas that compressed the remaining cortical tissue or the medulla (Fig. 18). They consisted of sheets of cortical cells with a strongly eosinophilic cytoplasm and loss of the foamy vacuolation, typical for normal cortical cells. They were observed in three males and one female with a range of age from 4 years 11 months to 6 years 5 months. The fifth tumor was a hemangioma (Fig. 19) that unilaterally had completely replaced the adrenal gland. It was seen in a 10 years 9 months old female and consisted of large, blood filled, endothelial lined caverns with only few islands of cortical cells remaining between them. Hemosiderin pigment in the interstitium of the tumor indicated former episodes of hemorrhage. In some of the blood filled spaces, thrombi were seen that were partly mineralized. Cortical adenomas have been observed before in cynomolgus monkeys and other primate species. Houser et al. (Houser *et al*., 1962) and Maruffo (Maruffo, 1967) found cortical adenomas in howler monkeys (*Alouatta villosa* and *Alouatta caraya*), but they were also observed in a blacktailed marmoset (*Callithrix melanura*) and a cottontop tamarin (*Saguinus oedipus*) by

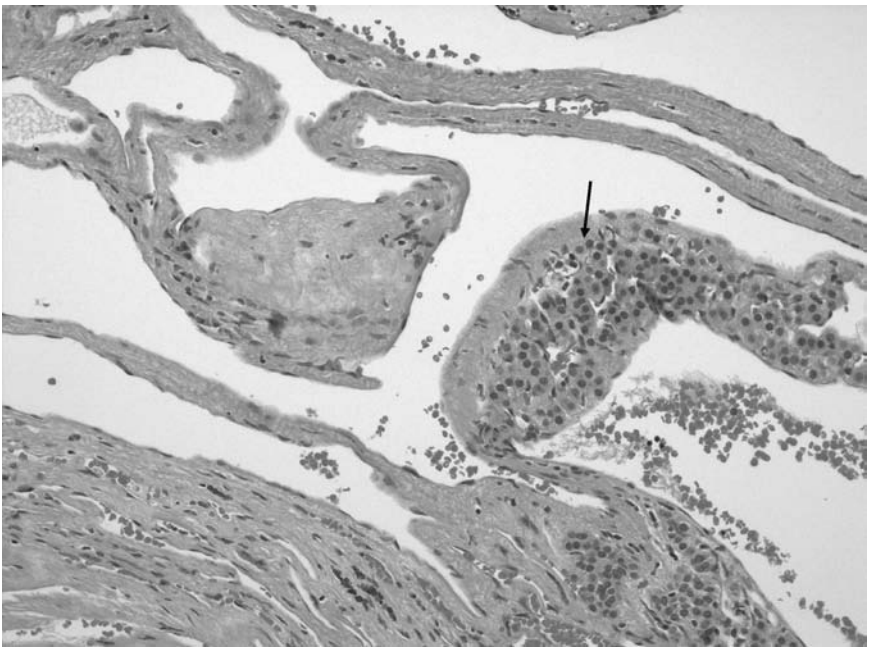

Figure 19 (*See color insert*) Cavernous hemangioma in *M. fascicularis*. Note also island of cortical cells within the tumor *(arrow*).

Dias et al. (Dias *et al*., 1996) and Brack (Brack, 2000). A malignant variant of cortical tumor was reported by Cicmanec et al. (Cicmanec *et al*., 1974), who found an adrenal cortical carcinoma with invasion of omentum, mesentery, kidney, and large intestine as well as distant metastasis to liver, lung, testis, and kidney in a 44 months old *M. fascicularis*. The unusual tumor type of myelolipoma (consisting of mature adipose tissue and focal collections of normal hematopoietic tissue) was repeatedly observed in marmoset species (Kakinuma *et al*., 1994; Pearson *et al*., 1987; Yanai *et al*., 1996), like *S. oedipus* and *C. jacchus*.

Tumors of the adrenal medulla have been also been described. They included pheochromocytoma (Brack, 2000; Dias *et al*., 1996; Seibold and Wolf, 1973; Vogel and Fritz, 2003) in cynomolgus monkey (*M. fascicularis*), golden lion tamarin (*Leontopithecus rosalia*), mantled howler monkey (*Aluoatta palliata*), brown spider monkey (*Ateles hybridus*), cottontop tamarin (*Saguinus oedipus*), and rhesus monkey (*M. mulatta*) and a ganglioneuroma in a golden lion tamarin (Dias *et al*., 1996).

REFERENCES

Andrews MC. The hematopoietic system. In: Turton J, Hooson J, eds. Target Organ Pathology London: Taylor & Francis; 1998:177.

Blanchard JL, Baskin GB, Watson EA. Generalized amyloidosis in rhesus monkeys. Vet. Pathol 1986; 23:425.
Brack M. Adrenal cortical epithelial cysts in two saddleback tamarins (*Saguinus fuscicollis*). J Comp Pathol 1998; 119:183.
Brack M. Adrenal gland tumours in two cotton-top tamarins (*Saguinus oedipus oedipus*). Lab Anim 2000; 34:106.
Brenner RM. Fine structure of adrenocortical cells in adult male rhesus monkeys. Dev Dyn 1966; 119:429.
Capen CC, deLellis RA, Yarrington YT. Endocrine system. In: Haschek WM, Rousseaux CG, eds. Handbook of Toxicologic Pathology. New York: NY, Academic press; 1991:675.
Cicmanec JJ, Neubauer RH, Wallen WC, et al. Spontaneous neoplasms of nonhuman primates. Attempted transmission to immunosuppressed hosts and in vitro characterization. Lab Anim Sci 1974; 24:233.
Conaway CH. Adrenal cortical rests of the ovarian hilus of the patas monkey. Folia Primatol 1969; 11:175.
Dias JLC, Montali RJ, Strandberg JD, et al. Endocrine neoplasia in New World primates. J Med Primatol 1996; 25:34.
Henderson JD. Naturally occurring lesions seen at necropsy in eight woolly monkeys (*Lagothrix sp*.), Lab Anim Care 1970; 20:1087.
Houser RG, Hartman FA, Knouff RA, et al. Adrenals in some panama monkeys. Anat Rec 1962; 142:41.
Ito T, Chatani F, Sasaki S, et al. Spontaneous lesions in cynomolgus monkeys used in toxicity studies. Exp Anim 1992; 41:455.
Kakinuma C, Harada T, Watanabe M, et al. Spontaneous adrenal and hepatic myelolipomas in the common marmoset. Tox Path 1994; 22:440.
Kaspareit J, Friderichs-Gromoll S, Buse E, et al. Background pathology of the common marmoset (*Callithrix jacchus*) in toxicological studies. Exp Tox Path 2006; 57:405.
Kaspareit J, Friderichs-Gromoll S, Buse E, et al. Spontaneous tumours observed in Cynomolgus monkeys (*Macaca fascicularis*) during a 15-year period. Exp Tox Path 2007; 59:163.
Kast A, Pfeil H, Weisse I. Calcified foci at the junction between adrenal cortex and medulla of rhesus monkeys. Lab Anim 1994; 28:80.
Levine J, Wolfe LG, Schiebinger RJ, et al. Rapid regression of fetal adrenal zone and absence of reticular zone in the marmoset. Endocrinology 1982; 111:1997.
Lowenstine LJ. A primer of primate pathology: Lesions and nonlesions. Tox Path 2003; 31:92.
Lundlage E, Murphy CL, Davern SM, et al. Systemic AA amyloidosis in the common marmoset. Vet Pathol 2005; 42:117.
Majeed SK, Gopinath C. Calcification in the adrenals and ovaries of monkeys. Lab Anim 1980; 14:363.
Maruffo CA. Spontaneous tumours in howler monkeys. Nature 1967; 213:521.
Mousa S, van Esch E. Two cases of adreno-hepatic fusion in cynomolgus monkeys (*Macaca fascicularis*). Tox Path 2004; 32:511.
Naumenko ES, Krylova RI. Amyloidosis in macaques in adler primatology center. Bull Exp Biol Med 2003; 136:80.
Okazaki Y, Kurata Y, Makinodan F, et al. Spontaneous lesions detected in common cotton-eared marmosets (*Callithrix jacchus*). J Vet Med Sci 1996; 58:181.

Pattisson JC. Male marmosets monkeys express an adrenal fetal zone at birth, but not a zona reticularis in adulthood. Endocrinology 2005; 146:364.
Pearson GR, Kirkwood JK, Scullon F. Adrenal myelolipoma in the cottontp tamarin (*Saguinus o. oeddipus*). Am J Primatol 1987; 13:203.
Penny DP, Brown GM. The fine structural morphology of adrenal cortices of normal and stressed squirrel monkeys. J Morph 1971; 134:447.
Prentice DE, Jorgeson W. Ectopic tissue in the kidney of rhesus monkeys (*Macaca mulata*). Lab Anim 1979; 13:221.
Schmidt IG. An accessory adrenal cortex in the kidney of a rhesus monkey and the effects of pyrimethamine on this tissue. Endocrinology 1956; 59:454.
Schmidt IG. Bone formation in the adrenal gland of a rhesus monkey. Endocrinology 1957; 61:781.
Seibold HR, Wolf RH. Neoplasms and proliferative lesions in 1065 nonhuman primate necropsies. Lab Anim Sci 1973; 23:533.
Skelton-Stroud PN, Ishmael J. Adrenal lesions in the baboon (*Papio spp*). Vet Pathol 1985; 22:141.
Slattum MM, Tsai CC, DiGiacomo RF, et al. Amyloidosis in pigtailed macaques (*Macaca nemestrina*): Pathologic aspects. Lab Anim Sci 1989; 39:567.
Takayama S, Makita T, Thorgeirsson U. Color Atlas of Nonhuman Primate Histology. Tokyo/Karger, Basel: Japan Scientific Societies Press; 1999:171.
Tucker MJ. A survey of the pathology of marmosets (*Callithrix jacchus*) under experiment. Lab Anim 1984; 18:351.
Vogel P, Fritz D. Cardiomyopathy associated with angiomatous pheochromocytoma in a rhesus macaque (*Macaca mulata*). Vet Pathol 2003; 40:468.
Weber HW, Greeff MJ. Observations on spontaneous pathological lesions in chacma baboons (*Papio ursinus*). Am J Phys Anthropol 1973; 38:407.
Yanai T, Tanigushi H, Sakai H, et al. Bilateral giant cell myelolipoma in the adrenal gland of a cotton-top tamarin (*Sanguinus oedipus*). J Med Primatol 1996; 25:309.

6

Hypothalamic-Pituitary-Adrenal Toxicity in Dogs

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UNIQUE FEATURES OF THE HPA AXIS IN THE DOG VS. OTHER MAMMALS

The hypothalamic-pituitary-adrenal gland (HPA) axis is a critical feedback system for maintaining homeostatic control of hormone synthesis and secretion. In the dog, as in human beings, the stress response is one physiological function that is dependent on proper functioning of the HPA axis. The hormonal connection with the hypothalamic-pituitary system is via production and/or secretion of glucocorticoids and mineralcorticoids by unique zones that comprise the cortex of the adrenal glands. The cortex is of mesodermal origin and is comprised of three functionally distinct but not morphologically distinct layers (zones) which surround the medulla. The outer layer (zona glomerulosa) produces the mineralcorticoid aldosterone, the principal hormone that orchestrates sodium and potassium concentrations in the maintenance of normal fluid balance and circulatory volume. The middle layer (zona fasciculata) secretes glucocorticoids and the inner layer of the cortex (zona reticularis) secretes sex steroids.

Adrenocorticotropin hormone (ACTH) is the major hormone secreted by the pituitary gland that modulates release of glucocorticoids, notably cortisol, by a negative feedback response. The production depends upon the blood level of the

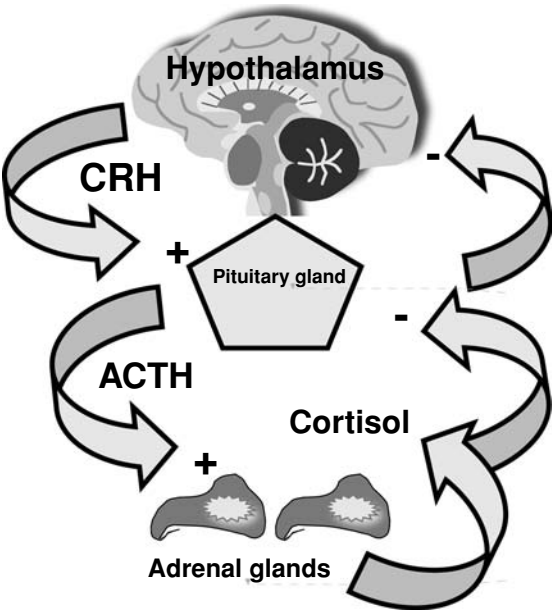

Figure 1 The diagram illustrates the hormonal interactions that regulate the hypothalamic-pituitary-adrenal axis. A reduction of the circulating cortisol concentration initiates the release of CRH which stimulates the pituitary gland to release ACTH into the peripheral circulation and activates the adrenal glands to release cortisol. A rise in the circulating cortisol inhibits their release (negative feedback).

hormone secreted by the target endocrine organ, the metabolic state of the animal, the control of the CNS through secretion of releasing factors, and factors inhibiting the release of pituitary hormones (Liwnicz and Liwnicz, 1984). Following physical or emotional stress, the HPA system regulates the release of hormones pivotal to the physiological responses needed to protect the organism. The stress response is initially driven by catecholamines secreted from the sympathetic nervous system located in the medulla of the adrenal glands followed rapidly by hypothalamic release of corticotropin-releasing hormone (CRH) (Sapolsky *et al*., 2000). Normally, CRH is released in response to endogenous rhythms in the brain for a diurnal pattern (Liwnicz and Liwnicz, 1984). The release of CRH acts on the anterior pituitary to secrete ACTH, which then stimulates the release of glucocorticoids (primarily cortisol in the dog and human beings) from the adrenal gland. Cortisol in turn inhibits CRH release by the hypothalamus which inhibits further ACTH release from the pituitary gland. (Fig. 1).

The dog is frequently used as a model of human adrenal disease as the species share similar pathways for steroid synthesis. Steroid synthesis is catalyzed by mitochondrial and microsomal Cytochrome P450s, isomerases, and dehydrogenases (Hallberg, 1990). All steroid synthesis in the adrenal gland

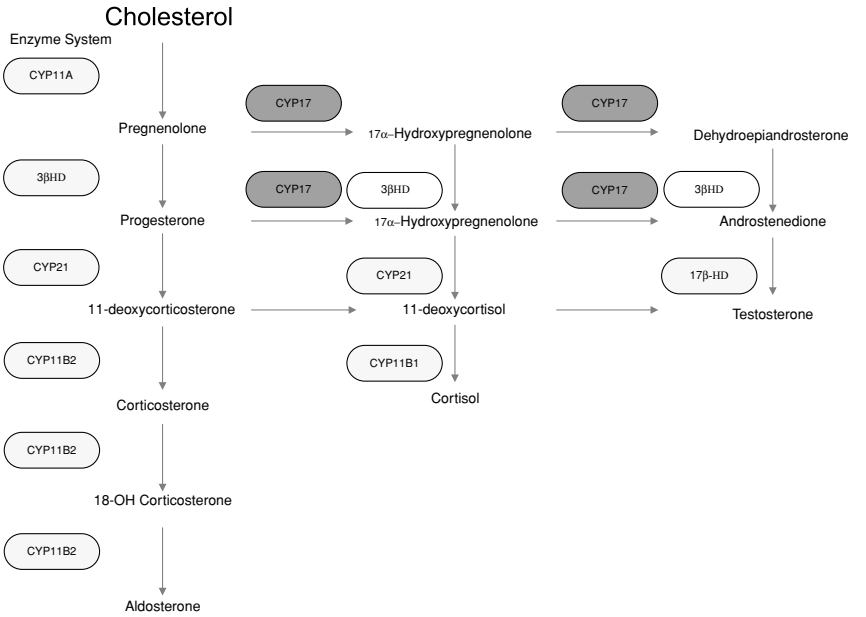

Figure 2 A schematic representation of steroid synthesis in the dog and rat. Comparison of the steroid biosynthesis pathways in the adrenal cortex in the dog and rat. CYP17 is not found in the rat. *Abbreviations*: 3βHD, 3β-Hydroxysteroid Dehydrogenase; 17βHD, 17-βHydroxysteroid Dehydrogenase; 5αR, 5α-reductase.

begins with cholesterol precursors stored as esters in cytosolic lipid droplets either synthesized directly by the adrenal or supplied by the liver via blood lipoproteins (Hallberg, 1990). The mitochondrial fraction of the subcellular tissue has the largest free cholesterol of any portion of the adrenal gland (Holzbauer, 1981). Cholesterol is both synthesized by the adrenal gland as well as taken up from the circulation (Temple and Liddle, 1970). Steroid synthesis is influenced by ACTH activity. Stimulation with ACTH leads to an increase in adrenal corticosterone levels which in turn upregulates progesterone and pregnenolone levels (Holzbauer, 1981).

In humans and dogs the main glucocorticoid produced in the adrenal cortex is cortisol. This is in contrast to rodents that primarily secrete corticosterone (Fig. 2). Adrenal steroid biosynthesis is different in the rat due to a lack of Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) expression (Hinson and Raven, 2006). CYP17 is a microsomal enzyme catalyzing two distinct activities, 17α-hydroxylase and 17,20-lyase, essential for the biosynthesis of 19-carbon androgens, androstenedione, and dehydroepiandrosterone (DHEA) (Brock and Waterman, 1999; Liu *et al*., 2005). Without this enzyme, there is minimal secretion of adrenal androgens; therefore, corticosterone is the primary glucocorticoid secreted in the rat rather than cortisol as in the dog and human (Hinson and Raven, 2006).

Aldosterone secretion is regulated by the renin–angiotensin system that involves interaction with the kidney (Temple and Liddle, 1970). The juxtaglomerular cells located in the wall of the afferent glomerular arteriole synthesize and secrete renin in response to several signals, such as changes in renal perfusion pressure (baroreceptor response). Renin is a proteolytic enzyme that catalyzes a chain reaction by converting hepatic angiotensinogen to angiotensin I followed by action of angiotensin converting enzyme to form angiotensinogen II. In addition to causing potent vasoconstriction, angiotensinogen II stimulates the release of aldosterone. The potassium concentration is an important regulator of aldosterone secretion. A rise in the potassium concentration causes secretion of aldosterone, while a reduction is inhibitory.

Canine Abnormalities

Disorders of the adrenal cortex are classified into those that decrease, increase, or have no effect on steroid release (Hoff, 1984). Excess production of cortisol or a deficiency of cortisol secretion can disrupt the HPA feedback loop. A decrease in steroid release, or hypocorticalism, can be caused by a number of factors including inflammatory disease, infectious agents, or neoplasia (Hoff, 1984). Primary hypoadrenocorticism, or Addison Disease, results when there is destruction of the adrenal cortices leading to clinical disease. A profound loss of cortisol can lead to deranged metabolism and an inability to manage stress and infections (Plechner, 2004). In addition to cortisol loss, reduced mineralocorticoid production and release can develop in some adrenal gland diseases as well. As a consequence of this, fluid and electrolyte loss may be a life-threatening pathophysiological event (Pinney, 2000).

An increase in cortisol production in dogs is classified as Canine Cushing syndrome. There are two main forms of excess cortisol production—pituitary-dependent and adrenal-dependent hyperadrenocorticism. By definition, with pituitary-dependent hyperadrenocorticism (PDH), the pituitary is over-secreting ACTH which overstimulates the zona fasciculata layer of the adrenal glands to secrete excessive quantities of cortisol while the feedback is impaired (Plechner, 2004). Bilateral adrenal hyperplasia is frequently a consequence of PDH. Adrenal-dependent hyperadrenocorticism (ADH) is caused by adrenal tumors secreting hormones independent of control by the pituitary gland. ADH may result from an adrenal adenoma or carcinoma and are almost always unilateral resulting in asymmetrical size on ultrasonography (Meijer, 1978). The excessive quantities of cortisol produced by an adrenal tumor inhibit further production of ACTH by the pituitary gland. The incidences of both PDH and ADH are much higher in dogs than human beings with the PDH form seen most frequently in dogs, particularly in the middle-aged years (Greco, 2007).

As glucocorticoids play a major role in glucose metabolism, hyperadrenocorticalism can stimulate hepatic gluconeogenesis (conversion of amino acids to glycogen). This response can enhance the effects of glucagon and epinephrine. To support this activity, glucocorticoids inhibit protein synthesis and, in excess, cause protein catabolism with subsequent breakdown and release of amino acids. The

loss of mass of the abdominal muscles contributes to the "pot-bellied" appearance of dogs with cortisol excess secondary to hyperadrenocorticism. Glucocorticoids have an "anti-insulin" effect, i.e., interfere with glucose uptake and metabolism by skeletal muscle and adipose tissue. Cortisol excess should be considered in a patient with diabetes mellitus when insulin regulation is problematic. Glucocorticoid excess, endogenous or exogenous, commonly causes excessive glycogen storage in the canine liver and abnormal hepatic enzyme tests (Meyer, 2004).

Symptoms of Canine Cushing disease that may bring a patient into the veterinarian's office include frequent drinking and urination, patchy fur and/or abdominal distension. A workup of the dog would include routine blood analysis and a urinalysis. The complete blood count may show a pattern of change in the white blood cell profile called a "stress leukogram"(Brooks, 2007). This term refers to the relative change in proportions of different white blood cell populations with a shift in the profile. Concurrent stress-induced sympathoadrenal-stimulated release of excess epinephrine can contribute to changes to the leukogram. An elevation in relative alkaline phosphatase and cholesterol may also be measured. The alkaline phosphatase increase is due to a response to cortisol in the liver and the elevation in total cholesterol concentration is a consequence of abnormal fat mobilization (Brooks, 2007). The urine of dogs with Cushing disease may be dilute due to excess water consumption.

Measurement of circulating ACTH can distinguish between ADH and PDH. Dogs with an adrenal tumor will have low- to undetectable levels of ACTH (Greco, 2007). The preferred veterinary tests to diagnose hyperadrenocorticism are : The ACTH stimulation test and the low-dose dexamethasone suppression test (Feldman *et al*., 1996). Only the former test is useful for diagnosing Iatrogenic Cushing disease, a condition caused by chronic glucocortoid administration. Prescription glucocorticoids, like the natural products, inhibit the release of CRH and ACTH from the hypothalamus and pituitary, respectively. When glucocorticoid treatment is either long term (greater than several weeks) or high dose, the normal HPA can be chronically inhibited. With no ACTH stimulation, adrenal glands atrophy and become inactive.

The ACTH stimulation test can reliably diagnose about 85% of PDH cases but only 50% of ADH cases. It is quick and simple to perform and is less affected by stress than measuring serum cortisol. The ACTH stimulation test does not distinguish PDH from ADH. With iatrogenic disease, dogs with excessive or prolonged administration of corticosteroid medications will have little or no stimulation when challenged by exogenous ACTH (Peterson, 2007). Some dogs with nonadrenal illness will show an exaggerated response to ACTH stimulation. With PDH, approximately 85% of dogs will show exaggerated response to stimulation with exogenous ACTH. With ADH, approximately 60% of dogs will show an exaggerated response to ACTH and 40% will have a normal response.

Synthetic ACTH (Cortrosyn® i.e., cosyntropin), can be used to stimulate adrenal gland secretion of cortisol before and following the administration of an agent to assess its effects. An IV injection of 250 μg Cortrosyn will rapidly influence cortisol levels. Blood samples for cortisol measurements can be

collected from the jugular vein prior to injection and 30 minutes and 60 minutes postinjection. Additional serum samples can be collected 3, 6, 10, and 14 days post-ACTH administration. A normal adrenal response would demonstrate a reduction in cortisol and ACTH levels. Over time dogs are stimulated with ACTH prior to agent administration and an increase in serum cortisol concentration should be observed if the agent does not influence the adrenal gland. This is a measure of cortisol release following synthetic ACTH stimulation.

The initial values may be used as a reference to monitoring effects. Normal dogs will show an increase in cortisol levels of up to 300 nmol/L poststimulation (Peterson, 2007). An exaggerated response is expected in animals with PDH and cortisol concentrations can rise above 600 nmol/L and often above 1000 nmol/L. Dogs with an adrenal tumor usually have basal cortisol greater than 250 nmol/L with little or no change after stimulation. However, the ACTH stimulation test is not as sensitive at detecting adrenal tumors, and results which are not diagnostic should be confirmed with a low-dose dexamethasone test, especially in animals showing clinical signs of Cushing disease.

The low-dose dexamethasone screening test is more reliable than the ACTH stimulation test in confirming hyperadrenocorticism, as all the results are diagnostic in ADH and for about 95% of PDH cases. It is a much longer procedure and can be severely affected by stress. In the test, a basal blood sample is collected in the morning. Dexamethasone is then injected intravenously and two blood samples are collected 3 hours and 8 hours later. Normal dogs will depress their cortisol concentrations by at least 50% at 3 hours and cortisol levels remain below 40 nmol/L at 8 hours (Liwnicz and Liwnicz, 1984; Meijer, 1978). Results indicative of Cushing disease involve no change in cortisol with low-dose dexamethasone. If the cortisol levels are unchanged by low- and high-dose dexamethasone, then a cortisol secreting adrenocortical tumor or ectopic ACTH syndrome is suspected. Dogs with PDH will depress their cortisol levels at 3 hours but will increase at 8 hours and will show an accentuated rebound of more than 40 nmol/L. Dogs with an adrenal tumor will not have depressed cortisol at 3 hours and will remain at more than 40 nmol/L at 8 hours (Brooks, 2007).

In addition to the ACTH stimulation test and low-dose dexamethasone test, measuring the concentration of endogenous plasma ACTH may be helpful in the diagnosis of iatrogenic disease. Synthetic ACTH is injected intramuscularly after obtaining a baseline cortisol level; then 1 hour later, another blood sample is obtained and a cortisol level is ascertained again looking for change. When measuring adrenal function in the dog, it is important to consider diurnal variation and ultradian rhythm (Kemppainen and Sartin, 1984). Examination of a single point in time can be very misleading and a time course must be assessed to truly understand "normal" variation from a pathologic change. The process of sample collection can cause undue stress to dogs and stress is a major activator of the HPA axis. A simple procedure, such as insertion of a gavage needle has been shown to alter plasma glucocorticoid levels (Colagiovanni, unpublished observation).

Basal cortisol levels alone are of little value in diagnosing Canine Cushing disease, as there is a lot of variation and overlap between healthy dogs. The

Cortisol/Creatinine Ratio is a very sensitive test to exclude hyperadrenocorticism but must not be used to diagnose hyperadrenocorticism as it is not specific, and nonadrenal illness will give a positive result. A morning urine sample is collected and this reflects the cortisol release over several hours.

Medical treatment in canines for these conditions include surgical hypophysectomy, destruction of the adrenal cortex with Mitotane, or blockage of adrenocortical steroid synthesis (Rijnberk *et al*., 2003). Mitotane (*o,p′*-DDD) is a tissue-selective toxicant. Following local metabolic activation and irreversible protein binding in the adrenal cortex of dogs, Mitotane is adrenocorticolytic (Lindhe *et al*., 2002). Another treatment option is Trilostane, a competitive inhibitor of 3β-hydroxysteroid dehydrogenase (3-βHD) (Reine, 2007). In addition to 3-βHD activity, Trilostane is thought to inhibit other enzymes in the hormone cascade, possibly including 11β-hydroxylase or 11β-hydroxysteroid dehydrogenase (Sieber-Ruckstuhl, 2006). Trilostane is not currently available in the United States, but is replacing Mitotane in countries where it has been approved due to ease of use and low rates of side effects (Reine, 2007).

AGE-RELATED EVALUATION OF THE HPA AXIS

The dog is an excellent natural model of age-related human neurodegenerative pathologies. Both the human and dog secrete cortisol as the primary glucocorticoid, and the main ACTH-secreting regions of the canine brain correspond with patterns in humans (Pesini, 2004). The glucocorticoids initiate primary molecular interactions in their target cells through binding to their nuclear receptors (Sapolsky *et al*., 2000). Feedback actions of glucocorticoids are mediated via glucocorticoid receptors (GRs) in the hypothalamus and pituitary (Rothuizen, 1991). In the brain, two corticosteroid receptors are involved with homeostasis—the mineralocorticoid receptor (MR) and GR (Rothuizen, 1991). Various factors influence the sensitivity of cells to glucocorticoids. The magnitude of hormone activity is influenced in a cell by glucocorticoids through MRs and GRs and depends upon how many ligand–receptor complexes are formed in the cell (Sapolsky *et al*., 2000). This, in turn, is due to the number of inherent receptors in a tissue and the corresponding free ligand concentrations available. As cortisol and corticosterone are the glucocorticoids which bind with the greatest affinity to the MR, evaluation of their activity is important for understanding age-related changes. The canine MR is found widely throughout the HPA, primarily in the septohippocampal region, then the hypothalamus and cortical regions (Reul and De Kloet, 1991). The GR are evenly distributed in canine brain tissue. Both MR and GR are abundant in anterior and neurointermediate lobes of the canine pituitary (Reul and De Kloet, 1991). Progestins are known to exert effects on the HPA axis by means of the glucocorticoid receptor (Selman, 1994).

Areas of ACTH production have been mapped in the canine brain. Using Anti-ACTH antibody stains in dogs, ACTH-secreting cells were noted to be concentrated in the hypothalamus in the arcuate nucleus and periventricular nucleus. Low concentrations in the ventral tegmental area of the mesencephalon and

along a strip from the raphe to the cerebral penduncle were seen (Pesini, 2004). Differences with dogs and other mammals were evident in the hypothalamus, where there is a moderate to high density of labeled fibers and there are presumed terminals in the medial and lateral preoptic area, in the ventromedial hypothalamic area, and in the supramammillary nuclei (as in the cat) (Pesini, 2004). There may be some similarities in carnivores as opposed to other mammals, as both the dog and cat lack ACTH innervation in the substantia nigra (Pesini, 2004).

Differences in neuroendocrine regulation and central corticosteroid receptors in dogs with aging have been reported (Reul and De Kloet, 1991). In aged dogs, MR concentrations were markedly decreased in extrahypothalamic regions. This could lead to saturation of peripheral MR receptors and a reduction in aldosterone release. Glucocorticoid receptor concentrations were not reduced compared to the brain tissues in young animals; and in the pituitary, GR levels were increased by approximately 70% compared to young dogs. This corresponds with observed hypercortisolemia under basal cortisol and stress challenged versus young animals (Reul and De Kloet, 1991). It is the glucocorticoids that regulate the stress response by terminating feedback via GR in the hypothalamus and hypophysis (Rothuizen, 1991). In a time-course study with 11 beagles beginning at approximately the age of 4 years and ending at approximately the age of 9 years, no significant cortisol hypersensitivity to ACTH stimulation was seen (Goy-Thollot *et al*., 2007). These data suggest that basal cortisol was negatively correlated with adrenal responsiveness for cortisol (i.e., perhaps in the zona fasciculata, ACTH reactivity could depend upon baseline cortisol levels limiting the amplitude of response to stimulation). While in dogs, the feedback mechanisms remain unchanged in aging, secretion of ACTH and cortisol are increased by emotional stress or when induced by immobilization (Rothuizen, 1991). No apparent differences in age-related effects on corticosteroid receptors were seen with different strains of dogs, nor were sex differences noted in studies of young (18 to 24 months) versus aged ($\geq$ 11 years of age) dogs (Reul and De Kloet, 1991). As a consequence of hypercortisolemia, serum chemistry abnormalities may be seen in dogs. These changes can include increases in alkaline phosphatase, alanine aminotransferase, cholesterol, and glucose (Greco, 2007).

KNOWN AGENTS THAT TARGET THE HPA AXIS IN THE DOG

Adrenal steroids are metabolized in the liver and other tissues prior to excretion. The enzymes that metabolize steroids are also a potential site for xenobiotic toxic effects. The adrenal gland is the site in the endocrine system most frequently noted for toxicity (Hinson and Raven, 2006; Ribelin, 1984). Because of the rich blood supply to this organ, it facilitates delivery of toxins to the tissue. Accumulation of xenobiotics can occur in the adrenal cortex. These effects include lipid accumulation or endothelial damage in the mitochondria, endoplasmic reticulum, or lysosomes (Ribelin, 1984). Aliphatic compounds of 3 or 4 carbon-chain lengths with electronegative groups at one or both ends have shown necrosis of zona fasciculata and reticularis. Three carbon length molecules appear to be the most potent

Table 1 Known Canine Adrenotoxic Agents

Agent	Effect	Adrenal location
Aminoglutethimide	Cellular swelling, lipidosis, focal cellular loss	ZF, ZR, ZG
Clotrimazole	Cloudy swelling, lipidosis	ZF, ZR
Etomidate	Irreversible binding	ZF, ZR
o,p′-DDD	Lipidosis, cytotoxic cellular atrophy	ZF/ZR
DMNM	Vacuolar generation, atrophy	ZF,ZR
Hexadimethrine bromide	Infarcts	ZF, ZR, ZG
Mefloquine	Minimal necrosis	ZF, ZR
Ponceau SX (FD&C Red No. 4)	Atrophy	ZG
Reserpine	Cellular Proliferation	Medulla
Medroxyprogesterone	Atrophy	ZF, ZR

Source: Adapted from Ribelin, 1984.
Abbreviations: ZF, zona fasciculata; ZR, zona reticularis; ZG, zona glomerulosa.

(Ribelin, 1984). The PCBs, DDT, PBBs, and toxaphene are known to accumulate to a greater degree in the adrenal gland than other tissues, including the liver (Hallberg, 1990). Mammalian adrenal glands are more susceptible than those of birds or fish due to the discrete location of this highly vascularized organ (Hinson and Raven, 2006).

The homeostatic mechanisms of the HPA axis regulate the system in such a balance, that often, only the most catastrophic effects are recognized as adrenal disruption. To assess toxicity to the HPA axis, it is useful to measure ACTH and corticosteroid concentrations, as well as assess adrenal weight relative to a control population and tissue histopathology. Reductions in adrenal weight have been seen with synthetic progestins in dogs. Medroxyprogesterone causes adrenal atrophy (up to 40%) with repeated administration (Selman, 1994). Medroxyprogesterone and other progesterone derivatives, such as proligestone, also suppress ACTH and cortisol levels in plasma (Selman, 1994). These glucocorticoid agonist effects were long lasting, resulting in suppression of the HPA axis.

One of the most potent identified adrenotoxic agents is the human drug etomidate. Etomidate is an intravenous anesthetic used for prolonged sedation (Wagner, 1984). Etomidate causes adrenal insufficiency via direct inhibition of CYP11B1, thus blocking cortisol synthesis (Hinson and Raven, 2006). Another well characterized direct inhibitor of adrenal function is a derivative of the pesticide DDT, Mitotane (*o,p′*-DDD or Lysodren), used in veterinary practice to treat Cushing syndrome. It is a tissue selective toxicant that irreversibly binds proteins in the adrenal cortex (Lindhe *et al*., 2002). Many agents can adversely affect adrenal function but may act in different zones of the tissue (Table 1) (Ribelin, 1984). If toxicity is suspected from a pharmaceutical or chemical agent, assessment of ACTH and cortisol pre- and postexposure can offer insight into tissue targets.

A case study of the dog as a model for assessing potential adrenal toxicity can be found with the oncology agent OSI-7836. In human clinical trials, data indicated fatigue as a common dose-limiting toxicity with repeated OSI-7836 administration (de Jonge, 2003; Siu, 2003). The mechanism was not understood and the clinical team asked the nonclinical scientists for possible animal models to evaluate the observed effects. Was the test article affecting adrenal function directly? As a reduction in red blood cell oxygen-carrying capacity has been observed with other chemotherapy agents, could this be the cause for fatigue? Was the thyroid gland targeted or was OSI-7836 possibly causing a centrally mediated effect? The red blood cell hypothesis was eliminated as a cause of the observed weakness, as no clinically relevant reductions in the circulating red cell mass were observed. The beagle dog was selected as a model for assessment of effects on the HPA axis as well as adrenal and thyroid function. Thyroid function was evaluated because hypothyroidism can cause lethargy in the dog (Meyer and Harvey, 2004). The pituitary-adrenal axis was assessed by measurement of serum cortisol and ACTH concentrations. Thyroid function was evaluated by measurement of serum thyroxine (T_4) concentration prior to the administration of OSI-7836 and at time points following its administration. The thyroid and adrenal functions were evaluated in four female dogs following the administration of a single IV dose of OSI-7836 at 12 mg/kg. All hormones were measured by an Immunolite® Chemiluminescent System (DPC, Los Angeles, California, U.S.A.).

Initially, the T_4, ACTH, and cortisol were measured prior to and 6 days after the IV bolus injection of 12 mg/kg OSI-7836. Pre- and post-OSI-7836 administration hormone values were compared to determine an effect. To normalize for treatment-related effects, blood was collected for cortisol measurements prior to and 4 hours after an IV bolus injection of normal saline (2 mL/kg). Cortrosyn® was used to stimulate adrenal gland secretion of cortisol before and following the administration of OSI-7836. Blood samples for cortisol measurements were collected from the jugular vein prior to and 30 and 60 minutes post- Cortrosyn® injection. Additional serum samples were collected out to 14 days postsynthetic ACTH administration. All samples were stored frozen until assayed for cortisol.

The cortisol levels in all animals were markedly reduced following the administration of OSI-7836 and remained reduced to 144 hours post-OSI-7836 administration at levels that were below the limit of quantitation of the assay (1 μg/dL). The ACTH levels were also markedly reduced at 144 hours; all values were below the limit of quantitation of the assay at 72 hours (Table 2). When dogs were stimulated with ACTH prior to OSI-7836 administration, there was an approximately 5-fold increase in serum cortisol levels. The adrenal response to a second ACTH stimulation immediately (and also several days) following the administration of OSI-7836 was also normal (Fig. 3). OSI-7836 did not seem to alter thyroid function but did cause a profound reduction in the circulating cortisol and ACTH levels. The response of the adrenal gland to the ACTH

Table 2 Mean Serum Levels for Cortisol and ACTH in Beagle Dogs Following 12 mg/kg OSI-7836 by IV Administration

Hormone	No. of samples	Time (hours)					
		0	24	72	96	120	144
Cortisol (μg/dL, Reference range = 1.0–6.0)[a]	4	2.675	3.525	1.525	1.8	<1	<1
ACTH (pg/mL, Reference range = 10–110)[b]	4	12.6	10.125	<10	<10	<10	<10

[a] Analytical limit of detection, 1.0 μg/dL.
[b] Analytical limit of detection, 10 pg/dL.
Abbreviation: ACTH, Adrenocorticotropin hormone.

stimulation test was normal. Therefore, the administration of OSI-7836 did not alter cortisol release following synthetic ACTH stimulation. These findings suggest that the lowered serum cortisol levels were likely not due to inhibition of cortisol production by the adrenal gland, but, due to an impairment of the HPA axis that appeared to be centrally mediated (Colagiovanni, 2006).

The dog has proven to be a useful species for evaluating HPA axis function. The similarities with the human system offer a relevant model to evaluation of proper function and degenerative disease. In nonclinical evaluations of pharmaceutical agents, the dog rather than the rat should be evaluated when investigating toxicity potential for humans. Age of the dogs used should be considered as hypercortisolemia is a frequent consequence of aging (Reul, 1991).

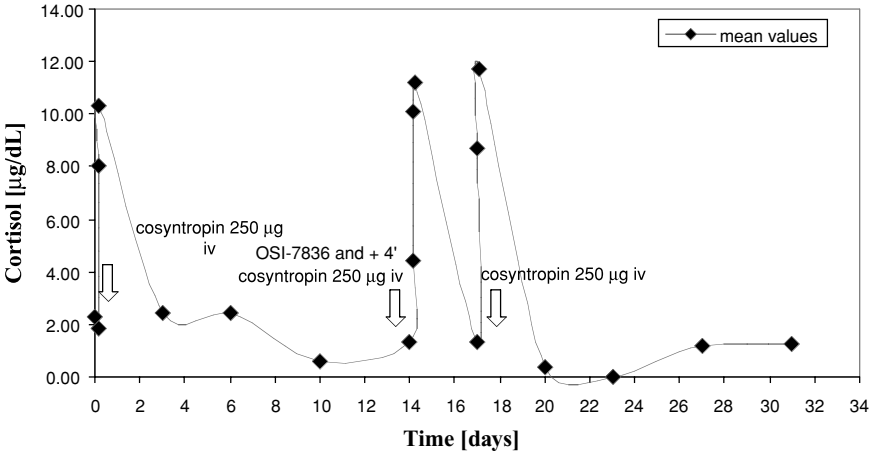

Figure 3 Mean (SD) serum cortisol levels following ACTH stimulation ($n = 4$ dogs/time point) and OSI-7836 administration.

REFERENCES

Holzbauer M. Effect of ACTH on the subcellular distribution of steroids in the adrenal gland. J Steroid Biochem 1981; 14:1189.

Brock BJ, Waterman MR. Biochemical differences between rat and human cytochrome p450c17 support the different steriodogenic needs of these two species. Biochemistry 1999; 38:1598.

Brooks WC. 3 testing: Laboratory hints suggesting Cushing's syndrome. http://www.veterinarypartner.com/Content.plx?P=A&C=&A=628&SourceID=, 2007.

Colagiovanni DB et al. Safety assessment of 4′-thio-beta-D-arabinofuranosylcytosine in the beagle dog suggests a drug-induced centrally mediated effect on the hypothalamic-pituitary-adrenal axis. Int J Toxicol 2006; 25:119.

de Jonge MJ et al. Phase I evaluation of OSI-7836—a new nucleoside analogue—using two administration schedules. Clin Cancer Res 2003; 9:6080.

Feldman EC, Nelson RW, Feldman MS. Use of low- and high-dose dexamethasone tests for distinguishing pituitary-dependent from adrenal tumor hyperadrenocorticism in dogs. J Am Vet Med Assoc 1996; 209:772.

Goy-Thollot I, Decosne-Junot C, Bonnet JM. Influence of aging on adrenal responsiveness in a population of eleven healthy beagle dogs. Res Vet Sci 2007; 82:195.

Greco DS. Hyperadrenocorticism associated with sex steroid excess. Clin Tech Small Anim Pract 2007; 22:12.

Hallberg E. Metabolism and toxicity of xenobiotics in the adrenal cortex, with particular reference to 7,12-dimethylbenz(a)anthracene. J Biochem Toxicol 1990; 5:71.

Hinson JP, Raven PW. Effects of endocrine-disrupting chemicals on adrenal function. Best Pract Res Clin Endocrinol Metab 2006; 20:111.

Hoff R. Adrenal hormones. In Kaplan LA, Pesce AJ, eds. Clinical Chemistry theory, analysis, and correlation. St. Louis, MO: The C. V. Mosby Company; 1984: 43.

Kemppainen RJ and Sartin JL. Evidence for episodic but not circadian activity in plasma concentrations of adrenocorticotrophin, cortisol and thyroxine in dogs. J Endocrinol 1984; 103(2): 219–226.

Lindhe O, Skogseid B, Brandt I. Cytochrome P450-catalyzed binding of 3-methylsulfonyl-DDE and o,p′-DDD in human adrenal zona fasciculata/reticularis. J Clin Endocrinol Metab 2002; 87:1319.

Liu Y, Yao Z, Papadopoulos V. Cytochrome P450 16 hydroxylase/17,20-lyase (CYP17) function in cholesterol biosynthesis: Identification of squalene monooxygenase (epoxidase) activity associated with CYP17 in leydig cells. Mol Endocrinol 2005; 19:1918.

Liwnicz BH, Liwnicz RG. Endocrine function. In: Kaplan LA, Pesce AJ, eds. Clinical Chemistry theory, analysis, and correlation. St. Louis, MO: The C. V. Mosby Company 1984:38.

Meijer JC et al. Biochemical characterization of pituitary-dependent hyperadrenocorticism in the dog. J Endocrinol 1978; 77:111.

Meyer DJ, Harvey JW. Evaluation of endocrine function, bone and mineral metabolism, and lipid metabolism. In: Winkel A ed. Veterinary Laboratory Medicine-Interpretation and Diagnosis. Philadelphia, PA: W.B. Saunders Co.; 2004:200–224.

Pesini P et al. Distribution of ACTH immunoreactivity in the diencephalon and the brainstem of the dog. J Chem Neuroanat 2004; 27:275.

Peterson ME. Diagnosis of hyperadrenocorticism in dogs. Clin Tech Small Anim Pract 2007; 22:2.

Pinney CC. The Illustrated Veterinary Guide, 2nd ed. Highstown, NJ, McGraw-Hill, 2000:315–318.

Plechner AJ. Adrenal toxicity in dogs and cats as a contributing cause of hormonal and immune destabilization. J Appl Toxicol 2004; 24:53.

Reine NJ. Medical management of pituitary-dependent hyperadrenocorticism: Mitotane versus trilostane. Clin Tech Small Anim Pract 2007; 22:18.

Reul JMHM, Rothuizen J, De Kloet ER. Age-related changes in the dog hypothalamic-pituitary-adrenocortical system: Neuroendocrine activity and corticosteroid receptors. J Steroid Biochem Mol Biol 1991; 40:63.

Ribelin WE. The effects of drugs and chemicals upon the structure of the adrenal gland. Fundam Appl Toxicol 1984; 4:105.

Rijnberk A, Kooistr HS, Mol JA. Endocrine diseases in dogs and cats: Similarities and differences with endocrine diseases in humans. Growth Horm IGF Res 2003; 13:S158.

Rothuizen J et al. Aging and the hypothalamus-pituitary-adrenocortical-axis, with special reference to the dog. Acta Endocrinol 1991; 125:73.

Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 2000; 21:55.

Selman PJ et al. Progestin treatment in the dog II. Effects on the hypothalamic-pituitary-adrenocortical axis. Eur J Endocrinol 1994; 131:422.

Sieber-Ruckstuhl NS et al. Cortisol, aldosterone, cortisol precursor, androgen and endogenous ACTH concentrations in dogs with pituitary-dependent hyperadrenocorticism treated with trilostane. Domest Anim Endocrinol 2006; 31:63.

Siu L et al. A phase I trial of the novel nucleoside analog OSI-7836 given on days 1 and 8 every 3 weeks. Eur J Cancer 2003; 1:165.

Temple TE, Liddle GW. Inhibitors of adrenal steroid biosynthesis. Amnnu Rev Pharmacol 1970; 10:199.

Wagner RL et al. Inhibition of adrenal steroidogenesis by anesthetic etomidate. N Engl J Med 1984; 310:1415.

