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Human Growth Hormone

Research and Clinical Practice

Edited by
Roy G. Smith, PhD
Michael O. Thorner, MD

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PREFACE

In *Human Growth Hormone: Research and Clinical Practice*, we have been fortunate to be able to convince many of the leaders in the field to write about the recent developments in the understanding of basic and clinical research in the field of human growth hormone. During the last few years, there have been major advances in this field, one that has been dramatically enhanced by the discovery of the growth hormone-releasing peptide. This spawned much novel research, and ultimately led to the cloning of the receptor for the growth hormone secretagogues. The understanding of the molecular biology, structure, and function of growth hormone and the growth hormone receptor complex has also set a new standard in the understanding of the structural biology of cell signaling.

Growth hormone secretagogues and GHRH offer new possibilities for the treatment of growth hormone deficiency states. Growth hormone has an important role, not only in stimulating growth, but also in the control of metabolism. With the major recent advance in understanding the molecular mechanisms of the growth hormone axis, it is now possible to identify molecular defects in the axis.

It is our intention that *Human Growth Hormone: Research and Clinical Practice* should serve as an up-to-date summary of the field and should be of benefit both to the basic and clinical researchers as well as clinical endocrinologists who are now beginning to use growth hormone, not only in growth hormone-deficient states in childhood, but in the adult and also for dealing with metabolic derangements associated with catabolic disease.

Roy G. Smith

CONTENTS

Part II Clinical Practice

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1 Overview of Human Growth Hormone

Research and Clinical Practice

Roy G. Smith, PhD

Contents

INTRODUCTION

Investigations into the potential clinical applications of growth hormone (GH) has increased over the past 10 years, following the availability of human recombinant GH (hGH). Although GH replacement had proven to be effective and well-tolerated in GH-deficient children, its use was not widespread until the mid-1980s. Until then, supply had been limited because GH was obtained by extraction from pituitary glands of human cadavers. The purity of the extracted GH became an issue because of an association with outbreaks of Jacob-Creutzfeldt disease, a fatal neurodegenerative disorder. However, the manufacture of recombinant hGH in the 1980s prompted investigators to evaluate uses of GH beyond the treatment of GH-deficient children. As a result, investigators were encouraged to learn more about the basic mechanisms controlling the episodic nature of GH release and about the function of GH at the cellular level. In this introductory chapter, I first review the regulatory role of growth hormone releasing-hormone (GHRH), somatostatin (sst or SRIF), and the GH-secretagogue receptor (GHS-R), referring to relevant chapters in this volume. I then review the content of the remaining chapters. This will provide a brief summary of the current molecular understanding of the GH receptor and its potential role in the central nervous system (CNS) followed by an overview of the clinical aspects of GH deficiency, indications for its replacement, and associated benefits of direct and indirect GH replacement.

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THE ROLES OF GHRH, SST, AND GHS-R IN REGULATING GH RELEASE

GH Release Is Pulsatile

Like many hormones, GH is released episodically and is regulated by tightly controlled feedback pathways. This tightly controlled process provides a mechanism that has presumably been optimized biologically for hormone-receptor interactions resulting in activation, inactivation, and reactivation of signal transduction cascades. The frequency of GH pulses appears to be conserved in all mammalian species, occurring at approx 3-h intervals. How this frequency is regulated is a topic that continues to hold great fascination for scientists. Historically, GH secretion was considered to be regulated by a positive/negative feedback loop controlled by the two hypothalamic hormones, GHRH and sst. GHRH is released from arcuate neurons into the median eminence and transported through the portal vessels to the pituitary gland, where it stimulates GH release from somatotrophs. Negative feedback is thought to be mediated by GH stimulating the release of sst from hypothalamic neurons. sst acts to inhibit GH release from the pituitary gland.

The discovery of the GH-releasing peptides (GHRPs) by Bowers was a very important episode in defining the characteristics of the regulation of GH secretion, because the GHRPs do not act directly through the GHRH or sst receptors (Chapter 2). Based on Bowers' discovery, a series of nonpeptide mimetics were designed to allow optimization of oral bioavailability and other pharmacokinetic properties, resulting in the development of MK-0677 (Chapter 3). A single oral dose of MK-0677 amplified GH pulses for 24 h *(1)*. To distinguish the GHRPs and their mimetics from the natural hormone GHRH, the synthetic molecules were termed "GH-secretagogues" (GHS).

GHS-Receptor (GHS-R)

Early studies had suggested that GHRPs and their mimetics elicited their effects on GH release through a pathway distinct from that of GHRH *(2)*. Characterization of the receptor was frustrated by the very low abundance of binding sites in the pituitary gland. However, by incorporating ^{35}S into the MK-0677 molecule, a high specific activity radioligand was synthesized *(3)*. Using this ligand, Pong et al. showed that MK-0677 bound with high affinity (Kd = 200 p*M*) to the plasma membrane fraction of pituitary and hypothalamic tissues *(2,4)*. The concentration of binding sites was remarkably low (2 fmole and 6 fmole/mg protein in pituitary and hypothalamic membranes, respectively). ^{35}S-MK-0677 binding was competitively inhibited by L-692,429, MK-0677, and GHRPs, but not by GHRH or sst; thus, this new receptor is selective for this specific class of synthetic growth hormone secretagogues (GHS). High affinity binding was inhibited noncompetitively by GTP-γ-S, suggesting that the receptor was G-protein coupled *(2,4)*.

The identification of the first of the optimized drug candidates, MK-0677, led to the cloning and molecular characterization of a new orphan G-protein coupled receptor for the GH-secretagogues, GHS-R. Activation of the GHS-R by synthetic ligands initiates and amplifies pulsatile GH release in animals, including humans *(5)*. This new receptor was cloned using a strategy that exploited the observation that ligands for this receptor activated IP_3-induced release of intracellular Ca^{2+} (Chapter 4). Based on this property, the receptor was expression-cloned from a pituitary cDNA library using *Xenopus* oocytes (Chapter 4). The predicted amino-acid sequence of the receptor (GHS-R) was consistent

with biochemical studies that predicted it belonged to the G-protein coupled receptor family. Based on knowledge derived from the structure of other G-protein coupled receptors, the X-ray structure of MK-0677 and energy calculations derived from nuclear magnetic resonance (NMR) studies with MK-0677, a three-dimensional model for the ligand-receptor complex was proposed *(6,7)*. A series of experiments were designed to test the validity of the model. The orientation of the receptor in the cell membrane was examined using antibodies generated to peptide sequences that, according to the model, were in the extracellular and intracellular loops. Immunofluorescence studies on intact and permeablized cells expressing GHS-R confirmed the orientation of the extracellular and extracellular loops *(6)*.

The binding pocket occupied by MK-0677 was characterized using selected site-directed mutagenesis based on a computer-generated, space-filling model (Fig. 1) for the receptor–ligand complex *(7)*. The consequences of mutating each amino acid were evaluated by measuring the ability of different ligands to activate the mutated receptors *(6)*. The model illustrated in Fig. 2 suggests that E124 in helix 3 serves as a counter-ion to the basic N in the MK-0677 side chain, and this was confirmed by site-directed mutagenesis. Docking the structurally distinct agonists L-692,585 and GHRP-6 into the pocket also supported this hypothesis *(6)*.

Localization of GHS-R Expression

In situ hybridization studies using selective nonoverlapping radiolabeled oligonucleotides showed that GHS-R was expressed in the pituitary gland and brain *(8)*. In the rat pituitary gland, expression of GHS-R was confined to the anterior lobe *(8)*. This observation was consistent with experiments showing that GHRP-6 and its mimetics selectively activate somatotrophs and somatomammotrophs, and that a fluorescently tagged analog of MK-0677 selectively binds to GH-producing cells *(2,5)*. Based on a combination of RNAse protection assays and *in situ* hybridization to different tissues, it appears that GHS-R expression is confined to the anterior pituitary gland and CNS *(8)*.

In the brain, the GHS-R is widely expressed *(8)*. Intriguingly, although we anticipated expression in those nuclei that play a role in the control of GH release, the receptor is also expressed in areas of the brain that affect mood, cognition, memory, learning, feeding, and sleep. The GHS-R mRNA is expressed in multiple hypothalamic nuclei including anteroventral preoptic nucleus, anterior hypothalamic area, suprachiasmatic nucleus, lateroanterior hypothalamic nucleus, supraoptic nucleus, ventromedial hypothalamic nucleus, arcuate nucleus, paraventricular nucleus, and tuberomamillary nucleus *(8)*. Expression is also found in the dentate gyrus of the hippocampal formation, the CA2 and CA3 areas of the hippocampus, the pars compacta of the substantia nigra, ventral tegmental area, dorsal and medial raphae nuclei, and Edinger-Westphal nucleus *(8)*. The functional significance of expression of the GHS-R in areas of the brain other than those involved in GH release is critical for our understanding of the complete physiological significance of the GHS-R. Evidence for activation of this receptor in the CNS by GHRPs and mimetics is presented in Chapter 5.

Interaction of GHS-R, GHRH, and SST Hypothalamic Neurons

The key role of the hypothalamic hormone GHRH in stimulating GH release from the pituitary gland is well-established *(9)*. However, because its biological half-life is short, a series of analogs have been synthesized in attempts to develop compounds with

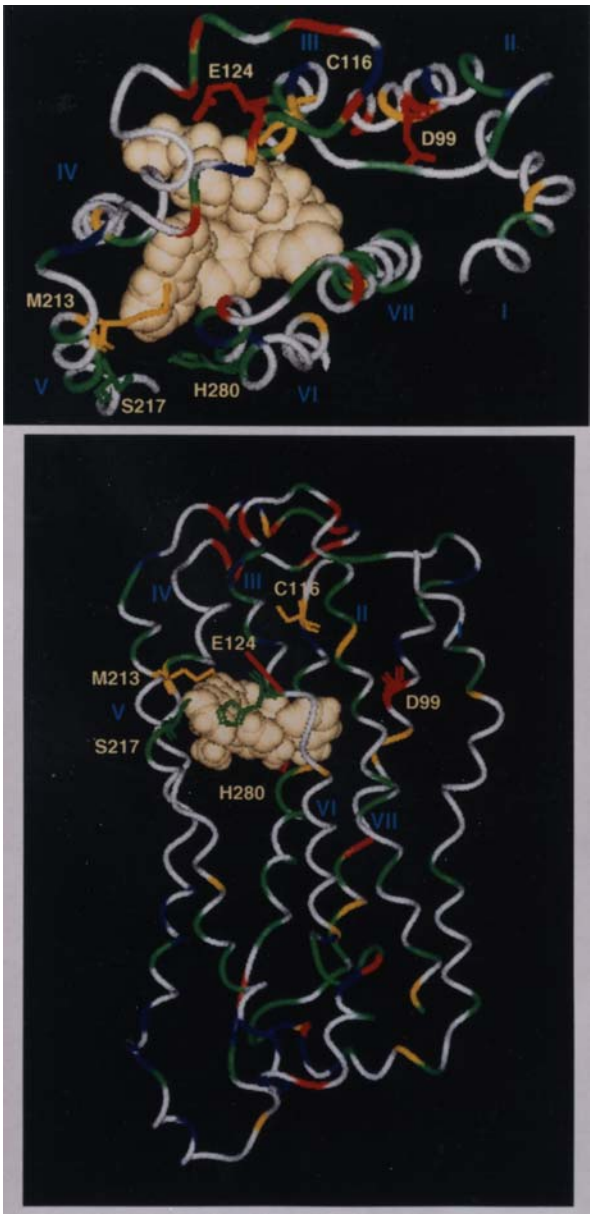

Fig. 1. Computer-generated, space-filling model of GHS-R. Ligand complex shows proximity of critical amino acids *(7)*.