7

Adrenocortical Toxicology In Vitro: Assessment of Steroidogenic Enzyme Expression and Steroid Production in H295R Cells

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THE H295 AND H295R CELL LINE

The H295 and H295R human adrenocortical carcinoma (a subpopulation of H295 that forms adherent monolayers in culture) cell lines have been characterized in detail and shown to be useful tools for the study of adrenocortical function and steroidogenesis. The H295R cell line is preferably used as its adherent properties make it easier to culture and more suitable for experimental manipulation. The H295 and H295R cell lines express all the key enzymes necessary for steroidogenesis (Gazdar *et al*., 1990; Rainey *et al*., 1993; Rainey *et al*., 1994; Sanderson *et al*., 2000; Staels *et al*., 1993). These include CYP11A (cholesterol side-chain cleavge), CYP11B1, CYP11B2 (aldosterone synthetase), CYP17 (17α-hydroxylase and 17,20-lyase), CYP19 (aromatase), CYP21, type 2 3β-HSD (hydroxysteroid dehydrogenase), and types 1 and 4 17β-HSD. The cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells (Gazdar *et al*., 1990; Staels *et al*., 1993), with the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex (Neville and O'Hare, 1985). These are the zona granulosa that produces the mineralocorticoid aldosterone, the zona fasciculata that produces the glucocorticoid cortisol, and the zona reticularis that produces the weak androgens

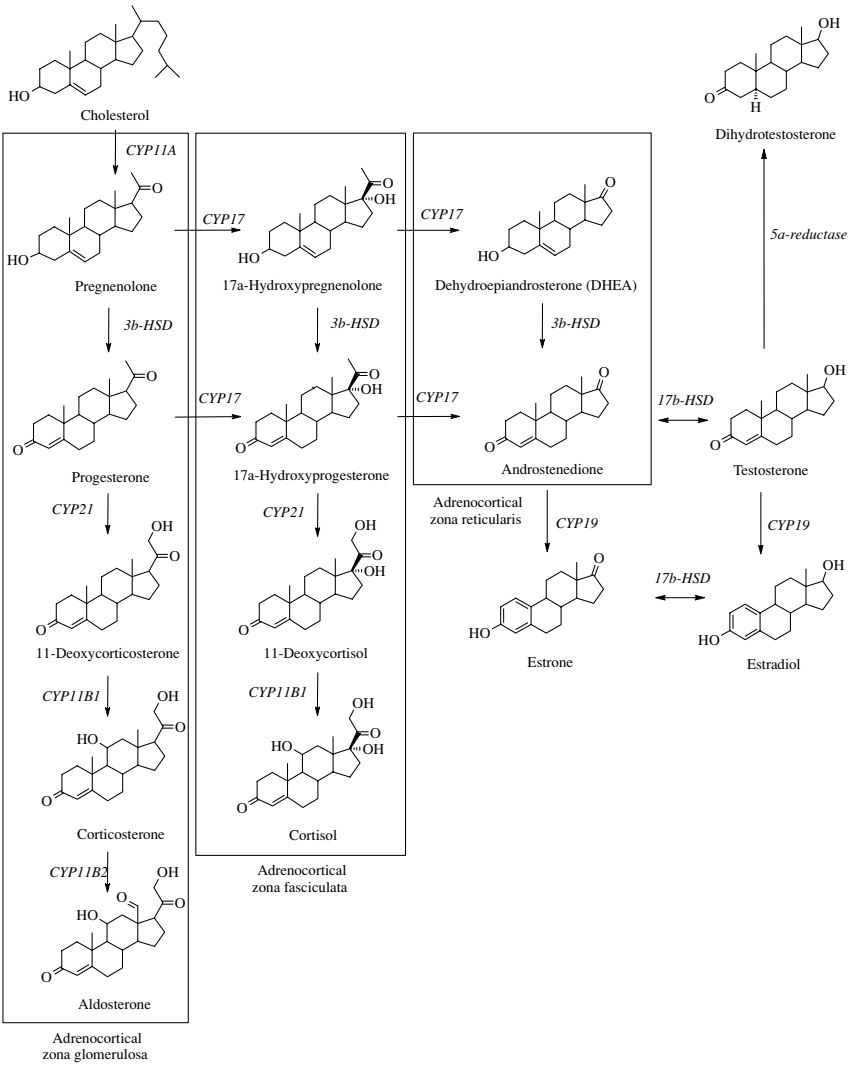

Figure 1 Enzyme expression and steroid production in the zona glomerulosa, zona fasciculata, and zona reticularis of the adrenal cortex.

DHEA (dehydroepiandrosterone) and androstenedione (Miller and Tyrell, 1995). H295R cells are further capable of producing testosterone, estrogens, as they express aromatase, and possibly DHT (dihydrotestosterone) as they express type 1 SRD5A (steroid 5α-reductase, preliminary results by Sanderson). Figure 1 shows the enzymes and steroids produced in the various zones of the adrenal cortex.

DE NOVO SYNTHESIS AND CONVERSION OF CHOLESTEROL

H295R cells produce cholesterol de novo, as steroid production occurs in the presence of defined cholesterol-free medium, although the major source of steroid synthesis would be exogenous cholesterol or cholesterol precursors administered via the culture medium (Gazdar *et al*., 1990). Recently, H295R cells have indeed been shown to express hydroxymethylglutaryl CoA reductase mRNA (Hilscherova *et al*., 2004), which converts acetyl coenzyme A into mevalonate and is the rate-limiting step in the synthesis of cholesterol. H295R cells express steroidogenic acute regulatory protein (StAR) which, when activated, rapidly mobilizes cholesterol from intracellular stores (Stocco, 2001), making it available to mitochondrial CYP11A for conversion to pregnenolone. Cholesterol mobilization occurs via the rapid activation of cholesterol esterases, which are expressed in all cholesterol utilizing tissues, including H295R cells, although direct evidence does not appear to have been published. Forskolin and cAMP analogues activate StAR expression as well as that of CYP11A.

MINERALOCORTICOID SYNTHESIS

Aldosterone synthesis by H295(R) cells is stimulated by angiotensin II (AII) and K^+ (Bird and Hanley, 1993; Bird *et al*., 1998; Holland *et al*., 1993; Rainey *et al*., 1994). AII activates AII receptors of which type 1 is expressed in H295(R) cells (Bird *et al*., 1994). AII receptors are coupled to phosphoinositidase C which, once activated, produces inositol triphosphate (IP3) and diacylglycerol (DAG). DAG stimulates protein kinase C (PKC), whereas IP3 stimulates Ca^{2+} release from intracellular stores. K^+ directly causes membrane depolarization resulting in rapid influx of Ca^{2+} through voltage-gated calcium channels. Both AII and K^+ preferentially increase the expression of aldosterone synthase (CYP11B2), and this appears to be, in large part, by calcium-mediated activation of the calmodulin (CaM) kinase pathway, as blockers of CaM kinase prevent induction of CYP11B2 (Bird *et al*., 1998; Pezzi *et al*., 1997). There appears to be a built-in negative feedback of AII-stimulated aldosterone production and CYP11B2 transcription, as the second messenger DAG through slower-acting PKC-signaling results in downregulation of AII receptor expression over a period of 4 days (Bird *et al*., 1994; Bird *et al*., 1998). Another key aspect of AII-signaling is that it has little effect on CYP17 expression, which remains low in the zona glomerulosa, thus favoring mineralocorticoid synthesis over glucocorticoid or androgen formation.

GLUCOCORTICOID SYNTHESIS

Cortisol synthesis is controlled through the concerted action of 3β-HSD, CYP17 (hydroxylase activity), CYP21, and CYP11B1. In human adrenal cortex glucocorticoid synthesis is regulated by adrenocorticotropin (ACTH) originating from the pituitary. ACTH receptors are primarily expressed in zonae fasciculata and

reticularis and are coupled to G_s-proteins and adenylate cyclase. ACTH receptor agonism results in increased production of cAMP, which in turn activates the protein kinase A (PKA) pathway, ultimately increasing transcription of various genes including those coding for CYP11A, 3β-HSD, CYP17, CYP21, and CYP11B1. H295R cells are relatively unresponsive to ACTH, but the production of cortisol is greatly enhanced by activators of PKA that bypass membrane receptor activation, such as cAMP analogues and the adenylate cyclase stimulant forskolin (Rainey *et al*., 1993). This reflects the low level of ACTH receptor expression found in this cell line (Samandari *et al*., 2007). Stimulation of the PKA pathway in H295R cells results in predominant induction of CYP17 hydroxylase activity (up to 10-fold) resulting in preferential synthesis of cortisol. The induction of CYP17 appears to involve PKA-mediated increased transcription of steroidogenic factor 1 (SF-1) and subsequent attachment of SF-1 protein to phosphatidic acid, a complex that appears to be directly involved in coactivator recruitment and enhancement of CYP17 promoter activity (Li *et al*., 2007). Induced 3β-HSD and CYP17 (hydroxylase) activity greatly increases the availability of 17α-progesterone to the unique adrenocortical enzyme CYP21. CYP21 converts 17α- progesterone to deoxycortisol, which in turn undergoes 11β-hydroxylation to cortisol. Stimulation of PKA also increases CYP21 and CYP11B1 expression in H295R cells, although to a lesser extent than CYP17.

SEX STEROID SYNTHESIS

H295R cells produce weak androgens such as DHEA and androstenedione, but also testosterone and estradiol. The relative production of these sex steroids compared with glucocorticoids and mineralocorticoids is influenced by culture conditions and subsequent preferential selection of certain phenotypes in culture, and by exposure to various pharmacological stimulants. In the adult adrenal cortex, the synthesis of DHEA and androstenedione are important functions, as they act as precursors for local synthesis of the more potent androgens and estrogens in various peripheral tissues, such as bone, adipose, prostate, and breast. The key enzyme in adrenal androgen synthesis is CYP17 (17,20-lyase) which takes 17α-hydroxylated pregnenolone and progesterone and converts them to DHEA and androstenedione, respectively. Although not well understood in human (adult) adrenocortical function, H295(R) cells are clearly capable of producing the potent sex steroids, estradiol and testosterone. Preliminary results from our laboratory showing SRD5A1 (although not SRD5A2) expression further indicate that they may also produce dihydrotestosterone, a potent endogenous androgen affecting gene-regulation in many tissues in males. H295R cells express mRNA for types 1 and 4 17β-HSD (Hilscherova *et al*., 2004). Although their functional presence (catalytic activity) has not been confirmed, it is likely that these enzymes are responsible for the interconversion of estradiol and estrone in H295R cells.

The expression of the enzyme aromatase has been well documented in H295R cells. It was initially suggested to be present in the original H295 cells, as

these were capable of secreting small quantities of estradiol (Gazdar *et al*., 1990). It was confirmed by northern blotting that H295 cells expressed CYP19 mRNA after induction with 8-bromo-cAMP (Staels *et al*., 1993). In H295R cells, CYP19 gene expression and presence of catalytically active aromatase enzyme was first reported by our laboratory (Sanderson *et al*., 2000), where it was shown to be inducible by stimulants of the PKA pathway (Heneweer *et al*., 2004; Sanderson *et al*., 2000; Sanderson *et al*., 2002). We further showed that upregulation of CYP19 is controlled mainly by the pII- and to a lesser extent the I.3 promoter (Sanderson *et al*., 2004), both of which are involved in the normal regulation of aromatase expression in gonads, via LH- and/or FSH receptor-mediated PKA-activation. Forskolin-induced upregulation of pII- and I.3-derived CYP19 transcript in H295R cells was further confirmed by Watanabe and coworkers (Watanabe and Nakajin, 2004). It has also been found that aromatase expression is induced by dexamethasone and phorbol-12-myristate-13-acetate (Heneweer *et al*., 2004). Glucocorticoids and phorbol esters are stimulants of aromatase expression in adipose tissue (breast) (via the 1.4 promoter) and placenta (via the 1.1 promoter), respectively. However, 1.4 promoter-specific transcript was not detected (Heneweer *et al*., 2004), suggesting this promoter region may have been altered in this cancer cell line. It has further been shown that glucocorticoids and phorbol esters increase cortisol secretion in H295R cells as well, and that this is due to the induction of type 2 3β-HSD (Feltus *et al*., 2002).

H295R CELLS AS A SCREENING TOOL FOR ENDOCRINE DISRUPTORS AND ADRENAL TOXICANTS

Given the plethora of steroidogenic enzymes expressed and catalytically active in H295R cells, it is a highly useful and versatile biological system for the study of interactions of xenobiotics, whether drugs, toxicants, or natural compounds, with steroidogenesis. Aromatase has been given most initial attention, as it is a key enzyme in estrogen synthesis and in controlling the balance between estrogen and androgen levels in tissues. H295R cells are suitable for the detailed evaluation of mechanisms of inhibition (competitiveness and/or reversibility) (Heneweer *et al*., 2004; Sanderson *et al*., 2002; Sanderson *et al*., 2004) and induction (involvement of signaling pathways and tissue-specific promoters) of aromatase. The H295R cell line has also been used to develop a quantitative RT-PCR method for the detection of chemicals that can up- or downregulate the mRNA expression of 11 steroidogenic enzymes and related genes (Hilscherova *et al*., 2004; Zhang and Yu, 2005). These included CYP11A, CYP11B1, CYP11B2, CYP17, CYP19, CYP21, 3β-HSD1, 3β-HSD2, 17β-HSD1, 17β-HSD4, StAR, and hydroxymethylglutaryl-CoA reductase. These studies demonstrate the versatility of the H295R cell line as a bioassay tool for the assessment of effects on the catalytic activities and gene-regulation of numerous steroidogenic enzymes.

Steroidogenic enzyme expression in H295R cells has been reported to be affected by various xenobiotics including pesticides (Heneweer *et al*., 2004;

Sanderson *et al*., 2000; Sanderson *et al*., 2001; 109; Sanderson *et al*., 2002), flavonoids (Sanderson *et al*., 2004), and persistant organic pollutants (Canton *et al*., 2005; Canton *et al*., 2006; Heneweer *et al*., 2005; Li *et al*., 2004). Atrazine and the related herbicides simazine and propazine were found to be inducers of aromatase activity and CYP19 gene expression (Sanderson *et al*., 2000; Sanderson *et al*., 2001; 109). The same was found for the fungicide vinclozolin. Ability to induce aromatase activity correlated well with ability to increase intracellular cAMP levels, suggesting that the pesticides either stimulate cAMP synthesis or inhibit its breakdown. It was indeed confirmed that atrazine, for example, was an inhibiter of phosphodiesterase, at least in bovine heart (Roberge *et al*., 2004). Recently, atrazine-mediated aromatase induction was shown to be dependent on activation of SF-1, an essential factor for stimulation of pII-promoter activity (Fan *et al*., 2007). Cells that do not express SF-1 were not responsive to atrazine-mediated–aromatase induction, but were rendered responsive by exogenous addition of SF-1 (Fan *et al*., 2007). Several flavonoids found in fruits and vegetables were able to inhibit the catalytic activity of aromatase in H295R cells, such as chrysin, apigenin, and naringenin (Sanderson *et al*., 2004). In addition, the flavone quercetin and isoflavone genistein were found to induce aromatase activity and CYP19 gene-transcription in these cells. This induction coincided with significant increases in pII- and I.3-promoter-derived CYP19 mRNA levels. It is known that genistein and quercetin are phospohodiesterase inhibitors and can increase intracellular cAMP levels. It is thus plausible that these flavonoids cause CYP19 induction via the mechanism of increased cAMP-mediated activation of the PKA-signaling pathway (Sanderson *et al*., 2004) or, as suggested by Li and coworkers, cAMP-dependent, but PKA-independent activation of SF-1 (Fan *et al*., 2007).

FUTURE DIRECTIONS

The H295R cell line is proving itself as a useful and versatile tool for the study of human adrenocortical function, which includes the regulation of mineralocorticoid, glucocorticoid, and sex steroid synthesis. The ability of this cell line to stably express almost all known steroidogenic, as well as several steroid metabolizing enzymes is an enormous asset to many areas of endocrine research. The relative production of the steroid hormones in question is not necessarily an exact reflection of what happens in vivo in the adult human adrenal gland. Yet, the fact that the numerous second messenger pathways, reflecting regulation of steroidogenesis in various peripheral tissues, appear to be all functionally active in this cell line, makes it a biological system of choice for the study of interferences between xenobiotics and steroidogenesis in general. It is particularly important in the study of regulation and disruption of the vital mineralocorticoid synthesis, as with the exception of the rare possibility of obtaining primary human adrenocortical tissue no other options are currently available.

REFERENCES

Bird IM, Hanley NA Word RA, et al. Human NCI-H295 adrenocortical carcinoma cells: A model for angiotensin-II-responsive aldosterone secretion. Endocrinology 1993; 133:1555–1561.

Bird IM, Mason JI, Rainey WE. Regulation of type 1 angiotensin II receptor messenger ribonucleic acid expression in human adrenocortical carcinoma H295 cells. Endocrinology 1994; 134:2468–2474.

Bird IM, Mason JI, Rainey WE. Battle of the kinases: Integration of adrenal responses to cAMP, DG and Ca^{2+} at the level of steroidogenic cytochromes P450 and 3beta-HSD expression in H295R cells. Endocr Res 1998; 24:345–354.

Canton RF, Sanderson JT, Letcher RJ, et al. Inhibition and induction of aromatase (CYP19) activity by brominated flame retardants in H295R human adrenocortical carcinoma cells. Toxicol Sci 2005; 88:447–455.

Canton RF, Sanderson JT, Nijmeijer S, et al. In vitro effects of brominated flame retardants and metabolites on CYP17 catalytic activity: A novel mechanism of action? Toxicol Appl Pharmacol 2006; 216:274–281.

Fan W, Yanase T, Morinaga H, et al. Herbicide atrazine activates SF-1 by direct affinity and concomitant co-activators recruitments to induce aromatase expression via promoter II. Biochem Biophys Res Commun 2007; 355:1012–1018.

Fan W, Yanase T, Morinaga H, et al. Atrazine-induced aromatase expression is SF-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans. Environ Health Perspect 2007; 115:720–727.

Feltus FA, Cote S, Simard J, et al. Glucocorticoids enhance activation of the human type II 3beta-hydroxysteroid dehydrogenase-Delta5-Delta4 isomerase gene. J Steroid Biochem Mol Biol 2002; 82:55–63.

Gazdar AF, Oie HK, Shackleton CH, et al. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Res 1990; 50:5488–5496.

Heneweer M, Van Den Berg M, Sanderson JT. A comparison of human H295R and rat R2 C cell lines as in vitro screening tools for effects on aromatase. Toxicol Lett 2004; 146;183–194.

Heneweer M, Van Den Berg M, de Geest MC, et al. Inhibition of aromatase activity by methyl sulfonyl PCB metabolites in primary culture of human mammary fibroblasts. Toxicol Appl Pharmacol 2005; 202:50–58.

Hilscherova K, Jones PD, Gracia T, et al. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol Sci 2004; 81:78–89.

Holland OB, Mathis JM, Bird IM, et al. Angiotensin increases aldosterone synthase mRNA levels in human NCI-H295 cells. Mol Cell Endocrinol 1993; 94:R9–R13.

Li LA, Wang PW, Chang LW. Polychlorinated biphenyl 126 stimulates basal and inducible aldosterone biosynthesis of human adrenocortical H295R cells. Toxicol Appl Pharmacol 2004; 195:92–102.

Li D, Urs AN, Allegood J, et al. Cyclic AMP-stimulated interaction between steroidogenic factor 1 and diacylglycerol kinase theta facilitates induction of CYP17. Mol Cell Biol 2007; 27:6669–6685.

Miller W, Tyrell J. The adrenal cortex. In: Felig P, Baxter J, Frohman L, eds. Endocrinology and Metabolism. New York, NY: McGraw-Hill Press; 1995:555–711.

Neville AM, O'Hare MJ. Histopathology of the human adrenal cortex. Clin Endocrinol Metab 1985; 14:791–820.

Pezzi V, Clyne CD, Ando S, et al. Ca(2+)-regulated expression of aldosterone synthase is mediated by calmodulin and calmodulin-dependent protein kinases. Endocrinology 1997; 138:835–838.

Rainey WE, Bird IM, Sawetawan C, et al. Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. J Clin Endocrinol Metab 1993; 77:731–737.

Rainey WE, Bird IM, Mason JI. The NCI-H295 cell line: A pluripotent model for human adrenocortical studies. Mol Cell Endocrinol 1994; 100:45–50.

Roberge M, Hakk H, Larsen G. Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the estrogen receptor. Toxicol Lett 2004; 154:61–68.

Samandari E, Kempna P, Nuoffer JM, et al. Human adrenal corticocarcinoma NCI-H295R cells produce more androgens than NCI-H295 A cells and differ in 3beta-hydroxysteroid dehydrogenase type 2 and 17,20-lyase activities. J Endocrinol 2007; 195:459–472.

Sanderson JT, Seinen W, Giesy JP, et al. 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: A novel mechanism for estrogenicity? Toxicol Sci 2000; 54:121–127.

Sanderson JT, Letcher RJ, Heneweer M, et al. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ Health Perspect 2001; 109;1027–1031.

Sanderson JT, Boerma J, Lansbergen GW, et al. Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Toxicol Appl Pharmacol 2002; 182:44–54.

Sanderson JT, Hordijk J, Denison MS, et al. Induction and Inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxicol Sci 2004; 82:70–79.

Staels B, Hum DW, Miller WL. Regulation of steroidogenesis in NCI-H295 cells: A cellular model of the human fetal adrenal. Mol. Endocrinol 1993; 7:423–433.

Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 2001; 63:193–213.

Watanabe M, Nakajin S. Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R. J Endocrinol 2004; 180:125–133.

Zhang X, Yu RM, Jones PD, et al. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environ Sci Technol 2005; 39:2777–2785.

8

Comparisons of Adrenocortical Cell Lines as In Vitro Test Systems

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INTRODUCTION

The adrenal gland is a compound endocrine organ separated into two distinct, developmentally unrelated tissues— an adrenal medulla and an adrenal cortex. The adrenal medulla is the core of the adrenal gland; the chromaffin cells of the medulla are the body's main source of the catecholamines. The cells comprising the adrenal cortex are derived from mesoderm of the dorsal coelomic wall and thus have common features to the steroidogenic cells within the gonads. In 1866, Arnold first described the histology of the adrenal cortex and noted that it was divided into three concentric zones which he named the zona glomerulosa, zona fasciculata, and zona reticularis (Arnold, 1866). While this description was based on the histological organization, it is now accepted that these zones have functionally distinct roles in steroid hormone production. Namely, the glomerulosa synthesizes mineralocorticoids, the fasciculata produces glucocorticoids, and in the human, the zona reticularis produces C_{19} steroids, including DHEA and DHEA-sulfate. Molecular mechanisms leading to zone specific expression of these steroids are yet to be defined. Each adrenocortical zone synthesizes its steroid products from the common substrate cholesterol. Steroidogenic cholesterol can arise from endogenous cholesterol stores, from serum derived lipoprotein or from de novo synthesis. Within the human adrenal cortex, steroid synthesis involves coordinated actions of five forms of cytochrome P450 and the enzyme 3β-hydroxysteroid dehydrogenase (Figure 1); these enzymes are distributed between the mitochondria and the

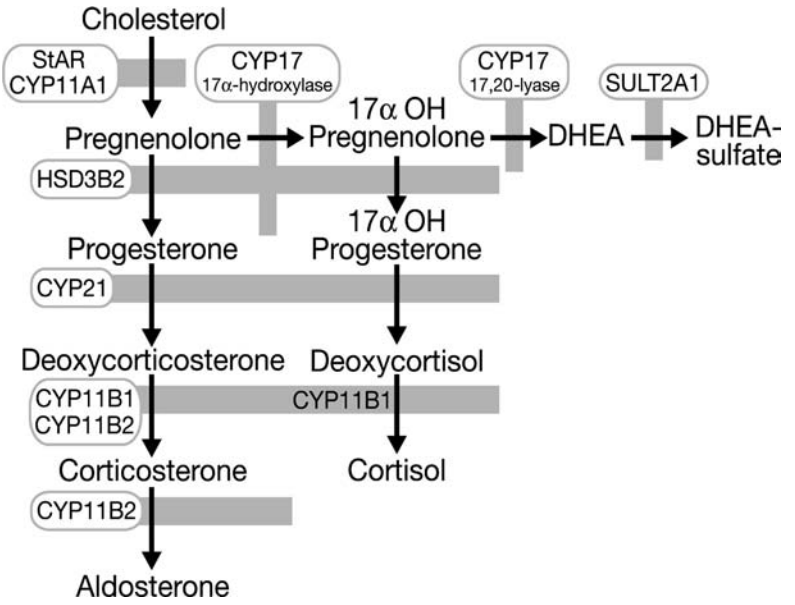

Figure 1 Human adrenal steroid biosynthetic pathways illustrating the three main products of the adrenal (aldosterone, cortisol, and DHEA/S) and the enzymes that synthesize these products. Classification for the enzymes in the P450 superfamily follow the guidelines previously reported (Nelson *et al*., 1996). *Abbreviations*: StAR, steroidogenic acute regulatory protein; CYP11A1, cholesterol side-chain cleavage; CYP17, 17α-hydroxylase 17,20-lyase; SULT2A1, DHEA-sulfotransferase; HSD3B2, 3β-hydroxysteroid dehydrogenase type II; CYP21, 21-hydroxylase; CYP11B1, 11β-hydroxylase; CYP11B2, aldosterone synthase.

endoplasmic reticulum (Simpson, 1988). It is the differential expression of these enzymes within the three adrenocortical zones that allows for the wide array of steroid hormones secreted by this gland.

Although the enzymes that are differentially expressed between the zones have been identified, the molecular mechanisms causing zone-specific expression patterns are yet to be fully understood. The mechanism regulating production of steroids from each distinct zone is quite different and therefore, extremely complex. Angiotensin II (ANG II) and potassium (K^+) are the major regulators of steroidogenesis in the zona glomerulosa, while ACTH is the principal hormone regulating steroidogenesis in the fasciculata and reticularis (Fig. 2). In each case, the rate-limiting step in steroid hormone biosynthesis is the translocation of substrate cholesterol from the outer mitochondrial membrane to cholesterol side-chain cleavage enzyme (CYP11A)—the first enzyme in the steroidogenic pathway, which is located inside the mitochondrion. In addition, regulatory mechanisms

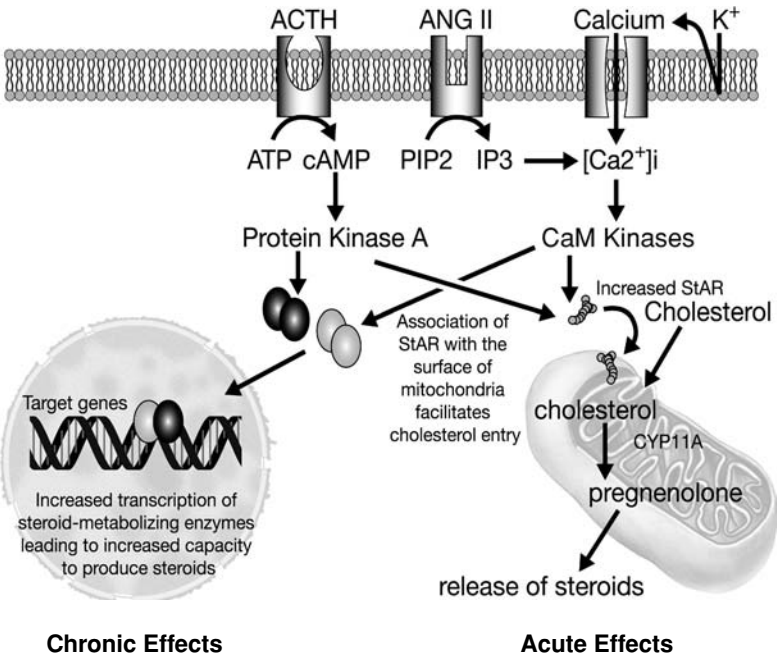

Figure 2 Signaling pathways that regulate steroid production in human adrenal cells. The primary regulators of adrenal steroid hormone biosynthesis are ACTH, angiotensin II, and potassium, which work at the level of the cell membrane. Both the calmodulin-dependent protein kinases (CaM Kinases) and protein kinase A pathways are able to activate acute and chronic steroid hormone production.

control the bioavailability of substrate cholesterol, the synthesis of the enzymes required for steroidogenesis, and the size and structural integrity of the gland.

Investigations into the complexity of the adrenal cortex have been hampered by the unavailability of adrenal tissue samples and cell cultures. This has made the development of viable in vitro cell models an attractive alternative to studies using whole animals. A number of in vitro systems have been investigated, including cell suspensions from acutely dispersed tissue, primary cultures from normal adrenal glands and adrenal tumors, (Gospodarowicz *et al*., 1977; O'Hare and Neville, 1973), and established cell lines from tumors (Rogriquez *et al*., 1997; Yasumura *et al*., 1966) or from immortalized cells (Auersperg *et al*., 1990; Mellon *et al*., 1994; Mukai *et al*., 2002; Pan *et al*., 1995). Primary cultures of adrenocortical cells have proven useful for examining mechanisms controlling the many aspects of adrenal physiology but the constant requirement for freshly acquired tissue and the difficulties associated with the isolation of cells has increased the demand for alternatives. Adrenal cell lines, which allow large numbers of functional cells to be propagated without the need for animal sacrifice or acquisition of human tissue, have yielded considerable progress in defining basic mechanisms involved

in adrenal function. Several issues become important for these adrenal model systems, including cell growth, response to agonists, and maintenance of steroidogenic capacity. The ability of adrenal primary cultures and cell lines to produce steroids or to respond to ACTH, ANG II, and K^+ can change over time in culture. Therefore, steroid synthesis and cellular responses to agonists must be monitored continuously. The expression of the enzymes involved in steroid hormone biosynthesis can also change under culture conditions. As a result, the steroids produced by cells in culture can be quite different from that seen in vivo. Thus, each of these criteria must also be considered when developing or evaluating cell lines to study the adrenal.

AVAILABLE RODENT ADRENOCORTICAL CELL LINES (Table 1)

The Y1 Adrenal Cell Line

The Y1 adrenal cell line was derived from an adrenal tumor that developed in a LAF1 (C57L × A/HeJ) mouse following exposure to an atomic blast; a direct relationship of tumor development to the radiation is not clear (Cohen *et al*., 1957). Gordon Sato and colleagues (Buonassisi *et al*., 1962) adapted the transplantable tumor to grow in vitro by alternately propagating dispersed tumor cells as monolayer cultures and as tumors in mice. One of the clones developed from the mixed population of tumor cells was named Y1, and it was this clone that was deposited at the American Type Culture Collection (ATCC CCL-79) (Yasumura *et al*., 1966).

Major steroid pathways present in cultured Y1 cells are shown in Figure 3. In contrast to the normal mouse adrenal gland that produces corticosterone as the major steroid product, Y1 cells in culture produce 20α-hydroxy-Δ^4-pregnen-3-one (20α-dihydroxyprogesterone) and 11β,20α-dihydroxy-Δ^4-pregnen-3-one (11β,20α-dihydroxyprogesterone) (Kowal and Fiedler, 1968; Pierson, 1967). This abnormal steroid profile relates to the deficiency in 21-hydroxylase (CYP21) (Parker *et al*., 1985) coupled with an increase in 20α-hydroxysteroid dehydrogenase activity (Pierson, 1967).

The ability of ACTH to regulate Y1 cell steroid synthesis is similar to that seen for normal mouse adrenal cells and other agents that raise intracellular levels of cAMP, which leads to an induction of a number of genes that are supportive for steroid hormone biosynthesis. Examples include genes encoding the ACTH receptor (Mountjoy *et al*., 1994; Schimmer *et al*., 1995), CYP11a (Black *et al*., 1993; Guo *et al*., 1993; Lin *et al*., 2001; Wong *et al*., 1989), 11β-hydroxylase (CYP11b1) (Mitani *et al*., 1998; Rice *et al*., 1989; Wong *et al*., 1989), steroidogenic acute regulatory protein (StAR) (Lin *et al*., 1995; Lin *et al*., 2001; Lopez *et al*., 2001), adrenodoxin (Black *et al*., 1993; Guo *et al*., 1993), adrenodoxin reductase (Black *et al*., 1993), and the HDL receptor (SR-BI) (Temel *et al*., 1997). As opposed to the robust stimulation of steroid hormone

Table 1 Summary of Information on Rodent and Bovine Adrenal Cell Lines

Cell line name	ACTH	K^+	ANG II	cAMP	Steroids produced	Availability	Reference
Y1 and its mutant strains	+	–	–	+	Progesterone metabolites	ATCC and Schimmer Lab	Schimmer, 1979; Schimmer and Zimmerman, 1976; Rainey *et al*., 2004
Rat adrenal cells—2FASC and 7GLOM	+	–	–	+	Progesterone metabolites	Auersperg Lab	Roskelly and Auersperg, 1995; Auersperg, 1990
Rat adrenocortical line—TRA	+	–	–	+	Progesterone metabolites	Auersperg Lab	Auersperg, 1978; Auersperg *et al*., 1977
Mouse adrenal cell-lines—ATC1 and ATC7-L	+	ND	–	+	Reported glucocorticoids	Martinez lab	Ragazzon *et al*., 2006
Immortalized mouse adrenal cell lines—AcA201, AcE60 and AcA101	–	–	–	+	Progesterone metabolites	Ishimura lab	Mukai *et al*., 2002
Immortalized mouse adrenal cell lines—ST2, ST5-R, and ST5-L	–	ND	ND	+	Progesterone metabolites	Mellon Lab	Mellon *et al*., 1994
Immortalized mouse adrenal cell line—ST5Lc	–	ND	ND	+	Progesterone metabolites and Glucocorticoids	Mellon Lab	Compagnone *et al*., 1997
SBAC and other bovine adrenocortical cell lines	–	ND	+	+	None reported	ATCC	Cheng and Hornsby, 1992

Abbreviation: ND, Not documented.

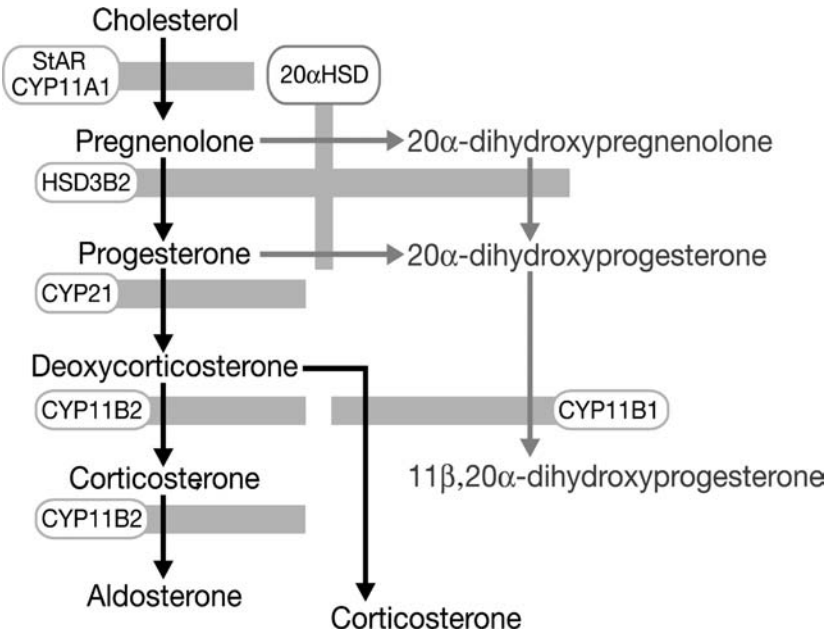

Figure 3 Rodent adrenal steroid biosynthetic pathways for the production of aldosterone and corticosterone. Also illustrated are the variations seen in Y-1 adrenal cell steroid production, which produces primarily 20α-dihydroprogesterone and 11β,20α-dihydroxyprogesterone. *Abbreviations*: StAR, steroidogenic acute regulatory protein; CYP11A1, cholesterol side-chain cleavage; CYP17, 17α-hydroxylase 17,20-lyase; SULT2A1, DHEA-sulfotransferase; HSD3B2, 3β-hydroxysteroid dehydrogenase type II; CYP21, 21-hydroxylase; CYP11B1, 11β hydroxylase; CYP11B2, aldosterone synthase.

production by ACTH, Y1 cells do not respond to ANG II, although transgene expression of the ANG II receptor in Y1 cells has been described (Tian *et al*., 1996).

Y1 cells are grown under a humidified atmosphere of 5% CO_2 and 95% air in a variety of growth media supplemented with serum. The cells' approximate doubling time is 30 to 40 hours (Schimmer, 1979; Schimmer, 1985). Y1 cells, for the most part, grow in culture as flat, adherent cells with polyhedral shapes maintained by a network of stress fibers and focal adhesions near the cell surface. In response to ACTH or to the adenylyl cyclase activator forskolin, which raises intracellular levels of cAMP, Y1 cells retract their extended plasma membranes, become spherical with short processes, and detach easily from the substratum on which they are grown (Cuprak *et al*., 1977; Mattson and Kowal, 1978; Voorhees *et al*., 1984; Yasumura *et al*., 1966). The effects of ACTH and cAMP on cell shape are closely linked to the effects of these agents on steroidogenesis and possibly reflect an involvement of the cytoskeleton in the delivery of cholesterol to the mitochondria (Cortese and Wolff, 1978; Osawa *et al*., 1984; Shiver *et al*., 1992; Whitehouse *et al*., 2002).

The Y1 cell line, as maintained in continuous culture, started to accumulate in clonal variants with markedly different steroidogenesis (Schimmer, 1979). Among the variants, some appeared to be completely resistant to ACTH (Schimmer, 1969; Schimmer, 1979; Schimmer, 1985; Yasumura, 1968) and some were resistant to ACTH-induced desensitization of adenylyl cyclases (Colantonio *et al*., 1998). Also, a family of ACTH receptor deficient mutants were developed (Qiu *et al*., 1996; Schimmer *et al*., 1995), and some mutants harbored a dominant inhibitory protein kinase A (Olson *et al*., 1993; Rae *et al*., 1979). These mutants have proved to be very valuable in assessing the roles of cAMP, protein kinase A, adenylyl cyclases, ACTH, and ACTH receptor in adrenocortical study.

Use of Viral Oncogenes to Develop Rodent Adrenal Cell Lines

Ras Transformed Rat Adrenal Cells

Auersperg et al. explored the possibility of establishing rat adrenal cell lines by infection with Kirsten murine sarcoma virus (Auersperg, 1978; Auersperg *et al*., 1977; Auersperg *et al*., 1981; Pan *et al*., 1995). Primary cultures of rat adrenal cells in early passage were infected with virus and allowed to overgrow the normal cell population. The transformants metabolized pregnenolone to progesterone and 20α-dihydroxyprogesterone, indicating that they retained some steroidogenic potential. However, they did not synthesize 11- or 21-hydroxylated steroids nor was their capacity to form steroids from endogenous cholesterol measured. A similar strategy was used to establish an adrenal cell line from rat zona glomerulosa cells (Pan *et al*., 1995). These cells produced basal levels of steroids and increased steroid production in response to ACTH or cAMP. However, the stimulated levels of steroid production as estimated by radioimmunoassay were very low (8 ng per 10^5 cells per 24 hours), and the identity of the steroid product was not rigorously identified.

Cell Lines from Tumors in Transgenic Animals

Several investigators established immortalized adrenal cell lines from adrenal tumors in mice carrying the Simian Virus 40 T-antigen as a transgene (Compagnone *et al*., 1997; Kananen *et al*., 1996; Mellon *et al*., 1994; Mukai *et al*., 2002; Rilianawati *et al*., 1998). Using a human CYP11A promoter to target expression of T-antigen to the adrenal cortex, Mellon et al. (Mellon *et al*., 1994; Compagnone *et al*., 1997) generated adrenal tumors in female mice and used these tumors to establish cell lines that produced progesterone as the major steroid product. Progesterone synthesis was not responsive to ACTH or ANG II but was stimulated by 8-bromo-cAMP. Progesterone was synthesized at a rate of 100 ng/μg of DNA per 24 hours, under stimulated conditions. The cells expressed CYP11A and CYP11B1 but did not synthesize 11-hydroxylated steroids. Although the cells produced small amounts of deoxycorticosterone, they did not express measurable amounts of CYP21, suggesting that another enzyme carried out the steroid 21-hydroxylation reaction. Curiously, these cells also expressed renin-1 mRNA (Compagnone *et al*., 1997).

The mouse inhibin α-subunit promoter also was used to drive T-antigen expression and to generate adrenal tumors. These tumors were postulated to originate from the X-zone of the adrenal cortex (Kananen *et al*., 1996; Rilianawati *et al*., 1998). A cell line derived from one of these tumors (clone Cα1) produced progesterone as the major steroid product. These cells expressed LH receptors, rendering them modestly responsive to stimulation by hCG. Unstimulated Cα1 cells produced progesterone at a relatively high rate (350 ng/10^6 cells per hour), and treatment with hCG produced a small but statistically significant (≤40%) increase in progesterone production. Effects of ACTH on steroid production were not documented. Treatment of Cα1 cells with inhibin decreased T-antigen levels slowed the rate of proliferation. Effects of inhibin on steroidogenesis were not reported, although it would have been interesting to determine if suppression of T-antigen expression affected the rate or profile of steroid production.

Several immortalized adrenocortical cell lines were generated from mice bearing a transgene for a temperature-sensitive form of T-antigen under control of its own promoter (Mukai *et al*., 2002). At permissive temperatures, one group of cells expressed SF-1, CYP11A, StAR, and CYP11B1, suggesting that they were of fasciculata-reticularis origin. A second group of cells expressed SF-1, CYP11A, and StAR but not CYP11B1, suggesting that they had lost differentiated function or were derived from an undifferentiated cell layer situated between the glomerulosa zone and the fasciculata zone. Mukai et al. suggested that these adrenal cell isolates may be useful in exploring the basis for functional zonation of the adrenal cortex. Major steroid products of these cells appeared to be pregnenolone and progesterone. None of the cells were responsive to ACTH or ANG II and none of the cells expressed aldosterone synthase (CYP11B2). At permissive temperatures, the phenotypes of the cell lines remained stable for ≥ 200 passages. Shifting cells to nonpermissive temperatures or treating cells with dibutyryl cAMP had variable effects on gene expression; either treatment converted the undifferentiated cells to fasciculata-like phenotypes and also induced CYP21 and HSD3B expression.

Perhaps, the most successfully established mouse adrenocortical tumor cell lines were recently described and characterized by Ragazzon et al. (2006). Using genetically targeted oncogenesis, the first immortalized cell lines, ATC1 and ATC7-L, were developed. Both ATC1 and ATC7-L have retained a complete ZF phenotype. This phenotype was manifested at multiple levels—steroidogenic capacity, ACTH responsiveness, and expression profile of specific genes. The two cell lines were established from adrenal tumors of two transgenic mice harboring the large T-antigen of SV40 under the control of adrenocortical-specific promoter of Akr1b7. Both lines showed a significant stimulation of corticosterone secretion upon treatment with 10^{-11} M ACTH which then increased in a dose-dependent manner, reaching the highest values at 10^{-7} M ACTH. Detailed examination of expression of hormonal responsiveness of the mRNAs for the genes involved in the different steps in glucocorticoid synthesis revealed detectable levels of Mc2r (ACTH receptor mRNA) and CYP21a1 mRNAs, but were unresponsive to ACTH in both cell lines. By contrast, mRNA levels for Sr-b1, StAR, CYP11a1, CYP11b1, and Akr1b7 were strongly induced in a time-dependent manner by ACTH

treatment, and these inductions were not abolished by the protein synthesis inhibitor, cycloheximide. Thus, the effect of ACTH on accumulation of these mRNAs is essentially transcriptional. The ATC cell lines have been developed as novel in vitro models, maintaining differentiated endocrine functions of ZF adrenocytes. They have proven very useful in investigating the mechanisms of steroidogenic gene regulation in response to the physiological activator of glucocorticoid synthesis ACTH.

BOVINE ADRENOCORTICAL CELL LINES (Table 1)

Primary cultures of bovine adrenal cells have been a favored model to study adrenal cell function. This is likely due to the fact that bovine adrenal glands are readily available at abattoirs and the large size of the gland allows for isolation of sufficient cells for large experiments. Bovine adrenal cells were the first adrenal model system to be used for the development of cell lines through viral gene transformation. Hornsby and colleagues used SV40 to create a series of clonal bovine adrenal cell lines (Cheng *et al*., 1989). The addition of SV40 to the cells greatly enhanced the cell culture lifespan of the cells. The cells continued to respond to cAMP-related agonists by induction of 17α-hydroxylase 17,20-lyase (CYP17) and CYP11A. However, cellular expression of CYP21 and CYP11B were found to require specialized growth conditions (Chang *et al*., 1991).

More recent studies by Hornsby and colleagues have combined human telomerase reverse transcriptase with SV40T antigen and *ras* oncogene to immortalize bovine adrenocortical cells (Thomas *et al*., 2002). The immortalized bovine adrenal cells have been used in vivo to rescue adrenalectomized SCID (Severe combined immunodeficient) mice from glucocorticoid insufficiency through the production of cortisol. These data suggest that such strategies could be used in the future as a means of adrenocortical replacement.

HUMAN ADRENOCORTICAL CELL LINES (Table 2)

Development of Human Adrenal Cell Lines Using Viral Oncogenes

In a strategy similar to that used for bovine adrenal cells, Hornsby and colleagues used the SV40 T-antigen to isolate clonal populations of proliferating human fetal adrenal cells. These human adrenal clones responded to cAMP by increasing both CYP17 and CYP11A, but levels of CYP21 and CYP11B1 were low. Transformed cells could be maintained in culture for 30 to 40 population doublings after isolation, but would then enter "crisis" and stop dividing. The cause of this late passage crisis is the result of telomere shortening that occurs with each population doubling and is normally not corrected by SV40 expression (Cong *et al*., 2002).

The NCI-H295 Cell Line and Related Strains

Origins and Steroid Synthesis

A 48-year-old African-American female from whom the NCI-H295 cell line was established, had the typical clinical, biochemical, and pathological profile of a

Table 2 Summary of Information on Human Adrenal Cell Lines

Cell line name	ACTH	K^+	ANG II	cAMP	Steroids produced	Availability	Reference
NCI-H295	–	ND	ND	ND	Mineralocorticoids Glucocorticoids Adrenal Androgens	ATCC	Gazdar *et al*., 1990
NCI-H295A	–	ND	–	+	Mineralocorticoids Glucocorticoids Adrenal Androgens	Miller Lab	Huang *et al*., 2005; Samandari *et al*., 2007
NCI-H295R	–/+	+	+	+	Mineralocorticoids Glucocorticoids Adrenal Androgens	ATCC and Rainey Lab	Bird *et al*., 1993b; Bird *et al*., 1995b; Bird *et al*., 1996b; Clark *et al*., 1995; Denner *et al*., 1996
HAC15	+	+	+	+	Mineralocorticoids Glucocorticoids Adrenal Androgens	Rainey Lab	Parmar *et al*., 2008

Abbreviation: ND, Not documented.

steroidogenic adrenocortical carcinoma. She was diagnosed with acne, facial hirsutism, edema, diarrhea, weight loss, and a recent cessation of menses (Gazdar *et al*., 1990). Computer assisted tomography revealed a large, locally invasive malignant tumor, which later metastasized to the lungs and liver, had clinical and biochemical evidence of excessive secretion of glucocorticoids and ketosteroids. The excised tumor was 14 × 13 × 11 cm and was used to establish the original NCI-H295 cell line. Tumor tissue was finely minced and the resulting suspension was maintained in various serum-containing and serum-free culture media for 1 year (Gazdar *et al*., 1990). Gas chromatography/mass spectroscopy and radioimmunoassay were used to identify the production of steroids by these cells. Of the 30 steroids detected, approximately 20 were identified. Based on secreted steroids, these cells appeared to contain all of the adrenocortical enzyme systems which presumably were present in the original tumor, including CYP11A, HSD3B2, CYP11B1, CYP21, CYP17, CYP11B2, 3β-hydroxysulfotransferase, and low levels of aromatase (CYP19). Cytogenetically, the H295 cell is highly aneuploid and hypertriploid, with 30% of the cells containing a modal chromosome number of 62. This cell line is presently available from the American Type Culture Collection as ATCC CRL-10296 and will grow in suspension.

Growth and Morphology

Initial tumor cell growth in conventional serum-containing medium was unsuccessful due to fibroblast overgrowth. The culture was later adapted to grow in such media. Initial or continued growth of NCI-H295 cells did not require fibroblast growth factor, a potent stimulator of normal and cultured adrenocortical cells. Due to continuous growth and development, multiple substrains have been adapted from the NCI-H295 cell line using alternative growth conditions to encourage substrate attachment and shorter cell cycle times. The cell culture medium growth supplement, Ultroser G (2%—BioSepra SA, Villeneuve la Garenne Cedex, France) is a relatively defined bovine derived serum substitute that was used to increase cell growth rate. This supplement was selected due to previous reports suggesting that it helped retain steroidogenic cell function (Hornsby and McAllister, 1991; McAllister *et al*., 1994). NCI-H295 cells grown in Ultroser-supplemented medium demonstrated increased growth rate, but the cells continued to grow as floating aggregates or loosely attached cells. Over a 3-month period, changing culture medium every three days and discarding unattached cells, cells that maintained attachment to plastic culture dishes were selected. After characterization, it was subsequently designated as H295R to differentiate this strain from the original cells. In comparison to the parent H295 cell line, H295R cells grow as an adherent monolayer, and population doubling time was reduced from 5 to 2 days. This substrain is propagated in Dulbecco's modified Eagle's and Ham's F12 medium, 1% ITS plus (Collaborative Biomedical Products, Bedford, Massachusets, U.S.A.), 1% penicillin/streptomycin (Gibco), 0.01% gentamicin, and 2% Ultroser G (BioSepra SA, Villeneuve la Garenne Cedex, France). Because of the difficulty in the importation of the Ultroser G serum substitute, a population

of H295R cells was selected to grow in a commercially available serum substitute (Nu-Serum type I—Collaborative Biomedical Products, Bedford, Massachusets, U.S.A.). The strain that can be grown in Nu-Serum is available from the American Type Culture Collection as ATCC CRL-2128. These cells have decreased responses to ANG II and K^+ but continue to respond to agonists of the protein kinase A signaling pathways. In an attempt to develop a strain of H295 cells that would grow in commercial serum and retain ANG II and K^+ treatment, a series of sera were tested. Growth of cells in DME/F12 medium with 10% supplementation with Cosmic Calf Serum (CCS) (Hyclone, Logan, UT) was found to maintain cell growth and responses to ANG II and K^+. This strain of H295R is advantageous because the growth medium is considerably less expensive and no supplementation with ITS-plus is needed. Also, in comparison to the original H295 cells that grew in suspension, these also grow as a monolayer.

Subsequently, another strain of cells, designated NCI-H295A, have been described which also grow as a monolayer (Rogriquez *et al*., 1997). The method for isolation of the NCI-H295A strain is similar to that described above, relying on the removal of nonattached cells with medium changes and, therefore, selecting a population of cells that grow as a monolayer. It has been recently proposed that NCI-H295A cells produce more mineralocorticoids than NCI-H295R cells (Fluck *et al*., 2002). The same study also showed a lack of ANG II stimulated steroidogenesis in NCI-H295A and the angiotensin type 1 receptor was highly expressed in NCI-H295R strain compared to NCI-H295A. This study did not show any K^+ (one of the primary mineralocorticoid regulators) regulated steroidogenesis nor did it show any CYP11B2 expression by NCI-H295A. The study failed to show any legitimate conclusive evidence about NCI-H295A strain's steroidogenesis.

Hormonal Response and Expression of Hormone Receptors on H295 Cells

Peptide hormones, ANG II and ACTH are the primary physiological regulators of steroid hormone production in the human adrenal gland, along with circulating levels of K^+ (Fig. 2). The original description of NCI-H295 cells did not report on the hormonal responsiveness of these cells (Gazdar *et al*., 1990). Subsequently, the responses to ANG II, K^+, and ACTH treatment, as well as expression of trophic hormone receptors, were characterized for the NCI-H295R cell strain. In vivo ANG II acts on the adrenal zona glomerulosa to increase production of aldosterone through type 1 ANG II (AT1) receptors. Studies of [^{125}I] radiolabeled ANG II binding to H295R cells in the presence of antagonists to the AT1 and AT2 receptors established that the H295R cells express AT1 receptors almost exclusively (Bird *et al*., 1993a; Bird *et al*., 1994). The AT1 receptor is coupled to phosphoinositidase C and increases the production of inositol phosphates in H295R cells (Bird *et al*., 1993b). ANG II, through the AT1 receptor, also increases H295R cell production of aldosterone. ANG II-mediated production of aldosterone has been shown to be differentially regulated by the dopamine

receptors D2 and D4. Both receptors are found in H295 cells with D2 inhibiting and D4 activating aldosterone production via ANG II (Wu *et al*., 2001).

Under appropriate culture conditions, as described above, the H295R cell line has shown sustained regulation of AT1 receptor expression and activity. Regulation of aldosterone production in H295R cells is mediated via the transcription factors Sp1 and Sp3, which are required for AT1 receptor expression (Zhao *et al*., 2001). These cells readily exhibit detectable levels of AT1 receptor mRNA, as well as [^{125}I] ANG II binding (Bird *et al*., 1993a; Bird *et al*., 1994; Bird *et al*., 1995a). Expression of transcript and binding appears to be affected in parallel (Bird *et al*., 1994; Bird *et al*., 1995a). Thus, the H295R cell line may be useful to define the mechanisms regulating adrenal cell responsiveness to ANG II.

Extracellular K^+ is the other major physiological regulator of adrenal aldosterone production.K^+ increases intracellular calcium levels in H295R cells, which appears to be the mechanism to increase aldosterone biosynthesis. Recent studies have also shown that K^+ stimulation increases the production of both ANG I and ANG II, suggesting that these cells may provide a model to study the hypothesis that an intra-adrenal renin–angiotensin system may exist (Hilbers *et al*., 1999).

An alternate pathway for the regulation of adrenal aldosterone production is through the action of parathyroid hormone (PTH) and parathyroid hormone-related peptides (PTHRP), which have been shown to regulate aldosterone production in freshly isolated glomerulosa cells (Isales *et al*., 1991). PTH and PTHRP also stimulate H295R cells to increase aldosterone synthesis (Hanley *et al*., 1993). PTH and PTHRP activate steroidogenesis in a cAMP-dependent manner in normal cells and the H295R cell line. Considering these observations, the H295R cell strain appears to be an appropriate model to study several of the major and minor physiological regulators of aldosterone biosynthesis.

The primary hormonal regulator of adrenal cortisol production is ACTH. The H295R cell line is only mildly responsive to ACTH, and most strains are completely resistant. While ACTH treatment did cause an acute increase in aldosterone synthesis, long-term stimulation could not be maintained (Hanley *et al*., 1993). The low response to ACTH may reflect the low level of ACTH receptor expression in the H295R cell (Mountjoy *et al*., 1994). Therefore, most experiments designed to examine the cAMP-dependent pathway requires the addition of either forskolin (to activate adenylyl cyclase) or cAMP analogues (Bird *et al*., 1993b; Bird *et al*., 1994; Bird *et al*., 1995a; Bird *et al*., 1996b; Rainey *et al*., 1993a; Rainey *et al*., 1994). The low response to ACTH is therefore, a drawback of this cell model. Studies directed toward ACTH action would need to be pursued using either primary cultures of adrenal cells or the Y1 mouse adrenal cell line, which retains ACTH responsiveness (Schimmer, 1979). An alternate strategy would be to use transgenic technology to reinstate ACTH receptor expression in the H295 cell line.

The responsiveness of the H295R cell strain is highly dependent on growth conditions. Thus, when adapting the cells to alternate growth conditions, a characterization of responsiveness may be necessary. Because of the bovine components of Ultroser G, its importation into the United States from France requires

a specific permit from the U.S. Department of Agriculture. For that reason, the selection of a strain of H295R cells that grow in CCS (commercially available from Hyclone) has made it very easy to maintain the cells.

Steroid Biosynthesis

As previously noted, adrenocortical steroid hormone biosynthesis is complex and varies dramatically between the glomerulosa, fasciculata, and reticularis zones. The clinical features of the patient with the adrenal tumor that gave rise to the H295 cell line indicated that steroids which normally arise from each of the adrenal zones were produced by the tumor. The steroids produced by the original H295 cell line, as well as the H295R strains, maintain this diversity. The ability of the cells to produce steroids which originate from multiple zones of the adrenal suggests that the H295R cell line remains pluripotent with regard to adrenocortical differentiation. In Gazdar's initial report (Gazdar *et al*., 1990) concerning these cells, a broad spectrum of basal steroid hormone synthesis was noted (30 steroids). The steroid profile was greatly influenced by serum conditions. Using the H295R cell line we have shown that these cells also produce an array of steroids even under basal conditions (Bird *et al*., 1993b; Rainey *et al*., 1993b). However, treatment with agonists appears to selectively promote the synthesis of certain zone-specific steroid hormone groups. Specifically, treatment with ANG II or K^+ will promote the cells to produce aldosterone (Bird *et al*., 1993b; Clark *et al*., 1995; Rainey *et al*., 1994). While aldosterone does not constitute the major product, even under these conditions, the H295R cell can be used to study aldosterone synthesis and factors which preferentially activate its synthesis. This is particularly important when one considers the difficulty in obtaining primary cultures of aldosterone-producing cells. Treatment of the H295R cell strain with agonists working through the cAMP pathway produce a pattern of steroids approaching those of the zona fasciculata and reticularis. Steroids produced during treatment with forskolin include cortisol, 11β-hydroxyandrostenedione, DHEA, DHEA-sulfate, corticosterone, 11-deoxycortisol, and androstenedione (Rainey *et al*., 1993b). These data support the proposition that the H295R cell line can act as an appropriate model for adrenocortical steroid hormone biosynthesis and may be useful in defining the various mechanisms causing the synthesis of the "zone-specific" steroids.

Expression of Steroid-Metabolizing Enzymes in H295 Cells

The original NCI-H295, as well as the H295R and H295A cell strains, have been used as genetic models for studying steroidogenic enzyme gene expression. The NCI-H295 adrenocortical cells express all of the enzymes participating in normal human adrenal steroidogenesis (Staels *et al*., 1993). In addition, the genes that encode these enzymes respond to the same second messengers controlling normal human adrenocortical function. Expression of genes encoding CYP11A, CYP17, and CYP21, together with CYP11B1 and CYP11B2, were first studied in the NCI-H295 cells (Staels *et al*., 1993;

Winqvist *et al*., 1992). The transcripts encoding these enzymes accumulated in response to agonists (8-bromo-cAMP, forskolin, cholera toxin, and 3-isobutyl-1-methylxanthine), which activate the protein kinase-A pathway. Consistent with normal adrenocortical tissue, stimulating the protein kinase-C pathway using phorbol esters resulted in decreased CYP11A1 and CYP17, but accumulation of CYP21. In a similar manner, 8-bromo-cAMP increased CYP11B1, CYP11B2, and unexpectedly, aromatase (CYP19) mRNA levels. Recent studies have demonstrated that mRNAs encoding the StAR gene as well as the five forms of cytochrome P450 known to be involved in adrenal steroidogenesis (CYP11A1, CYP17, CYP21, CYP11B2, and CYP11B1) are also detectable in the NCI-H295R cell substrain (Bird *et al*., 1993a; Bird *et al*., 1993b; Bird *et al*., 1994; Bird *et al*., 1995a; Bird *et al*., 1995b; Bird *et al*., 1996b; Denner *et al*., 1996; Hanley *et al*., 1993; Rainey *et al*., 1993a; Rainey *et al*., 1993b; Rainey *et al*., 1994).

Similar to other mammalian adrenal cells (McAllister and Hornsby, 1988; Rainey *et al*., 1991), the levels of mRNA encoding CYP17 and HSD3B2 appear to be differentially regulated in NCI-H295R cells (Bird *et al*., 1996a). ANG II promoted a significant increase in mRNA level for HSD3B2 and CYP11A1 but only marginally increased the mRNA levels of CYP17. Potent activators of the protein kinase A pathway (forskolin and dbcAMP), while increasing the message for HSD3B2, have a greater effect on levels of CYP17 mRNA. Thus, activation of the protein kinase C and calcium signaling pathways tend to support a glomerulosa-like steroidogenic enzyme expression, while activation of the protein kinase A pathway promotes fasciculata-like enzyme expression.

Another pair of steroidogenic enzymes that exhibit an adrenal zone-specific distribution includes CYP11B2 and CYP11B1. CYP11B2 is expressed only in the zona glomerulosa and is essential for corticosterone conversion to aldosterone (Fig. 1). On the other hand, CYP11B1 is expressed at higher levels in the zona fasciculata/reticularis and is essential for the production of cortisol from deoxycortisol (Fig. 1). The transcripts encoding these two enzymes are increased by treatment of H295R cells with activators of the protein kinase-A pathway, although the effect on CYP11B1 mRNA levels is greater. In addition, levels of CYP11B2 mRNA are increased in H295R cells by treatment with ANG II (Bird *et al*., 1993b; Denner *et al*., 1996; Holland *et al*., 1993; Pezzi *et al*., 1997) or K^+ (Bird *et al*., 1993b; Denner *et al*., 1996; Pezzi *et al*., 1997). The effects of ANG II on the expression of CYP11B1 were not as pronounced as was observed for CYP11B2, suggesting that the protein kinase-A and calcium pathways differentially regulate the expression of CYP11B2 and CYP11B1.

The H295R and H295A cell lines have also been shown to be useful model systems to define the mechanisms controlling transcription of the steroid-metabolizing genes. Fusion genes containing the 5′-flanking DNA from CYP11B1, CYP11B2, CYP17 and HSD3B2 have been studied using these cell lines (Clyne *et al*., 1997; Leers-Sucheta *et al*., 1997; Rogriquez *et al*., 1997). Further regulation of genes in the steroidogenic pathway has been demonstrated by a number of transcription factors identified in H295 cells (Gizard *et al*., 2002; Guo *et al*.,

1995; Vilain *et al*., 1997). The H295R cell line is the first steroidogenic cell line to maintain the full complement of adrenal steroid-metabolizing enzymes and therefore may continue to be useful in defining the elements in the 5′-flanking region of these genes that regulate transcription.

The SW13 Human Adrenal Carcinoma-Derived Cell Line

These cells were derived from a human adrenal carcinoma (Leibovitz *et al*., 1973) and are available from the American Type Culture Collection (CCL-105). The SW13 is an interesting cell culture model that has a mosaic pattern of vimentin expression and is deficient in the mammalian homologues of Brahma genes, Brm and BRG1 (Mizutani *et al*., 2002). Although adrenal in origin, SW13 cells produce no steroids and it is not clear that it is derived from steroidogenic adrenal cells. Therefore, its usefulness as an adrenocortical model system is limited.

Human Adrenocortical Carcinoma (HAC) Cell Line and Related Clones

The above mentioned NCI-H295 cell line and its strains have proven useful due to the retention of ANG II dependent aldosterone production; however, they lack ACTH response. In addition, the H295 models are not clonal but represent a mixture of cells isolated from primary cultures of the original tumor. In an attempt to develop a HAC cell line, our group recently isolated clonal populations of cells from an adrenal tumor. Primary adrenocortical carcinoma cells were isolated from a tumor removed from a child diagnosed with hypertension, obesity, and hirsutism. Cells were maintained in DME/F12 medium supplemented with 10% CCS, ITS+ premix, penicillin, streptomycin, and gentamicin. Parental tumor cells were used to develop 47 discrete clones, six of which grew well enough for further characterization. Preliminary results suggest that ACTH was effective at increasing cortisol production in at least one of the clones (Parmar *et al*., 2008). The cells also retained responsiveness to Ang II and K^+ through the production of aldosterone (Parmar *et al*., 2008). Further characterization is ongoing, but these cell lines promise to provide an improvement in the available systems to study human adrenal steroid production.

SUMMARY

The mechanisms involving hormonal regulation of adrenocortical cell growth, morphology, and steroidogenesis remain a topic of research. The murine Y1 and human H295 adrenocortical cell lines are the most widely used adrenal cell culture models. Together, they provide unique systems to study both the molecular and biochemical characteristics of adrenal function. The Y1 cell line has provided a unique experimental system to dissect the mechanisms of ACTH action on steroidogenesis, cell proliferation, morphology, and the factors that govern the expression of genes required for steroidogenesis. The ability of H295 cells to produce mineralocorticoids, glucocorticoids, and C19 steroids based on culture

conditions has provided a valuable model to study the processes involved in adrenocortical differentiation. However, both model systems have limitations. The Y1, due to the deficiency of 21-hydroxylase, cannot produce corticosteroids. The H295 models have low or no ACTH response. The recent development of a corticosterone-producing mouse adrenal cell line and an ACTH responsive human adrenal cell line should improve our ability to better study adrenal toxicology and cellular physiology.

REFERENCES

Arnold J. Ein Beitrag zu der feiner Struktur und dem Chemismus der Nebennieren. Virchows Arch 1866; 35:64–107.

Auersperg N, Hudson JB, Goddard EG, et al. Transformation of cultured rat adrenocortical cells by Kirsten murine sarcoma virus (Ki-MSV). Int J Cancer 1977; 19:81–89.

Auersperg N. Effects of culture conditions on the growth and differentiation of transformed rat adrenocortical cells. Cancer Res 1978; 38:1872–1884.

Auersperg N, Wan MW, Sanderson RA, et al. Morphological and functional differentiation of Kirsten murine sarcoma virus-transformed rat adrenocortical cell lines. Cancer Res 1981; 41:1763–1771.

Auersperg N, MacLaren IA, Bissell MJ.V-K-ras transformation induces reversion to an earlier developmental form in adult rat adrenal cells. Differentiation 1990; 43:29–36.

Bird IM, Hanley NA, Word RA, et al. Human NCI-H295 adrenocortical carcinoma cells: A model for angiotensin-II-responsive aldosterone secretion. Endocrinology 1993; 133:1555–1561.

Bird IM, Mason JI, Oka K, et al. Angiotensin-II stimulates an increase in cAMP and expression of 17 alpha-hydroxylase cytochrome P450 in fetal bovine adrenocortical cells. Endocrinology 1993; 132:932–934.

Bird IM, Mason JI, Rainey WE. Regulation of type 1 angiotensin II receptor messenger ribonucleic acid expression in human adrenocortical carcinoma H295 cells. Endocrinology 1994; 134:2468–2474.

Bird IM, Mason JI, Rainey WE. Hormonal regulation of angiotensin II type 1 receptor expression and AT1-R mRNA levels in human adrenocortical cells. Endocr Res 1995; 21:169–182.

Bird IM, Word RA, Clyne C, et al. Potassium negatively regulates angiotensin II type 1 receptor expression in human adrenocortical H295R cells. Hypertension 1995; 25:1129–1134.

Bird IM, Pasquarette MM, Rainey WE, et al. Differential control of 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. J Clin Endocrinology Metab 1996; 81:2171–2178.

Bird IM, Imaishi K, Pasquarette MM, et al. Regulation of 3 beta-hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. J Endocrinol 1996; 150(Suppl):S165–S173.

Black SM, Szklarz GD, Harikrishna JA, et al. Regulation of proteins in the cholesterol side-chain cleavage system in JEG-3 and Y-1 cells. Endocrinology 1993; 132:539–545.

Buonassisi V, Sato G, Cohen AI. Hormone-producing cultures of adrenal and pituitary tumor origin. Proc Natl Acad Sci U S A 1962; 48:1184–1190.

Chang CW, Naseeruddin SA, Hornsby PJ. The response of 21-hydroxylase messenger ribonucleic acid levels to adenosine 3′,5′-monophosphate and 12-*O*-tetradecanoylphorbol-13-acetate in bovine adrenocortical cells is dependent on culture conditions. Endocrinology 1991; 128:604–610.

Chang SF, Chung BC. Difference in transcriptional activity of two homologous CYP21A genes. Mol Endocrinol 1995; 9:1330–1336.

Cheng CY, Ryan RF, Vo TP, et al. Cellular senescence involves stochastic processes causing loss of expression of differentiated function genes: Transfection with SV40 as a means for dissociating effects of senescence on growth and on differentiated function gene expression. Exp Cell Res 1989; 180:49–62.

Cheng CY, Hornsby PJ. Expression of 11 beta-hydroxylase and 21-hydroxylase in long-term cultures of bovine adrenocortical cells requires extracellular matrix factors. Endocrinology 1992; 130:2883–2889.

Clark BJ, Pezzi V, Stocco DM, et al. The steroidogenic acute regulatory protein is induced by angiotensin II and K^+ in H295R adrenocortical cells. Mol Cell Endocrinol 1995; 115:215–219.

Clyne CD, Zhang Y, Slutsker L, et al. Angiotensin II and potassium regulate human CYP11B2 transcription through common cis-elements. Mol Endocrinol 1997; 11:638–649.

Cohen AI, Bloch E, Celozzi E. In vitro response of experimental adrenal tumors to corticotropin (ACTH). Proc Soc Exp Biol Med 1957; 95:304–309.

Colantonio CM, Kwan WK, Czerwinski W, et al. Altered G protein activity in a desensitization-resistant mutant of the Y1 adrenocortical tumor cell line. Endocrinology 1998; 139:626–633.

Compagnone NA, Bair SR, Mellon SH. Characterization of adrenocortical cell lines produced by genetically targeted tumorigenesis in transgenic mice. Steroids 1997; 62:238–243.

Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. Microbiol Mol Biol Rev 2002; 66:407–425.

Cortese F, Wolff J. Cytochalasin-stimulated steroidogenesis from high density lipoproteins. J Cell Biol 1978; 77:507–516.

Cuprak LJ, Lammi CJ, Bayer RC. Scanning electron microscopy of induced cell rounding of mouse adrenal cortex tumor cells in culture. Tissue Cell 1977; 9:667–680.

Denner K, Rainey WE, Pezzi V, et al. Differential regulation of 11 beta-hydroxylase and aldosterone synthase in human adrenocortical H295R cells. Mol Cell Endocrinol 1996; 121:87–91.

Fluck CE, Martens JW, Conte FA, Clinical, genetic, and functional characterization of adrenocorticotropin receptor mutations using a novel receptor assay. J Clin Endocrinol Metab 2002; 87:4318–4323.

Gazdar AF, Oie HK, Shackleton CH, et al. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Res 1990; 50:5488–5496.

Gizard F, El-Alfy M, Duguay Y, et al. Function of the transcriptional regulating protein of 132 kDa (TReP-132) on human P450scc gene expression. Endocr Res 2002; 28:559–574.

Gospodarowicz D, ll CR, Hornsby PJ, et al. Control of bovine adrenal cortical cell proliferation by fibroblast growth factor. Lack of effect of epidermal growth factor. Endocrinology 1977; 100:1080–1089.

Guo IC, Huang C, Chung BC. Differential regulation of the CYP11A1 (P450scc) and ferredoxin genes in adrenal and placental cells. DNA Cell Biol 1993; 12:849–860.

Guo W, Burris TP, McCabe ER. Expression of DAX-1, the gene responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism, in the hypothalamic-pituitary-adrenal/gonadal axis. Biochem Mol Med 1995; 56:8–13.

Hanley NA, Wester RM, Carr BR, et al. Parathyroid hormone and parathyroid hormone-related peptide stimulate aldosterone production in the human adrenocortical cell line, NCI-H295. Endocr J 1993; 1:447–450.

Havelock JC, Rainey WE & Carr BR. Ovarian granulosa cell lines. Mol Cell Endocrinol 2004; 228:67–78.

Hilbers U, Peters J, Bornstein SR, et al. Local renin-angiotensin system is involved in K^+-induced aldosterone secretion from human adrenocortical NCI-H295 cells. Hypertension 1999; 33:1025–1030.

Holland OB, Mathis JM, Bird IM, et al. Angiotensin increases aldosterone synthase mRNA levels in human NCI-H295 cells. Mol Cell Endocrinol 1993; 94:R9–R13.

Hornsby PJ, McAllister JM. Culturing steroidogenic cells. Methods Enzymol 1991; 206:371–380.

Huang N, Dardis A, Miller WL. Regulation of cytochrome b5 gene transcription by Sp3, GATA-6, and steroidogenic factor 1 in human adrenal NCI-H295A cells. Mol Endocrinol 2005; 19:2020–2034.

Isales CM, Barrett PQ, Brines M, et al. Parathyroid hormone modulates angiotensin II-induced aldosterone secretion from the adrenal glomerulosa cell. Endocrinology 1991; 129:489–495.

Kananen K, Markkula M, Mikola M, et al. Gonadectomy permits adrenocortical tumorigenesis in mice transgenic for the mouse inhibin alpha-subunit promoter/simian virus 40 T-antigen fusion gene: Evidence for negative autoregulation of the inhibin alpha-subunit gene. Mol Endocrinol 1996; 10:1667–1677.

Kowal J, Fiedler R. Adrenal cells in tissue culture. I. Assay of steroid products steroidogenic responses to peptide hormones. Arch Biochem Biophys 1968; 128:406–421.

Leers-Sucheta S, Morohashi K, Mason JI, et al. Synergistic activation of the human type II 3β-hydroxysteroid dehydrogenase delta 5-delta 4 isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester. J Biol Chem 1997; 272:7960–7967.

Leibovitz A, McCombs WM, III, Johnston D, et al. New human cancer cell culture lines. I.SW-13, small-cell carcinoma of the adrenal cortex. J Natl Cancer Inst 1973; 51:691–697.

Lin D, Sugawara T, Strauss JF, III, et al. Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 1995; 267:1828–1831.

Lin X, Takemori H, Katoh Y, et al. Salt-inducible kinase is involved in the ACTH/cAMP-dependent protein kinase signaling in Y1 mouse adrenocortical tumor cells. Mol Endocrinol 2001; 15:1264–1276.

Lopez D, Nackley AC, Shea-Eaton W, et al. Effects of mutating different steroidogenic factor-1 protein regions on gene regulation. Endocrine 2001; 14:353–362.

Mattson P, Kowal J. The ultrastructure of functional mouse adrenal cortical tumor cells in vitro. Differentiation 1978; 11:75–88.

McAllister JM, Hornsby PJ. Dual regulation of 3 beta-hydroxysteroid dehydrogenase, 17 alpha-hydroxylase, and dehydroepiandrosterone sulfotransferase by adenosine

3′,5′-monophosphate and activators of protein kinase C in cultured human adrenocortical cells. Endocrinology 1988; 122:2012–2018.

McAllister JM, Byrd W, Simpson E. The effects of growth factors and phorbol esters on steroid biosynthesis in isolated human theca interna and granulosa-lutein cells in long term culture. J Clin Endocrinol Metab 1994; 79:106–112.

Mellon SH, Miller WL, Bair SR, et al. Steroidogenic adrenocortical cell lines produced by genetically targeted tumorigenesis in transgenic mice. Mol Endocrinol 1994; 8:97–108.

Mitani F, Mukai K, Miyamoto H. et al. (Localization of replicating cells in rat adrenal cortex during the late gestational and early postnatal stages. Endocr Res 1998; 24:983–986.

Mizutani T, Ito T, Nishina M, et al. Maintenance of integrated proviral gene expression requires Brm, a catalytic subunit of SWI/SNF complex. J Biol Chem 2002; 277:15859–15864.

Mountjoy KG, Bird IM, Rainey WE, et al. ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. Mol Cell Endocrinol 1994; 99:17–20.

Mukai K, Nagasawa H, Agake-Suzuki R, et al. Conditionally immortalized adrenocortical cell lines at undifferentiated states exhibit inducible expression of glucocorticoid-synthesizing genes. Eur J Biochem 2002; 269:69–81.

Nelson DR, Koymans L, Kamataki T, et al. P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 1996; 6:1–42.

O'Hare MJ, Neville AM. Morphological responses to corticotrophin and cyclic AMP by adult rat adrenocortical cells in monolayer culture. J Endocrinol 1973; 56:529–536.

Olson MF, Krolczyk AJ, Gorman KB, et al. Molecular basis for the 3',5'-cyclic adenosine monophosphate resistance of Kin mutant Y1 adrenocortical tumor cells. Mol Endocrinol 1993; 7:477–487.

Osawa S, Betz G, Hall PF. Role of actin in the responses of adrenal cells to ACTH and cyclic AMP: Inhibition by DNase I. J Cell Biol 1984; 99:1335–1342.

Pan J, Roskelley CD, Auersperg N. Influence of cell type on the steroidogenic potential and basal cyclic AMP levels of ras-oncogene-transformed rat cells. Differentiation 1995; 58:321–328.

Parker KL, Chaplin DD, Wong M, et al. Expression of murine 21-hydroxylase in mouse adrenal glands and in transfected Y1 adrenocortical tumor cells. Proc Natl Acad Sci U S A 1985; 82:7860–7864.

Parmar J, Rainey WE Development of an ACTH responsive human adrenocortical carcinoma cell line. J Clin Endocrinol Metab. 2008, in Press.

Pezzi V, Clyne CD, Ando S, et al. Ca^{2+}-regulated expression of aldosterone synthase is mediated by calmodulin and calmodulin-dependent protein kinases. Endocrinology 1997; 138:835–838.

Pierson RWJ. Metabolism of steroid hormones in adrenal cortex tumor cultures. Endocrinology 1967; 81:693–707.

Qiu R, Tsao J, Kwan WK, Mutations to forskolin resistance result in loss of adrenocorticotropin receptors and consequent reductions in levels of G protein alpha-subunits. Mol Endocrinol 1996; 10:1708–1718.

Rae PA, Gutmann NS, Tsao J, et al. Mutations in cyclic AMP-dependent protein kinase and corticotropin (ACTH)-sensitive adenylate cyclase affect adrenal steroidogenesis. Proc Natl Acad Sci U S A 1979; 76:1896–1900.

Ragazzon B, Lefrancois-Martinez AM, Val P, et al. Adrenocorticotropin-dependent changes in SF-1/DAX-1 ratio influence steroidogenic genes expression in a novel model of glucocorticoid-producing adrenocortical cell lines derived from targeted tumorigenesis. Endocrinology 2006; 147:1805–1818.

Rainey WE, Naville D, Mason JI. Regulation of 3 beta-hydroxysteroid dehydrogenase in adrenocortical cells: Effects of angiotensin-II and transforming growth factor beta. Endocr Res 1991; 17:281–296.

Rainey WE, Bird IM, Sawetawan C, et al. Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. J Clin Endocrinol Metab 1993; 77:731–737.

Rainey WE, Bird IM, Byrd W, et al. Effect of angiotensin II on human luteinized granulosa cells. Fertil Steril 1993; 59:143–147.

Rainey WE, Bird IM, Mason JI. The NCI-H295 cell line: A pluripotent model for human adrenocortical studies. Mol Cell Endocrinol 1994; 100:45–50.

Rainey WE, Saner K, Schimmer BP. Adrenocortical cell lines. Mol Cell Endocrinol 2004; 228:23–38.

Rice DA, Aitken LD, Vandenbark GR, et al. A cAMP-responsive element regulates expression of the mouse steroid 11 beta-hydroxylase gene. J Biol Chem 1989; 264:14011–14015.

Rilianawati, Paukku T, Kero J, et al. Direct luteinizing hormone action triggers adrenocortical tumorigenesis in castrated mice transgenic for the murine inhibin alpha-subunit promoter/simian virus 40 T-antigen fusion gene. Mol Endocrinol 1998; 12:801–809.

Rogriquez H, Hum DW, Staels B, Transcription of the human genes for cytochrome P450scc and P450c17 is regulated differently in human adrenal NCI-H295 cells than in mouse adrenal Y1 cells. J Endocrinol Metab 1997; 82:365–371.

Roskelley CD, Auersperg N. Density separation of rat adrenocortical cells: Morphology, steroidogenesis, and P-450scc expression in primary culture. In Vitro Cell Dev Biol 1990; 26:493–501.

Roskelly CD, Auersperg N. Rapid ras-oncogene-mediated transformation maintains steroidogenic differentiation in adrenocortical parenchymal cells. Differentiation 1995; 59:103–111.

Samandari E, Kempna P, Nuoffer JM, et al. Human adrenal corticocarcinoma NCI-H295R cells produce more androgens than NCI-H295A cells and differ in 3 beta-hydroxysteroid dehydrogenase type 2 and 17,20-lyase activities. J Endocrinol 2007; 195:459–472.

Schimmer BP. Phenotypically variant adrenal tumor cell cultures with biochemical lesions in the ACTH-stimulated steroidogenic pathway. J Cell Physiol 1969; 74:115–122.

Schimmer BP, Zimmerman AE. Steroidogenesis and extracellular cAMP accumulation in adrenal tumor cell cultures. Mol Cell Endocrinol 1976; 4:263–270.

Schimmer BP. Adrenocortical Y1 cells. Methods Enzymol 1979; 58: 570–574.

Schimmer BP. Isolation of ACTH-resistant Y1 adrenal tumor cells. Methods Enzymol 1985; 109:350–356.

Schimmer BP, Kwan WK, Tsao J, et al. Adrenocorticotropin-resistant mutants of the Y1 adrenal cell line fail to express the adrenocorticotropin receptor. J Cell Physiol 1995; 163:164–171.

Shiver TM, Sackett DL, Knipling L, et al. Intermediate filaments and steroidogenesis in adrenal Y-1 cells: Acrylamide stimulation of steroid production. Endocrinology 1992; 131:201–207.

Simpson ER WM. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu Rev Physiol 1988; 50:427–440.

Staels B, Hum DW, Miller WL. Regulation of steroidogenesis in NCI-H295 cells: A cellular model of the human fetal adrenal. Mol Endocrinol 1993; 7:423–433.

Temel RE, Trigatti B, DeMattos RB, et al. Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. Proc Natl Acad Sci U S A 1997; 94:13600–13605.

Thomas M, Suwa T, Yang L, et al. Cooperation of hTERT, SV40T antigen and oncogenic Ras in tumorigenesis: A cell transplantation model using bovine adrenocortical cells. Neoplasia 2002; 4:493–500.

Tian Y, Baukal AJ, Sandberg K, et al. Properties of AT1a and AT1b angiotensin receptors expressed in adrenocortical Y-1 cells. Am J Physiol 1996; 270:E831–E839.

Vilain E, Guo W, Zhang YH, et al. DAX1 gene expression upregulated by steroidogenic factor 1 in an adrenocortical carcinoma cell line. Biochem Mol Med 1997; 61:1–8.

Voorhees H, Aschenbrenner J, Carnes J, et al. Rounding and steroidogenesis of enzyme- and ACTH-treated Y-1 mouse adrenal tumor cells. Cell Biol Intl 1984; 8:483–497.

Whitehouse BJ, Gyles SL, Squires PE, et al. Interdependence of steroidogenesis and shape changes in Y1 adrenocortical cells: Studies with inhibitors of phosphoprotein phosphatases. J Endocrinol 2002; 172:583–593.

Winqvist O, Karlsson FA, Kampe O. 21-hydroxylase, a major autoantigen in idiopathic Addison's disease. Lancet 1992; 339:1559–1562.

Wong M, Rice DA, Parker KL, et al. The roles of cAMP and cAMP-dependent protein kinase in the expression of cholesterol side chain cleavage and steroid 11 beta-hydroxylase genes in mouse adrenocortical tumor cells. J Biol Chem 1989; 264:12867–12871.

Wu KD, Chen YM, Chu TS, et al. Expression and localization of human dopamine D2 and D4 receptor mRNA in the adrenal gland, aldosterone-producing adenoma, and pheochromocytoma. J Clin Endocrinol Metab 2001; 86:4460–4467.

Yasumura Y, Buonassisi V, Sato G. Clonal analysis of differentiated function in animal cell cultures. I. Possible correlated maintenance of differentiated function and the diploid karyotype. Cancer Res 1966; 26:529–535.

Yasumura Y. Retention of differentiated function in clonal animal cell lines, particularly hormone-secreting cultures. Am Zoologist 1968;285–305.

Zhao X, Martin MM, Elton TS. The transcription factors Sp1 and Sp3 are required for human angiotensin II type 1 receptor gene expression in H295-R cells. Biochim Biophys Acta 2001; 1522:195–206.

9

Glucocorticoid Pharmacotoxicological Interactions and Modulation of Toxicity

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INTRODUCTION

The historical breakthrough of glucocorticoids as a pharmacological agent can be traced back to 1929. At the time, Dr. Philip S. Hench developed the concept that steroids could alleviate symptoms of rheumatoid arthritis (Glyn, 1998). During 1930–1938, Drs. Edward C. Kendall and Tadeus Reichstein independently isolated and identified the adrenal steroids. Following the ground-breaking discovery of cortisone as an effective therapeutic agent against rheumatoid arthritis in 1949 (Hench *et al*., 1949), a variety of synthetic glucocorticoids (GCs) have been made with different potency, half-life, and administering methods. Among the most commonly used synthetic GCs for therapeutic purposes are dexamethasone, prednisone, and methylprednisolone. While dexamethasone has a long half-life and therefore is long acting, both prednisone and methylprednisolone exhibit an intermediate half-life resembling endogenous GCs. These synthetic GCs can be administered topically, orally, intramuscularly, or intravenously. Methylprednisolone can also be administered intralesionally, intraarticularly, and via nasal spray or inhalation. Like cortisone, which is a prodrug of cortisol, prednisone is a prodrug being converted to prednisolone rapidly in the body.

Endogenous GCs are produced in the adrenal glands. Although the adrenal glands also make mineralocorticoids, the term "corticosteroid" often denotes a

synonym of GCs. The principal form of GC produced in humans is cortisol, i.e., hydrocortisone, while its counterpart in rodents is corticosterone. Synthesis of GCs occurs in the zona fasciculata of the adrenal glands, where cholesterol is converted to pregnenolone by Cytochrome P450 11 A enzyme. Pregnenolone can be either dehydrogenated to form progesterone or hydroxylated to form 17α-hydroxypregnenolone, which becomes cortisol with 17α-hydroxyprogesterone and 11-deoxycortisol as intermediates. In rodents, corticosterone is derived from progesterone with 11-deoxycorticosterone as an intermediate. While the majority of cortisol binds to either globulin or albumin in the circulating system, 5% to 10% of cortisol exists freely in the blood. The protein bound steroid cannot enter into cells and, therefore, is biologically inactive.

The plasma concentration of cortisol is tightly controlled via the hypothalamic-pituitary-adrenal (HPA) axis. The level of GCs varies per diurnal cycle, peaks in early morning between approximately 6 and 8 am from 0.3 to 0.7μM, followed by a decline throughout the day, and reaches the nadir of 0.05 to 0.1μM at the midnight (Retana-Marquez *et al*., 2003). Either physical or psychological stress increases the synthesis of GCs via stimulation of the HPA axis. After a transient physical stress, such as intense exercise, GC levels rise within 5 to 10 minutes, peak around 30 minutes to 1.1 μM or higher, and return to normal within 60 to 90 minutes (de Kloet *et al*., 2005; Deuster *et al*., 2000). Chronic psychological stresses disturb the diurnal cycle, stimulate HPA axis constitutively, and increase the overall level of circulating GCs.

Two isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD) represent two key enzymes regulating GC metabolism. 11β-HSD1 converts inactive GCs to their biologically active forms, for example, from cortisone to cortisol, while 11β-HSD2 converts active GCs, i.e., cortisol or corticosterone, to inactive 11-keto analogues. 11β-HSD1 is widely expressed in the liver, lung, adipose tissue, vasculature, ovary, and the central nervous system. In contrast, 11β-HSD2 is primarily expressed in the kidney, colonic mucosa, salivary glands, and placenta. The tissue-specific expression of 11β-HSDs contributes to the pathogenesis of diseases related to disturbance of GCs in a tissue-specific manner (Tomlinson and Stewart, 2001).

GCs regulate a variety of physiological functions, from energy metabolism and biochemical homeostasis to immune responses. In addition to what the name stands for, i.e., regulating glucose metabolism, GCs also regulate the metabolism of amino acids, fatty acids, and nonglucose carbohydrates. To maintain the plasma concentration of glucose, GCs stimulate gluconeogenesis in the liver, inhibit glucose uptake by muscle or adipose tissue, and increase protein or fat breakdown. At the organismic level, GCs regulate nerve, cardiovascular, endocrine, and immune systems. GCs alter mood swing through its effect on central nervous system. In the cardiovascular system, GCs influence the synthesis of prostaglandins, angiotensinogen, and atrial natriuretic peptide, all of which modulate the vascular tone. GCs influence fetal lung development and promote gastric acidity. Given the

wide array of GC functions in various organs, it is expected that disturbance of GC homeostasis provokes numerous deleterious effects.

Sustained deficiency or overproduction of GCs results in diseases. Deficiency of cortisol causes Addison's disease or hypoadrenocorticism, which is manifested by fatigue, weight loss, low blood pressure, skin darkening and diarrhea. Cushing disease, the most prevalent disorder of excessive cortisol, leads to symptoms of immunosuppression, delayed wound healing, hypertension, obesity, edema, fatigue, muscle wasting, skin thinning, diabetes, and osteoporosis. Overdose of GCs, as a result of therapeutic application, leads to toxicity with symptoms of Cushing disease.

INTERACTION WITH CHEMOTHERAPEUTIC AGENTS

Chemotherapy for many types of cancer is most effective with a combination of antineoplastic drugs. Synthetic GCs are prescribed along with cytotoxic chemotherapeutic drugs for treatment of hematopoietic malignancies involving the growth of lymphoid tissue. The chemotherapeutic regiments for leukemia, Hodgkin or non-Hodgkin lymphoma, and myeloma contain prolonged treatment of prednisone or dexamethasone (Table 1). In addition to combination therapies, dexamethasone is often prescribed as an antiemetic agent during chemotherapy. Although a single dose and short-course dexamethasone treatment can be effective in inhibiting nausea and vomiting, administering to patients during chemotherapy feeds the probability of GC interacting with additional chemotherapeutic agents not listed in Table 1

Three types of cytotoxic agents are commonly used along with prednisone or dexamethasone for lymphoid malignancies—alkylating agents (cyclophosphamide, cisplatin, carmustine, etoposide, mechlorethamine, melphalan, nitrogen mustard, procarbazine, vincristine), antimetabolites (asparaginase, cytarabine, methotrexate, leucovorin), and antibiotics (doxorubicin, daunorubicin, bleomycin). Cyclophosphamide is a prodrug, being converted to phosphoramide mustard following Cytochrome p450 (CYP) enzymatic reaction and chemical rearrangement. Cisplatin intercalates DNA and causes DNA–DNA or protein–DNA crosslinks. The antimetabolite methotrexate inhibits dihydrofolate reductase, a key enzyme for purine and pyrimidine synthesis. Doxorubicin and daunorubicin are related anthracyclines that intercalate DNA, inhibit topoisomerase II, and generate free radicals. Bleomycin causes sequence specific single- and double-strand DNA breaks. GCs act synergistically with these agents to produce cytostatic or cytotoxic effect in lymphoid tumor cells.

Paclitaxel is a semisynthetic compound derived from plant Taxus brevifolia metabolite taxoids (Pang *et al*., 2006; Wu *et al*., 2005). Paclitaxel binds to microtubules to induce apoptosis and represents a front line chemotherapeutic agent against breast cancer and ovarian cancer. Paclitaxel is also a key component of

Table 1 Combination Cancer Chemotherapy Containing Glucocorticoids

Cancer	Regimen	GC Form & dose	Combination drug
Hodgkin's lymphoma	MOPP	Prednisone 40 mg/m^2 day 1–14/4 wks	Nitrogen mustard Vinblastine Procarbazine
	Stanford V	Prednisone 40 mg/m^2 Every 2 days/4 wks 3 cycles	Doxorubicin Vinblastine Mechlorethamine Vincristine Bleomycin Etoposide
Leukemia	BFM induction	Prednisone 60 mg/m^2 day 1–28	Vincristine Daunorubicin L-Asparaginase
	BFM consolidation	Dexamethasone 10 mg/m^2 day 1–28	Vincristine Doxorubicin
	ECOG induction	Prednisone 60 mg/m^2 day 1–28	Daunorubicin Vincristine Methotrexate
	ECOG Cycle I consolidation	Dexamethasone 10 mg/m^2 day 1–28	Cytarabine Etoposide Vincristine
Myeloma	MP	Prednisone 60 mg/m^2 day 1–4/4 wks 1–2 years	Melphalan
	VBMCP	Prednisone 40 mg/m^2 day 1–7/ 5 wks 20 mg/m^2 day 8–14/5 wks, 1–2 years	Vincristine Carmustine Melphalan Cyclophosphamide
	VMCP/VBAP/ ABCM	Prednisone 60 mg/m^2 day 1–4 and 22–25	Vincristine Cyclophosphamide Melphalan Carmustine Doxorubicin
	VAD	Dexamethasone 40 mg/m^2 day 9–12 and 17–20	Vincristine Doxorubicin
Non-Hodgkin's lymphoma	CVP	Prednisone 100 mg/m^2 day 1–5/ 28 days	Cyclophosphamide Vincristine
	CHOP	Prednisone 100 mg/m^2 5 days/ 3–4wks	Cyclophosphamide Hydroxydaunorubicin Vincristine

Table 1 Combination Cancer Chemotherapy Containing Glucocorticoids (*Continued*)

Cancer	Regimen	GC Form & dose	Combination drug
	PROMACE-CYTABOM	Prednisone 60 mg/m^2 day 1–14 /3 wks >6 cycles	Doxorubicin Cyclophosphamide Etoposide Cytosine Arabinoside Bleomycin Vincristine Methotrexate Leucovorin
	DHAP	Dexamethasone 40 mg day/m^2 1–4	Cisplatin Cytarabine
	Vanderbilt	Prednisone 60 mg/m^2 day 1,2,8,29–36	Cyclophosphamide Etoposide Vincristine Bleomycin Methotrexate Leucovorin

drug-eluting stents for angioplasty to treat coronary artery disease. An interaction of dexamethasone with paclitaxel has been reported (Moran *et al*., 2000; Wu *et al*., 2004).

Most chemotherapeutic drugs produce a number of adverse effects in patients, including alopecia, myelosuppression, and gastrointestinal toxicity such as nausea, vomiting, and stomatitis. While dexamethasone inhibits nausea and vomiting, whether dexamethasone or prednisone influences chemotherapy induced alopecia has not been demonstrated. As myelosuppression remains a common toxic effect of most chemotherapeutic agents, one piece of evidence suggests that methylprednisolone and prednisone reverse bone marrow necrosis (Santana *et al*., 2005). High dose methylprednisolone may reduce chemotherapy induced leukopenia by increasing the number of hematopoietic progenitor cells (Cetin *et al*., 1996). These reports suggest that GCs may ease myelosuppression induced by cytotoxic chemotherapeutic agents.