increased potency and improved pharmacokinetics for use in the clinic (Chapter 6). An alternative clinical approach is to identify a compound that induces the release of GHRH from arcuate neurons in the hypothalamus. The localization of *GHS-R* in the arcuate nucleus implicated the GHS-R ligands as regulators of GHRH release. Intravenous administration of the ligands results in stimulation of c-*fos* and electrical activity in arcuate neurons that project to the median eminence (Chapter 5; *[10]*). *In situ* hybridization studies to monkey and rat brains showed that *GHS-R* is expressed in

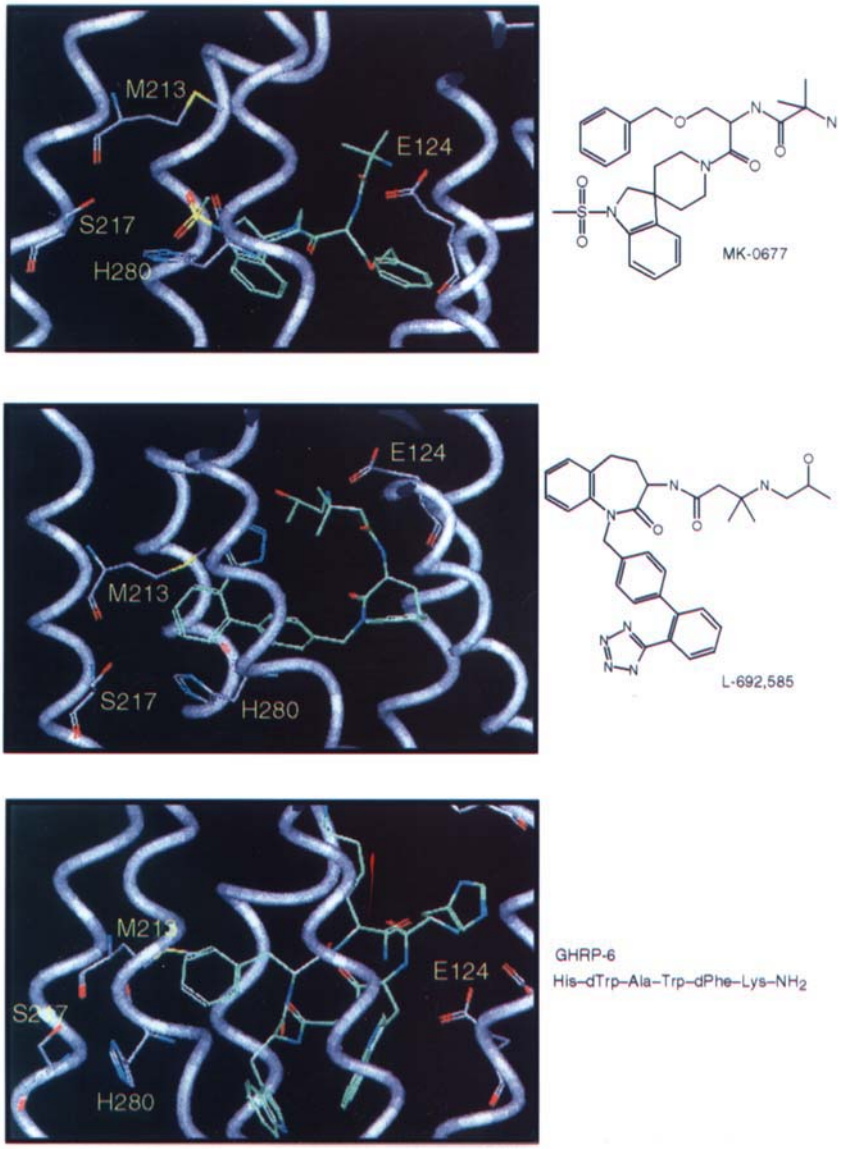

Fig. 2. Three-dimensional structure of ligand-binding pocket of the human GHS-R with MK-0677, L-692,585, and GHRP-6 docked *(6)*.

arcuate neurons, suggesting that the GHS-R ligands stimulate these neurons directly *(5)*. A recent study using immunohistological techniques showed that the *GHS-R* is expressed in GHRH neurons *(11)*. Furthermore, studies in sheep have shown that GHRH is released into the portal vessels immediately following treatment with a GHS-R hexapeptide ligand *(12)*. Collectively, these data are consistent with GHS-R ligands acting directly on arcuate neurons to stimulate the release of GHRH. Activation of these neurons by MK-0677 can be inhibited by sst or pretreatment with GH *(13)*. These important studies link the action of the GHS-R ligands with the two known endogenous regulators of GH-release GHRH and somatostatin.

GH self-entrains the ultradian rhythm of episodic GH release *(14,15)*. Presumably, the negative feedback phase is either mediated directly by GH, or indirectly by causing the release of sst. Ligand binding to GHS-R results in functional antagonism of sst by depolarization of target cell membranes *(16)*. Hence, if sst is involved in sustaining the rhythm of GH pulsatility, it follows that the rhythm can be interrupted by ligands that activate the GHS-R. Indeed, this is precisely what is observed in animals. Administration of GHS-R ligands immediately stimulate GH release and, as a consequence, the GH ultradian rhythm is reset *(16,17)*. These observations support the suggestion that sst plays a critical role in the control of the pulse frequency of GH release *(18,19–25)*.

It is reasonable to speculate that feedback control is regulated through GHRH containing arcuate neurons. Negative feedback could be regulated directly by GH or indirectly by GH causing the release of sst from hypothalamic neurons. To determine whether sst is involved, we generated transgenic mice in which the *somatostatin receptor subtype-2 (sstr2)* gene was selectively inactivated. Of the five sst subtypes (Chapter 7), we selected sstr2 because this particular subtype had already been implicated in the regulation of GH release *(26)*. Subsequent studies with more selective nonpeptide sstr2 agonists support these earlier studies *(27,28)*. The *sstr2* was inactivated by homologous recombination in mouse embryonic stem cells *(13)*. Mice homozygous for the deleted *sstr2* allele appeared normal and healthy and were indistinguishable in appearance from *sstr2* intact animals. The central effects of GH and MK-0677 were evaluated using induction of Fos immunoactivity to monitor activation of hypothalamic neurons *(13)*. In parallel, wild-type and *sstr2* null mice were treated with GH or placebo 10 min prior to injection with MK-0677 or vehicle. Thirty minutes later, the mice were sacrificed and brain sections isolated to measure Fos expression by immunohistochemistry. In both wild-type and *sstr2*, knockout mice treatment with GH caused expression of Fos in the periventricular nucleus, but not in the arcuate nucleus. In wild-type mice, pretreatment with mouse GH completely prevented MK-0677 activation of Fos in arcuate neurons. By contrast, in *sstr2* null mice, pretreatment with GH failed to prevent activation of Fos in arcuate neurons. These results are consistent with GH-mediated negative feedback on GHRH neurons being regulated through sst and specifically sstr2. However, more work is needed before concluding that GH induced sst release is sufficient to explain entrainment of the 3 h pulses of GH.

The results summarized above with the *sstr2* $^{-/-}$ and wild-type mice suggest that GH pulsatility is regulated at the hypothalamic level through GH receptors on periventricular neurons. Activation of these neurons results in the release of sst to suppress the activity of GHRH neurons in the arcuate nucleus. This interpretation is supported by electrophysiology experiments where stimulation of periventricular neurons results in inhibition of arcuate neurons *(29,30)*. However, histological evidence for the projection of periventricular neurons to the arcuate nucleus is lacking at this time. An alternative explanation is that the negative feedback signal is mediated from periventricular to the arcuate nucleus indirectly through the basal lateral amygdala (BLA); electrical stimulation of BLA neurons also inhibits activity of arcuate neurons *(29,30)*. The amplitude of GH pulses is apparently regulated through the GH receptor. When GH-receptor antisense RNA is administered to rats by intracerebral ventricular injection, the amplitude of GH pulses increases *(31)*. Whether the effect is mediated indirectly by sst at the hypothalamic or pituitary level is not clear; however, the GH receptor apparently plays a pivotal role in the regulation.

Figure 3 illustrates a model of GH/sst mediated feedback regulation through arcuate neurons that express GHRH, GHS-R, and sstr2. Although not illustrated in Fig. 3, sst

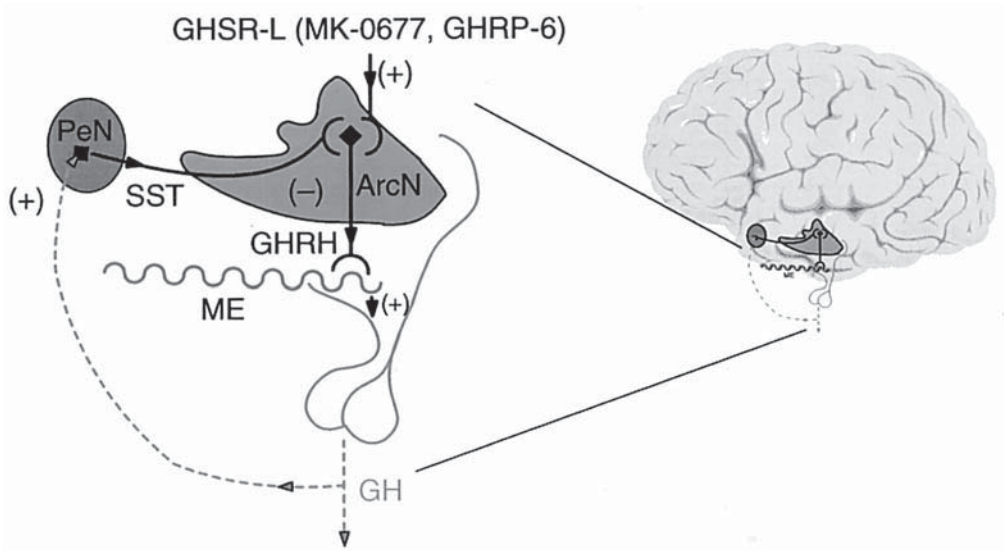

Fig. 3. Model of interaction among GH, sst, and GHS-R ligands and their potential role in the entrainment of GH pulsatility *(13)*.

also acts directly on the pituitary gland. However, we have speculated that modulation of neuronal activity in the hypothalamus via GHS-R and sstr2 is the primary mechanism regulating the timing of the GH pulses. Mathematical modeling of the GHRH, GH, and sst feedback pathway is presented in Chapter 8.

Potential Significance of GHS-R Expression in the Suprachiasmatic Nucleus (SCN)

GHS-R is expressed at relatively high levels in the SCN, suggesting it may play a role in the control of circadian rhythms *(8)*. Ligand activation of GHS-R in all species studied causes immediate GH release, which initiates a new pulsatile cycle *(5)*. This is perhaps not surprising because GH self-entrains the GH rhythmicity *(14)*. In elderly humans who were treated chronically with the GHS-R ligand MK-0677, a change in sleep patterns was noted, with a 50% increase in REM sleep *(32)*. These findings suggested that MK-0677 simultaneously improves the quality of sleep and corrects the relative hyposomatotropism of senescence. Whether these effects on sleep are mediated directly by stimulation of receptors in the SCN or indirectly through the GH pathway has yet to be elucidated.

DEVELOPMENTS IN BASIC SCIENCE OF GH

GH Receptor and Signal Transduction

The receptor for GH is localized on the plasma membrane of GH-responsive cells. The receptor consists of a large extracellular domain, a single transmembrane helix, and an intracellular domain. Until relatively recently, the cellular mechanisms by which GH transduced its signal following binding to the extracellular domain was unknown. Elucidation of the pathway has involved an extremely elegant combination of biochemistry, mutagenesis, and crystallographic studies (Chapter 9). It has been concluded that a single GH molecule binds asymmetrically to the extracellular domain of two receptor molecules, causing the receptor to dimerize. This dimerization process triggers tyrosine phosphoryla-

tion of the intracellular domains of the receptor, the JAK2 kinase, and several STAT proteins. Chapter 9 provides a detailed account of the nature of the GH/GH-receptor complex and the signal transduction pathway activated following receptor dimerization.

Traditionally, we consider that the main targets of GH action are liver, muscle, and bone, where the action is partially mediated indirectly through increases in IGF-1. However, evidence is emerging that supports a role for GH in the brain (Chapter 10). GH receptors are developmentally regulated in the CNS and GH deficiency has been implicated in deficits in brain development. Furthermore, GH treatment has been shown to result in changes in neurotransmitters. Expression of GH-receptors in the hippocampus suggests a role for GH in memory and learning. In Chapter 10, experimental approaches to investigate the CNS as a target for GH action are reviewed.

CLINICAL APPLICATIONS OF GH REPLACEMENT

Background

Chapter 11 reviews the molecular elements affecting different parts of the somatotropic axis that result in GH deficiency. The process by which each of these various elements can be diagnosed is also included. Genetic causes of GH deficiency are relatively rare. Traditionally, the deficiency was classified based on mode of inheritance. However, now that we have a better understanding of the elements controlling the GH axis and knowledge of the molecular genetics involved, GH deficiency can be classified according to molecular abnormalities. For example, three important factors involved at the level of the pituitary gland are the GHRH-receptor, GH, and pit-1. Abnormalities in genes encoding these key factors will be expressed as some form of functional GH-deficiency. A normal GHRH receptor is critical for the signal to release GH from the somatotroph. The GH that is released must be functional. A normal pit-1 protein is essential because this specific transcription factor is required for differentiation to produce somatotrophs and for GH and GHRH-receptor gene transcription. Mutations in GHRH-receptor, GH, and pit-1 have indeed been identified in humans exhibiting a GH deficient phenotype (Chapter 11). We also assume that a functional GHS-R is important, because, as previously described, ligands for this receptor control the amplitude of GH release. However, we might anticipate that because the GHS-R pathway appears to play a permissive role in concert with GHRH, a GH deficiency involving a nonfunctional GHS-R would result in a less obvious phenotype than that from a nonfunctional GHRH receptor. Complete evaluation of the significance of the GHS-R pathway as it relates to GH deficiency awaits identification of the endogenous ligand for the GHS-R.

GH Replacement in the Clinic

Traditionally, GH use was confined to treatment of GH-deficient children because of the importance of this hormone in maintaining normal growth velocity. The pathophysiology of GH deficiency in children and the ethical issues relating to treatment of short-statured children is discussed in Chapter 12. Until relatively recently, many physicians considered that GH was unimportant after linear growth had ceased. However, the availability of recombinant hGH has provided the physician with a valuable product to investigate the potential of GH replacement in a variety of clinical situations. The rationale for GH replacement in adults is presented in Chapter 13. For example, it is well established that GH plays an important role in determining body composition to maintain a beneficial ratio between skeletal muscle and fat. GH-deficient adults have reduced exercise toler-

ance; hence, GH also appears to be important for muscle function. It is becoming clear that GH provides an important function during adulthood. The preliminary reports of the benefits of GH treatment in age-related disorders such as frailty associated with progressive muscle loss, and accelerating recovery from hip fractures, are encouraging *(33)*.

It is well-known that during aging, there is a progressive decline in the amplitude of the episodic release of GH from the pituitary gland. In humans, the most marked change begins around age 30 yr and is accompanied by an age-related loss in muscle mass and an increase in the fat/lean mass ratio. These changes in body composition might be a consequence of metabolic changes that occur as a result of the age-related reduction in GH secretion. Whether this observation means that GH deficiency is a disease of aging and that GH replacement would prevent the physical decline associated with aging are provocative issues. Carefully controlled clinical studies are needed to address this question.

Rudman's group was the first to report beneficial effects of GH replacement in the geriatric population *(34)*. In 21 healthy men 61–81-yr-old, GH administration over a 6-mo period produced marked improvements in muscle tone, skin thickness, lean body mass, and density of the lumbar vertebrae. Accompanying the increases in lean body mass was a significant loss in fat mass. Based on these results, it was concluded that 6 mo of GH treatment reversed the effects of 20 yr of aging on lean muscle mass and adipose tissue. Rudman's publication prompted additional investigations. Although improvements in lean muscle mass have been a consistent finding, investigators have been unable to reproducibly document clear improvements in muscle function. Furthermore, in many cases, GH treatment is often poorly tolerated in the elderly. However, when lower doses are used, the incidence of adverse side effects is significantly reduced. Long-term, well-controlled studies in the geriatric population are now needed before deciding who will benefit most from GH replacement.

It is well-established that GH promotes longitudinal bone growth, and it has become apparent that GH also plays a role in bone metabolism. GH stimulates the proliferation of osteoblasts and promotes bone formation. In humans, GH deficiency is associated with osteoporosis; Chapter 14 addresses this relationship.

An association between a series of risk factors involved in noninsulin dependent diabetes and myocardial infarction (MI) is well known. The term "Syndrome X" is commonly used to describe the link between insulin resistance and hypertension. A critical factor relating to this syndrome is believed to be the mass of intra-abdominal fat. Since reduced GH secretion is associated with obesity and GH administration results in a preferential reduction in abdominal fat mass, GH treatment may prove beneficial in prevention and treatment of Syndrome X (Chapter 15).

GH secretion is markedly stimulated during sleep. Indeed, in humans the relationship between sleep onset and GH secretion is remarkably consistent. Intriguingly, a number of studies suggest that stimulation of GH release and promotion of sleep represent independent outputs from distinct populations of hypothalamic GHRH-containing neurons (Chapter 16). Indeed, it can be hypothesized that the well-documented age-related decrease in the amplitude of GH is associated with the changes in sleep patterns in the elderly. Sleep fragmentation has been linked to decreases in nocturnal GH secretion. A relationship between components of the somatotropic axis and sleep patterns is suggested by studies in transgenic mice as well as humans. For example, transgenic mice with a deficiency in this axis experienced a reduction in non-REM sleep and treatment of GH-deficient subjects with exogenous GH produced increased REM sleep. The precise

relationship between components of the somatotropic axis and sleep is complex. A critical review of the basic science and clinical studies is provided in Chapter 16.

GH administration appears to have beneficial effects in reversing catabolic states caused by malnutrition and severe illness. Alterations in the GH/IGF-1 axis that are associated with metabolic disturbances have been described in subjects with HIV infection. For example, increasing energy intake does not consistently restore lean body mass in HIV patients. Therefore, based on the knowledge that GH is effective in stimulating nitrogen retention in catabolic patients, GH and IGF-1 treatment have been used to treat wasting associated with HIV infection. A summary of these clinical studies is reviewed in Chapter 17.

Clinical Applications of GHRH

GHRH treatment is a more physiological method for treating GH deficiency where reduced GH secretion is owing to hypothalamic causes (Chapter 18). Its advantage—compared to direct GH replacement—is that it stimulates an episodic pattern of endogenous GH release. However, like GH, it must be administered by injection, although oral and long-acting formulations are under development. Compared to the GHS-R ligands, GHRH itself has a very short half-life, but analogs that have increased potency and longer half-lives are being optimized as potential clinical candidates (Chapter 6). The advantage of GHRH treatment over treatment with an orally active GHS-R ligand such as MK-0677 is apparent in cases where there is a lesion in the hypothalamic-pituitary stalk. For optimal activity, the GHS-R ligands require the presence of GHRH; however, in those subjects not having an intact hypothalamic/pituitary axis, the synergy between GHRH and GHS-R ligands could be used to advantage.

Clinical Applications of GHS-R Ligands

The inevitable age-related reduction in the amplitude of GH pulses is explained either by decreased production or reduced secretion of GH by the pituitary gland. In elderly humans, GHRH administration increases GH release. Hence, the decrease in GH during aging is explained by a change in the factor(s) that stimulates GH secretion. This interpretation has been confirmed by treating elderly subjects chronically with the synthetic GH-secretagogue MK-0677 for up to 12 mo. Taken orally, once daily, MK-0677 produced a pulsatile profile of GH release in this elderly population (70–89 yr) that was typical of that of subjects in their late twenties *(1)*. When GH deficiency in the elderly was replaced in this more physiological way, it was well-tolerated. It is worth noting that although the use of agents such as MK-0677 is a more natural way to treat GH deficiency, the amount of GH released from the pituitary gland is limited by feedback inhibition, consequently, pharmacological intervention is not feasible *(5)*. Moreover, optimal stimulation of the physiological pathway by MK-0677 requires an intact hypothalamic/pituitary axis.

In the clinic, the GHRPs and their nonpeptide mimetics present a number of potential therapeutic opportunities for treating GH-deficient states by activating the release of endogenous GH (reviewed in Chapter 19). In particular, MK-0677 offers an alternative form of therapy to injectable GH, because it offers the advantage of oral dosing and a physiological GH profile. In the elderly, the physiologic GH profile appears to translate into improved tolerability compared to GH injections *(1,35,36)*. MK-0677 was effective in increasing GH, IGF-1, and IGFBP-3 in a selected group of severely GH-deficient men, suggesting MK-0677 may have a role in the treatment of GH deficiency of childhood

onset *(35)*. In a model of short-term, diet-induced nitrogen wasting in healthy young men, 7 d treatment with MK-0677 resulted in a reversal of nitrogen wasting, suggesting that MK-0677 may be useful in treating catabolic conditions. Again, this beneficial effect was associated with sustained increases in serum concentrations of GH, IGF-1, and IGFBP-3 *(37)*. The effects of MK-0677 were evaluated in a population of obese men. Interestingly, although the men failed to lose weight during 2-mo treatment with MK-0677, they experienced an increase in fat-free mass, and increased energy expenditure *(38)*.