Some chemotherapeutic agents produce dermal toxicity (e.g., cyclophosphamide, cisplatin, doxorubicin), peripheral neuropathy (e.g., cisplatin), hepatotoxicity (e.g., cyclophosphamide, methotrexate), nephrotoxicity (e.g., cisplatin), and cardiotoxicity (e.g., doxorubicin). Topical application of GCs relieves skin inflammation, leading to the assumption that systemic administration of GCs likely counteracts inflammation associated with skin lesion prompted by chemotherapy. The role of GCs in hepatotoxicity remains largely undefined. One study indicates that dexamethasone increases hepatotoxicity of methotrexate during treatment of childhood brain tumors (Wolff *et al*., 1998). It is not known whether

dexamethasone or prednisone increases hepatotoxicity of adult patients during methotrexate chemotherapy. A large volume of literature suggests that reactive oxygen species (ROS) mediate nephrotoxicity of cisplatin. In experimental animals, a transient increase of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione S-transferase has been observed in the kidney following dexamethasone administration (Rajashree and Puvanakrishnan, 1998), suggesting that dexamethasone may be protective against nephrotoxicity of cisplatin. In terms of cardiac toxicity of doxorubicin, if the data from cell culture or experimental animals can be extrapolated (Bruynzeel *et al*., 2007; Chen *et al*., 2005), GCs may be protective against doxorubicin induced cardiomyopathy. Added together, GC administration may reduce dermal toxicity, nephrotoxicity, and cardiotoxicity of chemotherapeutic agents.

The mechanism of GC interaction with chemotherapeutic agents involves altering the metabolism and enhancing or inhibiting apoptosis. Changes in the activity of chemotherapeutic agents due to biotransformation have to be taken into consideration regarding the clinical outcome of chemotherapy. While augmenting cytotoxicity in tumor cells is indeed desirable, preventing apoptosis of normal nontumor cells becomes essential for reducing adverse effects of chemotherapeutic agents. Despite heterogeneous outcomes of GCs among different cell types, GCs draw the biological effect ultimately by modulating gene expression.

REGULATION OF GENE TRANSCRIPTION

The glucocorticoid receptor (GR) mediates most biological functions of GCs. The hydrophobic nature of GC molecules allows them to diffuse across the plasma membrane and bind to GR in the cytoplasm. GR is expressed in almost all cell types of mammalian species and belongs to a family of nuclear receptors, which include receptors for mineralocorticoids, sex hormones, thyroid hormones, vitamin D, retinoids, and peroxisome proliferators (Whitfield *et al*., 1999). The human GR gene encodes two splice variants—GRα and GRβ. The mRNA of each variant is translated into multiple proteins from distinct initiation sites. The classic GRα and GRβ contain 777 and 742 amino acids, respectively. While the α-isoform becomes active upon ligand-binding, the β-isoform does not bind to GCs and is thought to act as a dominant negative regulator. Therefore, GR in a broader sense is usually referred to as the GRα.

Upon ligand binding, GR activates or suppresses gene expression through three modes—binding to DNA, i.e., the genomic effect; interacting with transcription factors, transcriptional coactivators/corepressors, or chromatin remodeling factors; and activating signaling pathway, independent of GR nuclear translocation, i.e., the nongenomic effect. The genomic effect of GR has been well characterized such that the binding of GCs promotes GR dissociation from HSP90 in the cytoplasm and subsequently, nuclear translocation. Ligand-bound GR forms a homodimer, activating transcription via binding to the promoter of target genes at the glucocorticoid response element (GRE), a palindromic sequence consisting of

5'-AGAACA-3' with a spacer of three nucleotides between the repeat. In contrast to GRE mediated transcription, monomeric GR can bind to the negative glucocorticoid response element (nGRE) in the promoter of certain genes to suppress transcription.

GR also activates or suppresses gene expression through interaction with transcriptional coactivators/corepressors. The ligand bound GR recruits transcriptional coactivators, such as SRC-1, p/CAF, and CBP/p300. Some of these coactivators have intrinsic histone acetyltransferase activity and can acetylate core histones. In addition, GR interacts with SWI/SNF, an ATP-dependent–chromatin remodeling factor. Histone acetylation and chromatin remodeling lead to increased promoter accessibility and transcriptional initiation (Deroo and Archer, 2001). On the other hand, instead of recruiting coactivators, ligand binding of GR can result in interaction with transcriptional corepressors such as NCoR/SMRT and RIP140 (Christian *et al*., 2006; Jones and Shi, 2003), which exhibit histone deacetylase activity and restore the folding of chromatin structure by histone deacetylation, keeping DNA sequences inaccessible from the transcriptional machinery. Therefore depending on its partners, GCs can activate the expression of one set of genes while suppress the expression of another set of genes.

GR can counteract the activity of certain transcription factors through physical interactions. Antagonizing NF-κB, AP-1, NFAT, and STAT transcription factors by ligand-bound GR results in suppressed expression of the inflammatory genes under the control of these transcription factors. Contrary to transcriptional suppression, GR also acts as a transcriptional coactivator upon ligand binding by cooperating with certain transcription factors, such as C/EBPs, Sp1, Ets, and Oct. In this manner, GR regulates target genes through indirect binding to the promoter of these genes.

Increasing evidence suggests that GCs can produce biological changes through nongenomic means (Ito *et al*., 2006; Leung and Bloom, 2003; Necela and Cidlowski, 2004; Rhen and Cidlowski, 2005). In response to GC exposure, rapid signaling events can occur prior to alteration of gene transcription (Limbourg and Liao, 2003; Stellato, 2004; Wehling, 1997). Activation of kinases may result from nonspecific action of GCs on the plasma membrane, specific interaction of GCs with a hypothetical membrane associated GR, or binding of GCs with the classical intracellular GR (Buttgereit and Scheffold, 2002; Stellato, 2004). The classical GR has been reported to interact with key proteins regulating signal transduction pathways. For example, GCs activate phosphatidylinositol 3-kinase through physical contact of GR with the p85 regulatory subunit of phosphatidylinositol 3-kinase (Hafezi-Moghadam *et al*., 2002). GC exposure has been linked to activation of p38 Mitogen Activated Protein Kinase (MAPK), increased inositol trisphosphate, inhibiting cytosolic phospholipase A2, and blocking JNK signaling (Limbourg and Liao, 2003; Sun *et al*., 0000). Unlike the genomic effect, which typically takes hours to alter gene expression, the nongenomic effect occurs within minutes (Buttgereit and Scheffold, 2002; Limbourg

and Liao, 2003; Stellato, 2004). Although the nongenomic effect is transcription independent, by intersecting signal transduction pathways, GCs modulate gene expression via the downstream transcription factors of the signaling transduction pathways.

INDUCTION OF TRANSPORTER AND DRUG-METABOLISM ENZYMES

Several genes induced by GCs regulate transport, metabolism, and detoxification of pharmaceutical agents. These genes include P-glycoprotein (Pgp), CYP, and glutathione S-transferases. Pgp encodes a transporter located across the plasma membrane of mammalian cells. Such transporter pumps a variety of hydrophobic compounds out of cells, including the alkylating agents, antimetabolites, and antibiotics commonly prescribed for cancer chemotherapy. Although Pgp can transport steroids (Meijer *et al*., 1998; Webster and Carlstedt-Duke, 2002), the binding of GCs to GR appears to inhibit the activity of Pgp (Gruol and Bourgeois, 1997). Adding to the complexity of the relationship between GCs and Pgp, dexamethasone has been shown to induce the expression of Pgp gene and activate the activity of Pgp transporter (Lin *et al*., 1999; Pavek *et al*., 2007; Perloff *et al*., 2004; Regina *et al*., 1999). Such induction involves GR-dependent transcriptional activation of Pgp gene (Pavek *et al*., 2007). In cell lines and experimental animals, Pgp gene induction requires hours to days. This leads to the assumption that GCs, when first administered along with chemotherapeutic agents, may increase cellular accumulation by blocking Pgp activity. The time-frame required for Pgp gene to increase expression suggests that chronic GC administration may reduce cytosolic concentration of pharmacological agents in the later time-point due to elevated expression of Pgp gene.

CYPs encode an essential component for metabolism of xenobiotics and endogenous compounds. The CYP enzyme system, i.e., CYP plus NADPH–cytochrome p450 reductase, carries several types of oxidation reactions, from hydroxylation to oxidative deamination or desulfuration. At least 12 isoforms of CYPs have been reported to increase expression as a result of GC exposure. These include CYP1A1, CYP2B, CYP2C8, CYP2C9, CYP2D18, CYP2E, CYP2J2, CYP3A1, CYP3A2, CYP3A4, CYP3A6, and CYP3A44 (Table 2) (Attar *et al*., 2005; Bhadhprasit *et al*., 2007; Chen *et al*., 2005; Eliasson *et al*., 1994; Ged and Beaune, 1991; Hoen *et al*., 2000; Jarukamjorn *et al*., 1999; McCune *et al*., 2000; Mei *et al*., 2004; Pascussi *et al*., 2001; Pavek *et al*., 2007, Li *et al*., 1995; Rodrigues *et al*., 2003; Sampol *et al*., 1997; Schuetz *et al*., 2000; Wu *et al*., 2004). These isoforms belong to 3 out of 8 main families of CYPs and participate in phase I biotransformation of xenobiotics. While several CYP genes contain GRE in the promoter and respond to GCs by increasing transcription due to the genomic effect of GR, evidence also suggests that GR collaboration with the orphan nuclear receptor, e.g., pregnane X receptor (PXR), or activation of PXR by GC induces CYP gene transcription (Bhadhprasit *et al*., 2007; Pascussi *et al*.,

Table 2 Cytochrome p450 Gene Isoforms Induced by Glucocorticoids

GC Forms	Species	Tissue/Cells	Isoform	Mechanism	References
Clobetasol	Human	Skin	CYP1A1 mRNA & protein	N.D.	(Li *et al.*, 1995)
Pregnenolone	Mouse	Liver	CYP2B Protein	GR dependent	(Schuetz *et al.*, 2000)
	Human		CYP2C8	GRE	(Ged and Beaune, 1991)
Dexamethasone	Mouse	Liver hepatocytes	CYP2B10 mRNA	N.D.	(Jarukamjorn *et al.*, 1999)
Dexamethasone	Human	Placental trophoblasts	CYP2C9 Promoter, mRNA	Hepatocyte nuclear factor4a dependent	(Pavek *et al.*, 2007)
Corticosterone	Rat	Cardiomyocytes	CYP2D18 mRNA	N.D.	(Chen *et al.*, 2005)
Dexamethasone	Mouse	Skin	CYP2E1 mRNA & protein	N.D.	(Sampol *et al.*, 1997)
Dexamethasone	Human	Mammary epithelial cells	CYP2J2 mRNA	N.D.	(Wu *et al.*, 2004)
Dexamethasone	Rat	Hepatocytes	CYP3A1 protein	Protein kinase A/protein stabilization	(Eliasson *et al.*, 1994)
Dexamethasone	Rat	Liver	CYP3A1 Promoter mRNA protein	c/EBPα	(Rodrigues *et al.*, 2003)
Dexamethasone	Rat	Hepatocytes	CYP3A1 mRNA protein	N.D.	(Hoen *et al.*, 2000)
Dexamethasone	Rat	Liver, Small intestine, colon, kidney, brain	CYP3A1 mRNA & protein	N.D.	(Mei *et al.*, 2004)
Dexamethasone	Rat	Colon, Kidney	CYP3A2 mRNA	N.D.	(Mei *et al.*, 2004)
Dexamethasone	Human	Hepatocytes	CYP3A4 activity	N.D.	(McCune *et al.*, 2000)
Dexamethasone	Human	Hepatocytes	CYP3A4	PXR	(Pascussi *et al.*, 2001)
Dexamethasone	Human	Placental trophoblasts	CYP3A4 promoter mRNA	N.D.	(Pavek *et al.*, 2007)
Dexamethasone	Rabbit	Lacrimal glands	CYP3A6 activity	N.D.	(Attar *et al.*, 2005)
Dexamethasone	Mouse	Hepatocytes	CYP3A44	GR/PXR	(Bhadhprasit *et al.*, 2007)

Abbreviation: N.D., Not determined.

2001). In some cases, the transcription factor CCAAT/enhancer-binding protein-α regulates GC induced CYP gene transcription (Rodrigues *et al*., 2003). Regardless of the mechanism of CYP induction, each of the CYPs has substrate specificity and tissue or species specific expression. The diverse forms of CYPs induced by GCs suggest a wide spread potential of GCs in modulating drug metabolism.

Among the antineoplastic agents that are coadministered with dexamethasone or prednisone during combination chemotherapy, cyclophosphamide, vincristine, etoposide, methotrexate, and cisplatin have been characterized as substrates for CYP metabolism. As described earlier, CYP metabolism of cyclophosphamide is essential for its therapeutic efficacy, since cyclophosphamide is a prodrug of phosphoramide mustard. On the other hand, CYPs catalyze cyclophosphamide disposition and elimination in the liver, reducing hepatotoxicity of this drug (Pass *et al*., 2005). CYP3A4-dependent clearance has been reported for vincristine and etoposide (Villikka *et al*., 1999; Zhuo *et al*., 2004). There is evidence that CYPs—for example, CYP2E1—enhances the cytotoxicity of methotrexate (Neuman *et al*., 1999). The metabolism of cisplatin by CYPs, mainly CYP2E1, magnifies its hepatotoxicity and nephrotoxicity (Liu and Baliga, 2003; Lu and Cederbaum, 2006). Therefore, by inducing CYPs, GCs increase the effectiveness of those drugs that are dependent on CYPs for activation, enhancing the toxicity of certain chemotherapeutic agents by producing reactive intermediates while reducing the toxicity of others by increasing hepatic disposition and elimination.

In addition to CYPs, GCs induce the expression of glutathione S-transferases, aryl sulfotransferases, and flavin-containing monooxygenases (Chen *et al*., 2005; Falkner *et al*., 2001; Fan *et al*., 1992; Fang *et al*., 2003). Glutathione S-transferases add glutathione to compounds exhibiting some hydrophobicity and having an electrophilic carbon. The resulting glutathione conjugates often undergo cleavage in the kidney to form cysteine derivatives, which are subjected to urinary excretion after N-acetylation. Aryl sulfotransferases decrease the toxicity of pharmaceutical agents by transferring inorganic sulfate to the hydroxyl group of substrates, resulting in ionized organic sulfate for urinary excretion. Flavin containing monooxygenases oxidize amine, sulfur, and organophosphorus compounds (Cashman, 2000). The resulting polar products can be excreted in the urine. Therefore, by inducing glutathione S-transferases, aryl sulfotransferase, or flavin containing monooxygenase, GC may exaggerate urinary excretion of certain pharmaceutical agents.

In cardiomyocytes, microarray experiments have found that GCs induce the expression of metal-binding antioxidant protein metallothioneins and the antioxidant enzyme glutathione peroxidase 3 (Chen *et al*., 2005). Reactive oxygen species (ROS) often play an important role in the overall toxicity of alkylating agents and certain antibiotics. Detoxification of alkylating agents via glutathione conjugation causes depletion of GSH and, therefore, an increased accumulation of ROS from endogenous sources. In parallel with increasing endogenous sources of ROS, anthracycline quinones are known to produce ROS. Doxorubicin accepts electrons

from oxoreductive enzymes in the mitochondria to form semiquinone-free radicals, which initiate a chain of redox reactions (Davies and Doroshow, 1986). Doxorubicin has been shown to produce superoxide and H_2 O_2, when incubated with tissue extracts (Doroshow, 1986). Induction of antioxidant proteins and enzymes alleviates the toxicity of those antineoplastic agents prone to produce ROS.

INHIBITING TOXICITY BY IMMUNE SUPPRESSION

GCs can attenuate chemical toxicity by inhibiting inflammatory response. For cancer patients, individual differences in inflammatory responses affect the effectiveness of chemotherapy (Alexandre *et al*., 2003; Slaviero *et al*., 2003). Alkylating agents and antibiotics often cause acute cell injury. Necrosis of tissue results in local inflammatory response, whereas certain chemotherapeutic agents can trigger a systematic inflammatory response. Among the common side effects of cancer chemotherapy are mild fever and fatigue. While the fever results from activated cyclooxygenases and systematic immune response, there is evidence that fatigue involves increased levels of cytokines and systematic inflammatory response. Cyclophosphamide, nitrogen mustard, melphalan, 5-fluorouracil, anthracyclines. and bleomycin elicit systematic inflammatory response in patients or in experimental systems. Paclitaxel administration causes flu-like symptoms involving elevated plasma levels of IL-10, IL-8, and IL-6 (Pusztai *et al*., 2004). High dose of carmustine for breast cancer treatment frequently produces inflammatory response in the lung (Abushamaa *et al*., 2002). Dexamethasone has been postulated to offer protection against sulfur mustard toxicity by reducing inflammatory response based on the data from macrophage cells in culture (Amir *et al*., 2000). Inhibiting inflammatory response with dexamethasone reduces cardiomyopathy resulting from doxorubicin administration in mice (Bruynzeel *et al*., 2007). Therefore, one can expect that GCs reduce some side effects of those chemotherapeutic drugs, eliciting inflammatory response.

GCs suppress inflammation through intercepting proinflammatory signaling cascade at cellular and molecular levels. GCs are among the most frequently prescribed pharmacological agents due to their potent immunosuppressive effects. Among the diseases commonly treated with GCs are rheumatoid arthritis, asthma, inflammatory bowel disease, chronic obstructive pulmonary disease, lupus, multiple sclerosis, and dermatitis. For organ transplant, immune suppression by GCs is essential for avoiding tissue rejection. Regional inflammatory response results from release of proinflammatory mediators at the injury site, increased vasodilation and capillary permeability, and migration of leukocytes from the blood to the injured tissue. At the molecular level, increased expression of proinflammatory factors, such as cytokines, chemokines, kinins, growth factors, adhesion molecules, and inflammatory enzymes e.g., iNOS and COX-2, promote the proliferation, maturation, activation, and migration of immune cells.

GCs increase the expression of anti-inflammatory proteins while suppressing the expression of proinflammatory genes (Clark, 2007). Among key regulators of

immune response, NF-κB transcription factor acts as a master switch by regulating the expression of a long list of cytokines, chemokines, immunoreceptors, and cell adhesion molecules. Induction of IkB gene by GCs causes an inhibition of NF-κB transcription factor (Scheinman *et al*., 1995). GCs also restrain NF-κB activity through physical interaction of GR with p65 subunit of NF-κB. In addition, GCs induce the expression of "glucocorticoid inducible leucine zipper," a protein that interacts with and inhibits NF-κB and AP-1 transcription factors. Therefore, GCs inhibit NF-κB transcription factor through three different means.

Among a long list of genes typically induced by GCs, dual specificity phosphatase 1 , i.e., MAPK phosphatase 1, dephosphorylates, and inactivated MAPK family members, including JNKs, p38, and ERKs. These MAPK family members regulate the expression of proinflammatory genes, such as cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6). The p38 MAPK phosphorylates and inactivates tristetraprolin (TTP), an mRNA destabilizing protein (Stoecklin *et al*., 2004). Recently, it has been shown that dexamethasone promotes transcriptional activation of TTP gene (Smoak and Cidlowski, 2006). TTP recognizes adenylate/uridylate-rich elements (AREs) in the 3' untranslated region of TNFα, interferon γ, and interferon β (Brewer *et al*., 2004; Worthington *et al*., 2002), resulting in an increased degradation of these mRNA species.

Destabilizing mRNA comprises an important mechanism of suppressed gene expression by GC exposure. Reduced levels of proinflammatory proteins, including COX-2, interferon β, IL-1α, IL-1β, IL6, and colony stimulating factor, have been reported in association with mRNA destabilization (Clark, 2007). In addition to destabilizing mRNA, GCs cause cytoplasm to plasma membrane translocation of Annexin A1, a calcium-dependent–phospholipid binding protein that inhibits inflammatory response through preventing the release of prostaglandins (Gerke *et al*., 2005). Therefore, multiple genes and pathways contribute to GC induced immune suppression and anti-inflammatory response.

INDUCTION OF APOPTOSIS

Apoptosis delineates a measure of cytotoxicity of GCs and pharmacological agents. Perhaps, the best known function of GCs at the cellular level is their ability to induce apoptosis of lymphocytes (Distelhorst, 2002; Herold *et al*., 2006). A large volume of literature has documented this phenomenon. (Brokaw *et al*., 1998; Dorscheid *et al*., 2001; Druilhe *et al*., 2003; Erlacher *et al*., 2005; Gao *et al*., 2003; Gohel *et al*., 1999; Gohel *et al*., 1999; Han *et al*., 2001; Hofmann *et al*., 1998; Katychev *et al*., 2003; Laane *et al*., 2007; Liu *et al*., 2004; Lu *et al*., 2003; Lu *et al*., 2006; Miller *et al*., 2005; Nakazawa *et al*., 2002; Nichols *et al*., 2005; Pace *et al*., 2004; Plotkin *et al*., 2007; Schmidt *et al*., 2001; Sionov *et al*., 2006; Tsujimoto *et al*., 2005; Valamanesh *et al*., 2007; Wang *et al*., 2003; Wang *et al*., 2006; Yao *et al*., 2004). Table 3 lists a few examples from the recent literature showing lymphocytes undergoing apoptosis and key molecular signals of GCs leading to apoptosis. Additional hematopoietic cells responding to GCs by

undergoing apoptosis include dendritic cells, monocytes, and human eosinophils (Table 3). GCs promote apoptosis of these cells by suppressing the expression of the growth factors, cytokines, and their receptors essential for maturation and survival (Druilhe *et al*., 2003; Heasman *et al*., 2003).

GCs also induce apoptosis of cells from several nonhematopoietic origins. Apoptosis of osteocytes depicts a major underlying cause of osteoporosis associated with Cushing disease or GC overdose. Induction of apoptosis in central airway epithelial cells by GC contact contributes to persistent airway epithelium damage (Dorscheid *et al*., 2001; Tse *et al*., 2003). Apoptosis of Leydig cells and testicular germ cells by corticosterone treatment prompts reduced testosterone synthesis and infertility in male experimental animals (Gao *et al*., 2003; Yazawa *et al*., 2000; Yazawa *et al*., 2001). Earlier studies have suggested that GCs induce hippocampal damage, a condition relevant to Alzheimer disease (Angelucci, 2000). Although it remains controversial whether GCs cause damage in certain areas of the brain, hippocampal cells and neuronal cells undergo apoptosis during GC exposure in vitro (Lu *et al*., 2003). GCs have been linked to apoptosis of skeletal muscle cells, chondrocytes, and retinal pigment epithelial cells (Table 3). These lines of evidence suggest that chronic administration or overdose of GCs due to necessary pharmacological applications produce unwanted side effects involving apoptosis in several tissues.

The cellular and molecular mechanism of GC-induced apoptosis remains controversial. While mounting evidence indicates reduced expression of prosurvival members of bcl-2 family in association with GC-induced apoptosis (Table 3), elevated expression of BH3—only proapoptotic member of bcl-2 family, such as bbc3, Bim, and PUMA have been found with treatment of GCs (Erlacher *et al*., 2005; Han *et al*., 2001; Wang *et al*., 2003). There is evidence that GR interacts with the death-associated protein 3 to accelerate apoptosis (Hulkko *et al*., 2000). A positive feedback of GR-gene expression with GC exposure also plays a role in cell death (Ramdas *et al*., 1999).

Loss of cell-survival genes comprises another important mechanism of GC-induced apoptosis. Suppression of AP-1, NF-κB, and c-myc transcription factors is a well established feature of agonist bound GR. Activated AP-1, NF-κB, and c-myc can be detected in many types of cancer cells and often mediate the resistance against apoptosis by chemotherapeutic agents. Unlike NF-κB, how AP-1 or c-myc mediates cell survival response remains controversial in most cases. Among a myriad of functions including promoting the expression of cytotoxic cytokines, NF-κB also regulates the expression of manganese superoxide dismutase (Mn-SOD), bcl-2, bcl-xl, and IAPs (Mattson and Meffert, 2006). Mn-SOD removes superoxide in the mitochondria, whereas accumulation of ROS in the mitochondria contributes to cytochrome c release, which triggers the assembly of apoptosomes and subsequent activation of an initiator caspase, caspase-9. While bcl-2 and bcl-xl block cytochrome c release from the mitochondria, IAPs participate in procaspase-9 ubiquitination and degradation. Mn-SOD, bcl-2, bcl-xL, and IAPs

Table 3 Examples of GC-Induced Apoptosis and Key Mediators

Form of GC	Cell Type	Key Mediator	References
Beclomethasone	Central airway epithelial cells	Caspase-9 activation	(Dorscheid *et al*., 2001)
Betamethasone	CEM C7 T lymphocytes	NF-κB inhibition	(Hofmann *et al*., 1998)
Budesonide	Lung tumor	Bim/Blk	(Yao *et al*., 2004)
	Central airway epithelial cells	caspase-9 activation	(Dorscheid *et al*., 2001)
Clobetasol	CEM C7 T lymphocytes	NF-κB inhibition	(Hofmann *et al*., 1998)
Corticosterone	Leydig cells	Fas-FasL	(Gao *et al*., 2003)
	Rat L6 skeletal muscle cells,	antioxidant genes	(Nichols *et al*., 2005)
	Mouse osteoblasts	Bcl-2/bax ratio	(Gohel *et al*., 1999)
Dexamethasone	Dendritic cells,	N.D.	(Brokaw *et al*., 1998)
	Human eosinophils,	Bcl-2, ROS, cytokines	(Druilhe *et al*., 2003)
	Lymphocytes,	Bcl-2 supression	(Laane *et al*., 2007)
	Lymphocytes,	Mitochondrial GR	(Sionov *et al*., 2006)
	Lymphocytes	p38/phospho GR	(Miller *et al*., 2005)
	Lymphocytes in mice,	Bim, Puma	(Erlacher *et al*., 2005)
	Murine lymphocyte cell lines,	Bim induction	(Wang *et al*., 2003)
	Murine lymphocyte cell line	txnip	(Wang *et al*., 2006)
	CCRF-CEM lymphocytes,	p38/Bim	(Lu *et al*., 2006)
	Human leukemic T cells,	GRautoregulation	(Ramdas *et al*., 1999)
	Thymocytes,	Bbc3 induction	(Han *et al*., 2001)
	Thymic lymphoma,	AP-4, Caspase-9	(Tsujimoto *et al*., 2005)
	Human Monocytes,	CD95/CD95 ligand	(Schmidt *et al*., 2001)
	Osteocytes,	Pyk2,JNK	(Plotkin *et al*., 2007)
	Osteoblasts,	Caspase-3	(Liu *et al*., 2004)
	Airway epithelial cells,	Caspase-9	(Dorscheid *et al*., 2001)
	Hippocampal cells,	Glutamate Receptor	(Lu *et al*., 2003)
	CNS pericytes	N.D.	(Katychev *et al*., 2003)
Fluticasone	Peripheral blood T Cells	Caspase 8, NF-κB	(Pace *et al*., 2004)
Hydrocortisone	CEM C7 T lymphocytes	NF-κB	(Hofmann *et al*., 1998)
	Mouse chondrocytes	N.D.	(Nakazawa *et al*., 2002)
Triamcinolone	Retinal pigment epithelial cells	Caspase independent	(Valamanesh *et al*., 2007)
	CEM C7 T lymphocytes	NF-κB	(Hofmann *et al*., 1998)

Abbreviations: Txnip, thioredoxin interacting protein; N.D., Not determined.

act independently or in concert to inhibit apoptosis. By inhibiting the expression of these genes due to NF-κB inactivation, GCs sensitize cells to undergo apoptosis.

INHIBITION OF APOPTOSIS

GCs have been shown to inhibit apoptosis in a few cell types. In the hematopoietic system, GCs appear to facilitate the survival of erythroblasts and neutrophils, which undergo apoptosis spontaneously (Cox, 1995; Kato *et al*., 1995; Kolbus *et al*., 2003). In nonhematopoietic systems, GCs increase cell survival of hepatic and ovarian tissue (Amsterdam and Sasson, 2002; Sasson and Amsterdam, 2003). Dexamethasone inhibits cytotoxicity of cisplatin and gemcitabine in lung cancer cells through blocking apoptosis (Gassler *et al*., 2005; Vander Els and Miller, 1998). In mammary epithelial cells, dexamethasone prevents apoptosis induced by paclitaxel (Pang *et al*., 2006; Wu *et al*., 2005). Inhibition of apoptosis in cancer cells has raised the concern about potential resistance against chemotherapy (Herr *et al*., 2003).

While inhibiting apoptosis in cancer cells decreases the effectiveness of chemotherapy, preventing apoptosis in normal cells helps to reduce adverse consequences of certain chemotherapeutic agents. For example, inhibiting apoptosis of cardiomyocytes prevents cardiac toxicity of doxorubicin. Cardiomyocytes have abundant mitochondria, and approximately 30% of cell volume is occupied by the mitochondria. This feature makes cardiomyocytes highly susceptible to generation of ROS when exposed to doxorubicin, since doxorubicin utilizes the mitochondrial respiration chain to produce oxidants (Davies and Doroshow, 1986; Doroshow, 1986). Patients administered with doxorubicin sometime develop cardiomyopathy years later after initial cancer chemotherapy (Singal *et al*., 2000; Wallace, 2003). Doxorubicin induces apoptosis of cardiomyocytes, an event contributing to cardiomyopathy. In cultured cardiomyocytes, corticosterone is capable of preventing doxorubicin from inducing apoptosis (Chen *et al*., 2005). Such a protective effect appears to be long-lasting, suggesting that GCs may attenuate cardiotoxicity of doxorubicin.

The mechanisms mediating GC-induced cytoprotection are summarized in Table 4, including activation of serum- and glucocorticoid-regulated protein kinase 1 (SGK1), induction of antiapoptotic genes, and elevation of antioxidant genes. (Chang *et al*., 1997; Costas *et al*., 0000; Evans-Storms and Cidlowski, 2000; Feng *et al*., 1995; Mikosz *et al*., 2001; Pelaia *et al*., 2003; Petrella *et al*., 2006; Urayama *et al*., 1998; Webster *et al*., 2002; Wen *et al*., 1997; Wu *et al*., 2006; Yamamoto *et al*., 1998; Das *et al*., 2007). SGK1 is a 49 kDa serine/threonine protein kinase that shares approximately 50% sequence homology with Akt/PKB, a kinase known to phosphorylate BAD, a proapoptotic member of the bcl-2 family. Since unphosphorylated BAD drives mitochondrial release of cytochrome c, phosphorylation of BAD cancels its apoptosis inducing effect. Like Akt, SGK1 can be a downstream target of phosphatidylinositol 3-kinase signaling and translocate to the nuclei upon activation. SGK1 activation mediates dexamethasone induced cell

Table 4 Glucocorticoid-Induced Cytoprotection

Form of GC	Cell type	Apoptosis inducers	Key mediator	References
Budesonide	Bronchial epithelial cells	TGF-β	Inhibiting p38	(Pelaia *et al*., 2003)
Corticosterone	Rat cardiomyocytes	Doxorubicin	Bcl-xl	(Chen *et al*., 2005)
	Rat hippocampus	Adrenalectomy	Neurotrophic factors	(Nichols *et al*., 2005)
Dexamethasone	Human peripheral blood neutrophils	TNF-α	Protein synthesis	(Kato *et al*., 1995)
	Lung epithelial cells	TNF-α	c-IAP2	(Webster *et al*., 2002)
	Lung epithelial cells	IFN-γ, Fas	c-IAP2	(Wen *et al*., 1997)
	Lung carcinoma cells	Cisplatin Gemcitabine	Not specified	(Gassler *et al*., 2005)
	Mammary epithelial cells	Paclitaxel	SGK1/FOXO3	(Mikosz *et al*., 2001; Wu *et al*., 2006)
	Mammary gland	Milk weaning	AP-1	(Feng *et al*., 1995)
	Gastric cancer cells	Protein synthesis inhibitor	Bcl-xl/bcl-xs	(Chang *et al*., 1997)
	Thyroid cancer cells	TRAIL	Bcl-xL	(Petrella *et al*., 2006)
	Rat intestinal IEC-18 epithelial cells	ROS	HSP72	(Urayama *et al*., 1998)
	Rat testicular germ cells	Ischemia	N.D.	(Yazawa *et al*., 2001)
	L-929 fibroblasts	TNF	Not NF-κB	(Costas *et al*., 2000)
	Rat hepatoma cells	Nutrient deprivation	NF-κB Mitochondria	(Evans-Storms and Cidlowski, 2000)
	Rat hepatoma cells	TGF-b1	Bcl-xL	(Yamamoto *et al*., 1998)
	Bovine glomerular endothelial cells	TNFα, LPS	Caspase-3 like protease	(Wen *et al*., 1997)
Methyl-prednisolone	Glioblastoma	ROS	Bax/Bcl2	(Das *et al*., 2007)

survival in mammary epithelial cells (Leong *et al*., 2003; Mikosz *et al*., 2001), in transformed human embryonic kidney 293 cells (Brunet *et al*., 2001), and in the myocardium (Aoyama *et al*., 2005). SGK1 modulates phosphorylation status of the Forkhead transcription factor FKHRL1 (FOXO3a), causing nuclear export thus inactivation of the transcription factor (Brunet *et al*., 2001; Leong *et al*.,

2003; Wu *et al*., 2006). FKHRL1 typically turns on the expression of proapoptotic factors such as TNF-related–apoptosis-inducing ligand (TRAIL), Noxa, and Bim (Ghaffari *et al*., 2003; Kikuchi *et al*., 2007; Obexer *et al*., 2007). Therefore, SGK1 activation results in reduction of these proapoptotic factors.

Induction of prosurvival members of bcl-2 family constitutes a key event of GC-induced cell survival. Elevated transcripts of bcl-xl have been observed in cardiomyocytes following corticosterone treatment (Chen *et al*., 2005). GR-dependent bcl-xl increase also occurs in thyroid cancer cells and in transformed mammary epithelial cells (Petrella *et al*., 2006; Schorr and Furth, 2000). Schorr et al. reveal that increased bcl-xL expression results from mRNA stabilization (Schorr and Furth, 2000). Recent work from our laboratory found corticosterone causes transcriptional activation of bcl-xl gene in cardiomyocytes (Morrissy and Chen, unpublished data). Although the mechanism of such transcriptional activation has not been fully characterized, as described earlier, bcl-xl blocks apoptosis by preventing cytochrome c release from the mitochondria.

Corticosterone induces the expression of antioxidant proteins and enzymes, including glutathione peroxidase-3, metallothionein I, and metallothionein II in cardiomyocytes (Chen *et al*., 2005). This serves as an additional means of GC-induced cytoprotection, since ROS are an important trigger of apoptosis. GCs have also been shown to induce Hsp 72 gene expression, which protects cardiac cells against oxidative injury (Urayama *et al*., 1998; Valen *et al*., 2000). While Hsp 72 elevation takes place through a nontranscriptional mechanism, the mechanism of GC-induced glutathione peroxide 3 expression has not been studied. Metallothionein gene contains GRE in the promoter and is a known target of GC-induced transcription (Karin, 1998). While Hsp 72 protects cells from oxidative stress through protein–protein interaction and function as a molecular chaperone, glutathione peroxidase removes hydrogen peroxide and metallothionein chelates iron to prevent redox cycling and ROS propagation.

CONCLUSION

The impact of GCs on the toxicity of pharmacological agents varies substantially per cell type and circumstance. While dexamethasone or prednisone is coadministered with alkylating agents, antimetabolites and antibiotics during combination chemotherapy against lymphoid malignancies, the antiemetic activity of dexamethasone allows it being prescribed roughly in 50% cases of chemotherapy, providing the probability of GC interacting with 23 commonly used chemotherapeutic agents. While GCs reduce toxicity of chemotherapeutic agents in certain organs or cell types by enhancing outward transport, biotransformation, and disposition of the drugs, GCs augment the toxicity through bioactivation and induction of apoptosis in other tissue or cell types. By elevating the expression of CYPs, GCs enhance the conversion of prodrugs to active forms (e.g., cyclophosphamide), increase the diversity of reactive species (e.g., cisplatin, methotrexate), and catalyze hepatic disposition of toxic metabolites (e.g., cyclophosphamide, vincristine

and etoposide). Induction of glutathione S-transferases, aryl transferase, and flavin containing monooxygenases likely facilitates urinary excretion of pharmacological agents.

GCs are well known to suppress the inflammatory response. This feature helps to reduce certain adverse effects of those chemotherapeutic agents that produce an inflammatory response. The mechanism of such immune suppression comprehends multifarious pathways, including inhibition of NF-κB transcription factor, induction of MAPK phosphatase 1, and mRNA destabilization. These molecular events lead to downregulation of cytokines and proinflammatory genes.

It remains a mystery why GCs induce apoptosis in one cell type but inhibit apoptosis in another cell type. While lymphocytes, eosinophils, osteocytes, airway epithelial cells, skeletal muscle cells, and retinal epithelial cells are among well-documented examples of GC-induced apoptosis, GC treatment leads to resistance against apoptosis in erythroblasts, neutrophils, hepatocytes, cardiomyocytes, ovarian cells, mammary epithelial cells, and lung cancer cells. To induce apoptotic response, GCs inhibit NF-κB transcription factor, reduce prosurvival members of bcl-2 family (bcl-2, bcl-xL), and elevate the expression of proapoptotic members of bcl-2 family (bbc3, Bim, PUMA). In contrast, cell survival response elicited by GCs involves activation of SGK1, elevated expression of bcl-xL, and induction of antioxidant proteins. Therefore, it appears that the profile of genes suppressed or induced by GCs determines whether the cells undergo apoptosis or become resistant to apoptosis.

Historically, GCs have been heavily studied in the immune system. The diverse molecular events induced by GCs in nonimmune systems clearly suggest cell type dependent response to GCs. Cell type–dependent GR splice variants, posttranslational modification, GR cofactors, and signaling web may all contribute to the ultimate end point of GC action.

REFERENCES

Abushamaa AM, Sporn TA, Folz RJ. Oxidative stress and inflammation contribute to lung toxicity after a common breast cancer chemotherapy regimen. Am J Physiol Lung Cell Mol Physiol 2002:283.

Alexandre J, Gross-Goupil M, Falissard B, et al. Evaluation of the nutritional and inflammatory status in cancer patients for the risk assessment of severe haematological toxicity following chemotherapy. Ann Oncol 2003; 14:36–41.

Amir A, Chapman S, Kadar T, et al. Sulfur mustard toxicity in macrophages: Effect of dexamethasone. J Appl Toxicol 2000; 20.

Amsterdam A, Sasson R. The anti-inflammatory action of glucocorticoids is mediated by cell type specific regulation of apoptosis. Mol Cell Endocrinol 2002; 189:1–9.

Angelucci L. The glucocorticoid hormone from pedestal to dust and back. Eur J Pharmacol 2000; 405:139–147.

Aoyama T, Matsui T, Novikov M, et al. Serum and glucocorticoid-responsive kinase-1 regulates cardiomyocyte survival and hypertrophic response. Circulation 2005; 111:1652–1659.

Attar M, Ling KH, Tang–Liu DD, et al. Cytochrome P450 3A expression and activity in the rabbit lacrimal gland glucocorticoid modulation and the impact on androgen metabolism. Invest Ophthalmol Vis Sci 2005; 46:4697–4706.

Bhadhprasit W, Sakuma T, Hatakeyama N, et al. Involvement of glucocorticoid receptor and pregnane X receptor in the regulation of mouse CYP3A44 female-predominant expression by glucocorticoid hormone. Drug Metab Dispos 2007; 35:1880–1885.

Brewer BY, Malicka J, Blackshear PJ, et al. RNA sequence elements required for high affinity binding by the zinc finger domain of tristetraprolin conformational changes coupled to the bipartite nature of Au-rich mRNA-destabilizing motifs. J Biol Chem 2004; 279:27870–27877.

Brokaw JJ, White GW, Baluk P, et al. Glucocorticoid-induced apoptosis of dendritic cells in the rat tracheal mucosa. Am J Respir Cell Mol Biol 1998; 19:598–605.

Brunet A, Park J, Tran H, et al. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol Cell Biol 2001; 21:952–965.

Bruynzeel AM, Abou El, Hassan MA, et al. Anti-inflammatory agents and monoHER protect against DOX-induced cardiotoxicity and accumulation of CML in mice. Br J Cancer 2007; 96:937–943.

Buttgereit F, Scheffold A. Rapid glucocorticoid effects on immune cells. Steroids 2002; 67:529–534.

Cashman JR. Human flavin-containing monooxygenase: substrate specificity and role in drug metabolism. Curr Drug Metab 2000; 1:181–191.

Cetin M, Hicsonmez G, Tuncer AM, et al. The effect of short-course high-dose corticosteroid therapy on peripheral blood CD34+ progenitor cells in children with acute leukemia. Exp Hematol 1996; 24:1191–1194.

Chang TC, Hung MW, Jiang SY, et al. Dexamethasone suppresses apoptosis in a human gastric cancer cell line through modulation of bcl-x gene expression. FEBS Lett 1997; 415:11–15.

Chen QM, Alexander D, Sun H, et al. Corticosteroids inhibit cell death induced by doxorubicin in cardiomyocytes: Induction of antiapoptosis, antioxidant, and detoxification genes. Mol Pharmacol 2005; 67:1861–1873.

Christian M, White R, Parker MG. Metabolic regulation by the nuclear receptor corepressor RIP140. Trends Endocrinol Metab 2006; 17:243–250.

Clark AR. Anti-inflammatory functions of glucocorticoid-induced genes. Mol Cell Endocrinol 2007; 275:79–97.

Costas MA, Muller Igaz L, Holsboer F, et al. Transrepression of NF-kappaB is not required for glucocorticoid-mediated protection of TNF-alpha-induced apoptosis on fibroblasts. Biochimica et Biophysica Acta 2000:11.

Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils: Separation of survival and activation outcomes. J Immunol 1995; 154:4719–4725.

Das A, Banik NL, Ray SK. Methylprednisolone and indomethacin inhibit oxidative stress mediated apoptosis in rat C6 glioblastoma cells. Neurochem Res 2007; 32:1849–1856.

Davies KJ, Doroshow JH. Redox cycling of anthracyclines by cardiac mitochondria. I. Anthracycline radical formation by NADH dehydrogenase. J Biol Chem 1986; 261:3060–3067.

de Kloet ER, Joels M, Holsboer F. Stress and the brain: From adaptation to disease. Nat Rev Neurosci 2005; 6:463–475.

Deroo BJ, Archer TK. Glucocorticoid receptor-mediated chromatin remodeling in vivo. Oncogene 2001; 20:3039–3046.

Deuster PA, Petrides JS, Singh A, et al. Endocrine response to high-intensity exercise: Dose-dependent effects of dexamethasone. J Clin Endocrinol Metab 2000; 85:1066–1073.

Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ 2002; 9:6–19.

Doroshow JH. Davies KJ. Redox cycling of anthracyclines by cardiac mitochondria. II. Formation of superoxide anion, hydrogen peroxide, and hydroxyl radical. J Biol Chem 1986; 261:3068–3074.

Dorscheid DR, Wojcik KR, Sun S, et al. Apoptosis of airway epithelial cells induced by corticosteroids.[see comment]. Am J Respir Crit Care Med 2001; 164:1939–1947.

Druilhe A, Letuve S, Pretolani M. Glucocorticoid-induced apoptosis in human eosinophils: mechanisms of action. Apoptosis 2003; 8:481–495.

Eliasson E, Mkrtchian S, Halpert JR, et al. Substrate-regulated, cAMP-dependent phosphorylation, denaturation, and degradation of glucocorticoid-inducible rat liver cytochrome P450 3A1. J Biol Chem 1994; 269:18378–18383.

Erlacher M, Michalak EM, Kelly PN, et al. BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo. Blood 2005; 106:4131–4138.

Evans-Storms RB, Cidlowski JA. Delineation of an antiapoptotic action of glucocorticoids in hepatoma cells: The role of nuclear factor-kappaB. Endocrinology 2000; 141:1854–1862.

Falkner KC, Pinaire JA, Xiao GH, et al. Regulation of the rat glutathione S-transferase A2 gene by glucocorticoids: Involvement of both the glucocorticoid and pregnane X receptors. Mol Pharmacol 2001; 60:611–619.

Fan W, Trifiletti R, Cooper T, et al. Cloning of a mu-class glutathione S-transferase gene and identification of the glucocorticoid regulatory domains in its 5′-flanking sequence. Proc Natl Acad Sci U S A 1992; 89:6104–6108.

Fang HL, Shenoy S, Duanmu Z, et al. Transactivation of glucocorticoid-inducible rat aryl sulfotransferase (SULT1A1) gene transcription. Drug Metab Dispos 2003; 31:1378–1381.

Feng Z, Marti A, Jehn B, et al. Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. J Cell Biol 1995; 131:1095–1103.

Gao HB, Tong MH, Hu YQ, et al. Mechanisms of glucocorticoid-induced Leydig cell apoptosis. Mol Cell Endocrinol 2003; 199:153–163.

Gassler N, Zhang C, Wenger T, et al. Dexamethasone-induced cisplatin and gemcitabine resistance in lung carcinoma samples treated ex vivo. Br J Cancer 2005; 92:1084–1088.

Ged C, Beaune P. Isolation of the human cytochrome P-450 IIC8 gene: Multiple glucocorticoid responsive elements in the 5' region. Biochim Biophys Acta 1991; 3:433–435.

Gerke V, Creutz CE, Moss SE. Annexins: Linking Ca^{2+} signalling to membrane dynamics. Nature Rev Mol Cell Biol 2005; 6:449–461.

Ghaffari S, Jagani Z, Kitidis C, et al. Cytokines and BCR-ABL mediate suppression of TRAIL-induced apoptosis through inhibition of forkhead FOXO3a transcription factor. Proc Natl Acad Sci U S A 2003; 100:6523–6528.

Glyn JH. The discovery of cortisone: A personal memory. Br Med J 1998; 317:822A.

Gohel A, McCarthy MB, Gronowicz G. Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts in vivo and in vitro. Endocrinology 1999; 140:5339–5347.

Gruol DJ, Bourgeois S. Chemosensitizing steroids: Glucocorticoid receptor agonists capable of inhibiting P-glycoprotein function. Cancer Research 1997; 57:720–727.

Hafezi-Moghadam A, Simoncini T, Yang Z, et al. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nat Med 2002; 8:473–479.

Han J, Flemington C, Houghton AB, et al. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc Natl Acad Sci U S A 2001; 98:11318–11323.

Heasman SJ, Giles KM, Ward C, et al. Glucocorticoid-mediated regulation of granulocyte apoptosis and macrophage phagocytosis of apoptotic cells: Implications for the resolution of inflammation. J Endocrinol 2003; 178:29–36.

Hench P, Slocumb C, Barnes A, et al. The effect of a hormone of the adrenal cortex, 17-hydroxy-11-dehydrocorticosterone (compound E), on the acute phase of rheumatic fevers. Proc Staff Meet Mayo Clin 1949; 24:277–297.

Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T-cell apoptosis and function. Cell Mol Life Sci 2006; 63:60–72.

Herr I, Ucur E, Herzer K, et al. Glucocorticoid cotreatment induces apoptosis resistance toward cancer therapy in carcinomas. Cancer Res 2003; 63:3112–3120.