A potential limitation of the clinical application of GHRPs and their mimetics compared with treatment with GH and GHRH is that the GHS-R pathway is subject to negative feedback *(2)*. Therefore, the sustained supraphysiological GH and IGF-1 levels that can be attained by injecting GH or GHRH are not possible with the GHS-R ligands. Moreover, the optimal effect on GH release requires an intact hypothalamic/pituitary axis *(39,40)*. For these reasons, the efficacy of the GHS-R ligands in some situations is likely to be limited. It is also unknown what type of pharmacodynamic profile will provide the broadest clinical utility. The GHRPs and the nonpeptides such as L-692,429, L-692,585, and L-163,540 are short-acting molecules that—during chronic once-daily dosing—provide an acute GH response without desensitization and without any appreciable increase in serum IGF-1 levels *(7)*. By contrast, MK-0677 is long-acting and produces a markedly reduced GH response during chronic administration. Furthermore, serum IGF-1 levels increase during the first 24 h following treatment and in spite of a reduced GH response during continued treatment, the increase in IGF-1 is sustained *(2)*. Another unanticipated benefit associated with chronic dosing of MK-0677 is that, in contrast to GHRPs and the short-acting mimetics, there are no measurable increases in serum cortisol and prolactin *(5)*. However, at this time, the selection of the ideal pharmacokinetic and pharmacodynamic properties of a GHS-R ligand for optimal treatment of a particular medical indication awaits the outcome of appropriate clinical trials.

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I RESEARCH

2

GHRP Historical Perspective

Basic and Clinical

C. Y. Bowers, MD

CONTENTS

INTRODUCTION

The growth hormone releasing peptides (GHRPs), in contrast to growth hormone releasing hormone (GHRH), were invented rather than discovered. "Reverse pharmacology," a term recently proposed by Michael Conn, was suggested to designate the developmental GHRP process *(1)*. GHRPs and their mimetics will undoubtedly have a clinical role in the future. Two immediate future objectives of salient importance will be isolation and identification of the putative native hormone for which GHRPs are mimetics and elucidation of its role in the physiological secretion of GH. Moreover, whether this hormone is involved in the pathophysiology of GH deficiency in children and adults is still to be determined.

HISTORICAL BACKGROUND

Between 1976–1980, during the development of GHRPs, a major impetus for the search was an unequivocal belief in the existence of a native (GHRH) in spite of the frustrations of unsuccessful, herculean efforts over a 15-year period (1962–1976). Interestingly, GH releasing activity was demonstrated in more than one partially purified fraction of porcine hypothalamic extracts, suggesting that perhaps more than one GHRH factor existed *(2–5)*.

Listed in Table 1 are the major GHRP milestones in chronological order. The first GHRP ($DTrp^2$) was developed in 1976 *(4,5)*. The amino-acid sequence is recorded in Table 2. Although $DTrp^2$ was not potent and was inactive in vivo, it released GH by a direct action on the pituitary. In addition, the GH action was specific in that LH, FSH, TSH, and PRL were not released (Fig. 1). This $DTrp^2$ pentapeptide, $TyrDTrpGlyPheMetNH_2$, evolved from the natural opiate Met enkephalin pentapeptide, TyrGlyGlyPheMet. Opiates and opiate peptides release GH via a hypothalamic action but not via a direct pituitary action. Furthermore $DTrp^2$ had no opiate activity.

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Table 1
Major GHRP Milestones

1976–77	First GHRPs
1978–80	New types of GHRPs
1980	In vivo active GHRPs
1984	Projection of a new hormone(s)
1989–92	GH release in humans
1992	Increased pulsatile GH secretion, young men
1992	First nonpeptidyl GHRP
1994–95	Increased body-growth velocity, children
1995	Potent new types of GHRP
1996	GHRP receptor cloned

Table 2
Key GHRPs[a]

Active only in vitro[b]		*Inactive in vitro*[b]
1. $TyrDTrpGlyPheMetNH_2$	($DTrp^2$)	1. $TyrGly^2GlyPheMetNH_2$
2. $TyrAlaDTrpPheMetNH_2$	($DTrp^3$)	2. Trp
3. $TyrDTrpDTrpPheNH_2$	($DTrp^{2,3}$)	3. Phe
4. $TyrDTrpAlaTrpDPheNH_2$	($DTrp^2LTrp^4$)	4. Pro
		5. Sar
		6. DVal
		7. DAla
		8. DLeu

[a]Reproduced with permission from ref. *64*.
[b]Dose: 100 μg/mL in vitro.

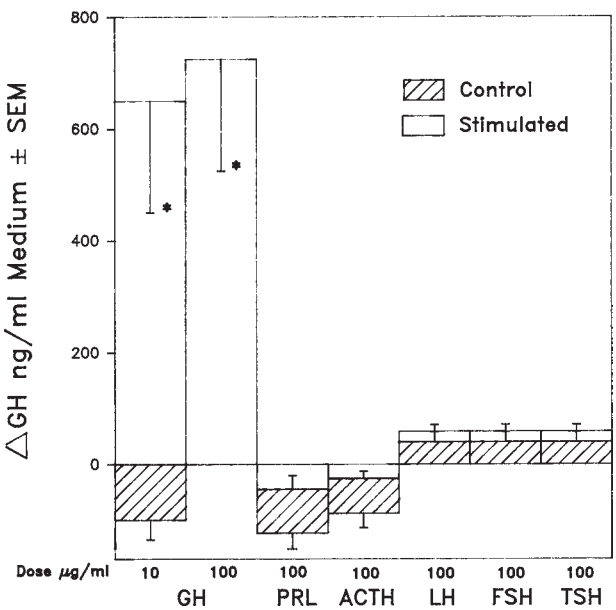


Fig. 1. Effect of $TryDTrp^2GlyPheMetNH_2$ on GH release in vitro. There was no effect on PRL, ACTH, LH, FSH, or TSH release. Pituitaries of 20-d-old female rats, $n = 6$, $*p = < 0.01$.

Table 3
The In Vitro and In Vivo Specificity of Activity of [His^{1}Lys^6]GHRP in Rats[a]

Peptide dose	*GH*	*TSH*	*LH*	*FSH*	*PRL*
μg/mL medium	In vitro (Δmg/mL serum ± SEM)				
—	–203 ± 109	–1372 ± 945	22 ± 11	248 ± 60	
0.03	2165 ± 407	–2308 ± 1230	9 ± 1	210 ± 67	
μg subcutaneously	In vivo (ng/mL serum ± SEM)				
—	6.1 ± 2.4	211 ± 32	0.05 ± 0.04	192 ± 23	4.7 ± 1.8
50	757 ± 40	210 ± 18	0.12 ± 0.04	199 ± 32	2.4 ± 0.2

[a]The in vitro studies (mean of 9 ± SEM) were performed using the pituitary incubate assay and the in vivo studies (mean of 10 ± SEM) using rats treated with 50 μg [His^{1}Lys^6]GHRP daily for 25 d sc at 1500 h. The aforementioned acute study was performed 24 h after the last injection of the peptide. Blood for hormone determinations was collected at +15 min after injection of saline or the peptide. Reproduced with permission from ref. *11*.

Between 1978 and 1980, four different major types of GHRPs were developed, including DTrp^2 (Table 2) *(6–9)*. Despite increased potency, none of these small peptides were active in vitro. Noteworthy was that GH releasing activity was strongly related to the position and stereochemistry of Trp residues. A series of detailed conformational studies by Momany helped to guide the development of the DTrp^{2}AlaLTrp^4 sequence of GHRP, which was valuable in the development of the in vitro and in vivo active GHRPs, i.e., GHRP-6, -1, -2 *(10–15)*. From desensitization crossover studies, and from synergistic or additive effects of the GHRPs, evidence strongly indicated that the same receptor and molecular mechanism was activated by structurally different GHRPs. A surprising exception, which suggested finding the possibility of a GHRP receptor subtype, was that in sheep pituitary cell cultures where GHRP-2, but not GHRP-6, raised intracellular cAMP levels; furthermore, a GHRH antagonist inhibited the GHRP-2 GH response *(16)*.

In 1976–77, early results of DTrp^2 were considered indicative that this pentapeptide may be acting via the putative GHRH receptor *(4,9)*. Subsequent studies with GHRP-6 in 1980–81 reinforced the notion. However, following the isolation of a native growth hormone releasing factor and its structural elucidation in 1982, it became apparent that the releasing factor was a natural growth hormone releasing hormone (GHRH) and that GHRP acted via a different receptor. Because GHRPs had characteristics of hypophysiotropic hormones, it was proposed in 1984 that they might mimic another native hormone different from GHRH *(11)*.

Results in Table 3 show that GHRP-6 specifically releases GH in vitro and in vivo *(11)*. The in vivo results were obtained after immature female rats were injected with GHRP-6 or saline once or twice daily subcutaneously (sc) for 25 d. After chronic administration of GHRP-6, the GH response and specificity as well as the increase in body weight gain were maintained (Table 3 and Fig. 2).

Between 1981–88, the interrelationship between the actions of GHRP-6, GHRH, and opiates or opiate peptides were studied *(10,17–28)*. Desensitization crossover studies of these three GH-releasing secretagogues revealed the independent action of all three peptides because when the GH response of one secretagogue was desensitized the other one was fully active. When these secretagogues were combined and administered to rats GH was released synergistically, and when all three were administered together, the

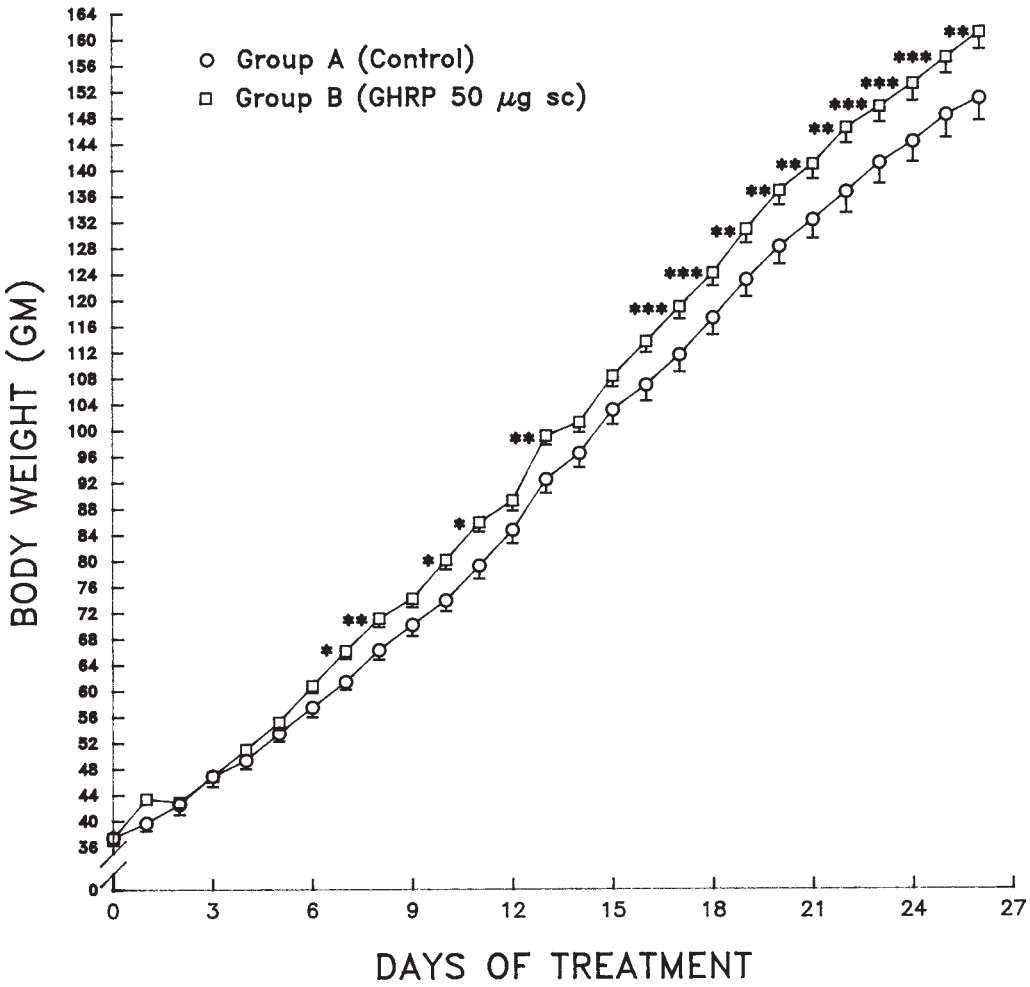
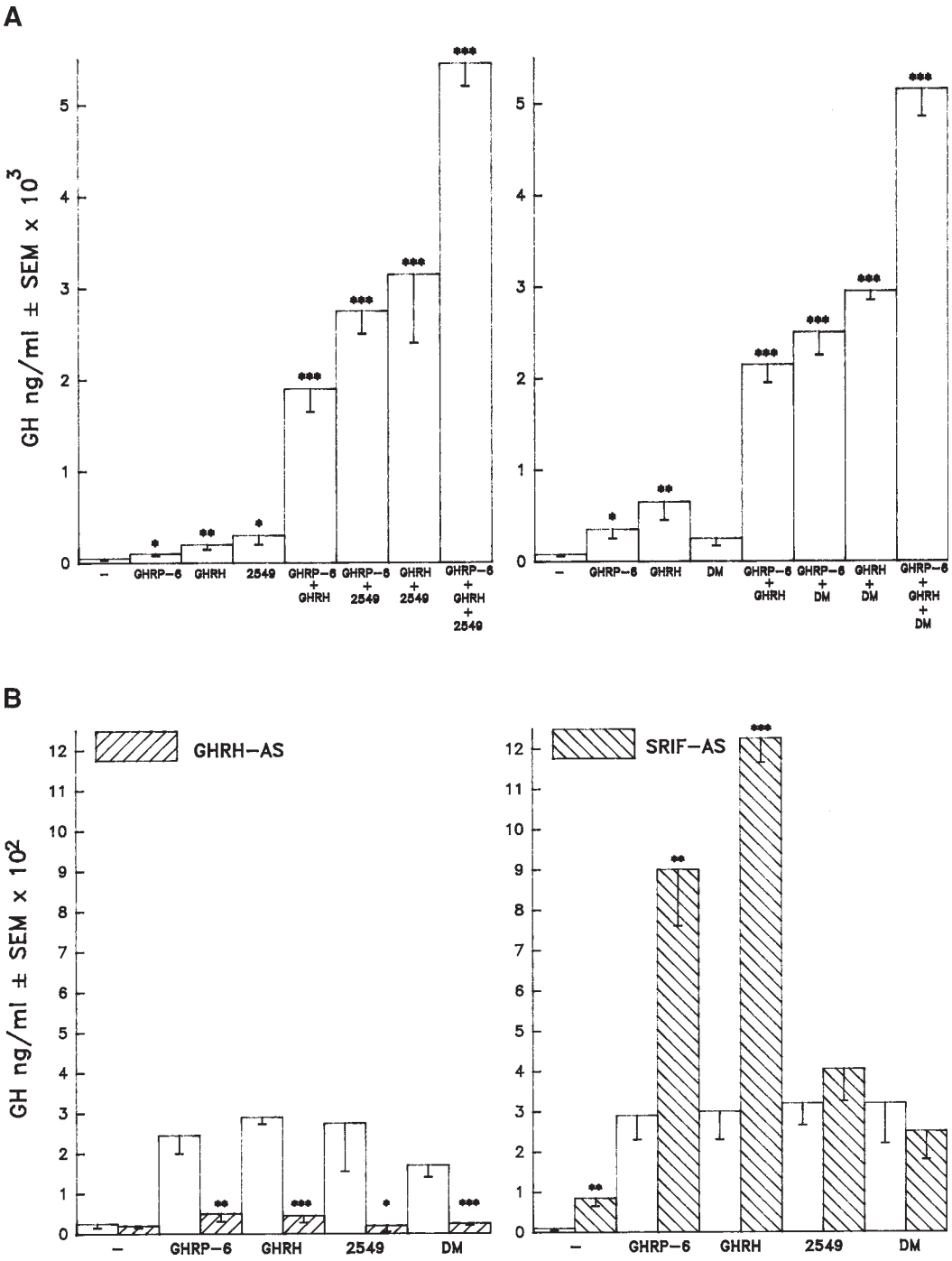


Fig. 2. Chronic treatment of immature female rats with [His^{1}Lys^6]GHRP. Initially immature female rats (16 d of age) were distributed among the mothers so that the BW in groups A and B would be the same, and treatment with saline **(A)** or the peptide **(B)** was started the next day. Saline or the peptide (50 μg) was injected sc daily at 1500 h for 25 d. Mean of 20 ± SEM. P values of treated vs untreated control group (A). *$p < 0.01$, **$p < 0.02$, ***$p < 0.05$. Reproduced with permission from ref. *11*.

synergism was even greater (Fig. 3A). Regardless of the apparent independent action of the three secretagogues, pretreatment with GHRH anti-serum markedly inhibited the GH response of each one of them (Fig. 3B). Complementary studies with somatotropin-release inhibiting factor (SRIF) antiserum pretreatment indicated that it increased the GH response to GHRP-6 and GHRH but not to the benzomorphan opiate 2549 or the opiate

Fig. 3. (A) In vivo studies on the synergistic release of GH in conscious 26-d-old female rats. At zero time, saline, 10 μg GHRP, 10 μg GHRH, 10 μg 2549 opiate, and/or 100 μg dermorphin (DM) were injected iv, and rats were killed at +10 min. Each value represents the mean of 6 ± SEM; P values are given for treated vs saline. *$p < 0.02$, **$p < 0.01$, ***$p < 0.001$. When P values were determined for the various groups vs GHRP, GHRH, plus dermorphin or 2549, the values ranged



from <0.01–<0.001. **(B)** In vivo GHRH and SRIF antiserum (AS) immunoneutralization studies on the GH responses of GHRP, GHRH 1-43OH, 2549 opiate, and dermorphin (DM) in rats. GHRH AS, SRIF AS, or normal rabbit serum (0.2 mL) was injected iv at –1 h into conscious 26-d-old female rats. At zero time, rats were injected in the tail vein with saline, 10 μg GHRP, 10 μg GHRH, 10 μg 2549 opiate, or 100 μg dermorphin and killed at +10 min. Each value represents the mean of 6 ± SEM. Peptide/2549 vs peptide/2549 plus antiserum: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table 4
GHRP Effect on GH Release in the Pituitary (P) vs Hypothalamus (H) and Hypothalamus Plus Pituitary Incubates In Vitro[a,b]

GHRP dose (ng/mL medium)	*Δ GH (ng/mL ± SEM)*		
	Pituitary	*Hypothalamus + pituitary*	*Hypothalamus[c]*
Control	7874 ± 674	15185 ± 2286	7314 ± 2079
1	9840 ± 1056	15471 ± 1612	5631 ± 805
3	10726 ± 1096	17766 ± 1574	7040 ± 1514
10	19366 ± 1325[d]	32336 ± 3161[d]	12970 ± 3001
30	22630 ± 2148[d]	38661 ± 2180[d]	16031 ± 3694[e]
100	18046 ± 2800[d]	39134 ± 1730[d]	21088 ± 4468[d]

[a]Reproduced with permission from ref. *26*.
[b]H and P from 26-d-old female rats. Values are the mean of 9 determinations. Each Δ value was calculated from three consecutive 1 h incubation periods (I_3–I_5) minus basal release of GH during the preincubation period.
[c]H + P – P.
[d]$p < 0.01$ vs control (by Newman-Keuls).
[e]$p < 0.05$ vs control (by Newman-Keuls).

peptide, dermorphin. These studies led to the conclusion that GHRP and the opiate GH responses were dependent on endogenous GHRH and since pretreatment with SRIF antiserum augmented the response of GHRP-6 and GHRH, neither one inhibited the release of SRIF. In contrast, because the SRIF antiserum pretreatment was without an effect on the GH response of the opiates, the opiates did appear to inhibit the release of endogenous SRIF. Thus, each of these three GH secretagogues—GHRP, GHRH, and the opiate peptide—was considered to release GH by a different mechanism and, in addition, the mechanisms or actions were complementary in releasing GH. Importantly, although response to the GHRP is dependent on endogenous GHRH, GHRH apparently plays a permissive role.

In addition to a direct action on the pituitary gland, a direct hypothalamic action of GHRP has been demonstrated (Table 4) *(26)*. In three different in vitro assay systems, pituitary incubate, dispersed pituitary monolayer cell culture, and perifusion of pituitary cells, the GH response to GHRP+GHRH was essentially additive or only slightly synergistic (≈30%), and thus the direct pituitary action of the two peptides was insufficient to account for the magnitude of the synergism induced by GHRP+GHRH *(26)*. Even when the in vitro GHRP+GHRH results in the pituitary cell culture and incubate assay were obtained under different experimental conditions and the time of the GH response was varied the effect on GH release was essentially additive. Other investigators have found synergism in vitro, but this has been the exception *(29)*.

The in vivo synergistic release of GH induced by GHRP+GHRH has been a hallmark of the GHRP effect on GH release in that it occurs in multiple animal species and in humans of all ages and both sexes. The exact mechanism(s) involved has not been elucidated. The fact that synergism has been such a consistent finding, even at very low dosages (≈2 μg) in humans, has led us to believe that understanding how this occurs will substantially aid in elucidation of the action of GHRP especially on the hypothalamus. Examples of the synergistic GH response induced by GHRP-6+GHRH in male and female rats, rhesus monkeys, and cows are recorded in Table 5.

Table 5
Synergistic GH Response[a]

Immature rat

Peptide	*Dose μg/kg*	*GH ng/mL ± SEM (n = 6)* *Male*	*Female*
Control	—	3 ± 1.7	10 ± 2
GHRP-6	200	289 ± 69	248 ± 31
GHRH-44	200	102 ± 19	173 ± 28
GHRP-6+GHRH-44	200 + 200	1063 ± 343	1184 ± 145

Monkey

Peptide	*Dose μg/kg*	*GH ng/mL ± SEM (n = 6)* *Male*	*Female*
Control	—	2 ± 1.6	6 ± 5
GHRP-6	5	1 ± 0.6	1 ± 0.5
GHRH	5	6 ± 2	2 ± 1
GHRP-6+GHRH	5 + 5	21 ± 8	26 ± 9

Nonlactating Holstein Cow

Peptide	*Dose μg/kg*	*GH ng/mL ± SEM (n = 4)*
Control	—	0.17 ± 0.19
Ala^1GHRP-6	3	8.6 ± 2.5
GHRH	3	5.7 ± 0.58
Ala^1GHRP-6+GHRH	3 + 3	88.0 ± 19.0

[a]Blood collected +10 (26-d-old rat), +20 (rhesus monkey), +15 (cow) min after iv peptide.