Hoen PA, Commandeur JN, Vermeulen NP, et al. Selective induction of cytochrome P450 3A1 by dexamethasone in cultured rat hepatocytes: analysis with a novel reverse transcriptase-polymerase chain reaction assay section sign. Biochem Pharmacol 2000; 60:1509–1518.

Hofmann TG, Hehner SP, Bacher S, et al. Various glucocorticoids differ in their ability to induce gene expression, apoptosis and to repress NF-kappaB-dependent transcription. FEBS Lett 1998; 441:441–446.

Hulkko SM, Wakui H, Zilliacus J. The pro-apoptotic protein death-associated protein 3 (DAP3) interacts with the glucocorticoid receptor and affects the receptor function. Biochem J 2000; 3:885–893.

Ito K, Chung KF, Adcock IM. Update on glucocorticoid action and resistance. J Allergy Clin Immunol 2006; 117:522–543.

Jarukamjorn K, Sakuma T, Miyaura J, et al. Different regulation of the expression of mouse hepatic cytochrome P450 2B enzymes by glucocorticoid and phenobarbital. Archives of Biochemistry & Biophysics 1999; 369:89–99.

Jones PL, Shi YB. N-CoR-HDAC corepressor complexes: Roles in transcriptional regulation by nuclear hormone receptors. Curr Top Microbiol Immunol 2003; 274:237–268.

Karin M. New twists in gene regulation by glucocorticoid receptor is DNA binding dispensable?[comment]. Cell 1998; 93:487–490.

Kato T, Takeda Y, Nakada T, et al. Inhibition by dexamethasone of human neutrophil apoptosis in vitro. Nat Immun 1995; 14:198–208.

Katychev A, Wang X, Duffy A, et al. Glucocorticoid-induced apoptosis in CNS microvascular pericytes. Dev Neurosci 2003; 25:436–446.

Kikuchi S, Nagai T, Kunitama M, et al. Active FKHRL1 overcomes imatinib resistance in chronic myelogenous leukemia-derived cell lines via the production of tumor necrosis factor-related apoptosis-inducing ligand. Cancer Sci 2007; 98:1949–1958.

Kolbus A, Blazquez-Domingo M, Carotta S, et al. Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: Molecular analysis by expression profiling. Blood 2003; 102:3136–3146.

Laane E, Panaretakis T, Pokrovskaja K, et al. Dexamethasone-induced apoptosis in acute lymphoblastic leukemia involves differential regulation of Bcl-2 family members. Haematologica 2007; 92:1460–1469.

Leong ML, Maiyar AC, Kim B, et al. Expression of the serum- and glucocorticoid-inducible protein kinase, Sgk, is a cell survival response to multiple types of environmental stress stimuli in mammary epithelial cells. J Biol Chem 2003; 278:5871–5882.

Leung DY, Bloom JW. Update on glucocorticoid action and resistance. J Allergy Clin Immunol 2003; 111:3–22.; quiz 23.

Li XY, Astrom A, Duell EA, et al. Retinoic acid antagonizes basal as well as coal tar and glucocorticoid-induced cytochrome P4501A1 expression in human skin. Carcinogenesis 1995; 16:519–524.

Limbourg FP, Liao JK. Nontranscriptional actions of the glucocorticoid receptor. J Mol Med 2003; 81:168–174.

Lin JH, Chiba M, Chen IW, et al. Effect of dexamethasone on the intestinal first-pass metabolism of indinavir in rats: Evidence of cytochrome P-450 3 A and p-glycoprotein induction. Drug Metab Dispos 1999; 27:1187–1193.

Liu H, Baliga R. Cytochrome P450 2E1 null mice provide novel protection against cisplatin-induced nephrotoxicity and apoptosis. Kidney Int 2003; 63:1687–1696.

Liu Y, Porta A, Peng X, et al. Prevention of glucocorticoid-induced apoptosis in osteocytes and osteoblasts by calbindin-D28k. J Bone Miner Res 2004; 19:479–490.

Lu Y, Cederbaum A I. Cisplatin-induced hepatotoxicity is enhanced by elevated expression of cytochrome P450 2E1. Toxicol Sci 2006; 89:515–523.

Lu J, Goula D, Sousa N, et al. Ionotropic and metabotropic glutamate receptor mediation of glucocorticoid-induced apoptosis in hippocampal cells and the neuroprotective role of synaptic N-methyl-D-aspartate receptors. Neuroscience 2003; 121:123–131.

Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. FEBS Lett 2006; 580:3539–3544.

Mattson MP, Meffert MK. Roles for NF-kappaB in nerve cell survival, plasticity, and disease. Cell Death Differ 2006; 13:852–860.

McCune JS, Hawke RL, LeCluyse EL, et al. In vivo and in vitro induction of human cytochrome P4503A4 by dexamethasone. Clin Pharmacol Ther 2000; 68:356–366.

Mei Q, Richards K, Strong–Basalyga K, et al. Using real-time quantitative TaqMan RT-PCR to evaluate the role of dexamethasone in gene regulation of rat P-glycoproteins mdr1a/1b and cytochrome P450 3A1/2 J Pharm Sci 2004; 93:2488–2496.

Meijer OC, de Lange EC, Breimer DD, et al. Penetration of dexamethasone into brain glucocorticoid targets is enhanced in mdr1 A P-glycoprotein knockout mice. Endocrinology 1998; 139:1789–1793.

Mikosz CA, Brickley DR, Sharkey MS, et al. Glucocorticoid receptor-mediated protection from apoptosis is associated with induction of the serine/threonine survival kinase gene, sgk-1. J Biol Chem 2001; 276:16649–16654.

Miller AL, Webb MS, Copik AJ, et al. p38 Mitogen-activated protein kinase (MAPK) is a key mediator in glucocorticoid-induced apoptosis of lymphoid cells: Correlation between

p38 MAPK activation and site-specific phosphorylation of the human glucocorticoid receptor at serine 211 Mol Endocrinol 2005; 19:1569–1583.
Moran TJ, Gray S, Mikosz CA, et al. The glucocorticoid receptor mediates a survival signal in human mammary epithelial cells. Cancer Res 2000; 60:867–872.
Nakazawa F, Matsuno H, Yudoh K, et al. Corticosteroid treatment induces chondrocyte apoptosis in an experimental arthritis model and in chondrocyte cultures. Clin Exp Rheumatol 2002; 20:773–781.
Necela BM, Cidlowski JA. Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells. Proc Am Thorac Soc 2004; 1:239–246.
Neuman MG, Cameron RG, Haber JA, et al. Inducers of cytochrome P450 2E1 enhance methotrexate-induced hepatocytotoxicity. Clin Biochem 1999; 32:519–536.
Nichols NR, Agolley D, Zieba M, et al. Glucocorticoid regulation of glial responses during hippocampal neurodegeneration and regeneration. Brain Res Brain Res Rev 2005; 48:287–301.
Obexer P, Geiger K, Ambros PF, et al. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. Cell Death Differ 2007; 14:534–547.
Pace E, Gagliardo R, Melis M, et al. Synergistic effects of fluticasone propionate and salmeterol on in vitro T-cell activation and apoptosis in asthma.[see comment]. J Allergy Clin Immunol 2004; 114:1216–1223.
Pang D, Kocherginsky M, Krausz T, et al. Dexamethasone decreases xenograft response to Paclitaxel through inhibition of tumor cell apoptosis.[see comment]. Cancer Biol Ther 2006; 5:933–940.
Pascussi JM, Drocourt L, Gerbal-Chaloin S, et al. Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. Eur J Biochem 2001; 268:6346–6358.
Pass GJ, Carrie D, Boylan M, et al. Role of hepatic cytochrome p450s in the pharmacokinetics and toxicity of cyclophosphamide: Studies with the hepatic cytochrome p450 reductase null mouse. Cancer Res 2005; 65:4211–4217.
Pavek P, Cerveny L, Svecova L, et al. Examination of Glucocorticoid receptor alpha-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines. Placenta 2007; 28:1004–1011.
Pelaia G, Cuda G, Vatrella A, et al. Effects of transforming growth factor-(beta) and budesonide on mitogen-activated protein kinase activation and apoptosis in airway epithelial cells. Am J Respir Cell Mol Biol 2003; 29:12–18.
Perloff MD, von Moltke LL, Greenblatt DJ. Ritonavir and dexamethasone induce expression of CYP3A and P-glycoprotein in rats. Xenobiotica 2004; 34:133–150.
Petrella A, Ercolino SF, Festa M, et al. Dexamethasone inhibits TRAIL-induced apoptosis of thyroid cancer cells via Bcl-xL induction. Eur J Cancer 2006; 42:3287–3293.
Plotkin LI, Manolagas SC, Bellido T. Glucocorticoids induce osteocyte apoptosis by blocking focal adhesion kinase-mediated survival. Evidence for inside-out signaling leading to anoikis. J Biol Chem 2007; 282:24120–24130.
Pusztai L, Mendoza TR, Reuben JM, et al. Changes in plasma levels of inflammatory cytokines in response to paclitaxel chemotherapy. Cytokine 2004; 25:94–102.
Rajashree S, Puvanakrishnan R. Dexamethasone induced alterations in enzymatic and nonenzymatic antioxidant status in heart and kidney of rats. Mol Cell Biochem 1998; 181:77–85.

Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res 1999; 59:1378–1385.

Regina A, Romero IA, Greenwood J, et al. Dexamethasone regulation of P-glycoprotein activity in an immortalized rat brain endothelial cell line, GPNT. J Neurochem 1999; 73:1954–1963.

Retana-Marquez S, Bonilla-Jaime H, Vazquez-Palacios G, et al. Body weight gain and diurnal differences of corticosterone changes in response to acute and chronic stress in rats. Psychoneuroendocrinology 2003; 28:207–227.

Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids : New mechanisms for old drugs. N Engl J Med 2005; 353:1711–1723.

Rodrigues E, Vilarem MJ, Ribeiro V, et al. Two CCAAT/enhancer binding protein sites in the cytochrome P4503A1 locus. Potential role in the glucocorticoid response. Eur J Biochem 2003; 270:556–564.

Sampol E, Mirrione A, Villard PH, et al. Evidence for a tissue-specific induction of cutaneous CYP2E1 by dexamethasone. Biochem Biophys Res Commun 1997; 235:557–561.

Santana AN, Ramos RG, Zanandrea EF, et al. Bone marrow necrosis successfully treated with corticosteroid. Eur J Haematol 2005; 74:75–76.

Sasson R, Amsterdam A. Pleiotropic anti-apoptotic activity of glucocorticoids in ovarian follicular cells. Biochem Pharmacol 2003; 66:1393–1401.

Scheinman RI, Cogswell PC, Lofquist AK, et al. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids.[comment]. Science 1995; 270:283–286.

Schmidt M, Lugering N, Lugering A, et al. Role of the CD95/CD95 ligand system in glucocorticoid-induced monocyte apoptosis. J Immunol 2001; 166:1344–1351.

Schorr K, Furth P. A. Induction of bcl-xL expression in mammary epithelial cells is glucocorticoid-dependent but not signal transducer and activator of transcription 5-dependent. Cancer Res 2000; 60:5950–5953.

Schuetz EG, Schmid W, Schutz G, et al. The glucocorticoid receptor is essential for induction of cytochrome P-4502B by steroids but not for drug or steroid induction of CYP3A or P-450 reductase in mouse liver. Drug Metab Dispos 2000; 28:268–278.

Singal PK, Li T, Kumar D, et al. Adriamycin-induced heart failure: Mechanism and modulation. Mol Cell Biochem 2000; 207:77–86.

Sionov RV, Cohen O, Kfir S, et al. Role of mitochondrial glucocorticoid receptor in glucocorticoid-induced apoptosis. J Exp Med 2006; 203:189–201.

Slaviero KA, Clarke SJ, Rivory LP. Inflammatory response: An unrecognised source of variability in the pharmacokinetics and pharmacodynamics of cancer chemotherapy. Lancet Oncol 2003; 4:224–232.

Smoak K, Cidlowski JA. Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. Mol Cell Biol 2006; 26:9126–9135.

Stellato C. Post-transcriptional and nongenomic effects of glucocorticoids. Proc Am Thorac Soc 2004; 1:255–263.

Stoecklin G, Stubbs T, Kedersha N, et al. MK2-induced tristetraprolin:14–3-3 complexes prevent stress granule association and ARE-mRNA decay. EMBO J 2004; 23:1313–1324.

Sun H, Xu BB, Inoue H, et al. p38 MAPK mediates COX-2 gene expression by corticosterone in cardiomyocytes. Cell Signaling, in press.

Tomlinson JW, Stewart PM. Cortisol metabolism and the role of 11beta-hydroxysteroid dehydrogenase. Best Pract Res Clin Endocrinol Metab 2001; 15:61–78.

Tse R, Marroquin BA, Dorscheid DR, et al. Beta-adrenergic agonists inhibit corticosteroid-induced apoptosis of airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 2003; 285.

Tsujimoto K, Ono T, Sato M, et al. Regulation of the expression of caspase-9 by the transcription factor activator protein-4 in glucocorticoid-induced apoptosis. J Biol Chem 2005; 280:27638–27644.

Urayama S, Musch MW, Retsky J, et al. Dexamethasone protection of rat intestinal epithelial cells against oxidant injury is mediated by induction of heat shock protein 72. J Clin Invest 1998; 102:1860–1865.

Valamanesh F, Torriglia A, Savoldelli M, et al. Glucocorticoids induce retinal toxicity through mechanisms mainly associated with paraptosis. Mol Vis 2007; 13:1746–57.

Valen G, Kawakami T, Tahepold P, et al. Glucocorticoid pretreatment protects cardiac function and induces cardiac heat shock protein 72. Am J Physiol Heart Circ Physiol 2000; 279.

Vander Els, NJ, Miller V. Successful treatment of gemcitabine toxicity with a brief course of oral corticosteroid therapy.[see comment]. Chest 1998; 114:1779–1781.

Villikka K, Kivisto KT, Maenpaa H, et al. Cytochrome P450-inducing antiepileptics increase the clearance of vincristine in patients with brain tumors. Clin Pharmacol Ther 1999; 66:589–593.

Wallace KB. Doxorubicin-induced cardiac mitochondrionopathy. Pharmacol Toxicol 2003; 93:105–115.

Wang Z, Malone MH, He H, et al. Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. J Biol Chem 2003; 278:23861–23867.

Wang Z, Rong YP, Malone MH, et al. Thioredoxin-interacting protein (txnip) is a glucocorticoid-regulated primary response gene involved in mediating glucocorticoid-induced apoptosis. Oncogene 2006; 25:1903–1913.

Webster JI, Carlstedt-Duke J. Involvement of multidrug resistance proteins (MDR) in the modulation of glucocorticoid response. J Steroid Biochem Mol Biol 2002; 82:277–288.

Webster JC, Huber RM, Hanson RL, et al. Dexamethasone and tumor necrosis factor-alpha act together to induce the cellular inhibitor of apoptosis-2 gene and prevent apoptosis in a variety of cell types. Endocrinology 2002; 143:3866–3874.

Wehling M. Specific, nongenomic actions of steroid hormones. Annu Rev Physiol 1997; 59:365–393.

Wen LP, Madani K, Fahrni JA, et al. Dexamethasone inhibits lung epithelial cell apoptosis induced by IFN-gamma and Fas. Am J Physiol 1997; 273.

Whitfield GK, Jurutka PW, Haussler CA, et al. Steroid hormone receptors: Evolution, ligands, and molecular basis of biologic function. J Cell Biochem 1999; (Suppl):110–122.

Wolff JE, Hauch H, Kuhl J, et al. Dexamethasone increases hepatotoxicity of MTX in children with brain tumors. Anticancer Res 1998; 18:2895–2899.

Worthington MT, Pelo JW, Sachedina MA, et al. RNA-binding properties of the AU-rich element-binding recombinant Nup475/TIS11/tristetraprolin protein. J Biol Chem 2002; 277:48558–48564.

Wu W, Chaudhuri S, Brickley DR, et al. Microarray analysis reveals glucocorticoid-regulated survival genes that are associated with inhibition of apoptosis in breast epithelial cells. Cancer Res 2004; 64:1757–1764.

Wu W, Pew T, Zou M, et al. Glucocorticoid receptor-induced MAPK phosphatase-1 (MPK-1) expression inhibits paclitaxel-associated MAPK activation and contributes to breast cancer cell survival. J Biol Chem 2005; 280:4117–4124.

Wu W, Zou M, Brickley DR, et al. Glucocorticoid receptor activation signals through forkhead transcription factor 3a in breast cancer cells. Mol Endocrinol 2006; 20:2304–2314.

Yamamoto M, Fukuda K, Miura N, et al. Inhibition by dexamethasone of transforming growth factor beta1-induced apoptosis in rat hepatoma cells: A possible association with Bcl-xL induction. Hepatology 1998; 27:959–966.

Yao R, Wang Y, Lemon WJ, et al. Budesonide exerts its chemopreventive efficacy during mouse lung tumorigenesis by modulating gene expressions. Oncogene 2004; 23:7746–7752.

Yazawa H, Sasagawa I, Nakada T. Apoptosis of testicular germ cells induced by exogenous glucocorticoid in rats. Hum Reprod 2000; 15:1917–1920.

Yazawa H, Sasagawa I, Suzuki Y, et al. Glucocorticoid hormone can suppress apoptosis of rat testicular germ cells induced by testicular ischemia. Fertil Steril 2001; 75:980–985.

Zhuo X, Zheng N, Felix CA, et al. Kinetics and regulation of cytochrome P450-mediated etoposide metabolism. Drug Metab Dispos 2004; 32:993–1000.

Part IV

Adrenal Dysfunction in Environmental Sentinel Species

10

Adrenocortical Toxicology in Fishes

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INTRODUCTION—OVERVIEW OF KEY FEATURES IN FISH ADRENALS

Teleost fishes, unlike mammals, do not have a discrete adrenal gland. In the piscine model, the adrenal tissue is located in the anterior or head portion of the kidney, a long thin organ lining the dorsal wall of the abdominal cavity. The steroidogenic (adrenocortical) and catecholaminergic (chromaffin) cells are intermingled in the head-kidney region (known as the interrenal tissue), mostly around the postcardinal vein, and there is no cortex or medulla (Norris, 1997; Wendelaar, 1997).

Even though the structure of adrenals in fish and in mammals is different, their biochemical and synthetic characteristics are rather similar. The main steroid produced by the adrenocortical cells in teleost fishes is cortisol, synthesized from cholesterol in a series of step-wise reactions similar to those in mammals. However, the inability to synthesize aldosterone in the adrenals of most fish is one important difference from the mammalian system. The chromaffin cells synthesize catecholamines, including adrenaline and noradrenaline (Reid *et al*., 1998). As in mammals, a major role for the hormones of the adrenal homologue in fish is in stress adaptation. Environmental stressors, including contaminants, activate the evolutionarily conserved adaptive physiological response characterized by an acute elevation in plasma catecholamine and cortisol levels. These primary adrenal hormones are thought to play an important role in the metabolic adjustments that allow the animals to cope with the acute stressor insult. Long-term exposure to

stressors, while activating the adaptive responses, may also lead to maladaptation, including reduced growth and mortality and increased disease incidences, but the mechanisms involved are far from clear (Barton and Iwama, 1991; Barton, 1991; Iwama *et al*., 2006; Mommsen *et al*., 1999; Barton *et al*., 2002; Wendelaar, 1997).

Unlike mammals, the lack of aldosterone is linked to another unique feature of the fish adrenals—the dual role of cortisol as both a glucocorticosteroid and mineralocorticosteroid. However, this view has recently been brought into question by the cloning and sequencing of a mineralocorticosteroid receptor (MR) in fish and the finding that 11-deoxycorticosterone binds with high affinity to this receptor in fish (Bury and Strum, 2007; Strum *et al*., 2005). Despite this information, there is little evidence as of yet for a ligand, other than cortisol, that is physiologically important in fish. In addition to its metabolic and osmoregualtory roles, cortisol also modulates most aspects of fish physiology, including development and growth, reproduction, immune function, and stress adaptation (Alsop and Vijayan, 2008; Mommsen *et al*., 1999).

Similar to other physiologically important tissues and organs, the adrenal tissue of fish is well vascularized. The cells are cholesterol-rich, a characteristic linked to their steroidogenic capabilities. The extensive vascularization and lipid content make the adrenal tissue of fish, similar to other vertebrates, highly vulnerable to contaminants, particularly those with a high K_{ow} (Colby, 1996). It has been documented that numerous contaminants, including metals, accumulate in the head kidney. Indeed, recent studies provided evidence that the adrenal tissue of fish is a target of environmental contaminants leading to steroid disruption (Hontela, 2005). Field studies with fish exposed chronically in their natural environment to pollutants, exhibit a blunted stress response characterized by a reduced capacity to either elevate plasma cortisol levels or to respond to acute adrenocorticotropic hormone (ACTH) stimulation (Hontela, 2005). Thus, while fish from clean lakes and rivers elevate plasma cortisol levels quickly in response to stressors, contaminated fish do not (Brodeur *et al*., 1997; Hontela, 2005). These studies provided evidence that the adrenal steroid biosynthetic pathway is disrupted by environmental contaminants in fish at specific intracellular sites, while the molecular mechanisms involved are just beginning to emerge. Specifically, cAMP production as well as abnormal gene expression of steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage (P450scc) have been identified as important targets for adrenal toxicants (Aluru and Vijayan, 2004; Aluru and Vijayan, 2006; Aluru and Vijayan, 2007; Gravel and Vijayan, 2006; Gravel and Vijayan, 2007; Hontela, 2005). These studies led to the development and validation of in vitro diagnostic tests using head-kidney tissues, which can be used both in the field and laboratory settings to detect and quantify adrenal dysfunction in sentinel species (Hontela, 2005). The following chapter will review adrenal toxicology in fish and outline the important differences and similarities between the mammalian and fish systems. The relevance of adrenal toxicology for

Ecological Risk Assessment will be also discussed. As very little is known about the impact of contaminants on catecholamine biosynthesis and action in fish, this review will focus mainly on the adrenal steroid disruption in fish.

STRUCTURE AND FUNCTION OF FISH ADRENALS

Anatomy of Teleost Adrenals

The anatomy of the adrenal tissue varies greatly among different groups of fish, ranging from paired strands of adrenocortical tissue within the posterior or main kidney in some cartilagineous fishes such as rays and sharks, to small islets of adrenocortical cells embedded in the head kidney in teleostean fishes such as trout and carp (Norris, 1997). Similar to mammals, the kidney and the adrenals in teleost fish are anatomically linked. While the kidney is a thin elongated organ lining the dorsal wall of the abdominal cavity from the anus to the heart, the head kidney wherein the adrenal tissue lies, is enlarged in many fish species. In some fish, including the rainbow trout, it has a butterfly-like shape. The head kidney or the pronephros of teleosts does not contain glomeruli or renal tubules, and does not have an osmoregulatory role. Instead, the matrix of the head kidney is composed of lymphoid tissue, generating blood cells. Small islets of steroidogenic cells are embedded in the proximity of blood vessels within the lymphoid tissue, which also contains packets of melanomacrophages, dark cells containing melanin pigment (Fig. 1). Chromaffin cells are localized in small clumps mainly in the walls of the blood vessels. It is important to note that the proportion of steroidogenic and chromaffin cells to the cells of the lymphoid matrix is small. Recent studies using Percoll gradients to isolate, count, and test the functional integrity of different cell populations within the head kidney of rainbow trout reported that steroidogenic cells in rainbow trout head kidney represent 1:8000 cells (Hontela *et al*., 2008). The anatomical organization of the adrenal tissue and the head kidney has important consequences for toxicological studies with fish adrenals. In contrast to mammalian studies where changes in the weight of the adrenals, cortex, or medulla can be used as indicators of functional alterations (Latendresse *et al*., 1994), it is not possible to weigh fish adrenals since the adrenal cells are embedded within the lymphoid tissue of the head kidney. Moreover, it is not possible to isolate the cortex from the medulla, a procedure that is frequently used in adrenal toxicology (Colby *et al*., 1994). At this time, histologic methods can be used to demonstrate changes in cell morphology and the only method to isolate steroidogenic cells from other cells in the head kidney are the Percoll gradients (Hontela *et al*., 2008). However, the anatomical organization of the teleostean adrenal lends itself very well to studies of paracrine interactions since different cell populations, including the immune cells, the steroidogenic, and the chromaffin cells, are intermingled in one tissue, the head kidney. Recent studies (Weyts *et al*., 1999) with this system provided new insights into the interactions between different cell types in fish adrenals.

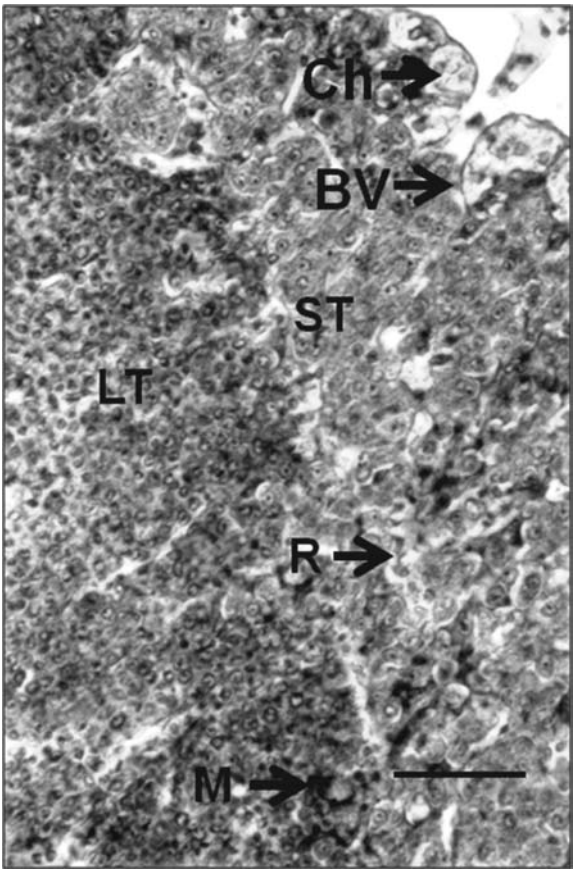

Figure 1 Adrenal tissue in the head kidney of yellow perch, *Perca flavescens*, stained with Trichrome de Masson. *Abbreviations*: Ch, chromaffin cells; LT, lymphoid tissue; M, – melanomacrophages; ST, steroidogenic tissue; R, red blood cells; BV, blood vessels. Magnified 100×, black bar measures 10 μm.

Synthesis and Signaling Pathways of Adrenal Steroid Hormones in Teleost Fish

The pathways and processes leading to the synthesis of cortisol, the main corticosteroid hormone of teleost fish are identical to those seen in the mammalian cortex (Hsu *et al*., 2006). The synthesis of cortisol is under the control of the brain–pituitary axis, and ACTH, the proopiomelanocortin (POMC)-derived peptide from the pituitary gland, is the primary secretagogue (Iwama *et al*., 2006; Mommsen *et al*., 1999; Wendelaar, 1997). The sequence of events in corticosteroid biosynthesis involves the binding of ACTH to melanocortin 2 receptor (MC2R) (also called the ACTH receptor), a G-protein coupled receptor belonging to the family of

melanocortin receptors, and activation of adenylate cyclase and cAMP production. These events lead to the transport of cholesterol from outer to inner mitochondrial membrane, the primary step in steroid biosynthesis (Penhoat *et al*., 2001). The cholesterol transport is thought to involve two key proteins, the steroidogenic acute regulatory protein (StAR) and the peripheral-type benzodiazepine receptor (PBR), however, the mechanisms involved are not yet well understood (Papadopoulos, 1993; Stocco, 2000). Corticosteroid synthesis from cholesterol is well characterized and involves a series of enzymatic steps, including cytochrome P450 family proteins, dehydrogenases, and hydroxylases. The rate-limiting step in this cascade is the conversion of cholesterol to pregnenolone, which is catalyzed by CYPscc (Payne and Hales, 2004).

In fish, MC2R has been cloned and sequenced from *Takifugu rubripes* and *Takifugu nigroviridis* (Klovins *et al*., 2004), *Danio rerio* (Ringholm *et al*., 2002), *Cyprinus carpio* (Metz *et al*., 2005), and *Oncorhynchus mykiss* (Aluru and Vijayan, 2008b). The upregulation of MC2R transcript levels by stressor exposure and/or in vitro ACTH stimulation is similar to that in mammals and suggests a key role for this receptor signaling in stressor-induced cortisol response (Aluru and Vijayan, 2008b). Stimulation of the steroidogenic cells by ACTH activates the cAMP-PKA pathway (Lacroix and Hontela, 2001) and facilitates the entry of cholesterol into cells and synthesis of cholesterol de novo. Calcium is also an important second messenger in this pathway; ACTH stimulates the entry of calcium into the steroidogenic cells and the action of ACTH is calcium-dependent (Lacroix and Hontela, 2006). Recent studies have shown an upregulation of StAR, P450scc (the first enzymatic step of corticosteroidogenesis that yields pregnenolone), and 11β-hydroxylase (the terminal enzyme in cortisol biosynthesis) mRNA abundance in fish head kidney tissue in response to stressor exposure (Aluru and Vijayan, 2006; Geislin and Auperin, 2004; Hagen *et al*., 2006; Kusakabe *et al*., 2002).

Cortisol is not stored in the cells but rather released into the circulation following stimulation with ACTH; thus, plasma cortisol levels reflect the rate of synthesis. A large proportion of circulating cortisol is bound to plasma proteins, the most important one is the corticosteroid-binding globulin, but a similar protein with high affinity for cortisol in fish plasma is yet to be convincingly established (Mommsen *et al*., 1999). Corticosteroids, as well as other steroids synthesized in the body, are metabolized mainly by hepatic Phase I and Phase II enzymes. These enzymes increase the solubility of the molecules, facilitating their excretion through urine or feces. Phase I and II enzymes have been well characterized in teleosts (Stegeman and Hahn, 1994). Pottinger et al (Pottinger *et al*., 1992) used GC/MS and radioimmunoassay to demonstrate that the major conjugated steroids in bile of stressed rainbow trout are, in decreasing order of concentrations, tetrahydrocortisone, tetrahydrocortisol, cortisone, cortisol, and cortolone. It is important to note that, similar to mammals, the enzymes metabolizing corticosteroids are also the enzymes that metabolize a variety of other substrates including pollutants, and may detoxify or bioactivate the xenobiotics (Buhler and Wang–Buhler, 1998).

Functions of Adrenal Hormones in Fish

In teleosts, cortisol plays a key role in many aspects of animal function, including growth and metabolism, reproduction, water and salt balance, immune function, and stress-coping mechanisms (Barton *et al*., 2002; Iwama *et al*., 2006; Mommsen *et al*., 1999; Norris, 1997). However, the majority of studies have focused on the action of this hormone on metabolism as well as osmotic and ionic regulation (McCormick, 1995; McCormick, 2001; Mommsen *et al*., 1999). Early studies on cortisol receptors in fish utilized radiolabeled ligands to identify binding affinity and capacity in a variety of species and tissues, including liver, gut, adipose tissue, as well as gonads and osmoregulatory organs. These studies showed that cortisol-specific binding was present in the cytosol and to some extent in the nuclear fractions (Mommsen *et al*., 1999; Vijayan *et al*., 2005).

The first cloning and sequencing of a fish glucocorticoid receptor (GR) was carried out in rainbow trout (Ducouret *et al*., 1995), and this was followed by molecular characterization of GR and MR in several species of fish (Alsop and Vijayan, 2008; Bury and Strum, 2007; Bury *et al*., 2003). The unique feature of fish GR compared to mammals was the presence of multiple genes for this receptor in most teleost species examined, except zebrafish (Alsop and Vijayan, 2008; Bury and Prunet, 2007; Bury and Strum, 2007). While the functional significance of the two GRs in fish is unclear, the different sensitivity of these receptors to cortisol does suggest a potentially different role for the two receptors in activating pathways at rest or in stressed fish (Bury and Prunet, 2007). While the presence of multiple corticosteroid receptors in teleosts is thought to be due to the genome duplication event, the absence of a second GR in zebrafish points to either the loss of function associated with that receptor or being taken over by the single receptor (Alsop and Vijayan, 2008).

While a single MR has been cloned and sequenced in a few teleost fishes, a functional ligand for this receptor, other than cortisol, is still elusive. Recently, 11-deoxycorticosteone was shown to activate trout MR signaling using reporter assays (Strum *et al*., 2005), suggesting a role for this hormone as a potential candidate in MR activation. However, in vivo and in vitro physiological studies using 11-deoxycorticosterone are clearly needed to establish the significance of this molecule as an MR ligand in fish. Recently, we were unable to detect 11-deoxycorticosterone in the embryos of zebrafish, while de novo cortisol synthesis was evident only posthatch, suggesting that the cortisol seen during embryogenesis is maternally derived, in agreement with other teleostean species (Alsop and Vijayan, 2008). However, we showed for the first time that MR mRNA abundance increased while GR transcripts dropped during embryogenesis in zebrafish, suggesting a role for maternal cortisol stimulation of MR as a key developmental signal (Alsop and Vijayan, 2008). The GR mRNA abundance rebounded posthatch in zebrafish and is thought to play a key role in the metabolic adjustments that are associated with stress adaptation and feeding (Alsop and Vijayan, 2008).

Indeed, a well-characterized role for GR activation is the production of glucose, a key metabolic fuel to cope with the increased energy demand in fish (Aluru and Vijayan, 2007; Mommsen *et al*., 1999). Cortisol treatment increases tissue metabolic capacity, including enhanced gluconeogenesis and amino acid catabolism, leading to hepatic glucose mobilization (Mommsen *et al*., 1999). Recently, using pharmacological manipulation of GR with receptor antagonist RU486, along with a transcriptomics approach, we identified several metabolic genes that are GR-responsive in fish (Aluru and Vijayan, 2007). This along with the observation that some of the GR-responsive genes were also altered with an acute stressor suggests a key role for genomic cortisol signaling in the metabolic adjustments to stress in fish (Aluru and Vijayan, 2007; Vijayan *et al*., 2003; Wiseman *et al*., 2007).

Another key action of cortisol is in osmotic and ionic regulation in fish (Lyssimachou and Arukwe, 2007). Cortisol has been shown to regulate ion transporters in fish and this action is mediated by GR (McCormick, 2001). However, studies have also shown that MR antagonists can offset some of these ionoregulatory adjustments seen in fish (Scott *et al*., 2005; Sloman *et al*., 2001), suggesting functional role for MR signaling in ion regulation. However, the precise contribution of MR and GR signaling in regulating water and ion balance and/or acid base regulation, as well as the ligands, other than cortisol, that mediate these homeostatic responses, are unclear in fish.

Corticosteroids also play a role in regulation of the immune function and reproduction. While their role in reproduction is thought to involve suppression of sex steroids and exerting antigonadal effects, the mechanisms of action are far from clear (Mommsen *et al*., 1999; Pankhurst and Van Der Kraak, 2000). Thus, cortisol appears to have several important functions in teleosts, some with constitutive beneficial effects and some, usually those associated with prolonged elevated cortisol levels, leading to adverse effects. Consequently, any impact on cortisol production capacity will have serious implication for animal health, including stress adaptation in fish.

EFFECTS OF TOXICANTS ON FISH ADRENALS

Laboratory Studies

The increase in plasma cortisol levels is a key step in the neuroendocrine stress response elicited during exposure to stressful stimuli, including toxicants (Hontela, 2005; Iwama *et al*., 2006; Wendelaar, 1997). While catecholamines, especially epinephrine, are also sensitive indicators of stress, it is very difficult to obtain resting levels of this hormone in fish and, therefore, it is not commonly used as a tool to detect stress in piscine models (Iwama *et al*., 2006). Since cortisol has a lag time of several minutes before it increases in circulation, and we can obtain resting levels from anesthetized fish, it is the indicator of choice for stress detection in fish. While plasma cortisol level is a reliable indicator of an acute

stressor exposure, long-term stressor exposures are less reliable because of changes in cortisol dynamics, which may result in the levels of this steroid dropping to resting levels despite stressor exposure (Iwama *et al*., 2006). However, exposure of fish to contaminants, including cadmium, copper, and hydrocarbons, lasting hours to days elevate plasma cortisol levels in fish and consequently, plasma cortisol level is commonly used as a sensitive indicator of toxicity due to chemicals (Hontela, 2005).

However, this paradigm is flawed, as several studies have shown that contaminants, depending upon the concentration and duration of exposure, can either result in no changes in cortisol levels or reduce circulating cortisol levels in fish (Hontela, 2005). For instance, the organochlorine *o,p′*-DDD [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane] impaired adrenocortical function and reduced plasma cortisol levels after only few days of exposure in rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis mossambicus*) (Benguira *et al*., 2002; Ilan and Yaron, 1980, 1983). There is some evidence that adenylyl cyclase may be targeted by this organochlorine contaminant in teleost adrenocortical cells (Lacroix and Hontela, 2003). Moreover, Lindhe et al (Lindhe *et al*., 2003) used autoradiography and binding studies to demonstrate cell-selective binding of *o,p′*-DDD, an isomer of DDT (dichlorodiphenyltrichloroethane), in corticosteroid producing interrenal cells in Atlantic cod (*Gadus morhua*). Other contaminants that lower cortisol levels in fish exposed in vivo for relatively short periods (less than 30 days) are polychlorinated biphenyls (Quabius *et al*., 2000) or β-naphthaflavone (Aluru and Vijayan, 2004; Wilson *et al*., 1998). The mechanisms mediating the cortisol impairment are under investigation, as described later in this chapter. An important observation however, is the finding that exposure to contaminants does not always elevate plasma cortisol in fish, and in general, the extrapolation of data from acute laboratory exposures to the situation in the field is not straightforward (Campbell *et al*., 2008; Rasmussen *et al*., 2008).

Field Studies

A series of interrelated field studies in lakes situated in metal-mining regions provided unique data that characterized the adrenal responses in fish exposed to trace metals in the field. Native fish (yellow perch, *Perca flavescens*) were sampled in a series of lakes, some situated upwind from a large smelter, some near the smelter, and some at a distance downwind from the smelter. Analyses of metals, including Cd, Zn, Cu, and Ni in lake water, sediments, and fish tissues (kidney, head kidney including the adrenal tissue, liver, and gills) provided extensive chemical data to characterize a gradient of exposure, ranging from reference lakes (upwind from the smelter), intermediate lakes (at a distance downwind from the smelter), and contaminated lakes (at proximity to the smelter). These field studies, each with a complete set of exposure gradients, were executed in two different geographical locations in Canada, one in Quebec and one in Ontario. The highest concentrations

of metals were accumulated in the liver and the kidney, with the head kidney, a tissue not involved in filtration and osmoregulation, reaching 10% to 30% of metal burdens of the kidney (Laflamme *et al*., 2000; Levesque *et al*., 2002; Levesque *et al*., 2003; Rasmussen *et al*., 2008). The tissue burdens of metals followed closely the environmental metal concentrations, thus confirming that fish tissues can be used as indicators of environmental contamination in environmental monitoring and setting of water quality guidelines (Campbell *et al*., 2008; Rasmussen *et al*., 2008). A key findings of these field studies was, however, the diagnosis of an adrenal impairment in fish that accumulated significant concentrations of metals, in particular Cd, in the head kidney tissue. Thus, these field studies provided unique evidence that the adrenal tissue of fish is a vulnerable target of environmental toxicants. The adrenal dysfunction was initially characterized by lower plasma cortisol levels following standardized stress protocols in fish from the lakes most contaminated by metals compared to fish from the reference lakes. Similar findings were obtained for other fish species and other anthropogenically impacted aquatic systems investigated recently, or older studies that were reinterpreted in light of the new evidence for adrenal impairment. These studies include data for whitefish (*Coregonus levaretus*) sampled in waters impacted by pulp mill effluents (Lappivaara and Oikari, 1999), northern pike (*Esox lucius*) exposed to mercury discharged by pulp mills using mercury-based slimicides (Lockhart *et al*., 1972) or mixtures of organic pollutants and mercury (Hontela *et al*., 1992), and brown trout (*Salmo trutta*) from metal polluted sites (Norris *et al*., 1999). The idea that chronic field exposures to some pollutants cause an impairment of the adrenal function in fish was relatively novel and did not agree with the well-accepted notion that potentially toxic chemicals will activate the physiological stress response and elevate plasma cortisol. Moreover, the cortisol levels in the field-sampled fish, even at the contaminated sites where presumably adrenal impairment occurred, were much higher than levels usually measured in fish, such as goldfish or rainbow trout, sampled in the laboratory (Wendelaar, 1997). Thus, mechanistic evidence was urgently needed to establish the cause–effect link between the exposure to the adrenotoxicants and the adrenal impairment in fish. There is a great interest in mechanisms that impair steroidogenesis, be it in the adrenal tissue or the gonads since all steroidogenic pathways depend on similar transduction processes and enzymatic constituents. Thus, a better understanding of cellular and biochemical vulnerability of the adrenal pathways may increase the understanding of mechanisms mediating impairment of all steroidogenic pathways, including the synthesis of androgens, estrogens, and progestins, as well as corticosteroids.

ADRENAL IMPAIRMENT IN FISH

Detection of Adrenal Impairment in Fish

Several lines of evidence were used to elucidate the mechanisms of the pollutant-induced adrenal impairment in fish. Histopathology and morphometric data from

the early studies provided evidence that the pituitary–adrenal axis, specifically the pituitary corticotropes and the adrenal steroidogenic cells, are altered by chronic field exposures to specific pollutants, including pulp and paper effluents and metals (Hontela *et al*., 1997; Levesque *et al*., 2003; Norris *et al*., 1999). However, testing the functional integrity of the fish adrenals gave insight into the mechanisms of the adrenal impairment and revealed that the lower circulating cortisol levels in fish from impacted sites were not caused by a pituitary dysfunction and lower titers of plasma ACTH, but rather a decreased ability to respond to ACTH. Fish collected at reference and contaminated sites within a specific geographical location were either subjected to standardized confinement stress, a treatment that elevates plasma cortisol in physiologically competent individuals, or they were injected with ACTH, or in some experiments, their adrenal tissues were isolated and stimulated in vitro with ACTH (Girard *et al*., 1998; Laflamme *et al*., 2000; Rasmussen *et al*., 2008). Evidence provided by these studies clearly showed that the response to ACTH is blunted in fish from contaminated sites and that cortisol synthesis is disrupted. The field studies and testing of the adrenal functional integrity was complemented by laboratory studies in which fish were exposed to selected toxicants and their adrenal tissue was tested in vitro. Quabius et al (Quabius *et al*., 1997; Quabius *et al*., 2000) reported that the adrenal tissue of tilapia (*Oreochromis mossambicus*) exposed to PCB through diet was impaired. Similar results were obtained from feeding studies with pharmaceuticals in rainbow trout (Gravel and Vijayan, 2007). Taken together, the results from the field studies and laboratory studies indicated that the adrenal dysfunction in fish was caused by an impairment of the steroidogenic cells rather than lower circulating ACTH levels. To identify the specific steps where the toxicants exerted their effects, in vitro studies were initiated using controlled exposures with adrenal tissue from hatchery reared fish.

In Vitro Studies of Mechanisms of Adrenotoxicity in Fish

The mechanisms of adrenotoxicity in fish adrenals were investigated in vitro using a head kidney cell preparation (Leblond and Hontela, 1999) that facilitated experimental exposures in a simple system, similar to cell preparations used with mammalian adrenals (Das *et al*., 2000; Kossor *et al*., 1991; Mathias *et al*., 1998). Enzymatically dispersed head kidney cells were plated out in a microplate, exposed to test toxicants or MEM (controls) and, following exposure, cells were stimulated with ACTH to assess the maximal capacity to secrete cortisol. The ACTH-stimulated cortisol secretion and also viability were measured to assess the adrenotoxicity of a series of environmental pollutants relevant for the aquatic ecosystems.

Studies using the fish adrenal bioassay generated new toxicological data, specifically, the EC50s (Effective concentration of the test toxicant that inhibits cortisol secretion by 50%) and the LC50s (Lethal concentrations of the toxicant that kills 50% of the cells) for a series of toxicants (Table 1) (Hontela, 2005). A ratio LC50/EC50 of 1.0 suggests that the test toxicant is highly cytotoxic and the

Table 1 Adrenotoxicity, Expressed as the Ratio of LC50/EC50 of Selected Environmental Toxicants Tested In Vitro in Adrenal Cells Isolated from Rainbow Trout, *Oncorhynchus mykiss*

Compound	Chemical type (use)	LC50/EC50[a]
Diazinon	Organophosphate (insecticide)	1.68[a]
Dimethoate	Organophosphate (insecticide)	1.4 (Quinn, unpublished)
o,p'-DDD	Organochlorine (insecticide)	2.96[a]
$HgCl_2$	Metal	8.92[a]
CH_3HgCl	Metal	9.83[a]
Na_2SeO_3	Metalloid	>10 (Miller, unpublished)
Endosulfan	Organochlorine (insecticide)	17.85[a]
$CdCl_2$	Metal	64.29[a]

[a]Data selected from Hontela, 2005.
Abbreviations: LC50, lethal concentration or concentration that kills 50% of cells exposed in vitro; EC50, effective concentration or concentration that inhibits cortisol secretion by 50% in vitro.

loss of cortisol secretion following exposure to the test chemical is due to cell death. In contrast, a high-ratio LC50/EC50 of for example 100, suggests that the test toxicant has a high endocrine disrupting potential, rather than cytotoxicity, as a higher concentration (by a factor of 100) is required to kill the endocrine cells than to disrupt cortisol secretion. Toxicants with a high endocrine disruptive potential include Cd, Zn (LC50/EC50 > 60) while highly cytotoxic toxicants (LC50/EC50 < 5) include *o,p'*-DDD and diazinon

The endocrine disrupting potential of Cd has been characterized not only in adrenal steroidogenesis in fish (Lacroix and Hontela, 2004; Leblond and Hontela, 1999) and mammals (Mathias *et al*., 1998), but also in gonadal steroidogenesis in both fish (Thomas and Wofford, 1993) and mammals (Gunnarsson *et al*., 2003; Laskey and Phelps, 1991). The toxic effects of Cd in steroidogenesis illustrate the vulnerability of an endocrine process (steroidogenesesis) across species and tissue systems, and the possibility to extrapolate knowledge of mechanisms in one system to other systems. Even though the adrenal bioassay does require further validation and development, it is an excellent tool for rapid screening of chemicals for adrenotoxic potential in fish. The bioassay is also a useful test system to further investigate the specific mechanisms of action and the effects of factors that may modifiy andrenotoxicity in the wild, including fish age, developmental stage, temperature, and diet.

CELLULAR AND MOLECULAR TARGETS OF TOXICANTS

Antioxidants

Oxidative stress and the imbalance among pro-oxidants, including reactive oxygen species (ROS) and antioxidants, is a mechanism of toxicity for many chemicals in all types of tissues (Johnson and Boldyrev, 2002), including the adrenals. Extensive evidence from laboratory studies indicates oxidative stress and the resulting

increase in lipid peroxidation, disrupt the functional integrity of mammalian adrenals (Colby *et al*., 1984; Brogan *et al*., 1984). Recent evidence from studies with fish adrenal cells identified oxidative stress as a mechanism mediating the effects of an organochlorine pesticide endosulfan (Dorval *et al*., 2003). Head kidney cell were exposed in vitro to endosulfan, a potent adrenotoxicant in both fish (Bisson and Hontela, 2002; Leblond *et al*., 2001) and amphibians (Goulet and Hontela, 2003) and a suite of indicators of oxidative status were measured. The loss of capacity to respond to ACTH and to secrete cortisol was linked to a decrease in reduced glutathione (GSH), a key antioxidant, and an increase in lipid peroxidation. Interestingly, substitution with pregnenolone reversed the effects of endosulfan within the toxic range of endosulfan concentrations. The importance of GSH was further investigated in a later study (Dorval and Hontela, 2003) where the levels of GSH, catalase, and glutathione peroxidase were experimentally manipulated with N-acetyl-cysteine, a precursor for GSH, aminotriazole, an inhibitor of catalase, and L-buthionine sulfoximine, an inhibitor of glutathione peroxidase. These specific treatments had significant effects on the EC50 and LC50 for endosulfan, demonstrating that GSH protects cells from the damaging effects of Endosulfan (Dorval and Hontela, 2003). Additional evidence for the importance of oxidative stress as a mechanism of adrenotoxicity in fish was provided by a field study with white sucker, *Catostomus commersoni*, sampled along a gradient of contamination with agricultural chemicals, including pesticides, in a river drainig an area of intensive agriculture (Dorval *et al*., 2005). Impairment of cortisol secretion was linked to a loss of GSH and an increase in lipid peroxidation in the adrenals, as well as the liver. Major effects on thyroid status were also detected in the fish sampled at the most contaminated sites in the river. The field studies, together with laboratory exposures show that oxidative stress and alterations of cellular parameters, such as GSH reserves, may represent the very early responses of adrenal cells to toxicant-induced damage. The intracellular targets of oxidative stress may be highly localized, as suggested by the reversal with pregnenolone of the cortisol impairment in endosulfan-exposed cells (Dorval *et al*., 2003). These results suggest that the intracellular steps downstream from pregnenolone within the synthetic pathway for cortisol were not disrupted by endosulfan-induced oxidative stress. Further investigations are needed to elucidate the vulnerability of specific processes within the steroidogenic adrenal cells.

Intracellular Calcium

Calcium concentrations in all cells are maintained within a very narrow range, Ca^{2+} playing a key role as an intracellular messenger in signaling pathways of many cells (Clapham, 1995). The stimulatory action of ACTH in adrenal steroidogenesis, (Lacroix and Hontela, 2006; Omura *et al*., 2007) as well as gonadal steroidogenesis in fish is dependent on calcium. There is extensive evidence that numerous environmental pollutants interfere with calcium signaling in cells (Kass and Orrenius, 1999; Liu and Lin, 2002), and divalent trace metals such as Cd^{2+}, Pb^{2+}, or Hg^{2+} have been shown to disrupt calcium signaling in mammalian steroidogenic

adrenal and gonadal cells (Mathias *et al*., 1998; Thoreux–Manlay *et al*., 1995). The understanding of the intracellular calcium-sensitive processes targeted by cadmium (Cd^{2+}) in mammalian systems is extensive; the Cd/Ca interactions have been elucidated using calcium-channel agonists, antagonists, and calcium ionophores (Benters *et al*., 1996; Hinkle and Osborne, 1994; Powlin *et al*., 1997).

Investigation of the effects of toxicants on intracellular calcium homeostasis in fish systems is a rapidly expanding area of research. Calcium protects against toxicity of cadmium in vivo; it is well documented that fish tolerate higher concentrations of cadmium in waters of higher calcium content (Hollis *et al*., 2000). Recent studies using the adrenal cells in vitro elucidated some of the interactions of Cd^{2+} and Ca^{2+} in fish. Lacroix and Hontela (Lacroix and Hontela, 2006) demonstrated that Cd, administered to the cells as $CdCl_2$ interferes with Ca channels activated during cortisol secretion. Pharmacological blockers and agonists of calcium channels, specifically Bay K8644, nicardipine, and thapsigargin, along with manipulations of concentrations of Cd^{2+} and Ca^{2+}, were used to demonstrate that cadmium enters the adrenocortical steroidogenic cells in rainbow trout through Ca^{2+} channels and disrupts, in a concentration-dependent pattern, the ability of the cells to secrete cortisol in response to stimulation with ACTH. In a toxicokinetic study, the interactions of Cd^{2+} and Ca^{2+} were further investigated in fish adrenal cells. Raynal et al. (Raynal *et al*., 2005) measured membrane uptake of Cd in media of various calcium concentrations and provided further support for a Cd^{2+}/Ca^{2+} competition at the level of calcium membrane channels. The interactions were further quantified using V_{max} and K_m in experiments that manipulated under controlled speciation protocols, the concentrations of Cd^{2+} and Ca^{2+}, in presence or absence of specific blockers and agonists (Gagnon *et al*., 2007). A major challenge of these mechanistic toxicological studies is to integrate the knowledge of various toxicity mechanisms, including oxidative stress, disruption of calcium homeostasis, and other mechanisms, to gain a full understanding of the specific cellular processes targeted by adrenal toxicants, particularly those that are environmentally relevant.

Steroidogenic Acute Regulatory Protein

Steroidogenic acute regulatory protein (StAR) is considered to be a key rate-limiting step in the synthesis of steroid hormones, including corticosteroids, as this protein transports cholesterol from the outer mitochondrial membrane to the inner membrane (Stocco, 2000). The cholesterol is used as the substrate for the first rate-limiting step in steroidogenesis that is catalyzed by cytochrome P450scc, the enzyme that cleaves the side chain from cholesterol to form pregnenolone. In humans, a congenital dysfunction of the StAR protein causes adrenal lipoid hyperplasia (Caron *et al*., 1997), a disease characterized by symptoms, including sterility and hypotension, linked to abnormally low synthesis of key steroids. Steroidogenic cells of these patients accumulate cholesterol but have a diminished capacity to synthesize the steroid hormones. Recent studies reported that exposure to pesticides, such as Roundup or lindane, disrupts the transcription and expression

of the StAR protein gene, leading to low levels of steroids in mammalian models (Walsh and Stocco, 2000; Walsh *et al*., 2000a,b). Disruption of the function of this key steroidogenic protein is another mechanism of action of environmental adrenotoxicants.

While most studies on StAR regulation focused on mammalian models, recent studies clearly suggest a role for this protein in adrenal dysfunction in fish. While several studies have examined StAR transcript levels in fish interrenals in response to stressor exposure and ACTH stimulation (Alsop and Vijayan, 2008; Aluru and Vijayan, 2006; Geislin and Auperin, 2004; Kusakabe *et al*., 2002), only few studies examined the impact of toxicants on StAR regulation in fish (Table 2) (Lyssimachou and Arukwe, 2007; Aluru and Vijayan, 2006; Aluru and

Table 2 Impact of Chemical Exposure on mRNA Abundance of Key Proteins Involved in Steroidogenesis in Teleost Fishes

Chemical exposure	Gene expression	Species	References
Head kidney			
Salicylate	↓ StAR and PBR	*O. mykiss*	(Gravel and Vijayan, 2006; Gravel and Vijayan, 2007)
BNF (AhR-dependent)	↓ StAR and P450scc	*O. mykiss*	(Aluru and Vijayan, 2006; Aluru *et al*., 2005)
EE2	↑ StAR, P450scc, and CYP11β	*S. salar*	(Lyssimachou and Arukwe, 2007)
Brain			
BNF (AhR-dependent)	↓ StAR		(Aluru and Vijayan, 2007)
BNF (AhR-independent)	↓ P450scc	*O. mykiss*	(Arukwe, 2005)
NP	↑ StAR ↑↓ P450scc, and CYP11β	*S. salar*	(Lyssimachou and Arukwe, 2007)
		S. salar	(Kortner and Arukwe, 2007a)
Gonad			
EE2	↑ StAR, P450scc, and CYP11β	*G. morhua*	(Kortner and Arukwe, 2007b)
NP	No change in StAR; ↓ P450scc at high concentrations	*G. morhua*	(Sharpe *et al*., 2007)
17α-MT	↑ StAR and ↓ P450scc	*C. auratus*	
β-sitosterol	↓ StAR		

Abbreviations: StAR, steroidogenic acute regulatory protein; PBR, peripheral-type benzodiazepine receptor; P450scc, cytochrome P450 side-chain cleavage; CYP11β, cytochrome P450 11β-hydroxylase; BNF, β-naphthoflavone; NP, nonylphenol; EE2, ethinylestradiol.

Vijayan, 2007; Aluru *et al*., 2005; Arukwe, 2005; Gravel and Vijayan, 2006; Gravel and Vijayan, 2007; Kortner and Arukwe, 2007a,b; Sharpe *et al*., 2007). Indeed, these studies clearly showed suppression of StAR mRNA levels in the head kidney of trout exposed to β-naphthoflavone (BNF, an AhR agonist) (Aluru and Vijayan, 2006; Aluru and Vijayan, 2008a; Aluru *et al*., 2005) and salicylate (an NSAID that is detected in sewage outfalls) (Gravel and Vijayan, 2006; Gravel and Vijayan, 2007), both in vivo and in vitro. As these studies also showed a reduction in stressor-induced circulating cortisol levels as well as suppression of acute ACTH-stimulated cortisol production in vitro, it appears reasonable to hypothesize that inhibition of StAR transcription is a mechanism for corticosteroid disruption by BNF and salicylate in fish. Indeed, using the AhR antagonist resveratrol along with BNF, we were able to show for the first time that AhR-dependent signaling is responsible for the suppression of cortisol production with BNF in fish (Aluru and Vijayan, 2006). As resveratrol completely abolished BNF-mediated suppression of cortisol production and this corresponded with the lack of inhibition of StAR mRNA levels, it seems likely that StAR is a target for adrenotoxicants impairing steroid production in fish interrenals (Aluru and Vijayan, 2006). However, since ethinylestradiol (EE2), a known xenoestrogen, was shown to increase StAR mRNA levels in Atlantic salmon interrenals (Lyssimachou and Arukwe, 2007), but without the corresponding changes in plasma cortisol levels, it is difficult to assess the impact of this molecular response on steroid output. In addition to head kidney, toxicants also impacted StAR gene expression in other tissues, notably the gonads and the brain (Table 2). While BNF suppressed StAR expression in the brain of trout (Aluru and Vijayan, 2008a), sex steroid agonists, including nonylphenol (NP), EE2, and 17α-methyltestosterone (17α-MT) either did not modify StAR mRNA levels (Kortner and Arukwe, 2007a) or elevated StAR mRNA abundance in the brain, gonad, and head kidney of fish (Arukwe, 2005; Kortner and Arukwe, 2007a; Lyssimachou and Arukwe, 2007). However, β-sitosterol, a weak ER agonist, suppressed StAR mRNA level in goldfish (Sharpe *et al*., 2007). From these investigations, it is apparent that more studies using environmentally relevant concentrations of chemicals either singly or in combination with other chemical classes (for instance, AhR ligands and xenoestrogens) are necessary to establish a cause–effect relationship between StAR gene expression and steroid output in fish. In addition to StAR, other key proteins involved in steroidogenesis, including peripheral-type benzodiazepine receptor [PBR; also known as Translocator protein (Papadopoulos, 2004)] and P450scc, a key rate-limiting step in steroidogenesis, were also impacted by toxicants in fish (Table 2). Taken together, these studies suggest multiple targets for adrenotoxicants disrupting steroid production in fish (Fig. 2), while the mechanism(s) of action remains to be established.

Glucocorticoid Receptor

While disruption of Glucocorticoid receptor (GR) is not directly linked to adrenotoxicity, the action of cortisol, the steroid hormone produced in the adrenals, is

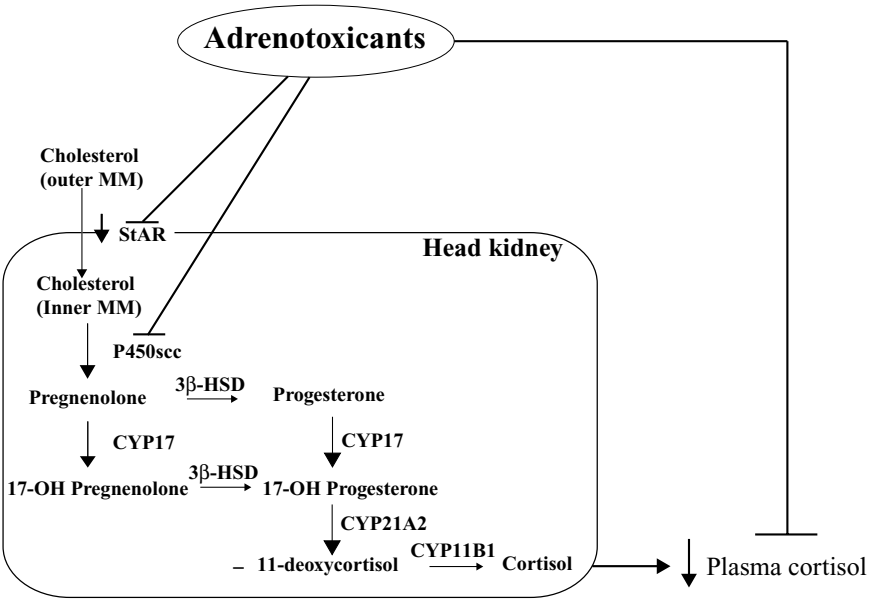

Figure 2 Schematic representation of toxicant impact on corticosteroid biosynthesis in fish. The adrenotoxicants that inhibit acute-stimulated cortisol production in fish include ligands that activate aryl hydrocarbon receptor (AhR) signaling, pharmaceuticals (salicylate, ibuprofen, acetaminophen), pesticides (endosulfan), and metals (cadmium and mercury). So far, AhR ligand (Aluru and Vijayan, 2006; Aluru and Vijayan, 2008a), salicylate (Gravel and Vijayan, 2006; Gravel and Vijayan, 2007), and cadmium (Sandhu and Vijayan, unpublished) have been shown to suppress gene expression of steroidogenic acute regulatory protein (StAR), a key rate-limiting step in steroidogenesis. This evidence led to the proposal that StAR is a target for adrenotoxicants disrupting steroid production in fish. *Abbreviations*: Outer MM, outer mitochondrial membrane; Inner MM. inner mitochondrial membrane; P450scc, cytochrome P450 side-chain cleavage (CYP11A1); 3b-HSD, 3b-hydroxysteroid dehydrogenase; CYP17, Steroid 17-alpha-hydroxylase/17,20-lyase; CYP21A2, steroid 21-hydroxylase; CYP11B1, 11beta-hydroxylase; OH, hydroxy.

indeed attributable to this receptor signaling. As GR in the brain and pituitary gland may be involved in the negative feedback regulation of cortisol, any impact on this receptor dynamics will lead to abnormal plasma cortisol levels. Indeed, disturbances in stressor-induced plasma cortisol levels were observed in Arctic char exposed to Aroclor 1254 for 4 months during their winter emaciation period (Jorgensen *et al*., 2002). The abnormal plasma cortisol dynamics seen in this fish exposed to high PCB levels coincided with a reduction in brain GR content, indicating disturbances in negative feedback regulation (Aluru *et al*., 2004). Moreover, recently we observed suppression of ACTH-mediated cortisol production in vitro by Ru486 (a GR antagonist), suggesting a key role for GR signaling in modulating

steroid output. Thus, there is strong evidence to indicate that disruption of GR signaling by toxicants will disturb the functioning of the hypothalamus-pituitary-interrenal (HPI) axis, as well as target tissue cortisol signaling in fish (Vijayan *et al*., 2005). Indeed, recent studies showed that PCBs, BNF, and metals affect GR protein content in fish (Vijayan *et al*., 2005). As cortisol is a key hormone essential for reestablishing homeostasis, any impact on this steroid hormone production and/or action will lead to homeostatic disruption.

USE OF ADRENOTOXICITY DATA IN ECOLOGICAL RISK ASSESSMENT

The evidence that toxicants released into aquatic habitats by human activities, such as agriculture, mining, or pulp and paper production impact fish populations and water quality is substantial (Campbell *et al*., 2008; Kilgour *et al*., 2007; Rasmussen *et al*., 2008; Yeom and Adams, 2007). It is well established that fish populations are excellent indicators of water quality; the physiological status of these sentinel species reflects the health and integrity of the ecosystems in which they live. The adrenal system is part of the physiological processes that enable an organism to maintain homeostasis and survive in an ever changing environment. The ability to respond to stressors, through an activation of the neuroendocrine response, including cortisol secretion, is a fundamental response of a healthy organism facing challenges such as presence of predators, changes in food availability, presence of potential mates and competitors, fluctuations in temperature, or water flow. Impairment of this response, through actions of adrenotoxicants on the steroidogenic cells in fish, is an important indicator of a toxicant-induced physiological dysfunction. There is an urgent need to develop and validate physiological and endocrine responses, that would help diagnose environments that are unsuitable for fish populations and through consumption of drinking water, for humans. Even though chemical analyses of contaminants are an important component of Ecological Risk Assessment, the temporal and qualitative aspects of sampling are a well-recognized challenge. A sentinel species and its physiological systems, including the adrenals, integrate damage over time and are not subject to temporal problems with water sampling and chemical analyses. Of course, the limitations of using sentinel species is often the lack of knowledge about the basic physiology and our limited capacity to interpret an alteration or impairment within the natural seasonal, sex-related, diet-related variability. Thus, fundamental research in comparative physiology and toxicology plays an important role in providing basic data to Risk Assessors who can then use the adrenotoxicity data as part of the dose–response relationships and risk evaluation (Campbell *et al*., 2003; Campbell *et al*., 2008; Campbell and Giguère, 2008; Rasmussen *et al*., 2008). Specific bioassays and markers, such as StAR protein and cortisol secretion, are simple tools usable by government agencies in charge of monitoring environmental quality. The research reviewed in this chapter includes some fundamental studies in adrenal toxicology of fishes, as well as some applications of this work.

CONCLUSION

Adrenal toxicology offers a unique area of investigation where comparative toxicology and physiology can provide data to advance the understanding of mechanisms of action of toxicants in an endocrine organ. Its anatomy evolves from a heterogenous tissue embedded in the head kidney of fishes to an encapsulated gland with a distinct medulla and cortex in mammals. Yet the cellular characteristics and vulnerabilities to toxic insults, such as oxidative stress or disruption of intracellular calcium homeostasis, are remarkably similar among species. Thus, the adrenals offer a system where comparative toxicologists and physiologists can exchange ideas and techniques to truly advance knowledge about the vulnerabilities of this organ and other steroidogenic tissues, and in the process, provide data that can be used in applications such as Ecological Risk Assessment and Environmental Monitoring.

REFERENCES

Alsop A, Vijayan MM. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am J Physiol Regul Integr Comp Physiol 2008; 294:R711–R719.

Aluru N, Vijayan MM. β-Naphthoflavone disrupts cortisol production and liver glucocorticoid responsiveness in rainbow trout. Aquat Toxicol 2004; 67:273–285.

Aluru N, Vijayan MM. Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate-limiting steps in steroidogenesis. Endocrinol 2006; 147:1895–1903.

Aluru N, Vijayan MM. Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout. Physiol Genom 2007; 31:483–491.

Aluru N, Vijayan MM. Brain transcriptomics in response to ß-naphthoflavone treatment in rainbow trout: The role of aryl hydrocarbon receptor signaling. Aquat Toxicol 2008a; 87:1–12.

Aluru N, Vijayan MM. Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. Endocrinol 2008b; In Press.

Aluru N, Jorgensen EH, Maule AG, et al. Aroclor 1254 disruption of the hypothalamus-pituitary-interrenal axis involves brain glucocorticoid receptor downregulation in anadromous Arctic charr. Am J Physiol Regul Integ Comp Physiol 2004; 287:R787–R793.

Aluru N, Renaud R, Leatherland JF, et al. Ah Receptor-mediated impairment of interrenal steroidogenesis involves StAR protein and P450scc gene attenuation in rainbow trout. Toxicol Sci 2005; 84:260–269.

Arukwe A. Modulation of brain steroidogenesis by affecting transcriptional changes of steroidogenic acute regulatory (StAR) protein and cholesterol side chain cleavage (P450scc) in juvenile Atlantic salmon (*Salmo salar*) is a novel aspect of nonylphenol toxicity. Environ Sci Technol 2005; 39:9791–9798.

Barton BA, Iwama GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann Rev Fish Dis 1991; 1: 3–26.

Barton BA, Morgan JD, Vijayan MM. Physiological and condition-related indicators of environmental stress in fish. In: Adams SM, ed. Biological Indicators of Stress in Fish. 2nd ed. Bethesda, MD: American Fisheries Society; 2002:111–148.

Barton BA. Stress in finfish: Past, present and future: A historical perspective. In: Iwama GK, Schreck CB, Pickering AD, and Sumpter J, eds. Fish Stress and health in aquaculture, Cambridge University press; 1991.

Benguira S, Leblond VS, Weber JP, et al. Loss of capacity to elevate plasma cortisol in rainbow trout (*Oncorhynchus mykiss*) treated with a single injection of *o,p'*-dichlorodiphenyldichloroethane. Environ Toxicol Chem 2002; 21:1753–1756.

Benters J, Schäfer T, Beyersmann D, et al. Agonist-stimulated calcium transients in PC12 cells are affected differentially by cadmium and nickel. Cell Calcium 1996; 20:441–446.

Bisson M, Hontela A. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicol Appl Pharmacol 2002; 180:110–117.

Brodeur JC, Sherwood G, Rasmussen JB, et al. Impaired cortisol secretion in yellow perch (*Perca flavescens*) from lakes contaminated by heavy metals: In vivo and in vitro assessment. Can J Fish Aquat Sci 1997; 54:2752–2758.

Brogan WCI, Eacho PI, Hinton DE, et al. Effects of carbon tetrachloride on adrenocortical structure and function in guinea pigs. Toxicol Appl Pharmacol 1984; 75:118–127.

Buhler DR, Wang–Buhler JL. Rainbow trout cytochrome P450s: Purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. Comp Biochem Physiol (C) 1998; 121:107–137.

Bury N, Prunet P. Steroid receptor signalling. Gen Comp Endocrinol 2007; 153:30.

Bury NR, Strum A. Evolution of the corticosteroid receptor signalling pathway in fish. Gen Comp Endocrinol 2007; 153:47–56.

Bury NR, Strum A, Le Rouzic P, et al. Evidence for two distinct functional glucocorticoid receptors in teleost fish. J Mol Endocrinol 2003; 31:141–156.

Campbell PGC, Kraemer LD, Giguère A, et al. Subcellular distribution of cadmium and nickel in chronically exposed wild fish: Inferences regarding metal detoxification strategies and implications for setting water quality guidelines for dissolved metals. Human Ecol Risk Assessment 2008; 14(2):290–316.

Campbell PGC, Hontela A, Rasmussen JB, et al. Differentiating between direct (physiological) and food-chain mediated (bioenergetic) effects on fish in metal-impacted lakes. Human Ecol Risk Assessment 2003; 9:847–866.

Caron KM, Soo S, Wetsel WC, et al. Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. Proc Natl Acad Sci U S A 1997; 94:11540–11545.

Clapham DE Calcium signaling. Cell Biol Toxicol 1995; 80:259–268.

Colby HD, Purcelli H, Kominami S, et al. Adrenal activation of carbon tetrachloride: Role of microsomal P450 isozymes. Toxicology 1994; 94:31–40.

Colby HD. The adrenal cortex as a toxicological target organ. In: Harvey PW, ed. The adrenal in Toxicology, Taylor & Francis Bristol, PA 19007, USA; 1996:131–163.

Das PC, McElroy WK, Cooper RL. Differential modulation of catecholamines by chlorotriazine herbicides in pheochromocytoma (PC12) cells in vitro. Toxicol Sci 2000; 56:324–331.

Dorval J, Hontela A. Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). Toxicol Appl Pharmacol 2003; 192:191–200.

Dorval J, Leblond VS, Hontela A. Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) exposed in vitro to endosulfan, an organochlorine pesticide. Aquat Toxicol 2003; 63:229–241.

Dorval J, Leblond V, DeBlois C, et al. Oxidative stress and endocrine endpoints in white sucker (*C. commersoni*) from a river impacted by agricultural chemicals. Env Toxicol Chem 2005; 24:1273–1280.

Ducouret B, Tujague M, Ashraf J, et al. Cloning of teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. Endocrinology 1995; 136:3774–3783.

Gagnon E, Hontela A, Jumarie C. Reciprocal inhibition of Cd and Ca uptake in isolated head-kidney cells of rainbow trout, *O. mykiss*. Toxicol In Vitro 2007; 21:1077–1086.

Geislin M, Auperin B. Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 2004; 135:70–80.

Girard C, Brodeur JC, Hontela A. Responsiveness of the interrenal tissue of yellow perch (*Perca flavescens*) from contaminated sites to an ACTH challenge test in vivo. Can J Fish Aquat Sci 1998; 55:438–450.

Goulet B, Hontela A. Toxicity of cadmium, endosulfan, and atrazine in adrenal steroidogenic cells of two amphibian species, *Xenopus laevis and Rana catesbeiana*. Environ Toxicol Chem 2003; 22:2106–2113.

Gravel A, Vijayan MM. Salicylate disrupts interrenal steroidogenesis and brain glucocorticoid receptor expression in rainbow trout. Toxicol Sci 2006; 93:41–49.

Gravel A, Vijayan MM. Salicylate impacts the physiological responses to an acute handling disturbance in rainbow trout. Aquat Toxicol 2007; 85:87–95.

Gunnarsson D, Nordberg G, Lundgren P, et al. Cadmium-induced decrement of the LH receptor expression and cAMP levels in the testis of rats. Toxicology 2003; 183: 57–63.

Hagen IJ, Kusakabe M, Young G. Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11beta-hydroxylase messenger RNAs in rainbow trout interrenal cells: Relationship with in vitro cortisol production. Gen Comp Endocrinol 2006; 145:254–262.

Hinkle PM, Osborne ME. Cadmium toxicity in rat pheochromocytoma cells: Studies on the mechanism of uptake. Toxicol Appl Pharmacol 1994; 124:91–98.

Hollis L, McGeer JC, McDonald DG, et al. Protective effects of calcium against chronic waterborne cadmium exposure to juvenile rainbow trout. Environ Toxicol Chem 2000; 19:2725–2734.

Hontela A, Rasmussen JB, Audet C, et al. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch Environ Contam Toxicol 1992; 22:278–283.

Hontela A, Daniel C, Rasmussen JB. Structural and functional impairment of the hypothalamo-pituitary-interrenal axis in fish exposed to bleached kraft mill effluent in the St Maurice River, Québec. Ecotoxicology 1997; 6:1–12.

Hontela A, Leblond VS, Chang JP. Purification and isolation of corticosteroidogenic cells from head kidney of rainbow trout *(Oncorhynchus mykiss)* for testing cell-specific effects of a pesticide. Comp Biochem Physiol Part C 2008; 147:52–60.

Hontela A. Adrenal toxicology: Environmental pollutants and the HPI axis. In: Mommsen TP, Moon TW, eds. Biochemistry and Molecular Biology of Fishes, Vol. 6, Environmental Toxicology, Elsevier B.V., Amsterdam, Netherlands 2005:331–363.

Hsu HJ, Hsu NC, Hu MC, et al. Steroidogenesis in zebrafish and mouse models. Mol Cell Endocrinol 2006; 248:160–163.

Ilan Z, Yaron Z. Suppression by organochlorines of the response to adrenocorticotropin of the interrenal tissue in *Sarotherodon aureus* (Teleostei). J Endocrinol 1980; 87:185–193.

Ilan Z, Yaron Z. Interference of *o,p'*-DDD with interrenal function and cortisol metabolism in *Sarotherodon aureus* (Steindacher). J Fish Biol 1983; 22:657–669.

Iwama GK, Afonso LOB, Vijayan MM. Stress in Fish. In: Evans DH, Claiborne JB, eds. The Physiology of Fishes. 3rd ed. Boca Raton, FL: CRC Press; 2006:319–342.

Johnson P, Boldyrev AA. Oxidative Stress at Molecular, Cellular and Organ Levels. Research Signpost; 2002:142.

Jorgensen EH, Vijayan MM, Aluru N, et al. Fasting modifies Aroclor 1254 impact on plasma cortisol, glucose and lactate responses to a handling disturbance in Arctic charr. Comp Biochem Physiol C 2002; 132:235–245.

Kass GEN, Orrenius S. Calcium signaling and cytotoxicity. Environ Health Perspect 1999; 107:25–32.

Kilgour BW, Dube MG, Hedley K, et al. Aquatic environmental effects monitoring guidance for environmental assessment practitioners. Environ Monit Assess 2007; 130:423–436.

Klovins J, Haitina T, Fridmanis D, et al. The melanocortin system in *Fugu*: Determination of POMC/AGRP/MCR gene repertoire and synteny, as well as pharmacology and anatomical distribution of the MCRs. Mol Biol Evol 2004; 21:563–579.

Kortner TM, Arukwe A. The xenoestrogen, 4-nonylphenol, impaired steroidogenesis in previtellogenic oocyte culture of Atlantic cod (*Gadus morhua*) by targeting the StAR protein and P450scc expressions. Gen Comp Endocrinol 2007a; 150:419–429.

Kortner TM, Arukwe A. Effects of 17alpha-methyltestosterone exposure on steroidogenesis and cyclin-B mRNA expression in previtellogenic oocytes of Atlantic cod (*Gadus morhua*). Comp Biochem Physiol C 2007b; 146:569–580.

Kossor DC, Kominami S, Takemori S, et al. Role of the steroid 17α–hydroxylase in spironolactone-mediated destruction of adrenal cytochrome P450. Mol Pharmacol 1991; 40:321–325.

Kusakabe M, Todo T, McQuillan HJ, et al. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. Endocrinology 2002; 143:2062–2070.

Lacroix M, Hontela A. Regulation of acute cortisol synthesis by cAMP-dependant protein kinase and protein kinase C in a teleost species, the rainbow trout (*Oncorhynchus mykiss*). J Endocrinol 2001; 169:71–78.

Lacroix M, Hontela A. The organochlorine *o,p'*-DDD disrupts the adrenal steroidogenic signaling pathway in rainbow trout (*Oncorhynchus mykiss*). Toxicol. Appl Pharmacol 2003; 190:197–205.

Lacroix A, Hontela A. A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, *Oncorhynchus mykiss*, and yellow perch, *Perca flavescens*. Aquat Toxicol 2004; 67:13–21.

Lacroix A, Hontela A. Role of calcium channels in cadmium-induced disruption of cortisol synthesis in rainbow trout, *Oncorhynchus mykiss*. Comp Biochem Physiol Part C 2006; 144:141–147.

Laflamme JS, Couillard Y, Campbell PGC, et al. Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. Can J Fish Aquat Sci 2000; 57:1692–1700.

Lappivaara J, Oikari A. Altered challenge response in whitefish subchronically exposed in areas polluted by bleached kraft mill effluents. Ecotoxicol Environ Safety 1999; 43:212–222.

Laskey JW, Phelps PV. Effect of cadmium and other metal cations on in vitro Leydig cell testosterone production. Toxicol Appl Pharmacol 1991; 108:296–306.

Latendresse JR, Brooks CL, Capen CC. Pathological effects of butylated triphenyl phosphate-based hydraulic fluid and tricresyl phosphate on the adrenal gland, ovary, and testis in the Fisher-344 rat. Toxicol Pathol 1994; 22:341–352.

Leblond V, Hontela A. Effects of in vitro exposures to cadmium, mercury, zinc, and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane on steroidogenesis by dispersed interrenal cells of rainbow trout (*Oncorhynchus mykiss*). Toxicol Appl Pharmacol 1999; 157:16–22.

Leblond VS, Bisson M, Hontela A. Inhibition of cortisol secretion in dispersed head kidney cells of rainbow trout (*Oncorhynchus mykiss*) by endosulfan, an organochlorine pesticide. Gen Comp Endocrinol 2001; 121:48–56.

Levesque HM, Moon TW, Campbell PGC, et al. Seasonal variation in carbohydrate and lipid metabolism of yellow perch (*Perca flavescens*) chronically exposed to metals in the field. Aquat Toxicol 2002; 60:257–267.

Levesque HM, Dorval J, Van Der Kraak GJ, et al. Hormonal, morpological, and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental exposures. J Toxicol Environ Health 2003; 66:657–676.

Lindhe O, Brandt I, Christiansen JS, et al. Irreversible binding of o,p'-DDD in interrenal cells of Atlantic cod (*Gadus morhua*). Chemosphere 2003; 50:1249–1253.

Liu P, Lin C. Phthalates suppress the calcium signaling of nicotinic acetylcholine receptors in bovine adrenal chromaffin cells. Toxicol Appl Pharmacol 2002; 183:92–98.

Lockhart WL, Uthe JF, Kenney AR, et al. Methylmercury in Northern pike (*Esox lucius*): Distribution, elimination, and some biochemical characteristics of contaminated fish. Can J FishAquat Sci 1972; 29:1519–1523.

Lyssimachou A, Arukwe A. Alteration of brain and interrenal StAR protein, P450scc, and Cyp11beta mRNA levels in atlantic salmon after nominal waterborne exposure to the synthetic pharmaceutical estrogen ethynylestradiol. J Toxicol Environ Health A 2007; 70:606–613.

Mathias SA, Mgbonyebi OP, Motley JR, et al. Modulation of adrenal cells functions by cadmium salts. 4. Ca^{2+}-dependant sites affected by $CdCl_2$ during basal and ACTH-stimulated steroid synthesis. Cell Biol Toxicol 1998; 14:225–236.

McCormick SD. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. In: Wood CM, Shuttleworth TJ, eds. Fish Physiology, Vol. XIV, Ionoregulation: Cellular and molecular approaches, New York: Academic Press; 1995:285–315.

McCormick SD. Endocrine control of osmoregulation in teleost fish. Amer Zool 2001; 41:781–794.

Metz JR, Geven EJW, vanden Burg EH, et al. ACTH, α- MSH and control of cortisol release: Cloning, sequencing and functional expression of the melanocortin-2 and melanocortin-5 receptor in *Cyprinus carpio*. Am J Physiol 2005; 289:R814–R826.

Mommsen TP, Vijayan MM, Moon TW. Cortisol in teleosts; dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish 1999; 9:211–268.

Norris DO, Donahue S, Dores RM, et al. Impaired adrenocortical response to stress by brown trout, *Salmo trutta*, living in metal-contaminated waters of the Eagle River, Colorado. Gen Comp Endocrinol 1999; 113:1–8.

Norris DO. Comparative aspects of vertebrate adrenals. In: Norris DO, ed. Vertebrate Endocrinology, Academic Press, San Diego, California, USA; 1997: 329–354.

Omura M, Suematsu S, Nishikawa T. Role of calcium messenger systems in ACTH-induced cortisol production in bovine adrenal fasciculo-reticularis cells. Endocrine J 2007; 54:585–592.

Pankhurst NW, Van Der Kraak G. Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout in vivo, through actions of cortisol. Gen Comp Endocrinol 2000; 117:225–237.

Papadopoulos V. Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: Biological role in steroidogenic cell function. Endocr Rev 1993; 14:222–240.

Papadopoulos V. In search of the function of the peripheral-type benzodiazepine receptor. Endocr Res 2004; 30:677–684.

Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 2004; 25:947–970.

Penhoat A, Naville D, Begeot M. The adrenocorticotrophic hormone receptor. Curr Opin Endocrinol Diabet 2001; 8:112–117.

Pottinger TG, Moran TA, Cranwell PA. The biliary accumulation of corticosteroids in rainbow trout, *Oncorhynchus mykiss*, during acute and chronic stress. Fish Physiol Biochem 1992; 10:55–66.

Powlin S, Keng PC, Miller RK. Toxicity of cadmium in human trophoblast cells (JAr choriocarcinoma): Role of calmodulin and the calmodulin inhibitor, zaldaride maleate. Toxicol Appl Pharmacol 1997; 144:225–234.

Quabius ES, Balm PHM, Wendelaar Bonga SE. Interrenal stress responsiveness of tilapia (*Oreochromis mossambicus*) is impaired by dietery exposure to PCB126. Gen Comp Endocrinol 1997; 108:472–482.

Quabius ES, Nolan DT, Allin CJ, et al. Influence of dietary exposure to polychlorinated biphenyl 126 and nutritional state on stress response in Tilapia *(Oreochromis mossambicus*) and rainbow trout (*Oncorhynchus mykiss*). Env Toxicol Chem 2000; 19:2892–2899.

Rasmussen JB, Gunn JM, Sherwood GD, et al. Direct and indirect (foodweb mediated) effects of metal exposure on the growth of yellow perch (*Perca flavescens*): Implications for Ecological Risk Assessment. Hum Ecol Risk Assessment 2008; 14(2):317–350.

Raynal N, Hontela A, Jumarie C. Cadmium uptake in isolated adrenocortical cells of rainbow trout and yellow perch. Comp Biochem Physiol C 2005; 140:374–382.

Reid SG, Bernier NJ, Perry SF. The adrenergic stress response in fish: Control of catecholamine storage and release. Comp Biochem Physiol C Toxicol Pharmacol 1998; 120:1–27.

Ringholm A, Fredriksson R, Poliakova N, et al. One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. J Neurochem 2002; 82:6–18.

Scott GR, Keir KR, Schulte PM. Effects of spironolactone and RU486 on gene expression and cell proliferation after freshwater transfer in the euryhaline killifish. J Comp Physiol [B] 2005; 175:499–510.

Sharpe RL, Woodhouse A, Moon TW, et al. Beta-sitosterol and 17beta-estradiol alter gonadal steroidogenic acute regulatory protein (StAR) expression in goldfish, *Carassius auratus*. Gen Comp Endocrinol 2007; 151:34–41.

Sloman KA, Desforges PR, Gilmour KM. Evidence for a mineralocorticoid-like receptor linked to branchial chloride cell proliferation in freshwater rainbow trout. J Exp Biol 2001; 204:3953–3961.

Stegeman JJ, Hahn ME. Biochemistry and molecular biology of monooxygenases: Current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In: Malins DC, Ostrander GK, eds. Aquatic Toxicology, CRC Press; 1994:87–206.

Stocco DM. Intramitochondrial cholesterol transfer. Biochim Biophysis Acta 2000; 1486:184–197.

Strum A, Bury NR, Dengreville L, et al. 11-deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. Endocrinol 2005; 46:47–55.

Thomas P, Wofford HW. Effects of cadmium and Aroclor 1254 on lipid peroxidation, glutathione peroxidase activity, and selected antioxidants in Atlantic croaker tissues. Aquat Toxicol 1993; 27:159–178.

Thoreux–Manlay A, Le Goascogne C, Segretain D, et al. Lead affects steroidogenesis in rat Leydig cells in vivo and in vitro. Toxicology 1995; 103:53–62.

Vijayan MM, Raptis S, Sathiyaa R. Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. Gen Comp Endocrinol 2003; 132:256–263.

Vijayan MM, Prunet P, Boone AN. Xenobiotic impact on corticosteroid signalling, Chapter 13, In: Mommsen TP, Moon TW, eds. Biochemistry and Molecular Biology of Fishes, Vol. 6, Environmental Toxicology Elsevier B.V., Amsterdam, Netherlands; 2005:365–394.

Walsh LP, Stocco DM. Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. Biol Reprod 2000; 63:1024–1033.

Walsh LP, McCormick C, Martin C, et al. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Env Health Perspect 2000a; 108:769–776.

Walsh LP, Webster DR, Stocco DM. Dimethoate inhibits steroidogenesis by disrupting transcription of the steroidogenic acute regulatory (StAR) gene. J Endocrinol 2000b; 167:253–263.

Wendelaar Bonga SE. The stress response in fish. Physiol Rev 1997; 77:591–625.

Weyts FAA, Cohen N, Verburg–Van Kemenade BML. Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. Fish Shellfish Immunol 1999; 9:1–20.

Wilson JM, Vijayan MM, Kennedy CJ, et al. Beta-naphthoflavone abolishes interrenal sensitivity to ACTH stimulation in rainbow trout. J Endocrinol 1998; 157:63–70.

Wiseman SB, Osachoff H, Bassett E, et al. Gene expression pattern in the liver during recovery from an acute stressor in rainbow trout. Comp Biochem Physiol Part D: Genomics Proteomics 2007; 2:234–244.

Yeom DH, Adams SM. Assessing effects of stress across levels of biological organization using an aquatic ecosystem health index. Ecotox Environ Safety 2007; 67:286–295.

11

Adrenal Toxicology in Birds: Environmental Contaminants and the Avian Response to Stress

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BIRDS AS SENTINEL SPECIES

Wildlife species show great potential as sentinels for the early detection of adverse health effects of chemicals present in the environment, and thus as potential protectors of human health (Fox, 2001; Sheffield *et al*., 1998). A common definition for a sentinel species is any life-being (prokaryotic or eukaryotic, natural or transgenic, plant or animal, feral or domesticated) that can be used as an indicator of exposure to or toxicity from environmental contaminants and, therefore, can help to assess potential impacts on similar organisms, on populations or on ecosystems (Lower and Kendall, 1992; Stahl, 1997). The concept is important in the environmental health sciences because sentinel species can provide integrated and relevant information on the types, amounts, availability, and effects of environmental contaminants. Therefore, we can consider, simplistically, that sentinels are signaling potential environmental hazards (LeBlanc, 1995, but see also Frame and Dickerson, 2006).

To be a sentinel, the species should be sensitive to the contaminant or contaminants of interest and, preferentially, have a wide geographical distribution, allowing the investigator to compare the response among individuals of the same species from multiple sites. Another consideration is the species' home range.

Migratory or wide-ranging species normally convey the problem of a difficult determination of the place and moment when exposure occurred. Therefore, it is generally advisable to select territorial, non-migratory species with a restricted home range. If the contaminant is biomagnified, the selection of a species that is on a higher trophic level is justified. However, predatory vertebrates such as hawks, eagles, owls, mink, seals, and alligators are frequently protected or sparsed over a targeted site of interest. Although protected and/or endangered species can still be used if non-lethal sampling methods are used, a scarce sample will make it difficult to obtain statistically valid results (Frame and Dickerson, 2006).

Most of the previous requisites are so far achieved by many avian species (Becker, 2003). Moreover, birds are conspicuous organisms and relatively easy to observe. The general biology, behavior, and ecology of birds is normally well known compared to other vertebrates, which enhances their usefulness as sentinels by reducing the risk of misinterpretations. Birds occupy different positions in the food chain with numerous species in the higher trophic levels, allowing assessment of chemical contamination in several compartments of the ecosystems as well as biomagnification of persistent chemicals. Blood collection is relatively easy, and numerous non-destructive sampling techniques for other tissues and substrates are currently available, avoiding harming the study specimens, which is always advisable and a necessity when working with protected or endangered species. Samples such as feathers, feces, or eggs reduce the sampling effort and are easy to collect. Furthermore, birds have the advantage compared to many other vertebrates that it is normally easy to gather information on demographic parameters, such as population size and reproductive success. Colonial species allow the collection of samples and data in relatively short time (Kushlan, 1993). Also important, compared to other taxa, birds possess unique aspects such as a high metabolic rate, and on a mass-size basis often have higher metabolism and food consumption rates than, for example, placental mammals of similar size (WHO/IPCS, 2002). These factors, together with increased rates of metabolic biotransformation of xenobiotics, may contribute to an increased exposure to environmental contaminants. Migration, courtship, breeding, and parental behaviors require high-energy expenditure and are often accompanied by periods of starvation. Birds respond to these situations by storing and mobilizing fat depots, thereby raising the potential of increased exposure to lipophilic contaminants that are subsequently released from the lipid-rich tissues where they have been accumulated.

Provided the advantages mentioned above, there are also drawbacks and limitations for the use of birds as sentinels that may deserve attention depending on the aim of study (Becker, 2003; Furness, 1993). For example, the longevity of birds, although can be seen as an advantage because a long lifespan implies that the individuals integrate the effects of environmental stress over time, it also makes more difficult to establish the effects of a short-term perturbation. Similarly, the mobility of birds implies an integrative value of bioindication over broad spatial scales, but can hinder their site-specific use as indicators. For example, the

sympatric occurrence of different populations of a given species during migration or staging at one site may obscure local sources of environmental stress and reduce their value as indicators. Bird numbers are regulated by density-dependent processes, and so their population sizes may be somewhat buffered against the impacts of environmental changes. Because a multitude of variables affect demographic parameters and stages, the effects of specific factors can be difficult to isolate. In addition, depending on the species, the maintenance of a captive population can be difficult or impossible to attain, constraining the design of experiments and laboratory tests.

The use of sentinel avian species to detect potential threats to human health is not recent. For example, canaries were used in coal mines for centuries to detect coal damp before this gas overcame coal miners (Burrell and Seibert, 1916; Schwabe, 1984). Birds drew great attention in the 1960s and 1970s as sentinel species for organochlorine pesticides, particularly DDT, when it was discovered that exposure to these pesticides resulted in eggshell thinning (Hickey and Anderson, 1968; Ratcliffe, 1970). Since then, a wide variety of avian species has been used as sentinels. These include raptors such as bald eagles (*Haliaeetus leucocephalus*), peregrine falcons (*Falco peregrinus*), American kestrels (*Falco sparverius*), and sparrow hawks (*Accipiter nisus*), and piscivorous species such as brown pelicans (*Pelecanus occidentalis*), great blue herons (*Ardea herodias*), cormorants, gulls, and terns, all useful due to their high position in the food web (e.g., Grasman *et al*., 1998). Owl species have also been suggested as good candidates for sentinels (Gervais and Anthony, 2003). Species such as the bobwhite quail (*Colinus virginianus*), Japanese quail (*Coturnix coturnix japonica*), Eastern bluebird (*Sialia sialis*), European starling (*Sturnus vulgaris*), tree swallow (*Tachycineta bicolor*), and various warblers that use natural or man-made cavities for nesting also can be useful sentinels (Mayne *et al*., 2004; McCarty, 2002; Romijn *et al*., 1995). A number of avian species are commercially available as either eggs or adults, including bobwhite quails, mallard ducks (*Anas platyrhynchos*), and ring-necked pheasants (*Phasianus colchicus*), allowing researchers to perform experimental exposure of adults to contaminants for single (see references in Table 1) or multigenerational studies (Heinz, 1979) and conduct egg-injection and incubation studies to assess developmental effects of environmental contaminants (e.g., Ottinger *et al*., 2001; Quinn *et al*., 2008). A number of these studies have focused on endocrine disruption. Indeed, a recent, specific use for wildlife sentinels is to detect contaminant exposure affecting the endocrine system, and this use deserves research emphasis and priority funding (DeRosa *et al*., 1998). However, there is a marked bias of this research in addressing the oestrogenic or antiandrogenic properties of pollutants and their subsequent effects on gender phenotype and on reproductive capability (Propper, 2005). As a consequence, there has been very little attention to other endocrine systems (see below), despite evidence indicates that adrenal function (as well as, e.g., thyroid function) may be adversely affected by chemicals in the environment (Table 1).