A series of GHRP antagonists were synthesized between 1980–83. The in vitro results of $HisDTrpDLysTrpDPheLysNH_2$, a GHRP antagonist, and the GHRH antagonist, $DArg^2,Ala^{8,9,15}$-GHRH, developed by Coy et al. are recorded in (Table 6) *(26)*. The GHRP antagonist inhibits the GH response to GHRP but not GHRH and the GHRH antagonist inhibits GHRH but not GHRP. In 1991, certain substance P antagonists were found to inhibit the GH response to GHRP *(30)* as well as labeled GHRP in the pituitary GHRP RRA *(31)*.

Clark and Robinson *(32)* continuously infused GHRP-6 to freely moving conscious rats over 8 h and a pulse of GHRH was administered each hour. GHRH pulses inconsistently released GH in saline treated control rats, whereas in the GHRP-6 treated rats, GH was consistently released by the pulses of GHRH. These results were interpreted to reflect a hypothalamic action of GHRP. Since exogenous GHRH was administered in this study, it is obvious that the hypothalamic action of GHRP is not due to the endogenous release of GHRH. Other results in support of a GHRP hypothalamic action include demonstration of high affinity binding studies in membranes of both the hypothalamus and pituitary by Codd et al. *(33)* and Sethumadhavan et al. *(31)* as well as those of Dickson et al. *(34)*, who showed that by both iv and icv routes, GHRP-6 increased c-*Fos* production in select neurons of the arcuate nucleus of the hypothalamus *(35,36)*, Guillaume et al. showed that GHRH was increased in hypophyseal portal blood of sheep following treatment with hexarelin *(37)*. Also, results of Mallo et al. *(38)* demonstrated a GHRP hypothalamic site of action following hypophyseal stalk section and pituitary transplan-

Table 6
In Vitro Effects of GHRH and GHRP Competitive Antagonists on the GH Responses of GHRP and GHRH

Peptide	*Dose ng/mL*	*$DArg^2Ala^{8,9,15}GHRH$ Dose μg/mL*	*GH (ng/mL ± SEM)*[a]
Control	—	—	179 ± 4
GHRP	10	—	930 ± 25
GHRP	10	1.0	856 ± 27
GHRP	10	3.0	933 ± 27
GHRP	10	10.0	920 ± 22
GHRH	10	—	1197 ± 17
GHRH	10	1.0	454 ± 23[b]
GHRH	10	3.0	309 ± 15[b]
GHRH	10	10.0	253 ± 15[b]
Peptide	*Dose ng/mL*	*$DLys^3$-GHRP Dose μg/mL*	*GH (ng/mL ± SEM)*[a]
Control	—	—	172 ± 15
GHRP	10	—	1020 ± 11
GHRP	10	1.0	626 ± 9[b]
GHRP	10	3.0	555 ± 36[c]
GHRP	10	10.0	357 ± 45[b]
GHRH	10	—	1197 ± 17
GHRH	10	1.0	1242 ± 18
GHRH	10	3.0	1206 ± 25
GHRH	10	10.0	1080 ± 52

[a] $n = 3$.
[b] $p = < 0.001$.
[c] $p < 0.01$ vs peptide alone.

tation. The recent important studies by Dickson et al. *(36)* demonstrate that GHRP-6 acts directly on the hypothalamus in vitro. Recently reported in vitro studies of Korbonits et al. failed to demonstrate that GHRP increased or decreased GHRH or SRIF release from the hypothalamus *(39)*. Furthermore, peptidomimetics of the GHRPs did not induce a reproducible rise of GHRH and/or fall in SRIF hypophyseal portal blood in vivo *(40)*.

Mechanism of action studies by Cheng in 1989 *(29)* demonstrated that GHRP-6 did not activate the adenyl cyclase cAMP pathway, but together with GHRH, synergistically raised intracellular cAMP levels by acting through the protein kinase C pathway. In 1983, we also reported that neither $DTrp^2$ nor $DTrp^3$ in vitro raised pituitary cAMP or cGMP levels *(9)*. Later results of Adams et al. *(41)* and Mau et al. *(42)* demonstrated that although GHRH stimulated the cAMP pathway GHRP-6 stimulated the phospholipase-C IP_3 (inositol triphosphate) pathway. In vitro results have supported the role of GHRP as a functional SRIF antagonist at the molecular level in that the peripheral membrane of the somatotroph is depolarized by GHRP by blocking the K^+ channels and inhibiting hyperpolarization by SRIF *(43,44)*. Intracellular Ca^{2+} is raised via voltage-activated L-type channels and by release from intracellular stores *(44,45)*. Recently, details of these studies were discussed by Smith et al. *(40)* and Chen *(46)*.

In 1989, our group, together with Michael Thorner's and as Ilson et al. at Smith-Kline Beecham, found that GHRP-6 very effectively released GH in normal young men *(47,48)*.

There was a small concomitant transient rise of serum PRL and cortisol, both of which were still within the normal range. Similar to that found in animal models, i.e., rats, monkeys, and cows, the combined administration of GHRP-6 and GHRH on GH release was synergistic in humans. These results underscore that, in humans also, GHRP and GHRH act differently. Another important property of the GHRPs was revealed when Huhn and Thorner et al. *(49)* and Jaffe and Barkan et al. *(50)* independently demonstrated that continuous iv infusion of GHRP-6 administered for 24–36 h to normal young men increased the amplitude of the spontaneous pulsatile secretion of GH. Because the GH response to GHRP-6 was readily desensitized after repeated administration to rats *(21)*, as well as by continuous administration during perifusion of dispersed rat pituitary cells *(18)*, these results in humans were surprising. However, the results of Clark and Robinson in conscious rats suggested that continuous infusion of GHRP-6 to humans might increase the amplitude of the spontaneous GH pulsatility and that this could occur despite desensitization of the GH response *(32)*.

Between 1991–1997, a series of detailed and noteworthy studies were performed with the very potent GHRP-6-like hexapeptide hexarelin, HisD2MeTrpAlaTrpDPheLysNH_2 that had been developed by Dengheni et al.: The effects of hexarelin essentially paralleled those of the other GHRPs *(51,52)*. Also, during this time, Walker and Bercu *(53)* reported the results of chronic administration of GHRP-6 to rats. They investigated the corrected effects of GHRP-6+GHRH co-administration, relationships to endogenous GHRH, TRH and GnRH secretion as well as secretion of PRL, body weight gain, and effect on serum lipids and hepatic mRNA levels for low-density lipoproteins (LDLs).

In 1992, a seminal accomplishment and a major GHRP milestone was the development of a substituted benzolactam peptidomimetic L-692,429 by Merck and Co. *(54)*. This was a special achievement because a peptidomimetic agonist was developed from a peptide agonist. In contrast, the development of a peptidomimetic antagonist from a peptide agonist is not such an unusual event. Undoubtedly this peptidomimetic will catalyze efforts to develop other peptidomimetic agonists that mimic the actions of small peptide hormones. A point of note has been the finding that the peptides and peptidomimetics act on the same receptor and activate GH release by the same intracellular signal transduction pathway *(55)*. An important improvement of the benzolactam GH secretagogue was reported by the Merck group in 1995. This spiroindoline derivative [MK-0677 (L-693,191)] is more potent, has higher oral bioavailability (≈60%) and increases pulsatile GH secretion with an associated increase of serum IGF-I levels during chronic oral administration to normal younger and older subjects *(56)*.

Also, in 1995, highly potent GHRPs were developed by the Genentech *(57,58)* and Novo Nordisk groups *(59,60)*. These GHRPs were developed primarily from the $DTrp^{2,3}$ type of GHRP with an aromatic core in the center of the molecule and special functional groups at each end. The Genentech group has reported potent GHRPs that are low in molecular weight ranging from 496 to 508. Gradually, small partial peptide GHRPs are being developed with more substitutions of the amino acids by organic chemical nonpeptide groups. Besides the four or more major types of GHRPs, there are now three major chemical classes of GHRPs, i.e., peptide, partial peptide, and peptidomimetics. Regardless of the broad range of the GHRP SARs, all of them appear to act on the same receptor and by the same molecular mechanism(s). What is different among these GH secretagogues is the pharmacokinetics. In principle, the pharmacokinetics do not alter the

action on GH release, but MK-0677, with a more prolonged serum half-life, appears advantageous in terms of increasing pulsatile GH secretion and serum IGF-I levels after oral administration. These same results have been observed with continuous infusion of GHRP-6 and GHRP-2 *(49,50,61,62)*.

In 1996, another seminal milestone was accomplished by the Merck group by cloning the MK-0677 receptor and characterizing it as the GHRP receptor *(63)*. This is a new seven transmembrane domain G-protein coupled receptor. Anatomically it has been localized in the hypothalamic arcuate nucleus and the infundibulum as well as in the pituitary on the somatotrophs. All of the various types and classes of GHRPs specifically bind to the transfected cloned receptor with high affinity. Genomic analysis of the receptor supports the presence of a single highly conserved gene in human, chimpanzee, swine, bovine, rat, and mouse genomic DNA.

The SARs of GHRP strongly support that the putative native GHRP-like hormone is a peptide. Because of the substitution of unnatural D amino acid stereoisomers in the GHRPs, it is probable that the amino acid sequence of the putative native GHRP-like hormone will not closely simulate the sequence(s) of the current peptide GHRPs. In regard to how GHRP releases GH, it is well established that it acts on both the hypothalamus and pituitary *(27)*. What is still unanswered is the relative importance of the action of GHRP at these two anatomical sites as well as the type of action(s) GHRP has on the hypothalamus, i.e., increased GHRH and/or decreased SRIF release or even the seemingly likely possibility of increased release of a yet unidentified factor. It has been postulated that the hypothalamic action of GHRP involves the release of U-factor (unknown factor) which in part mediates its effect on GH release *(27)*. The latter has been proposed because of an inability to explain the action of low dose GHRP via an effect on GHRH or SRIF release or as a functional SRIF-antagonist. Sequential events of the GHRP story also were outlined in 1996 *(64)*.

What has become gradually more apparent is that the type of action(s) induced by GHRP is probably dose dependent. High dosages are considered to reflect a pharmacological action and low dosages presumably a physiological action of a putative endogenous GHRP-like hormone. Conceptual models of the role of the putative GHRP system in the physiological regulation of GH secretion can be categorized in terms of three different types, hypothalamic, pituitary, and hypothalamic-pituitary *(27)*. Because GHRP acts on both the hypothalamus and pituitary, the hypothalamic-pituitary model is the most logical choice, but this model is particularly difficult to envision without knowing more about the hypothalamic action of GHRP and to what degree the quality and quantity of this effect is dosage-dependent.