Table 1 A Survey of Studies Reporting Adrenocortical Measurements in Birds Exposed to Environmental Contaminants

Chemical class	Chemical/s	Exposure	Species	Age status	Parameter		Stressor/s	Variables	Reference
					B	SI			
Petroleum hydrocarbons	Crude oil	E	Mallard (*Anas platyrhynchos*)	J	↓			Sex	(Rattner and Eastin, 1981)
	Crude oil	E	Mallard (*Anas platyrhynchos*)	A	○				(Rattner, 1981)
	Crude oil	E	Mallard (*Anas platyrhynchos*)	A	↓				(Harvey *et al*., 1981)
	Crude oil	E	Mallard (*Anas platyrhynchos*)	J	↓				(Gorsline and Holmes, 1981)
	Crude oil (DF)	E	Herring gull (*Larus argentatus*)	C	↑				(Peakall *et al*., 1981)
	Crude oil (DF)	E	Black guillemot (*Cepphus grylle*)	C	↑				(Peakall *et al*., 1981)
	Crude oil (DF)	E	Leach's storm-petrel (*Oceanodroma leucorhoa*)	A	○				(Peakall *et al*., 1981)
	Crude oil	E	Mallard (*Anas platyrhynchos*)	J	↓				(Gorsline and Holmes, 1982a)
	Crude oil (DF)	E	Mallard (*Anas platyrhynchos*)	J	↓				(Gorsline and Holmes, 1982b)
	Crude oil	E	Mallard (*Anas platyrhynchos*)	J	↓ [age]			Age	(Gorsline and Holmes, 1982c)
	Crude oil	E	Mallard (*Anas platyrhynchos*)	U	↓				(Gorsline, 1984)
	Crude oil	F	Magellanic penguin (*Spheniscus magellanicus*)	A	↑ [sex]			Sex	(Fowler *et al*., 1995)

Organochlorine compounds	*o,p′*-DDD	E	Chicken (*Gallus domesticus*)	C	↓				(Newcomer, 1959)
	DDTs	E	Chicken (*Gallus domesticus*)	J	↓				(Srebocan *et al*., 1971)
	DDE	E	Chicken (*Gallus domesticus*)	J		↓	ACTH		(Gross, 1990)
	PCBs	E	Mallard (*Anas platyrhynchos*)	A	○				(Fowles *et al*., 1997)
	PCBs, PCDDs, PCDFs, CHCs	F	Herring gull (*Larus argentatus*)	E	↓ [PCBs, PCDDs, PCDFs]				(Lorenzen *et al*., 1999)
	PCBs, DDE	F	Bald eagle (*Haliaeetus leucocephalus*)	C	○	↑ [iocation]	ACTH	Age, Sex, Location	(Bowerman *et al*., 2002)
	PCBs	E	American kestrel (*Falco sparverius*)	A	↓	↓	HR	Age, Body condition	(Love *et al*., 2003b)
	PCBs, PCDDs, PCDFs, DDE	F	Tree swallow (*Tachycineta bicolor*)	C	↓ [PCDFs]	○	HR	Body weight, Body lipids, Time of day, Year	(Martinovic *et al*., 2003)
	PCBs	F	Tree swallow (*Tachycineta bicolor*)	A		○	HR		(Franceschini *et al*., 2005)
	PCBs	F	Tree swallow (*Tachycineta bicolor*)	C		↑, ↓ [year]	HR, ACTH	Year	(Franceschini *et al*., 2005)
	p,p′-DDT	E	Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelli*)	A	○	○	FA, FL		(Scollon *et al*., 2004)
	p,p′-DDE	F	Tree swallow (*Tachycineta bicolor*)	C	○	○, ↑ [year]	HR, ACTH	Body weight, Sex, Daily temperature, Collection date, Year	(Mayne *et al*., 2004)
	p,p′-DDE	F	Eastern bluebird (*Sialia sialis*)	C	↑	↓	ACTH	Body weight, Sex, Daily temperature, Collection date	(Mayne *et al*., 2004)

(*continued*)

Table 1 A Survey of Studies Reporting Adrenocortical Measurements in Birds Exposed to Environmental Contaminants (*continued*)

Chemical class	Chemical/s	Exposure	Species	Age status	Parameter		Stressor/s	Variables	Reference
					B	SI			
Organophosphorus compounds	Parathion	E	Bobwhite quail (*Colinus virginianus*)	A	○	↑	C		(Rattner *et al.*, 1982a)
	Parathion	E	Bobwhite quail (*Colinus virginianus*)	A	○			Time of day	(Rattner *et al.*, 1982b)
	Fenthion	E	Black duck (*Anas rubripes*)	A	○	↓	SW		(Rattner *et al.*, 1983)
	Methyl parathion	E	American kestrel (*Falco sparverius*)	A	↑	○	C		(Rattner and Franson, 1983)
	Temephos	E	Mallard (*Anas platyrhynchos*)	C	↑	○	C		(Fleming *et al.*, 1985)
	Triorthotolyl phosphate	E	Chicken (*Gallus domesticus*)	A	↑				(Foil *et al.*, 1985)
	Mixture of nonpersistent pesticides	F	Tree swallow (*Tachycineta bicolor*)	C	○	○, ↑ [year]	HR, ACTH	Body weight, Sex, Daily temperature, Collection date, Year	(Mayne *et al.*, 2004)
	Mixture of non-persistent pesticides	F	Eastern bluebird (*Sialia sialis*)	C	↑	↓	ACTH	Body weight, Sex, Daily temperature, Collection date	(Mayne *et al.*, 2004)
Metals	Cd	E	Mallard (*Anas platyrhynchos*)	J	[↑]				(Di Giulio and Scanlon, 1984)
	Cd	E	Mallard (*Anas platyrhynchos*)	J		[↑]	FA		(Di Giulio and Scanlon, 1985)
	Al	E	Chicken (*Gallus domesticus*)	C	○			Food	(Capdevielle *et al.*, 1996)
	Hg	F	Bald eagle (*Haliaeetus leucocephalus*)	C	○	○	ACTH	Age, Sex, Location	(Bowerman *et al.*, 2002)

Cd, Hg, Se	F	Common eider (*Somateria mollissima*)	A		↑ [Cd], ↓ [Se] [year, sex]	HR	Sex, Year, Handling time	(Wayland *et al*., 2002)
Cd, Hg, Se	F	Common eider (*Somateria mollissima*)	A		[↓] [Se] [sex]	HR	Body weight, Sex, Handling time	(Wayland *et al*., 2003)
Hg	F	Tree swallow (*Tachycineta bicolor*)	C	○	○	HR	Body weight, Body lipids, Time of day, Year	(Martinovic *et al*., 2003)
Cu, Pb[a]	F	Great tit (*Parus major*)	A		○		Body weight, Handling time, Food	(Eeva *et al*., 2003)
Cu, Pb[a]	F	Great tit (*Parus major*)	C		○		Body weight, Handling time, Food	(Eeva *et al*., 2003)
Pb	E	Zebra finch (*Taeniopygia guttata*)	A	○			Sex, Ca	(Snoeijs *et al*., 2005)
Hg	F	White ibis (*Eudocimus albus*)	A		○		Sex, Reproductive stage	(Heath and Frederick, 2005)
Cu, Pb, Zn, Ni, As[a]	F	Pied flycatcher (*Ficedula hypoleuca*)	A		○		Handling time	(Eeva *et al*., 2005)
Cu, Pb, Zn, Ni, As[a]	F	Pied flycatcher (*Ficedula hypoleuca*)	C		○		Handling time	(Eeva *et al*., 2003)
Cu, Pb, Zn, Cd, As	F	White stork (*Ciconia ciconia*)	C	○	↑ [Pb] [brood size]	HR	Age, Body condition, Sex, Brood size, Location	Baos *et al*. (2006)

Indicated is the chemical class, chemical/s per se, the type of exposure (E vs. F), the species, age status (A, J, C, E, U), the adrenocortical parameter/s measured (B, SI), the type of experimental stressors, when applicable (HR, ACTH, C, SW, FA, FL), and the variables (factors and/or covariates) controlled for in statistical analysis, if any (e.g., age, sex, body condition, etc.). Significant ($P < 0.05$) associations with baseline, stress-induced, or both corticosterone measures are indicated as follows: ↑, increase cort; ↓, decrease cort; ○, no effect; symbols within brackets indicate marginally significant effect ($0.05 < P < 0.1$) as indicated by authors (e.g., [↑] marginally significant increase in cort levels). If results are constraint to a particular chemical (when more than a single chemical is considered in the same study) or variable tested, the chemical or variable involved is also specified between brackets. When the study includes more than one species and/or age status, and/or chemical class, separate rows are used for each species, status, and chemical class. Full references are given in the literature section at the end of the chapter.

We assume that cort levels measured in blood samples taken after 2 to 3 minutes post-capture indicate stress-induced responses (Romero and Reed, 2005). If no information is reported regarding the time elapsed since capture, we assume that blood samples were collected immediately after capture and, therefore, cort concentrations represent baseline levels.

[a]Metals reported in the area at higher concentrations.

Abbreviation: DF, distillation fractions; E, experimental; F, field; A, adult; J, juvenile; C, chick or nestling; E, embryo; U, unknown; B, baseline corticosterone levels; SI, stress-induced corticosterone levels; HR, handling and restraint; ACTH, adrenocorticotropic hormone; C, Cold; SW, Salt Water; FA, fasting; FL, flight.

ENDOCRINE TOXICOLOGY AND THE ADRENOCORTICAL RESPONSE TO STRESS

Endocrine toxicology is generally referred to the action of chemicals on the structure and function of a particular gland (commonly known as the target organ approach). However, the endocrine system, more than any other, regulates homeostatic balance and as a whole is sensitive to changes in the function of its constituent glands and non-endocrine organs such as the liver. Therefore, chemically induced changes in non-endocrine organs can affect the endocrine system, and thus compounds inherently toxic to the liver, kidney, or brain may also impair the normal functioning of the endocrine system (indirect toxicity). Furthermore, toxicological studies often focus on the damage induced by a chemical to an organ or tissue, leading to total or subtotal failure in function. In endocrine toxicology, as occurs in immunotoxicology, chemically induced increases in function can be as harmful as it is the loss or the decrease in function (see below). Provided these particularities, among the endocrine glands, the adrenals, and especially the cortex, is one of the organs most commonly affected by toxic substances (Ribelin, 1984). Without precluding direct toxicity, a reason why the adrenals are so commonly implicated in endocrine toxicological responses concerns their unique position in the regulation of the stress response. Endocrinologists have long been aware of the adrenal stress response and the functions that it serves in conditions of adversity (Selye, 1936).

What is a "Healthy" Response to Stress?

Animals have evolved physiological mechanisms to adjust their life cycle to a changing environment. Among birds, for example, life-history stages such as development, dispersal, reproduction, and migration normally follow a cyclic pattern in concert with predictable environmental changes (e.g., seasons, day-night, tides), and the endocrine system plays a fundamental role in adjusting behavior, morphology, and physiology to maximize life-time individual fitness (Jacobs and Wingfield, 2000). But in addition to cyclic, predictable changes in environmental conditions, all habitats suffer non-predictable perturbations that challenge individuals' homeostasis. Sudden weather inclemency, floods, droughts, decreased feeding resources, and outbreaks of parasites or predators, among others, can strongly modify environmental conditions and jeopardize individuals' development, reproduction, and survival. In order to maximize fitness, birds have also evolved endocrine mechanisms to cope with such noxious and energy demanding unpredictable situations (Romero, 2004; Wingfield, 2003). Activation of the Hypothalamus-Pituitary-Adrenal (HPA) axis constitutes a well-preserved emergency response in vertebrates, and it orchestrates physiological and behavioral changes adequate to cope with non-predictable changes in environmental conditions. Following exposure to a perturbation, the hypothalamus releases corticotropin-releasing hormone (CRH) and some other hormones (Fig. 1), which stimulate the pituitary to secrete adrenocorticotropic

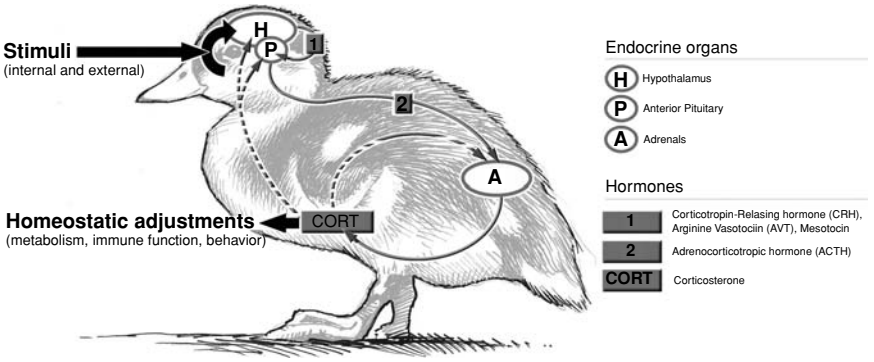

Figure 1 Schematic representation of the adrenocortical response to stress in birds. Following exposure to perturbations of exogenous or endogenous origin (e.g., predation attempts, energy imbalances; see upper black arrows), the hypothalamus (H) releases a number of hormones (1) including corticotropin-releasing hormone CRH. These in turn stimulate the anterior pituitary (P) to secrete adrenocorticotropic hormone ACTH (2) into circulation. In birds, the adrenals (A) respond to increased ACTH levels secreting corticosterone (CORT). Within minutes to hours following exposure to stress, the resulting corticosterone elevations promote multiple changes in physiology and behavior (lower black arrow) including increased gluconeogenesis, suppression of reproductive behaviors, regulation of immune function, irruptive migration, and increased night restfulness. These adjustments promote the maintenance of homeostasis through change (i.e., allostasis). Corticosterone secretion is subjected to negative feedback mechanisms, as indicated with dashed arrows. In addition to stress-related fluctuations, baseline corticosterone levels show circadian and circannual rhythms in birds, allowing endogenous regulation of numerous physiological processes. Drawing courtesy of J. A. Sencianes.

hormone (ACTH) into circulation (Sapolsky *et al*., 2000). In birds, the adrenals respond to increased ACTH levels secreting corticosterone. Within minutes to hours following exposure to stress, the resulting corticosterone elevations promote multiple changes in physiology and behavior including increased gluconeogenesis, suppression of reproductive behaviors, regulation of immune function, irruptive behavior, and increased night restfulness (Sapolsky *et al*., 2000; Wingfield and Ramenofsky, 1999; Wingfield and Romero, 2001). Increased corticosterone, therefore, constitutes a "healthy" or adaptive response to stress, as it promotes the maintenance of homeostasis through change [i.e., "allostasis" (McEwen and Wingfield, 2003)], priming physiological and behavioral adjustments aimed at maximizing immediate survival while suppressing non-essential activities. For this reason, experimental exposure to a number of physical noxious stimuli [such as capture and restraint, exposure to cold or heat, (Blas *et al*., 2005; Freeman and Manning, 1984; Ramade and Baylé, 1980)] is a frequently used protocol to assess adrenocortical function in birds (Fig. 2). Elevated corticosterone levels following short-term exposure to stress is therefore the expected response among healthy

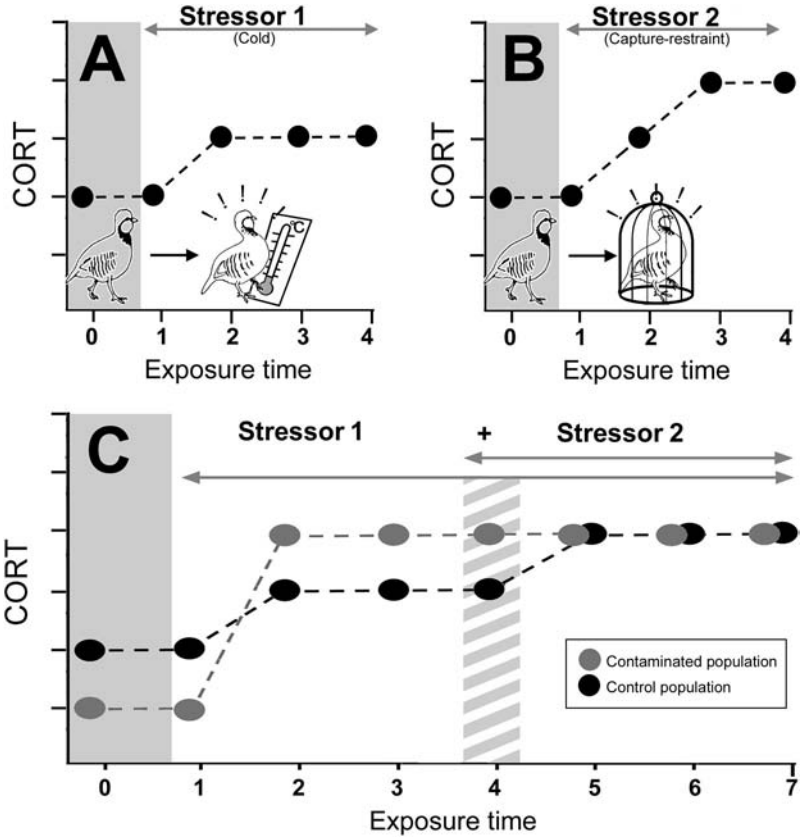

Figure 2 Assessing the response to stress: The importance of adequate baseline levels estimation. Exposure to a wide array of noxious stimuli activates the HPA axis of birds, which triggers a rapid elevation of the circulating levels of corticosterone. A widely used protocol aimed at assessing this response in birds consists on inducing experimental stress by means of exposure to a standardized perturbation, such as cold (**A**), or capture and restraint (**B**). This protocol is accompanied by the collection of several blood samples at predetermined time intervals (*X*-axes). Subsequent determination of the concentration of corticosterone (*Y*-axes) in the collected samples allows an objective assessment of the individuals' time-course patterns of response, providing an objective physiological record to establish comparisons among populations and to study associations with behavioral and toxicological data. The collection of an initial blood sample shortly following stress induction is required to assess baseline corticosterone titers (i.e., resting levels, gray area). Ideally, this sample should be collected before exposure to experimental stress, but because corticosterone elevations do not occur immediately, blood samples collected within the first 2 to 3 minutes provide a valid estimation of baseline titers. After this brief time lag, corticosterone levels rapidly elevate (i.e., stress-induced or acute levels) over the course of 30 to 60 minutes. The magnitude of the response depends upon the type of stressor (e.g., **A** and **B**; cold vs. capture-restraint); and experimental protocols sometimes

individuals. It is less clear, however, whether chronic corticosterone elevations in response to prolonged or repeated exposure to stressful situations constitutes an adaptive response. Under specific scenarios (e.g., a fish in a contaminated pond, a wild bird caged, exposed to intense parasitism or to severe food shortages, a mammal exposed to social subordination), the ability of an individual to avoid a perturbation may not be possible despite activation of emergency responses. Animals may then habituate to the perturbation and decrease activation of the HPA axis [e.g., habituation to capture and handling (Love *et al*., 2003a), or to captivity (Cabezas *et al*., 2006)], but depending on the quality of the stressful stimuli, this may not be possible (e.g., exposure to chronic malnutrition). Chronic (maintained from days to weeks) elevation of corticosterone levels may then promote catabolism up to the point of depleting fat stores and waste structural protein mass (e.g., muscle), and also inhibit the reproductive system, suppress growth and the immune system, disrupt second cell messengers, and provoke neuronal cell death (Sapolsky *et al*., 2000; Sapolsky, 1992; Wingfield and Romero, 2001). Numerous studies provide evidence for these and other deleterious effects of long-term experimentally elevated corticosterone (Joseph and Ramachandran, 1993; Kitaysky *et al*., 2003; Martin *et al*., 2005). However, it should be noted that this sort of experimental manipulation does not occur in a context of chronic stress: individuals subjected for a long-term to corticosterone elevations, normally show decreased fitness compared to controls (sham-implanted), provided that none of the experimental groups are exposed to a chronic perturbation. In other words, chronic corticosterone elevations may be maladaptive when there is no reason to activate emergency responses, but if the individuals are chronically exposed to a severe perturbation, this response could still be the best to a bad situation.

←

Figure 2 (*continued*) comprise different stressors sequentially applied to the same individuals (**C**). Despite acute corticosterone levels may be further elevated following exposure to a second source of stress (i.e., after time 4 in **C**), blood samples collected shortly after this time (dashed gray area) should not be considered true baseline levels. Such consideration would otherwise lead to an incorrect interpretation of the results, as illustrated in **C**; individuals from the population exposed to contaminants (gray dots) would be described as having higher baseline levels and being unable to respond to stress, when in fact they had a lower baseline and a faster corticosterone elevation compared to control birds (black dots). This observation is also relevant when comparing the stress response among populations, because uncontrolled local perturbations (e.g., inclement weather, parasites) may generate corticosterone elevations prior to the experimental exposure to stress (i.e., before the dashed gray area in **C**) potentially misleading the interpretation of contaminant-related effects. For all these reasons, it is always advisable to: (1) collect information on contaminant exposure on an individual basis (rather than just using a population mean), (2) work concurrently on the different study populations (to avoid seasonal and interannual variability), and (3) avoid intense sampling over a short period of time (to dilute the effects of episodic stress such as predation attempts in colonial birds, or weather-related variability).

Chemical Stressors or Endocrine Disruptors?

Field endocrinologists often assess circulating glucocorticosteroid levels (or related adrenocortical parameters, such as fecal corticosteroid metabolites) in wild vertebrates and use this endocrine parameter as a biomarker of exposure to environmental stress (Walker *et al*., 2005a). Despite increased baseline corticosterone levels may ultimately reflect activation of an emergency response, the adrenocortical system is not stressor-specific. Elevated baseline glucocorticosteroid levels are expected to occur among individuals or populations exposed to decreased food resources (Kitaysky *et al*., 1999; Kitaysky *et al*., 2001), reduced habitat quality (Marra and Holberton, 1998; Suorsa *et al*., 2003; Wasser *et al*., 1997), and increased anthropogenic pressure (Mullner *et al*., 2004; Walker *et al*., 2005a; Walker *et al*., 2005b), among others. Ultimately, all these perturbations share a common property: they increase the energy demands of the individual and, therefore, a corticosterone response helps to maintain homeostasis through promoting changes in physiology and behavior (McEwen and Wingfield, 2003). But what happens when the individual or population is exposed to environmental contaminants? Our literature survey shows that an array of chemicals also elicit corticosterone elevations in birds (Table 1); is this enough to label a chemical as "endocrine disruptor" or should we just consider it to be one more "stressor"? Let us compare these terms.

Stressors, Perturbations, Modifying Factors, and some other names

The terminology involved in the biomedical literature studying stress can be confusing, because the same term "stress" has been traditionally used to: (a) describe the noxious stimuli that an individual is exposed to, (b) the physiological and behavioral coping responses, and (c) the overstimulation of the coping responses that result in disease (Romero, 2004). But even constraining our discussion to definitions of the term "stressor" related the first meaning, i.e., "stressor is a noxious or unpredictable stimuli that causes a stress response" (Romero, 2004), different authors use a very varied terminology to define and classify stressors.

Romero (2004) differentiates between "acute stressors" (those that last a short period of time, such as predator attacks, dominance interactions, and storms) and "chronic stressors" (i.e., the latter ones when they persist on time, e.g., long-term subordinance, famine).

Wingfield (2003) refers to stressors using the terms "modifying factors" or "labile perturbation factors" that are defined as unpredictable events in the environment able to trigger a facultative emergency life-history stage, which redirects the individual away from the normal life-history stage (e.g., winter, breeding, moult) into a survival mode. The life-history emergency state is characterized by increased secretion of glucocorticosteroids, and some examples of these unpredictable events include severe storms, predator pressure, and human disturbance. These environmental perturbations can last long-term (e.g., human disturbance, global climate change) or be transient ("labile"). Within transient or labile perturbations, Wingfield (2003) discriminates two groups: Indirect (rapid events that do

not reduce food or access to it, e.g., nest predation, a sudden hail storm) and Direct (longer-term that force the individual to interrupt a previous life-history stage, e.g., reduced food supply, drop in social status, disease, predator influx), and includes pollution/endocrine disruptors into the latter category of direct labile perturbations.

McEwen and Wingfield (2003) present three new concepts that have generated some controversy (Dallman, 2003; Walsberg, 2003): "allostasis" (the maintenance of homeostasis through change), "allostatic load" (the measure of how hard an individual must work to accomplish a normal life-history task, such as the energy requirements for breeding), and "allostatic overload" (the state in which energy requirements exceed the capacity of an individual to replace that energy from the environment), and suggest to use the term "stress" only referred to stimuli that require an emergency energetic response [i.e., the equivalent to stressor according to Romero (2004)].

Pottinger (2003) defines stressor as a "destabilizing stimulus of external or internal origin", and classifies stressors as Physical (abiotic, such as temperature, wind, habitat alteration, etc., or biotic, such as conflict, predator, or parasite damage), Chemical (e.g., contaminants), Physiological (e.g. starvation, disease, dehydration), and psychological (e.g. threat of predation, intra- and interspecies conflict, territoriality).

Endocrine Disruptors

Originally, the concern over endocrine disruption was based almost entirely on perceived effects of chemicals on the reproductive system and it was usual to refer to these chemicals as estrogen mimics or estrogenic substances. Later, chemicals were found that could block oestrogenic responses (anti-oestrogenic) or androgenic responses (antiandrogens), and it was soon recognized that some substances could affect other elements of the endocrine system via interaction with hormones other than sex steroids (WHO/IPCS, 2002).

The term endocrine disruptor is now preferred because it allows inclusion of health effects thought to result from interference with any part of the endocrine system. Although there are several different definitions for this term in current use (Phillips and Harrison, 1999), the final report of the U.S. EPA's Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) (US EPA, 1998) defines an endocrine disruptor as "an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle".

A major difficulty that has been encountered with this definition (identified as a particular problem by EDSTAC) is the definition of the term "adverse". For a chemical to be judged as an endocrine disruptor, it is important to show that the recorded response has an adverse effect on the health or reproductive capability of affected organisms or populations and that this response does not fall within the normal range of physiological variation. This premise may help us to answer the question formulated above: as mentioned, the elevation of corticosterone levels

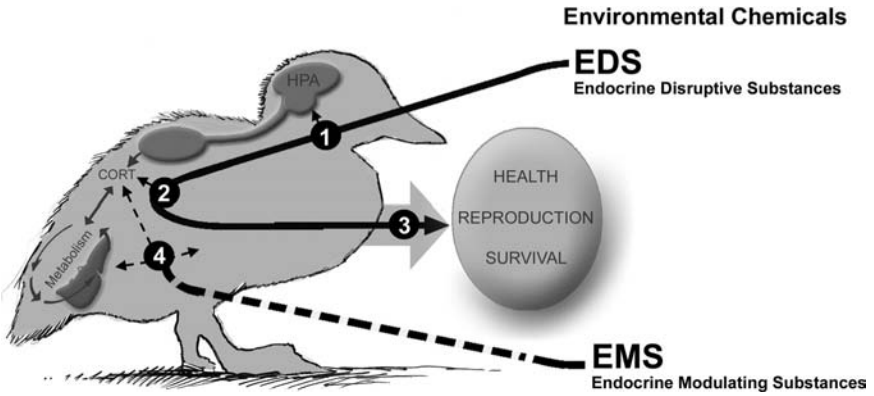

Figure 3 Environmental chemicals affecting the HPA axis. Although there is growing evidence that numerous human activities–derived chemicals present in the environment can affect the normal function of the stress axis, just a few of these substances can be categorized as endocrine disruptors following the current definition of this term (US EPA, 1998). Gray letters provide a schematic representation of the HPA system of a bird. Activation of this endocrine axis results in the secretion of corticosterone (CORT), which modulates numerous physiological functions such as metabolism (e.g., increasing hepatic glucose output through enhanced gluconeogenesis). Normal regulation of circulating corticosterone levels allows birds to maintain homeostasis, and therefore maximizes individual fitness, as represented with the broad gray arrow pointing to the main fitness traits (i.e., health, reproduction and survival). In order to classify a chemical as "endocrine disruptor" of the stress axis (EDS, see continuous black arrow) the following circumstances have to be proven: (1) the primary site of action is the endocrine system; (2) the structure or function(s) of the endocrine system is altered (e.g., corticosterone levels are abnormally increased or decreased); and (3) it causes adverse health effects at the level of the organism, its progeny, or the population. Demonstration of these three conditions requires an extensive knowledge of the specific mechanisms of action of each chemical, and this is extremely difficult to attain in field and environmental studies. In addition, the HPA-response is not stressor-specific and, therefore, can be indirectly activated if exposure to a given chemical jeopardizes homeostatic balance (e.g., exerting its action over other organs—such as the liver, or over other physiological processes—such as metabolism). As a consequence, despite many chemicals have been shown to elicit circulating corticosteroid levels above or below "normal" values, unless we relax the current definition of EDS they have to be considered as "chemical stressors" or "endocrine modulating substances" (EMS, see dashed black line).

following exposure to a chemical stressor could simply be the normal or expected stress response, and differ little from the response to capture or predation attempts. Thus, provided that such response does not exceed the homeostatic capacity of the individual and/or populations, the chemical might be just one more stressor, rather than an endocrine disruptor (Fig. 3).

A second problem associated to the definition of endocrine disruptor concerns the mechanism(s) of action of the chemical: disruptors should primar-

ily affect the endocrine system, thus excluding from this classification those chemicals causing overt toxicity in other body systems and indirectly affecting endocrine function. With regards to the HPA axis, the multiple levels of control over the stress response imply many potential sites of action for chemicals affecting or disrupting adrenal homeostasis (Pottinger, 2003). For example, chemicals may influence the negative feedback control loops of the adrenals through modifying hepatic metabolism, causing changes in glucocorticoid secretion (Rehulka and Kraus, 1987). Because such alterations in adrenal function constitute indirect responses (i.e., the primary site of action is not the endocrine system), these chemicals should not be called endocrine disruptors. In contrast, changes in ACTH secretion that result from chemical actions on the brain or pituitary gland and that will also alter secretion of glucocorticoids (Hadley *et al*., 1990; Spindel *et al*., 1983), constitute a secondary response to chemical actions at extra-adrenal endocrine sites and, therefore, might be considered endocrine disruption (provided that they cause adverse health effects on individuals and/or populations). At this point, it is important to note that the underlying mechanism(s) of action of most chemicals affecting the adrenocortical response to stress is still poorly understood, especially when the evidence of effects comes from field studies [e.g., Baos *et al*. (2006); Wayland *et al*. (2002)].

On the other hand, although transient elevations in circulating corticosterone concentrations are highly adaptive through facilitating short-term responses to stressors (by promoting behavioral changes or mobilizing energy reserves), chronic corticosterone elevations can have very relevant deleterious consequences such as fat-stores depletion, muscle waste, inhibition and suppression of growth, reproductive and immune systems, disruption of second cell messengers, or induction of neuronal cell death (Sapolsky *et al*., 2000; Sapolsky, 1992; Wingfield and Romero, 2001). Similarly, lower circulating levels of corticosterone can result in an inability to respond to stress, reduced gluconeogenesis, and stimulation of the immune system (Colby and Longhurst, 1996) that may ultimately affect fitness. Thus, abnormal responses in both directions, i.e., increasing or decreasing corticosterone levels as consequence of primary or secondary toxicity of chemicals acting directly or indirectly on the endocrine system might be equally harmful for individuals and/or populations. The lack of basic knowledge on the feedback loops and the boundaries of an organism's homeostatic range affects our ability to place in context the significance of a particular observation obtained in the field or even in the laboratory. Clearly, the boundaries of endocrine disruption, and particularly those concerning adrenocortical function cannot be clearly delineated.

For the purpose of our review, we will focus on chemicals in the environment that can increase or decrease adrenocortical function (i.e., plasma baseline and/or stress-induced corticosterone levels), using the term endocrine-modulating substance (EMS) to include the terms "chemical stressor", "endocrine active substance", and "endocrine disruptor" (Fig. 3). This is a relatively broad consideration of the chemicals of concern and reflects the difficulty to face research into adrenal "disruptors", especially when working with wildlife.

ENDOCRINE MODULATING SUBSTANCES IN THE ENVIRONMENT AND THE ADRENOCORTICAL RESPONSE TO STRESS

In this section, we will review the literature on adrenocortical stress response in birds exposed to environmental contaminants, with the goal of finding common patterns of responses that may allow us to reach conclusions and make inferences, as well as to identify research gaps and delineate future research directions.

A survey of the published studies reporting adrenocortical measurements [i.e., plasma baseline (or basal) and/or stress-induced (or acute) corticosterone concentrations] in avian species exposed to chemicals reveals that more than 90% of them ($N = 40$) deal with exposure to petroleum hydrocarbons, organochlorines (PCBs, PCDDs, PCDFs, and persistent organochlorine insecticides, such as DDT and its metabolites), metals, and organophosphorous compounds (Table 1).

Petroleum Hydrocarbons

The hydrocarbons considered in this review involve various compounds present in crude petroleum that are frequently released into the environment following major oil spills or slow seepage from natural deposits, oil industry storage, and extraction sites. Acute, subacute, and chronic exposure of birds may occur in nature through the oiling of plumage and through the ingestion of oil via feeding or preening.

The impact of petroleum hydrocarbons on avian adrenocortical function received considerable attention during the 1980s (Table 1). Most of the studies reporting plasma corticosterone in birds exposed to crude oil have been conducted experimentally on mallard ducks, and declines in circulating corticosterone concentrations have characterized their responses to ingested petroleum-contaminated food (see references cited in Table 1). Studies in vivo and in vitro have confirmed that the petroleum-induced decreases in plasma corticosterone concentration reflect diminished adrenocortical activities, due primarily to a suppression of the corticotropic responsiveness of cells in the inner zone of the adrenal gland (Gorsline and Holmes, 1982a). However, it is also feasible that petroleum-induced changes in liver function may indirectly influence adrenocortical function in contaminated birds. As in many other organisms exposed to hydrocarbon pollutants, the liver of birds consuming petroleum-contaminated food develops an increased ability to metabolize the circulating contaminants (Gorsline *et al*., 1981; Miller *et al*., 1978). This is accomplished through the action of a substrate-inducible mixed function oxidase system. However, although the primary function of this system is to rid the organism of the contaminants, it may also accelerate the turnover of some endogenous substrates, such as steroid hormones (Peakall, 1967). Thus, the low plasma corticosterone concentrations found in birds exposed to petroleum-contaminated food may have been caused by two distinct types of effects: one involving a diminished responsiveness to ACTH in the adrenals, and the other comprising an enhanced metabolism of circulating hormones (Gorsline and Holmes, 1981).

Although different crude oils seem to produce qualitatively similar effects on plasma corticosterone concentrations, the magnitude of the responses evoked in laboratory-maintained ducks varied considerably (Gorsline and Holmes, 1981; Harvey *et al*., 1981; Rattner and Eastin, 1981). Many factors may have been responsible for this variability. For example, the chemical composition of the crude oil and the environmental conditions under which the birds were maintained, could both contribute to the reported quantitative differences in response. Also, the magnitude of the perceived response may vary with the time of the day when blood samples were taken and hormone concentrations were compared (Gorsline and Holmes, 1981).

Unlike many other pollutants, crude oils are complex mixtures of different types of hydrocarbons, and thus it cannot be assumed that their toxicities are always attributable to a particular class of compounds. Gorsline and Holmes (1982b) found differences in plasma corticosterone concentrations among different distillation fractions of crude oils, but were unable to attribute this effect to specific hydrocarbon compounds present in any of the fractions.

The lower molecular weight constituents of crude oil, particularly the aromatic hydrocarbons, have often been assumed to be responsible for most of the adverse effects seen in contaminated organisms (Gorsline and Holmes, 1982b). In growing mallard ducks, decreases in plasma concentration of corticosterone after chronic ingestion of crude oil were most apparent in birds fed the oil with apparently greater aromatic content and was somewhat dose-dependent (Rattner and Eastin, 1981).

It is of particular interest that the extent of the reported decreases in adrenocortical function, even in response to the ingestion of a particular crude oil, may also differ among birds exposed to contaminated food for only a few days and those that have consumed the same food for several months (Gorsline and Holmes, 1981; Rattner and Eastin, 1981). In these instances, it is impossible to determine the exact reason for the differences in evoked change, as they may be due primarily to the duration of the exposure or reflect modifications in response due to aging (Gorsline and Holmes, 1982c). Gorsline and Holmes (1982c) reported larger decreases of plasma corticosterone in younger exposed ducks than in older birds, while no changes in plasma corticosterone concentration occurred with aging in the control birds. Thus, age seems to be an important factor determining the degree of the hypoadrenocorticalism developed following exposure to petroleum-contaminated food. Rattner (1981) showed no effects on corticosterone levels in adult mallards exposed to petroleum contaminated food for 7 days, suggesting that adults can tolerate oil-contaminated food better than hatchling and young growing birds. Similarly Peakall *et al.* (1981), failed to find effects on plasma corticosterone levels in adult Leach's petrels (*Oceanodroma leucorhoa*) dosed with weathered oil in a semi-field experiment where birds were recaptured, while nestling herring gulls (*Larus argentatus*) and black guillemots (*Cepphus grille*) sampled in the same study showed significant endocrine effects. However, contrary to previous experimental reports, oil-dosed nestlings of both species

of seabirds showed higher levels of corticosterone compared to control birds. Fowler *et al*. (1995) also found elevations of corticosterone levels in lightly oiled female Magellanic penguins (*Spheniscus magellanicus*) at the beginning of the breeding season following an accidental crude-oil spill. The authors argued that oiled penguins have to face heavy energetic demands and that elevated corticosterone levels are consistent with the role of this hormone in mobilizing energy substrates. Coincidentally, the latter two studies were the only ones that were conducted under field conditions, and both reported an elevation of corticosterone levels after exposure to petroleum hydrocarbons. In natural settings, birds must face considerable fluctuations in environmental conditions such as changes in food availability. The exposure to other stressors may interact with petroleum effects on the adrenocortical stress response, resulting in higher levels of several hormones. In this regard, Peakall *et al*. (1981) reported increases in both corticosterone and ACTH levels in oil-dosed nestling gulls. Adrenocorticotropic hormone is released by the pituitary in response to low glucocorticoid levels (Fig. 1). Because elevated glucocorticoids inhibit ACTH release (through negative feedback), in the absence of severe pathology, only "stress" will result in both elevated plasma corticosterone and elevated ACTH. On the other hand, the composition of the crude oil may also contribute to explain different results between field and experimental studies. Nestling herring gulls showed increased corticosterone levels only after exposure to certain crude oil or aromatic fractions (Peakall *et al*., 1981).

Organochlorine Compounds

Under this term, we will refer to polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and organochlorine insecticides such as DDT and its metabolites. In general, these chemicals are characterized by being highly lipophilic and showing low solubility in water, which facilitates their accumulation in fatty tissues and fat stores, often at increasing concentrations in animals occupying the higher levels of the food web (e.g., Borga *et al*., 2001). All share a high environmental persistence.

Polychlorinated biphenyls are commercial mixtures of related compounds (congeners), which were once used (in many countries, the use of PCBs is now banned or severely restricted) as dielectric fluids, heat transformer fluids, lubricants, vacuum pump fluids, as plasticizers (e.g., in paints), and for making carbonless copy paper. Major sources of pollution are or have been manufacturing wastes and the careless disposal or dumping of the liquids referred above (Waid, 1985–1987).

The best known member of PCDDs (there are 75 possible congeners of PCDDs) is 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD), usually referred to simply as "dioxin". This is a compound of extremely high toxicity to mammals. Polychlorinated dibenzofurans are similar to PCDDs both in structure and origin.

Both PCDDs and PCDFs are not produced commercially, but are unwanted by-products generated during the synthesis of other compounds. Dioxins are also formed during the combustion of PCBs (fires or chemical waste disposal) and by the interaction of chlorophenols (used as wood preservatives) during disposal of industrial wastes (e.g., pulp mill effluents). Like PCBs, PCDD residues have been detected widely in the environment (especially in the aquatic environment), albeit at low concentrations, e.g., in fish and fish-eating birds.

Organochlorine insecticides such as DDT are highly persistent in their original form or as stable metabolites. DDT was used mainly for vector control of insects transmitting diseases during the Second World War, but came to be very widely used thereafter for the control of agricultural pests, vectors of diseases (e.g., malarial mosquitoes), ectoparasites of farm animals, and insects in domestic and industrial facilities. By 1990s, the use of these compounds for most purposes had been banned on the grounds of perceived human health risks or hazards to the environment. However, some of these compounds continue to be used in some developing and tropical countries, for example, to control vectors such as the malarial mosquito. The very marked persistence of compounds such as *p,p′*-DDE has ensured that significant residues are still present in once heavily contaminated soils and/or sediments and will only slowly disappear over the decades to come. These residues are still slowly released into aquatic and terrestrial food webs and can reach significant concentrations in animals at higher trophic levels.

Among the environmental contaminants, persistent organochlorine compounds have received great attention in relation to endocrine modulation or disruption in avian species (WHO/IPCS, 2002). However, most of the studies in this regard have dealt with their well-known oestrogenic and/or antiandrogenic properties (Guillette *et al*., 2006; Vos *et al*., 2000) being comparatively scarce the literature published on organochlorine stress-related endpoints. Moreover, except for a couple of experimental studies with poultry (DDTs) carried out in late 50s (Newcomer, 1959) and early 70s (Srebocan *et al*., 1971), most research on the adrenocortical stress response in birds exposed to organochlorine contaminants have been conducted during the last decade. Over this period, more than a half of the published scientific reports correspond to field studies (Table 1) where, in addition to basal corticosterone levels, the authors have usually incorporated estimates of stress-induced response. Circulating corticosterone concentrations in blood collected immediately after capture (i.e., basal corticosterone) were assumed to reflect environmental stress as opposed to the stress-induced response measured after: (a) a standardized capture, handling, and restraint protocol; (b) exposure to a physical stressor designed to produce an increase in circulating corticosterone (e.g., cold); or (c) ACTH injection (Fig. 2). Stress-induced corticosterone concentration, usually in conjunction with basal corticosterone is used as a correlate for a functional HPA axis (Hinson and Raven, 2006; Norris, 2000). Furthermore, challenge with an exogenous ACTH injection allows distinguishing adrenal gland response from

nonadrenal causes of changes in circulating corticosterone concentrations, which might help to identify the mechanism(s) of toxicity.

Despite a prolific use of DDT for more than a quarter century, most of the residual DDT in the environment exists as *p,p′*-DDE (in commercial DDT, 70–80% corresponds to *p,p′*-DDT). Other metabolites such as *o,p′*-DDE, *p,p′*-DDD, and *o,p′*-DDD are also present in lesser amounts. Exposure to technical grade DDT (75% *p,p′*-DDT and 25% *o,p′*-DDT), *p,p′*-DDT, and *o,p′*-DDD has been shown to decrease basal corticosterone in chickens (Newcomer, 1959; Srebocan *et al*., 1971), with dose-dependent reductions in individuals fed technical grade DDT for several weeks (Srebocan *et al*., 1971). Dose (or the environmental level of exposure) and duration of exposure are factors argued by some authors to be the cause of failing to detect significant effects of DDTs on corticosterone concentrations either in experimental (Scollon *et al*., 2004) or field studies [Lorenzen *et al*. (1999); Mayne *et al*. (2004) in tree swallow]. Except for DDE, Lorenzen *et al*. (1999) found negative correlations between basal corticosterone in herring gull embryos from the Great Lakes and environmental levels of PCBs (total and non-ortho PCBs), PCDDs, and PCDFs measured in yolk sacs. Similarly, Martinovic *et al*. (2003) found negative correlations between basal corticosterone levels and PCDFs in nestling tree swallows sampled in two consecutive years, although they also reported no differences between exposed and reference sites in basal or stress-induced corticosterone concentrations after 10 minutes of handling and restraint. As mentioned earlier, the functionality of HPA axis through this procedure, or by the injection of ACTH, has been explored in several studies in which birds were exposed to organochlorine compounds, and their response compared with control or reference animals. In general, although both exposed and control (or reference) birds responded to the standardized handling and restraint procedure (or ACTH injection) by an increase in secretion of corticosterone (e.g., Love *et al*., 2003b), negative effects of PCBs and DDE on stress-induced response have been reported in both field and experimental studies (Gross, 1990; Love *et al*., 2003b; Mayne *et al*., 2004). Nevertheless, in some instances, associations are not straightforward. For example, Bowerman *et al*. (2002) found that exposure to DDE and PCBs in nestling bald eagles was associated with lesser induction of plasma corticosterone on a regional level (i.e., Great Lakes or Interior breeding area) when challenged with ACTH. However, they also reported that increases in corticosterone induction were positively related to increases in either DDE or PCBs. In two consecutive years of study, Franceschini *et al*. (2005) found that tree swallow nestlings chronically exposed to high PCB levels exhibited an increase in post-stress corticosterone concentrations in comparison with birds from reference sites during the first year; however, lower levels of corticosterone after ACTH injection were reported in the second year. In a study examining stress response in songbird nestlings co-exposed to *p,p′*-DDE residues and a mixture of non-persistent pesticides in apple orchards, Mayne *et al*. (2004) found interspecies differences in both basal and stress-induced response between tree swallows and Eastern bluebirds sampled in sprayed and reference sites (Table 1). Thus, while basal corticosterone

levels in nestling tree swallows was not affected by the exposure to pesticides, and levels of corticosterone secretion post-ACTH stimulation were increased in the sprayed orchards, exposed bluebird nestlings had higher levels of basal corticosterone and were less responsiveness to challenge with ACTH than reference birds (Table 1). Furthermore, stress-induced corticosterone concentrations in bluebirds were negatively associated with *p,p′*-DDE levels in eggs. From these results, and since eastern bluebird eggs contained much higher concentrations of *p,p′*-DDE than tree swallow eggs, authors concluded that modulation of HPA axis in the tested songbird chicks was mostly associated with high persistent pesticides, i.e., *p,p′*-DDE. Results from laboratory studies have established that DDT metabolites are potent toxicants in the adrenal cortex of birds (Jönsson *et al*., 1994). The high lipid content of the cortical tissue of the avian adrenals has a high affinity for the metabolites of DDT; the primary metabolite in adrenal tissue of chickens dosed with DDT was *p,p′*-DDE (Srebocan *et al*., 1971). Disruption of cortical cell activity by *p,p′*-DDE ultimately inhibiting steroidogenesis has been shown previously in mammals (Lund, 1994). Alternatively, mixed-function oxidase activity may be induced by high levels of *p,p′*-DDE. This, potentially, could increase the metabolic clearance rate of corticosterone, activating feedback mechanisms and prolonging the release of ACTH from pituitary corticotropes. Chronic stimulation of cortical tissue would have the effect of exhausting adrenal cortical cells and dampening the response to ACTH injection.

Organophosphorous Compounds

Organophosphorous compounds are organic esters of phosphorus acids that act as nerve poisons (neurotoxins) due to their ability to inhibit the enzyme acetylcholinesterase. Today, a large number of organophosphorous compounds are marketed as insecticides, being extensively used for the control of agricultural pests and disease vectors. They are more polar and water soluble than the main types of organochlorine insecticides, although their water solubility is highly variable. Despite their lipophilic character, they are, in general, less stable than organochlorine insecticides and more readily broken down by chemical or biochemical agents. Thus, they tend to be relatively short-lived in the environment and in the tissues of homeothermic animals, being environmental hazards largely, but not exclusively, associated with short-term (acute) toxicity. It is remarkable that, despite their short-lives, some organophosphorous insecticides are highly toxic to birds and small mammals for brief periods after application, occasionally affecting local wildlife populations [e.g., secondary poisoning in raptors (Mineau *et al*., 1999)].

Our literature survey reveals that a few number of studies have reported plasma corticosterone levels in birds exposed to organophosphorous compounds, and that almost all of them were published in a 4-year window (1982-1985), corresponding to experimental work conducted on adults of different avian species. The main aim of these investigations was to assess overt toxicity of organophosphorous

compounds on different aspects of avian physiology, either alone or in combination with physical stressors such as cold (e.g., Rattner and Franson, 1983; Rattner *et al*., 1982a). Corticosterone was determined in most cases within routine plasma chemistries and, overall, results showed a dose-dependent increase in corticosterone concentrations after organophosphorous exposure (Table 1). Thus, Fleming *et al*. (1985) found increased levels of corticosterone only in the ducklings exposed to the highest (100 ppm) dietary temephos concentration. Similarly, subchronic ingestion of 100 ppm parathion for 10 days followed by exposure to mild cold (6 °C) for up to 48 hours resulted in two- to five-fold elevation of plasma corticosterone concentration in female bobwhite quails, yet birds receiving 0 and 25 ppm parathion were not affected (Rattner *et al*., 1982a). Acute exposure to methyl parathion also elevated plasma corticosterone concentration in adult American kestrels (Rattner and Franson, 1983). In the single and most recent study performed in the wild, Mayne *et al*. (2004) investigated the combined effects of several non-persistent pesticides and the organochlorine insecticide *p,p′*-DDE on songbird nestlings' stress response. Tree swallow nestlings from pesticide-sprayed orchards showed higher levels of corticosterone after ACTH injection than chicks from reference sites; however, these results must be interpreted with caution since organophosphorous compounds were only one class of the chemicals included in the mixture of pesticides sprayed, and there was no correlation between stress-induced corticosterone concentrations and several estimates of pesticide exposure. Only Rattner *et al*. (1983) reported sustained corticosterone levels in ducks exposed to fenthion and receiving salt water for 12 days while non-exposed birds increased their levels of corticosterone. Thus, a reduced responsiveness of the HPA axis to organophosphorous exposure was suggested. In mammals, organophosphorous insecticides have been demonstrated to inhibit adrenal cholesterol esterification and hydrolysis, as well as hepatic steroid metabolism (Conney *et al*., 1971), and to reduce the rate of corticosteroidogenesis in vitro (Civen and Brown, 1974; Civen *et al*., 1977).

Metals

Metals are a large family of elements characterized by complex chemistry. Some metals are essential for normal physiological function, as integral parts of amino acids, nucleic acids, and structural compounds. Zinc, for example, is an essential component of at least 150 enzymes, Cu is essential for the normal function of cytochrome oxidase, and Fe is part of hemoglobin. All essential metals have a "window of essentiality", within which dietary concentrations in animals have to be maintained if the organism is to grow and reproduce normally. The window of essentiality for some elements is very narrow (e.g., Se). Metals such as Cd, Hg, Pb, or As are referred to as non-essential because they do not have a known physiological function. In addition to being toxic above certain levels, non-essential metals such as Hg or Cd, may also affect organisms by inducing deficiencies of

essential elements through competition at active sites in biologically important molecules. Such antagonism also occurs between essential elements.

The biological half-life varies for different metals. In mammals, the half-life of Cd is 20 to 30 years, while the half-life of As or Cr is a few hours or days. Their toxicity depends not only on dose and on length of exposure as occurs with other toxicants, but also on the ionic and chemical form (the species) of the metal and its bioavailability.

Metals are natural elements discharged into the environment by alteration of their geochemical cycles, through either human activities or natural processes such as volcanic eruptions or soil erosion. Mining and smelting activities, coal and petroleum combustion, and agricultural use of sludge from water treatment plants are important sources of contamination. The use of metal-based pesticides (e.g., lead arsenate) further contributes to environmental contamination. Acidification of watersheds by acid rain influences metal distribution in the ecosystem by promoting lixiviation of metals from soils into the aquatic compartment. Because their ecological half-life is long, and although their structure can be modified in the environment or in the animals by speciation and processes such as ionization, methylation, and binding to organic ligands, metals are classified as persistent contaminants. Moreover, their importance and widespread use in the manufacture of many products make contamination by metals ubiquitous.

Despite the extent to which biomarkers are able to provide unambiguous and ecologically relevant indicators of exposure to or effects of toxicants remains highly controversial (Forbes *et al*., 2006), over the past decade, the adrenocortical stress response has been widely considered within a suite of biomarkers chosen to reflect animal health and fitness in metal-exposed wild bird populations (Table 1). Our literature survey reveals that most of the studies performed in this regard have failed to detect significant effects of metal exposure on adrenocortical stress response (Table 1). However, it seems that the absence of significant results are more frequent for some elements, such as Hg (Bowerman *et al*., 2002; Heath and Frederick, 2005; Martinovic *et al*., 2003; Wayland *et al*., 2002; Wayland *et al*., 2003) than for others like Cd or Se (Di Giulio and Scanlon, 1984; Di Giulio and Scanlon, 1985; Wayland *et al*., 2002; Wayland *et al*., 2003). In any case, it is important to highlight that the number of publications per metal is very limited to reach definitive conclusions.

In a 3-year monitoring program of a breeding colony of common eiders (*Somateria mollissima*) in the Canadian Arctic, Wayland *et al*. (2002; 2003) found negative relationships between hepatic Se concentration and the stress-induced response in female eiders, while renal Cd concentration was reported to be positively related to plasma corticosterone levels in incubating fasted-females (Wayland *et al*., 2002). Although following the ecoepidemiological criteria (Fox, 1991), the lack of consistency in relationships among years was argued by the authors to avoid concluding that Cd exposure was related to the magnitude of the stress response in female eiders, experimental studies conducted on mallard ducks provide support for a positive association (Di Giulio and Scanlon, 1985). Di

Giulio and Scanlon, 1985 showed that mallards simultaneously food-restricted and exposed to dietary Cd had higher (although marginally significant) concentrations of corticosterone compared to non-Cd exposed and non-food-restricted counterparts.

The functional tests are highly relevant to assess the situation in the wild where birds from contaminated sites must not only cope with the contaminant(s), but also must react appropriately to predators, conspecifics, and various environmental stressors, either chronic or acute, such as food deprivation, harsh weather conditions, etc. In a recent study conducted on white stork (*Ciconia ciconia*) nestlings exposed to metals subsequent to a mining accident in southwestern Spain, Baos *et al*. (2006) showed no significant relationships between metals (Cu, Zn, Cd, Pb, As) and basal corticosterone concentration. However, maximum corticosterone concentration after a standardized handling and restraint protocol was positively related to low blood Pb levels, and singleton nestlings had higher levels of corticosterone than nestlings from multiple-chick broods. In addition, the interaction between Pb levels and brood size was also significant, suggesting that Pb had a greater impact on the stress-induced corticosterone of single nestlings than on those of multiple-chick broods. In a previous work, it was reported that single stork nestlings were reared in nests that experienced brood reduction, which suggested lower parental quality (Blas *et al*., 2005). Reduced attendance by young or inexperienced parents may lead singletons to suffer from environmental stressors other than Pb (e.g., a greater exposure to harsh weather conditions). This, in turn, may explain both their higher levels of maximum corticosterone, and the reported stronger relationship between the stress-induced response and Pb. Although similar (i.e., positive) associations between stress response and exposure to Pb have also been reported in rats (Cory-Slechta *et al*., 2004) and, more recently, in children exposed pre- and postnatally to low levels of Pb (Gump *et al*., 2008), experimental (Snoeijs *et al*., 2005) and field studies (Eeva *et al*., 2003; Eeva *et al*., 2005) on passerine birds have failed to detect Pb effects on either basal (Snoeijs *et al*., 2005) or stress-induced plasma corticosterone concentrations (Eeva *et al*., 2003; Eeva *et al*., 2005).

Finally, it is important to note that, although the study by Baos *et al*. (2006) is correlational in nature and comes from a small sample size, it would support the argument that contaminants acting in concert with other stressors may have a greater impact on individuals than the effects elicited by either the contaminants or other stressors acting alone.

SYNTHESIS, STUDY BIAS AND RESEARCH GAPS

Our review reveals that the assessment of adrenocortical parameters (plasma baseline and stress-induced corticosterone concentrations) might be a promising nondestructive biomarker of effect of environmental contaminants in birds. However, it also illustrates that despite a growing number of studies have been published during the last decade, the literature dealing with the impact of pollutants on the

stress response in avian species is still very scarce (Table 1), especially when compared with research assessing the impact of chemical contaminants on other physiological systems such as the immune system (Fairbrother *et al*., 2004). Moreover, certain biases and constraints deserving further attention have been identified in our review and are discussed below:

Study Models

A detailed analysis of the Table 1 reveals that the effects of petroleum hydrocarbons and organophosphorous compounds on adrenocortical stress response have been mostly studied on adults or juveniles of poultry species (e.g., chicken, mallard, bobwhite quail) experimentally exposed to variable doses of contaminants in controlled environments. Despite such studies represent a very valuable tool for characterizing the biological action of chemicals and understanding associated toxicity, results are constrained to a particular age segment (i.e., developing individuals are rarely studied), taxa (poultry species), and obtained under captive settings. These conditions imply that extrapolation to wild bird populations should be done with caution because: (1) captivity and domestication can strongly modify HPA function (Romero and Wingfield, 1999), (2) in general, developing birds are more vulnerable to toxicant effects than adults (Gochfeld, 1997), and this may differentially affect the adrenal stress response (Gorsline and Holmes, 1982c), (3) constant dosing regimes (acute exposure to relatively high doses of chemicals) differ from the irregular dietary intake of contaminants that very often characterize environmental exposure (chronic exposure to low levels of contaminants seems to be the general rule in wild birds, with acute exposure to high doses being restricted to certain hot spots or linked to accidental spills), and (4) the captive study models have limited exposure to real environmental perturbations (competition, weather inclemency, predation). Some of these conditions might explain the discrepancies regarding the effects of petroleum hydrocarbons in corticosterone secretion between studies performed in laboratory experiments and those performed on wild birds (Table 1).

Table 1 also reveals a temporal bias in the study of the stress response among classes of contaminants. While the studies on the effects of petroleum and organophosphorous compounds were mostly carried out during the 1980s, the majority of the recent reports deal with environmental exposure to organochlorines and metals. In the latter studies, corticosterone concentration is often measured in developing individuals of altricial or semialtricial species (i.e., nestlings) and within a broader set of biomarkers aimed at assessing the overall health status of exposed birds. It should be noted that the physiology, diet, and metabolism of young birds is substantially different compared to adults. These differences can modify the patterns of exposure and limit the applicability of data obtained from adult specimens (Burger *et al*., 2003). As mentioned, in general, developing organisms are more vulnerable to toxicant effects (Gochfeld, 1997), and this may differentially affect the adrenal stress response (Gorsline and Holmes, 1982c). The developmental stage of particular organs and tissues, and the maturation of

endocrine and nervous control can all interact in critical ways to influence the nature of the toxicant effect on the adrenal stress response. Endocrine systems mature at different rates in species showing altricial or precocial modes of development (Blas and Baos, 2008; Scanes and McNabb, 2003). Precocial birds (e.g., mallards, chickens, quails) hatch with sight, covered with down, and are able to thermoregulate, locomote, and feed independently of their parents. Adrenocortical function in response to stressors occurs as early as in 1-day old hatchlings, in contrast with altricial species (e.g., songbirds) which hatch almost naked, blind, unable to locomote or thermoregulate, and show little or no response to stress as nestlings (i.e., stress hyporesponsive period). Avian developmental modes vary along a continuum between true precocial and true altricial strategies, and age-related increases in stress-induced corticosterone elevations characterize growing (Blas and Baos, 2008; Blas *et al*., 2006). Thus, the timing of toxicant exposure during development (i.e., in ovo, at hatching, or during growing) may have different effects on the adrenal stress response and depends upon the species developmental mode.

Experimental Protocols

The use of standardized protocols aimed at estimating stress-induced responses such as handling and restraint or ACTH challenge have only been incorporated recently, and only among research focused on organochlorine and metal contamination (Table 1). As a consequence, the effects of petroleum hydrocarbons on stress-induced corticosterone levels remain totally unknown (Table 1). With regards to organophosphorous exposure, five studies have tested the effects on stress-induced responses. However, these reports used less conventional experimental stressors like cold temperature or salt water (e.g., Rattner and Franson, 1983; Rattner *et al*., 1982a; Rattner *et al*., 1983), increasing the methodological heterogeneity and making it more difficult to establish comparisons among contaminants. This problem also affects studies incorporating standardized handling and restraint protocols and ACTH challenge, because the sampling times following experimental treatments are highly variable, and sometimes corticosterone values are calculated as residuals between the observed levels and those expected from a linear regression with handling time (e.g., Eeva *et al*., 2003; Eeva *et al*., 2005). Although basal corticosterone levels constitute an important measure of general stress allowing comparisons within- and between populations, such a static measure is not sufficient to assess adrenocortical function (Norris, 2000), and the collection of additional information on the dynamics of the response to stress is strongly recommended (Table 2). In fact, the impact of contaminants may only become obvious on stress-induced responses with no effects on basal corticosterone levels, as reported in birds Baos *et al*. (2006); Bowerman *et al*., 2002; Franceschini *et al*., 2005) and other vertebrates (Norris, 1999).

An important question deserving special care is whether a given corticosterone measurement constitutes a reliable estimation of baseline levels rather than

Table 2 Recommendations for Studying the Adrenocortical Response to Stress in Wild Birds Exposed to Contaminants

- Collect both basal (within 2–3 min after capture) and stress-induced corticosterone concentrations.
- Use standardized protocols (e.g., HR, ACTH injection) to measure stress-induced response.
- Work concurrently on different study populations (to avoid seasonal and inter-annual variation).
- Measure contaminant exposure on an individual basis (rather than just using population means) both at contaminated and reference sites.
- The use of non-destructive (e.g., blood) or non-invasive (e.g., feathers) methods to estimate adrenocortical function and contaminant exposure is advisable (especially to estimate effects on long-term fitness components, i.e., survival, reproduction).
- Include host (e.g., age, sex, body condition, reproductive stage), ecological, and environmental factors in the statistical analyses.
- Examine how host factors interact with contaminant exposure.
- Consider potential interactions between host factors.
- If work is performed during development, be aware of the developmental mode (within the precocial–altricial spectrum of variation) and the timing of exposure to contaminants.

stress-induced response. Obtaining baseline samples is not always an easy task, and can be especially difficult when animals are captured in the field due to some methodological constraints illustrated in Figure 2. The collection of an initial blood sample shortly following induction of experimental stress is required to assess basal corticosterone concentrations, with time intervals of 2 to 3 minutes following capture yielding a widely accepted estimation of basal titers (Romero and Reed, 2005). However, after this brief time lag, corticosterone levels rapidly elevate to stress-induced or acute levels over the course of 30 to 60 minutes. A first consideration is that the magnitude of this response depends upon the type of stressor [Fig. 2(A) vs. (B)]; and, therefore, this source of variability handicaps adequate comparisons among studies. A second consideration regards experimental studies where different stressors are sequentially applied to the same individuals [Fig. 2(C)]. Despite acute corticosterone levels may be further elevated following exposure to a second source of stress [e.g., after time 4 in Fig. 2(C)], blood samples collected shortly after this time (even within 2–3 minutes, as represented in Fig. 2(C) by the dashed gray area) should not be considered true basal levels. Avoiding such consideration might lead to an incorrect interpretation of the results, as illustrated in Figure 2(C): individuals from the population exposed to contaminants (gray dots) could be erroneously described as having higher baseline levels and being unable to respond to stress, when in fact they had a lower basal corticosterone and a faster corticosterone elevation compared to control birds (black dots). This observation is also relevant when comparing the stress response among wild populations, because uncontrolled local perturbations (e.g., inclement weather, parasites) may generate corticosterone elevations prior to the experimental exposure to stress, potentially misleading the interpretation of contaminant-related effects.

Population versus Individual Approaches

Another important consideration regards the use of population mean levels of contaminant exposure rather than individual levels. The observation of adrenocortical differences among populations, even in conjunction with known differences in the presence or amount of certain contaminants, might not be enough to establish reliable associations. Numerous host, ecological, and environmental variables may account for population differences independent of toxic prevalence or exposure. For example, host factors such as age, sex, reproductive status, size and weight, body condition, nutritional status, genetics, and even behavioral interactions may differ among populations and confound results by being correlated with HPA axis activity (Harvey, 1996). In addition, these factors may as well influence the amount and degree of contaminant exposure, uptake, absorption, biokinetics, susceptibility, and toxicity (Peakall and Burger, 2003). Local environmental factors, like weather conditions, parasites, predation attempts, changes in food availability, density of conspecifics, and social competition may also affect the response to stress (Wingfield and Romero, 2001) and should be controlled in order to obtain a reliable estimate of the potential effects of contaminants on the stress response. Recent investigations with white stork nestlings have demonstrated that these factors have the potential to interact with contaminants modulating the stress response of young birds (Baos *et al*., 2006) making thus very advisable the assessment of contaminant exposure on an individual- rather than on a population basis.

Fitness Consequences and Extrapolation among Species

Two important questions deserving further attention are: (1) whether differences in the stress response associated to contaminant exposure are truly relevant if they do not translate into a proved impaired/reduced health, reproduction, or survival; and (2) whether demonstration of such effect in one species can be extrapolated to others. To date, the consequences of contaminants-related modulation of stress responses on unequivocal fitness traits remain largely unknown, possibly as a result of the difficulty of maintaining long-term programs of population monitoring in the wild. However, a recent series of investigations on HPA function in wild stork nestlings have provided interesting insights in this regard. In a long-term field study, Blas *et al*. (2007) found that individuals with a reduced stress response early in life (i.e., as nestlings) had a higher probability of survival and recruitment into the breeding population when adults, providing the first empirical evidence of a link between the physiological response to stress and long-term fitness components in a wild vertebrate. Because a positive relationship between blood Pb levels and the stress response had been previously reported in stork nestlings (Baos *et al*., 2006), it might be possible that metal contamination exerted some indirect effect on fitness. It is also important to highlight that the association between Pb and adrenocortical stress response reported by Baos *et al*. (2006) occurred at Pb levels below those considered to cause sublethal effects in birds.

Concerning the validity of extrapolation of results among species, it should be noted that the physiological ranges of baseline and stress-induced corticosterone levels show a strong interspecific variability. As a consequence, obtaining a species-specific reference value is always advisable to determine whether a response falls within the "normal" range of variation. However, rare or endangered species might not be available for sampling, and adverse effects on wildlife may constitute a useful warning signal to anticipate consequences of contaminant exposure on humans. These are strong practical arguments justifying the use of sentinel species, and thus the validity of extrapolation among taxa. An interesting example is a recent study performed on children (Gump *et al*., 2008) reporting a positive relationship between (relatively low) prenatal and postnatal blood Pb levels and adrenocortical response to acute stress, very similar to the results previously reported for wild white storks (Baos *et al*., 2006). From these reports we can conclude that white storks may be considered good sentinels for the detection of potential adverse effects of Pb on human health, providing support to the hypothesis that health effects observed in wildlife and in laboratory animals are predictive of similar health effects in humans (but see also Frame and Dickerson, 2006; Heindel *et al*., 1998). If we accept that white stork nestlings are sentinels for the Pb impact on the adrenal stress response of children, then the emerging data on the long-term consequences of such response on stork fitness deserve serious consideration.

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Compared to other biomarkers of environmental health, the number of publications on adrenal stress response in avian species exposed to environmental contaminants is relatively scarce, especially when restricted to a certain class of chemicals and even more when a particular substance is considered (Table 1). This fact contrasts with the recognition that the adrenal gland is the most frequently observed site of endocrine lesion (Ribelin, 1984) and the unquestioned pivotal role of the HPA axis in maintaining homeostasis. While it is clear that environmental chemicals can modulate the adrenal stress response in avian species, the underlying mechanisms are in most cases poorly understood. Mechanistic information is important to understand and ultimately reduce the uncertainties associated with the ecological risk to wildlife and ultimately to humans caused by EMSs. This type of information demands intense, detailed research.

Laboratory experiments are necessary to explore the relationships between different doses of contaminants and the stress response. One additional concern is the potential interactions among different EMSs on wild animals. When examining the impacts of a mixture of contaminants on endocrine endpoints, is the whole mixture greater than, less than, or equal to the sum of its constituent parts? This question is particularly intriguing when considering the widespread distribution of many EMSs in the environment that even at low levels may interact with the chemical of primary research interest, potentially leading to unexpected

responses and confounding results. Experimental work in this regard should be done. Recent studies in wild storks provide evidence that the concentrations of Pb positively affecting the stress response may be lower than the existing threshold reference levels, adding concern to the low-level exposure to many potential EMSs that are widely distributed in the environment. Only within the context of controlled laboratory conditions can individual effects be isolated and causality be established.

Laboratory experiments should nonetheless be combined with field studies. Despite the adrenocortical stress response is not stressor-specific, and can be affected by numerous host, ecological, and environmental factors (also potentially affecting the toxicity of any given substance), recent findings on several avian species provide evidence that this system can be a good biomarker of environmental health (e.g., Baos *et al*., 2006); Mayne *et al*., 2004). The potential application of field models as sentinels justify the need of extended research on the impacts of environmental contaminants on avian HPA function. Research on the many contaminants not tested yet (e.g., brominated flamed retardants) and on those having ubiquitous presence in the environment, even at low levels, constitutes a study priority that is especially encouraged. Although long-term field studies can be labor-intensive, expensive, and in some cases, logistically very difficult, they are necessary to determine the relevance of contaminant-induced stress responses on long-term fitness components (e.g., survival and reproduction). Recent investigations on white storks support the usefulness of this kind of field studies, which preferentially require long-lived species (e.g., seabirds, raptors) and non-destructive methods of sampling (e.g., blood or feathers). In this regard, a method for the assessment of adrenal function in feather samples has been recently published (Bortolotti *et al*., 2008). This novel approach has the great advantage of being non-invasive because feathers are naturally shed on a regular basis and bird capture is not required, thus reducing sampling effort and avoiding undesirable effects related to wildlife manipulation. On the other hand, feathers have also been used to examine levels of some metals (Burger, 1993), and recent investigations introduce this method as a promising biomonitoring tool for assessing organic pollutants (Jaspers *et al*., 2006; Van Den Steen *et al*., 2007). Therefore, the assessment of the impact of environmental contaminants on adrenocortical stress response using feather samples should prove fruitful in the near future.

REFERENCES

Baos R, Blas J, Bortolotti GR, et al. Adrenocortical response to stress and thyroid hormone status in free-living nestling white storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. Environ Health Perspect 2006; 114:1497–1501.

Becker PH. Biomonitoring with birds. In: Markert BA, Breure AM, Zechmeister HG, eds. Bioindicators and Biomonitors, Oxford, UK: Elsevier; 2003:677–736.

Blas J, Baos R. Stress in the nest: Causes and consequences of adrenocortical secretion in developing birds. In: Capaldo A, ed. Recent Advances in Non-mammalian Adrenal Gland

Research Trivandrum, Kerala, India: Research SignPost, 2008. In Press. at Available http://www.trnres. com/

Blas J, Baos R, Bortolotti GR, et al. A multi-tier approach to identifying environmental stress in altricial nestling birds. Func Ecol 2005; 19:315–322.

Blas J, Baos R, Bortolotti GR, et al. Age-related variation in the adrenocortical response to stress in nestling white storks (*Ciconia ciconia*) supports the developmental hypothesis. Gen Comp Endocrinol 2006; 148:172–180.

Blas J, Bortolotti GR, Tella JL, et al. Stress response during development predicts fitness in a wild, long-lived vertebrate. Proc Natl Acad Sci USA 2007; 104:8880–8884.

Borga K, Gabrielsen GW, Skaare JU. Biomagnification of organochlorines along a Barents Sea food chain. Environ Pollut 2001; 113:187–198.

Bortolotti GR, Marchant TA, Blas J, et al. (2008). Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. Func Ecol 2008; 22:494–500.

Bowerman WW, Mehne CJ, Best DA, et al. Adrenal corticotropin hormone and nestling bald eagle corticosterone levels. Bull Environ Contam Toxicol 2002; 68:355–360.

Burger J, Diaz-Barriga F, Marafante E, et al. Methodologies to examine the importance of host factors in bioavailability of metals. Ecotoxicol Environ Saf 2003; 56:20–31.

Burger J. Metals in avian feathers: Bioindicators of environmental pollution. Rev Environ Toxicol 1993; 5:203–311.

Burrell GA, Seibert FM. Gases found in coal mines. Miners' Circular 14. Bureau of Mines. Department of the Interior, Washington, DC; 1916.

Cabezas S, Blas J, Marchant TA, et al. Physiological stress levels predict survival probabilities in translocated wild rabbits. Horm Behav 2006; 51:313–320.

Capdevielle MC, Carsia RV, Scanes CG. Effect of acid or aluminum on growth and adrenal function in young chickens. Gen Comp Endocrinol 1996; 103:54–59.

Civen M, Brown CB. The effect of organophosphate insecticides on adrenal corticosterone formation. Pestic Biochem Physiol 1974; 4:254–259.

Civen M, Lifrack E, Brown CB. Studies on the mechanism of inhibition of adrenal steroidogenesis by organophosphate and carbamate compounds. Pestic Biochem Physiol 1977; 7:169–182.

Colby HD, Longhurst PA. Toxicology of the adrenal gland. In: Atterwill CK, Flack JD, eds. Endocrine Toxicology. Cambridge, UK: Cambridge University Press; 1996:243–281.

Conney AH, Welch RM, Kuntzman R, et al. Effect of pesticides on drug and steroid metabolism. Clin Pharmacol Ther 1971; 8:2–10.

Cory-Slechta DA, Virgolini MB, Thiruchelvam M, et al. Maternal stress modulates the effects of developmental lead exposure. Environ Health Perspect 2004; 112:717–730.

Dallman MF. Stress by any other name. . .? Horm Behav 2003; 43:18–20.

DeRosa C, Richter P, Pohl H, et al. Environmental exposure that affects the endocrine system: Public health implications. J Toxicol Environ Health Part B 1998; 1: 3–26.

Di Giulio RT, Scanlon PF. Sublethal effects of cadmium ingestion on mallard ducks. Arch Environ Contam Toxicol 1984; 13:765–771.

Di Giulio RT, Scanlon PF. Effects of cadmium ingestion and food restriction on energy metabolism and tissue metal concentrations in mallard ducks (*Anas platyrhynchos*). Environ Res 1985; 37:433–444.

Eeva T, Lehikoinen E, Nikinmaa M. Pollution-induced nutritional stress in birds: An experimental study of direct and indirect effects. Ecol Appl 2003; 13:1242–1249.

Eeva T, Hasselquist D, Langefors A, et al. Pollution related effects on immune function and stress in a free-living population of pied flycatcher *Ficedula hypoleuca*. J Avian Biol 2005; 36:405–412.

Fairbrother A, Smits J, Grasman KA. Avian immunotoxicology. J Toxicol Environ Health Part B 2004; 7:105–137.

Fleming WJ, Heinz GH, Franson JC. Toxicity of Abate 4E (temephos) in mallard ducklings and the influence of cold. Environ Toxicol Chem 1985; 4:193–199.

Foil L, Satterlee D, Jacobs-Perry L. Increased serum corticosterone levels in triorthotolyl phosphate-treated chickens. Bull Environ Contam Toxicol 1985; 34:780–786.

Forbes VE, Palmqvist A, Bach L. The use and misuse of biomarkers in ecotoxicology. Environ Toxicol Chem 2006; 25:272–280.

Fowler GS, Wingfield JC, Boersma PD. Hormonal and reproductive effects of low levels of petroleum fouling in Magellanic penguins (*Spheniscus magellanicus*). Auk 1995; 112:382–389.

Fowles JR, Fairbrother A, Trust KA, et al. Effects of Aroclor 1254 on the thyroid gland, immune function, and hepatic cytochrome P450 activity in mallards. Environ Res 1997; 75:119–129.

Fox GA. Practical causal inference for ecoepidemiologists. J Toxicol Environ Health 1991; 33:359–373.

Fox GA. Wildlife as sentinels of human health effects in the Great Lakes-St. Lawrence Basin. Environ Health Perspect 2001; 109(Suppl. 6):853–861.

Frame L, Dickerson RL. Fish and wildlife as sentinels of environmental contamination. In: Norris DO, Carr JA. eds. Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans. NY: Oxford University Press; 2006:202–222.

Franceschini MD, Custer CM, Custer TW, et al. Corticosterone stress response in tree swallows (*Tachycineta bicolor*) nesting near a PCB-contaminated river. Integr Comp Biol 2005; 45(6): 1132.

Freeman BM, Manning ACC. Re-establishment of the stress response in *Gallus domesticus* after hatching. Comp Biochem Physiol Part A 1984; 78:267–270.

Furness RW. Birds as monitors of pollutants. In: Furness RW, Greenwood JJD, eds. Birds as Monitors of Environmental Change. London, UK: Chapman & Hall; 1993:87–143.

Gervais JA, Anthony RG. Chronic organochlorine contaminants, environmental variability, and the demographics of a burrowing owl population. Ecol Appl 2003; 13:1250–1262.

Gochfeld M. Factors influencing susceptibility to metals. Environ Health Perspect 1997; 105(Suppl.4):817–822.

Gorsline J, Holmes WN. Effects of petroleum on adrenocortical activity and on hepatic naphthalene-metabolizing activity in mallard ducks. Arch Environ Contam Toxicol 1981; 10:765–777.

Gorsline J, Holmes WN. Suppression of adrenocortical activity in mallard ducks exposed to petroleum-contaminated food. Arch Environ Contam Toxicol 1982a; 11:497–502.

Gorsline J, Holmes WN. Adrenocortical function and hepatic naphthalene metabolism in mallard ducks (*Anas platyrhynchos*) consuming petroleum distillates. Environ Res 1982b; 28:139–146.

Gorsline J, Holmes WN. Variations with age in the adrenocortical responses of mallard ducks (*Anas platyrhynchos*) consuming petroleum-contaminated food. Bull Environ Contam Toxicol 1982c; 29:146–152.

Gorsline J, Holmes WN, Cronshaw J. The effects of ingested petroleum on the naphthalene-metabolizing properties of liver tissue in seawater adapted mallard ducks (*Anas platyrhynchos*). Environ Res 1981; 24(2):377–390.

Gorsline J. Impairment of adrenocortical function by ingested petroleum hydrocarbons. J Steroid Biochem Mol Biol 1984; 20(6B):1546.

Grasman KA, Scanlon PF, Fox GA. Reproductive and physiological effects of environmental contaminants in fish-eating birds of the Great Lakes: A review of historical trends. Environ Monit Assess 1998; 53:117–145.

Gross WB. Effect of adrenal blocking chemicals on the responses of chickens and turkeys to environmental stressors and ACTH. Avian Physiol 1990; 19:295–304.

Guillette LJ Jr, Kools SAE, Gunderson MP, et al. DDT and its analogues: New insights into their endocrine-disrupting effects on wildlife. In: Norris DO, Carr JA, eds. Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans. NY: Oxford University Press; 2006:332–355.

Gump BB, Stewart P, Reihman J, et al. Low-level prenatal and postnatal blood lead exposure and adrenocortical responses to acute stress in children. Environ Health Perspect 2008; 116:249–255.

Hadley AJ, Flack JD, Buckingham JC. Modulation of corticotrophin release in vitro by methylxanthines and adenosine analogues. Br J Pharmacol 1990; 100(Suppl): 337.

Harvey S, Klandorf H, Phillips JG. Reproductive performance and endocrine responses to ingested petroleum in domestic ducks (*Anas platyrhynchos*). Gen Comp Endocrinol 1981; 45:372–380.

Harvey PW. The Adrenal in Toxicology: Target Organ and Modulator of Toxicity. London, UK: Taylor & Francis; 1996.

Heath JA, Frederick PC. Relationships among mercury concentrations, hormones, and nesting effort of white ibises (*Eudocimus albus*) in the Florida Everglades. Auk 2005; 122:255–267.

Heindel JJ, Collman GW, Suk WA. Endocrine disruptors: Extrapolation from wildlife to human effects. In: Kendall RJ, Dickerson RL, Giesy J, Suk W, eds. Principles and Processes for Evaluating Endocrine Disruption in Wildlife. Pensacola, FL: SETAC Press; 1998:369–430.

Heinz GH. Methylmercury: Reproductive and behavioral effects on three generations of mallard ducks. J Wildl Manage 1979; 43:394–401.

Hickey JJ, Anderson DW. Chlorinated hydrocarbons and eggshell changes in raptorial and fish-eating birds. Science 1968; 162:271–273.

Hinson JP, Raven PW. Effects of endocrine-disrupting chemicals on adrenal function. Best Pract Res Clin Endocrinol Metab 2006; 20:111–120.

Jacobs LF, Wingfield JC. Endocrine control of life-cycle stages: A constraint on response to the environment? Condor 2000; 102:35–51.

Jaspers VLB, Voorspoels S, Covaci A, et al. Can predatory bird feathers be used as a non-destructive biomonitoring tool of organic pollutants? Biol Lett 2006; 2:283–285.

Jönsson CJ, Lund BO, Brunström B, et al. Toxicity and irreversible binding of two DDT metabolites—3-methylsulfonyl-DDE and *o,p′*-DDD—in adrenal interrenal cells in birds. Environ Toxicol Chem 1994; 13:1303–1310.

Joseph J, Ramachandran AV. Effect of exogenous dexamethasone and corticosterone on weight gain and organ growth in post-hatched white leghorn chicks. Indian J Exp Biol 1993; 31:858–860.

Kitaysky AS, Wingfield JC, Piatt JF. Dynamics of food availability, body condition and physiological stress response in breeding black-legged kittiwakes. Func Ecol 1999; 13:577–584.

Kitaysky AS, Kitaiskaia EV, Piatt JF, et al. Benefits and costs of increased levels of corticosterone in seabird chicks. Horm Behav 2003; 43:140–149.

Kitaysky AS, Kitaiskaia EV, Wingfield JC, et al. Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. J Comp Physiol Part B 2001; 171:701–709.

Kushlan JA. Colonial waterbirds as bioindicators of environmental change. Colonial Waterbirds 1993; 16:223–251.

LeBlanc GA. Are environmental sentinels signaling? Environ Health Perspect 1995; 103:888–890.

Lorenzen A, Moon TW, Kennedy SW, et al. Relationships between environmental organochlorine contaminant residues, plasma corticosterone concentrations, and intermediary metabolic enzyme activities in Great Lakes herring gull embryos. Environ Health Perspect 1999; 107:179–186.

Love OP, Shutt LJ, Silfies JS, et al. Repeated restraint and sampling results in reduced corticosterone levels in developing and adult captive American kestrels (*Falco sparverius*). Physiol Biochem Zool 2003a; 76:753–761.

Love OP, Shutt LJ, Silfies JS, et al. Effects of dietary PCB exposure on adrenocortical function in captive American kestrels (*Falco sparverius*). Ecotoxicology 2003b; 12:199–208.

Lower WR, Kendall RJ. Sentinel species and sentinel bioassays. In: McCarthy JF, Shugart LR, eds. Biomarkers of Environmental Contamination. Chelsea, MI: Lewis Publishers; 1992:309–331.

Lund B. In vitro adrenal bioactivation and effects on steroid metabolism of DDT, PCBs, and their metabolites in the gray seal (*Halichoerus gryus*). Environ Toxicol Chem 1994; 13:911–917.

Marra PP, Holberton RL. Corticosterone levels as indicators of habitat quality: Effects of habitat segregation in a migratory bird during the non-breeding season. Oecologia 1998; 116:284–292.

Martin II LB, Gilliam J, Han P, et al. Corticosterone supresses cutaneous immune function in temperate but no tropical house sparrows, *Passer domesticus*. Gen Comp Endocrinol 2005; 140:126–135.

Martinovic B, Lean DL, Bishop CA, et al. Health of tree swallows (*Tachycineta bicolor*) nestlings exposed to chlorinated hydrocarbons in the St. Lawrence River Basin. Part II. Basal and stress plasma corticosterone concentrations. J Toxicol Environ Health Part A 2003; 66:2015–2029.

Mayne GJ, Martin PA, Bishop CA, et al. Stress and immune responses of nestling tree swallows (*Tachycineta bicolor*) and Eastern bluebirds (*Sialia sialis*) exposed to nonpersistent pesticides and *p,p′*-dichlorodiphenyldichloroethylene in apple orchards of southern Ontario, Canada. Environ Toxicol Chem 2004; 23:2930–2940.

McCarty JP. Use of tree swallows in studies of environmental stress. Rev Toxicol 2002; 4:61–104.

McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. Horm Behav 2003; 43:2–15.

Miller DS, Peakall DB, Kinter WB. Ingestion of crude oil: Sublethal effects in herring gull chicks. Science 1978; 199:315–317.

Mineau P, Fletcher MR, Glaser LC, et al. Poisoning of raptors with organophosphorus and carbamate pesticides with emphasis on Canada, U. S. and U. K. J Raptor Res 1999; 33:1–37.

Mullner A, Linsenmair KE, Wikelski M. Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (*Opisthocomus hoazin*). Biol Conserv 2004; 118:549–558.

Newcomer WS. Adrenal and blood Δ^4-3-keto-corticosteroids following various treatments in the chick. Am J Physiol 1959; 196:276–278.

Norris DO. Endocrine disruptors of the stress axis in natural populations: How can we tell? Am Zool 2000; 40:393–401.

Norris, DO, Donahue S, Dores RM, et al. Impaired adrenocortical response to stress by brown trout, *Salmo trutta*, living in metal-contaminated waters of the Eagle River, Colorado. Gen Comp Endocrinol 1999; 113:1–8.

Ottinger MA, Abdelnabi MA, Henry P, et al. Neuroendocrine and behavioral implications of endocrine disrupting chemicals in quail. Horm Behav 2001; 40:234–247.

Peakall DB, Burger J. Methodologies for assessing exposure to metals: Speciation, bioavailability of metals, and ecological host factors. Ecotoxicol Environ Saf 2003; 56:110–121.

Peakall DB, Tremblay J, Kinter WB, et al. Endocrine dysfunction in seabirds caused by ingested oil. Environ Res 1981; 24:6–14.

Peakall DB. Pesticide-induced enzyme breakdown of steroids in birds. Nature 1967; 216:505.

Phillips B, Harrison P. Overview of the endocrine disrupters issue. In: Hester RE, Harrison RM, eds. Endocrine Disrupting Chemicals. Cambridge, UK: The Royal Society of Chemistry, 1999:1–25.

Pottinger TG. Interactions of endocrine disrupting chemicals with stress response in wildlife. Pure Appl Chem 2003; 75:2321–2333.

Propper CR. The study of endocrine-disrupting compounds: Past approaches and new directions. Inter Comp Bio 2005; 45: 194–200.

Quinn MJ Jr., Summitt CL, Ottinger MA. Consequences of in ovo exposure to *p,p′*-DDE on reproductive development and function in Japanese quail. Horm Behav 2008; 53:249–253

Ramade F, Baylé JD. Evaluation de l'activité hypophyso-corticosurrénalienne basale et de sa réponse au stress au cours de la période précédant et suivant l'éclosion chez la pigeon. J Physiol Paris 1980; 76:283–287.

Ratcliffe DA. Changes attributed to pesticides in egg breakage frequency and eggshell thickness in some British birds. J Appl Ecol 1970; 7:67–115.

Rattner BA, Franson JC. Methyl parathion and fenvalerate toxicity in American kestrels: Acute physiological responses and effects of cold. Can J Physiol Pharmacol 1983; 62:787–792.

Rattner BA, Sileo L, Scanes CG. Hormonal responses and tolerance to cold of female quail following parathion ingestion. Pestic Biochem Physiol 1982a; 18:132–138.

Rattner BA, Sileo L, Scanes CG. Oviposition and the plasma concentrations of LH, progesterone and corticosterone in bobwhite quail (*Colinus virginianus*) fed parathion. J Reprod Fertil 1982b; 66:147–155.

Rattner BA, Fleming WJ, Murray HC. Osmoregulatory function in ducks following ingestion of the organophosphorus insecticide fenthion. Pestic Biochem Physiol 1983; 20:246–255.

Rattner BA. Tolerance of adult mallards to subacute ingestion of crude petroleum oil. Toxicol Lett 1981; 8:337–342.

Rattner BA, Eastin, WC Jr. Plasma corticosterone and thyroxine concentrations during chronic ingestion of crude oil in mallard ducks (*Anas platyrhynchos*). Comp Biochem Physiol Part C 1981; 68:103–107.

Rehulka J, Kraus M. Regulation of corticosterone metabolism in liver cell fractions in young and adult rats: Cofactor requirements, effects of stress and phenobarbital treatment. Physiol Bohemoslov 1987; 36:21–32.

Ribelin WE. The effects of drugs and chemicals upon the structure of the adrenal gland. Fundam Appl Toxicol 1984; 4:105–119.

Romero LM, Reed JM. Collecting baseline corticosterone samples in the field: Is under 3 min good enough? Comp Biochem Physiol Part A 2005; 140:73–79.

Romero LM, Wingfield JC. Alterations in hypothalamic-pituitary-adrenal function associated with captivity in Gambel's white-crowned sparrow (*Zonotrichia leucophris gambelii*). Comp Biochem Physiol Part B 1999; 122:13–20.

Romero LM. Physiological stress in ecology: Lessons from biomedical research. Trends Ecol Evol 2004; 19:249–255.

Romijn CAFM, Grau R, Guth JA, et al. The use of Japanese and bobwhite quail as indicator species in avian toxicity test. Chemosphere 1995; 30:1033–1040.

Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 2000; 21:55–89.

Sapolsky RM. Neuroendocrinology of the stress-response. In: Becker B, Breedlove SM, Crews D, eds. Behavioural Endocrinology. Cambridge, MA: MIT Press; 1992:287–324.

Scanes CG, McNabb FMA. Avian models for research in toxicology and endocrine disruption. Avian Poult Biol Rev 2003; 14:21–52.

Schwabe CW. Animal monitors of the environment. In Veterinary Medicine and Human Health. Baltimore, MD: Williams & Wilkins; 1984:562–578.

Scollon EJ, Carr JA, Cobb GP. The effect of flight, fasting, and *p,p*'-DDT on thyroid hormones and corticosterone in Gambel's white crowned sparrow, *Zonotrichia leucophrys gambelli*. Comp Biochem Physiol Part C 2004; 137:179–189.

Selye H. A syndrome produced by diverse nocuous agents. Nature 1936; 138:32.

Sheffield SR, Matter JM, Rattner BA, et al. Fish and wildlife species as sentinels for environmental endocrine disrupters. In: Kendall RJ, Dickerson RL, Giesy J, Suk W, eds. Principles and Processes for Evaluating Endocrine Disruption in Wildlife. Pensacola, FL: SETAC Press; 1998:369–430.

Snoeijs T, Dauwe T, Pinxten R, et al. The combined effect of lead exposure and high or low dietary calcium on health and immunocompetence in the Zebra finch (*Taeniopygia guttata*). Environ Pollut 2005; 134:123–132.

Spindel E, Griffiths L, Wurtman RJ. Neuroendocrine effects of caffeine. II. Effects on thyrotropin and corticosterone secretion. J Pharmacol Exp Ther 1983; 225:346–350.

Srebocan V, Pompe-Gotal J, Adamovic V, et al. Effect of technical grade DDT and *p,p*′-DDT on adrenocortical function in chicks. Poult Sci 1971; 50:1271–1278.

Stahl, RG Jr. Can mammalian and non-mammalian "sentinel species" data be used to evaluate the human health application of environmental contaminants? Human Ecol Risk Assess 1997; 3:329–335.

Suorsa P, Huhta E, Nikula A, et al. Forest management is associated with physiological stress in an old-growth forest passerine. Proc R Soc Lond B 2003; 270:963–969.

US EPA, EDSTAC, Final Report, Washington, DC, 1998.

Van Den Steen E, Covaci A, Jaspers VLB, et al. Experimental evaluation of the usefulness of feathers as a non-destructive biomonitor for polychlorinated biphenyls (PCBs) using silastic implants as a novel method of exposure. Environ Int 2007; 33:257–264.

Vos JG, Dybing E, Greim HA, et al. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit Rev Toxicol 2000; 30:71–133.

Waid JS. PCBs in the environment (Vol. I–III). Cleveland, OH: CRC Press, 1985–1987.

Walker BG, Boersma PD, Wingfield JC. Field endocrinology and conservation biology. Integr Comp Biol 2005a; 45:12–18.

Walker BG, Boersma PD, Wingfield JC. Physiological and behavioral differences in Magellanic penguin chicks in undisturbed and tourist-visited locations of a colony. Conserv Biol 2005b; 19:1571–1577.

Walsberg GE. How useful is energy balance as an overall index of stress in animals? Horm Behav 2003; 43:16–17.

Wasser SK, Bevis K, King G, et al. Noninvasive physiological measures of disturbance in the Northern spotted owl. Conserv Biol 1997; 11:1019–1022.

Wayland M, Smits JEG, Gilchrist HG, et al. Biomarker responses in nesting, common eiders in the Canadian Arctic in relation to tissue cadmium, mercury and selenium concentrations. Ecotoxicology 2003; 12:225–237.

Wayland M, Gilchrist HG, Marchant T, et al. Immune function, stress response, and body condition in Arctic-breeding common eiders in relation to cadmium, mercury, and selenium concentrations. Environ Res 2002; 90:47–60.

WHO/IPCS. Global Assessment of the State-of-the-Science of Endocrine Disruptors. In: Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G, eds. WHO/IPCS/EDC/02.2, Geneva, Switzerland: WHO; 2002.

Wingfield JC, Ramenofsky M. Hormones and the behavioral ecology of stress. In: Balm PHN, ed. Stress Physiology in Animals. Sheffield, UK: Sheffield Academic Press; 1999:1–51.

Wingfield JC, Romero LM. Adrenocortical responses to stress and their modulation in free-living vertebrates. In: McEwen BS, ed. Handbook of Physiology. Section 7: The Endocrine System, Vol. 4: Coping with the Environment: Neural and Endocrine Mechanisms. Oxford, UK: Oxford University Press; 2001:211–236.

Wingfield JC. Control of behavioural strategies for capricious environments. Anim Behav 2003; 66:807–815.

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about the book...

Despite being regarded as the most common toxicological target in the endocrine system, the adrenal gland has often been neglected in regulatory testing. ***Adrenal Toxicology*** addresses the increased interest in adrenocortical toxicology and the need for a resource that makes techniques available to examine adrenal endocrine disruption.

Examining current techniques and the latest advancements, ***Adrenal Toxicology*** reviews the endocrinology, pharmacology, pathology and toxicology of the adrenal gland. This text provides information on the range of drugs and chemicals that affect adrenocortical function and suggests standardized approaches for in vivo and in vitro assessment. This volume also presents recent developments in the molecular mechanisms of toxicity to the adrenal cortex and medulla, and considers environmental adrenal endocrine disruption in sentinel species.

Adrenal Toxicology:

- reflects the major developments made over the past decade
- focuses on the latest research techniques, including their uses and limitations
- provides an integrated strategy for adrenal toxicology evaluation
- identifies knowledge and data gaps, providing impetus for regulatory consideration

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