Unusual and unexpected effects of GHRP in humans have been exemplified by the not infrequent unique actions of this new class of GH secretagogues. Figure 4 shows that each of the three GHRPs, GHRP-6, -1 and -2, increasingly released more GH in normal young men than GHRH when 1 μg/kg of the peptides was administered by iv bolus injection. Data recorded in Fig. 5 demonstrate another important aspect of these three initial GHRPs in that even though they are peptides they very effectively release GH after oral administration in normal young men. Figure 6 shows the high reproducibility and marked effect on GH release of four different oral formulations of GHRP-2 including small tablets at a dosage of 10 mg in normal young men. The low and consistent serum concentration of GHRP-2 after oral administration supports the consistency of the GH effect as well as the high potency of the peptide.

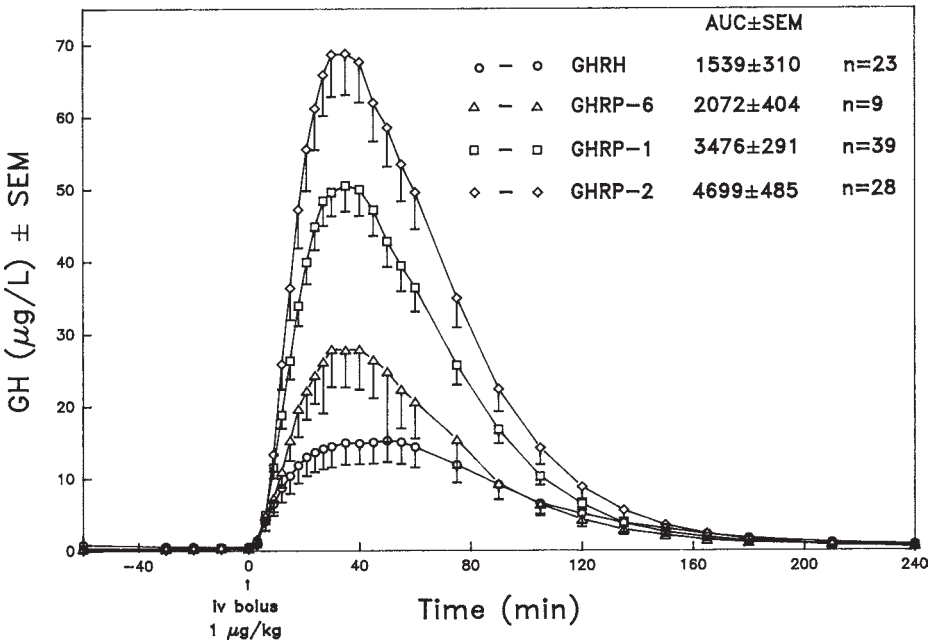


Fig. 4. Comparative mean responses to 1.0 μg/kg GHRH(1-44)NH2, GHRP-6, GHRP-1 and GHRP-2 in normal young men. Reproduced with permission from ref. *64*.

The results of a continuous infusion of GHRP-6 for 36 h to normal young men are recorded in Fig. 7 and demonstrate that the GH response to GHRP is both sensitized and desensitized *(50)*. The amplitude but not the frequency of the spontaneous GH pulses was increased during the infusion. Near the end of the infusion, the GH response to iv bolus GHRH was increased while that of iv bolus GHRP-6 was almost completely inhibited.

As recorded in Fig. 8, another dimension of the action of GHRP was observed when the effect of a very small amount of GHRP-2 (≈2 μg or 0.03 μg/kg) was administered to normal young men *(65)*. GHRP-2 alone in this small dosage was without an effect on GH release but when given together with 1 μg/kg GHRH, GH was synergistically released. When this study was performed in normal young women, essentially the same results were obtained. A number of interpretations and implications appear to evolve from this study. Because this was usually a subthreshold GH releasing dosage of GHRP-2, and because the in vitro effects of combined GHRP-2 and GHRH on GH release are usually additive or only marginally synergistic, the synergistic release of GH induced by low-dose GHRP-2+GHRH is unlikely mediated by the action of this small dose of GHRP-2 directly on the pituitary. Thus, the synergism is probably mediated via a hypothalamic action of GHRP. Because of the large amount of GHRH administered, the GHRP-2 hypothalamic action obviously is not mediated via an increased release of endogenous GHRH. In addition, the GHRP-2 hypothalamic action probably involves an action outside the blood brain barrier on the median eminence rather than on the arcuate nucleus because evidence indicates the blood brain barrier limits the access of GHRP *(35,66)* and a 2 μg dose of GHRP-2 is very low. Also, in a number of studies in which SRIF release was inhibited by different agents, i.e., pentobarbital, SRIF antiserum, opiates, pyridostigmine, the GH response to GHRP was increased, thus indicating GHRP does not

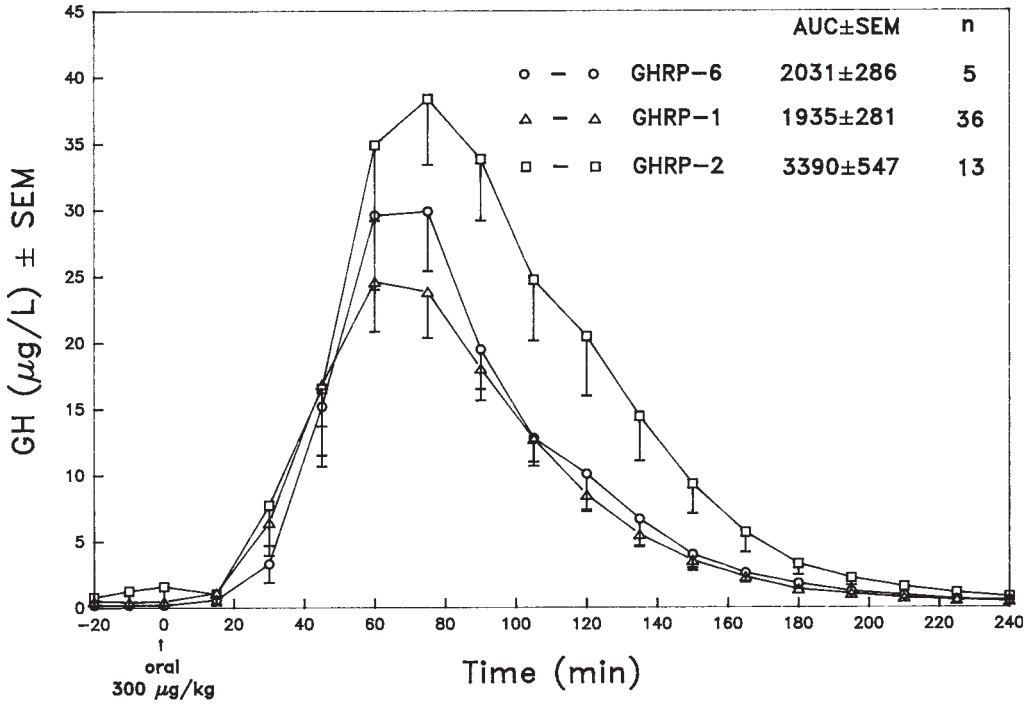


Fig. 5. Comparative mean GH responses to 300 μg/kg oral GHRP-6, GHRP-1, and GHRP-2 in normal young men.

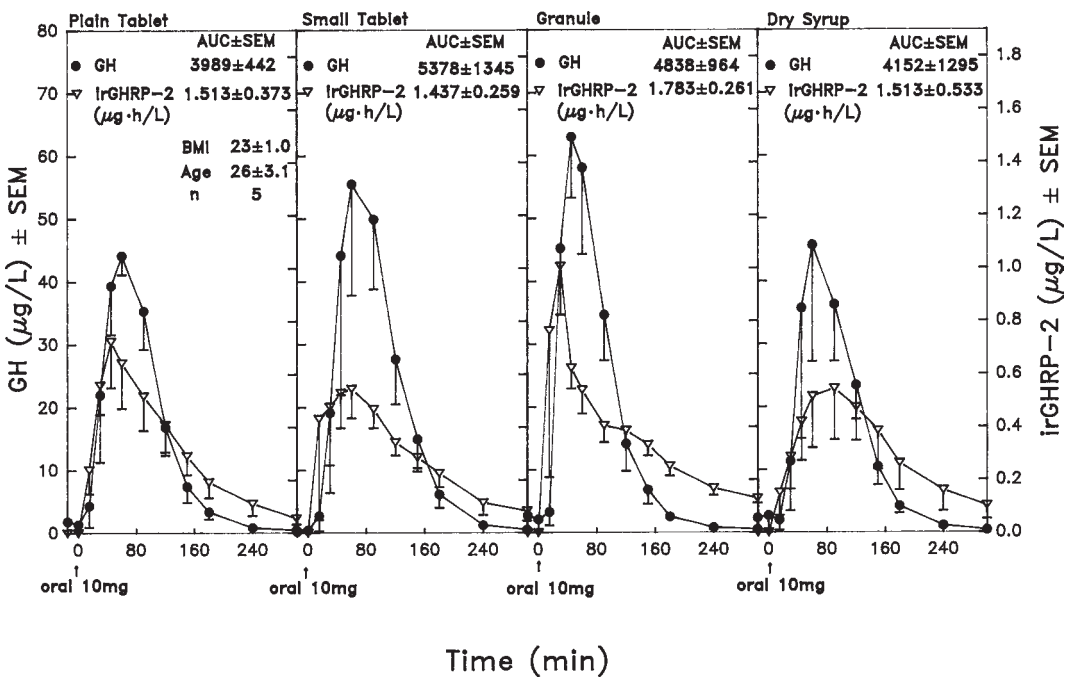


Fig. 6. GH and GHRP-2 concentration time-profiles after different formulations of 10 mg GHRP-2 orally on different occasions to the same five normal young men. Values are the mean ± SEM.

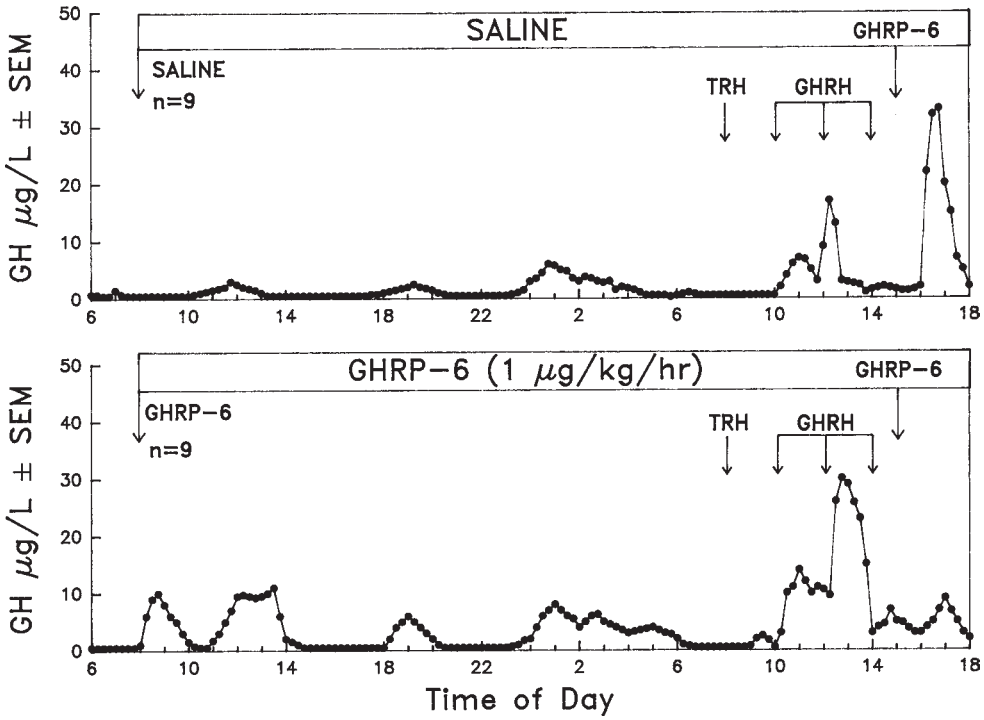


Fig. 7. Effect of continuous 36-h infusion of saline or GHRP-6 in normal young men. Reproduced with permission from ref. *50*.

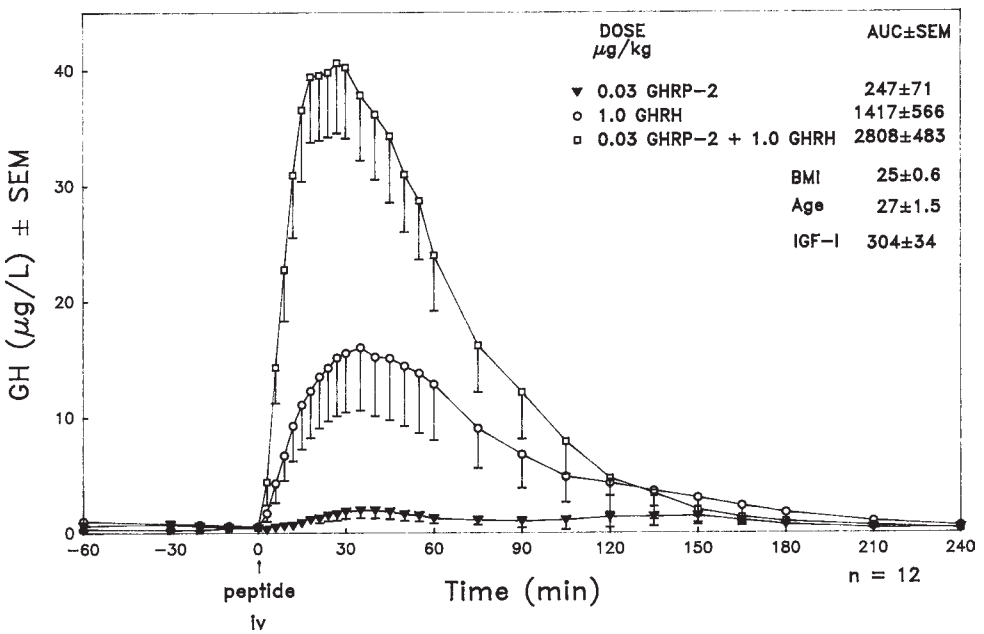


Fig. 8. Effect of a very low dosage of GHRP-2 (0.03 μg/kg) combined with a high dosage of GHRH (1.0 μg/kg) on the synergistic release of GH in normal young men. Reproduced with permission from ref. *81*.

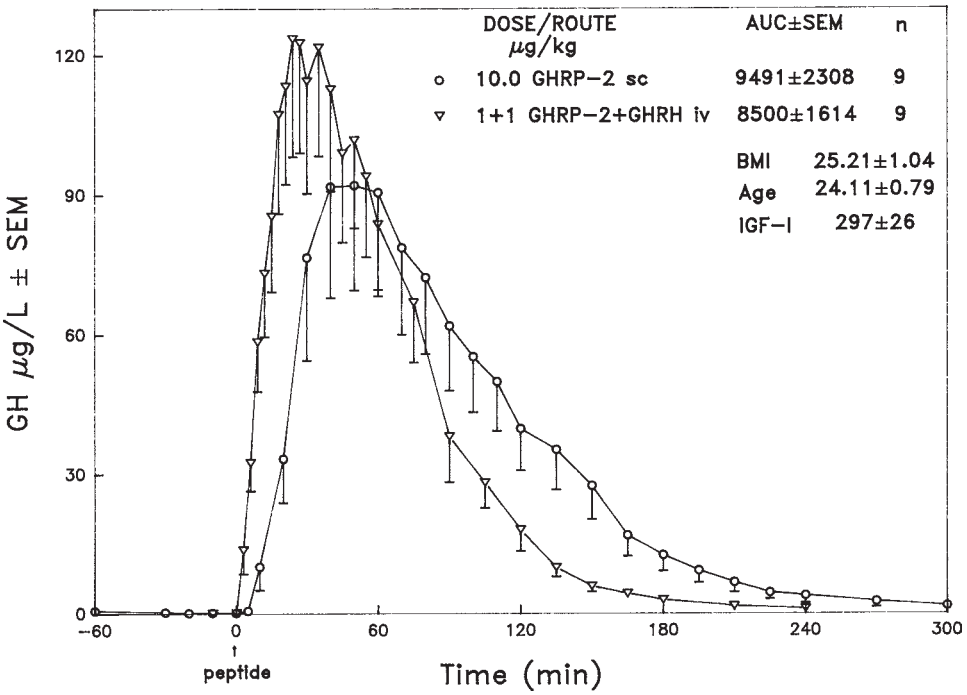


Fig. 9. Comparative effects on the GH release to 10 µg/kg sc high dose GHRP-2 vs 1+1 µg/kg iv bolus GHRP-2+GHRH in the same nine normal young men. Reproduced with permission from ref. *81*.

release GH by inhibition of SRIF release or by attenuation of the SRIF inhibitory action on the pituitary. Although GHRP can be categorized as a functional SRIF antagonist at the pituitary and possibly the hypothalamic level *(66,67)*, such a small dose of GHRP-2 would be unlikely to attenuate the pituitary or hypothalamic action of SRIF. A seemingly convoluted issue is to what degree is it possible to relate the hypothalamic action(s) of exogenous low dose GHRP-2 and a putative endogenous GHRP-like hormone. Because it has been impossible to explain the synergistic release of GH by a very low dosage of GHRP-2 via a hypothalamic action on the release of GHRH or SRIF, it has been hypothesized that a third factor designated U-factor mediates this synergism. U-factor is envisioned to be released from the hypothalamus via the action of GHRP. In concert with GHRH and sometimes with GHRP when higher dosages of GHRP are administered, U-factor acts on the pituitary to synergistically release GH, seemingly by a complementary intracellular signal transduction action and in part by possibly attenuating the pituitary inhibition of SRIF on GH release. A seemingly general valuable point is that GHRP studies alone and in combination with GHRH in humans can reveal new dimensions about the secretion of GH, as well as add new insight into the actions of GHRP.

Another dimension of the action of GHRP-2 on GH release was revealed when a large dose of 10 µg/kg was administered sc to normal young men (Fig. 9) *(65)*. Because this large dose of GHRP-2 alone released the same amount of GH as that induced by iv bolus 1+1 µg/kg GHRP-2+GHRH, GHRP-2 in this high dosage was considered to release endogenous GHRH from the hypothalamus and, in this way, release a large amount of GH possibly via the synergistic action of GHRP + endogenous GHRH.

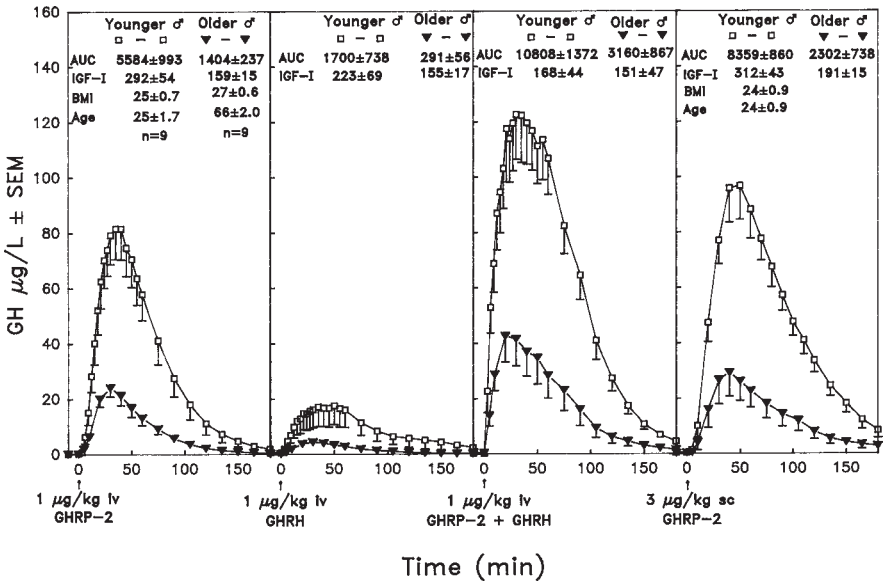


Fig. 10. Comparative effects on the GH response to GHRP-2, GHRH, and GHRP-2+GHRH in the same normal younger and the same normal older men. Three μg/kg sc GHRP-2 (last panel) was administered to these subjects, with the exception that there were only 5 of 7 younger female subjects.

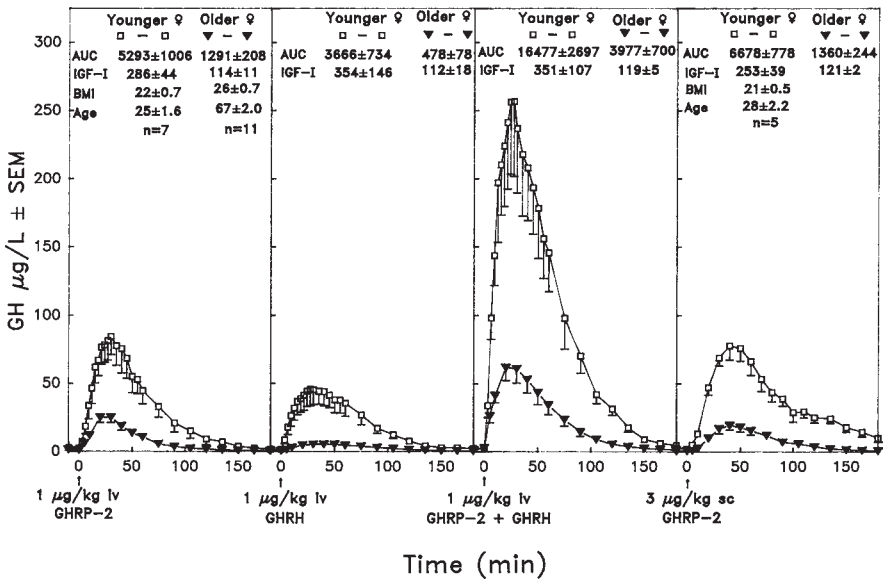


Fig. 11. Comparative effects on the GH response to GHRP-2, GHRH, and GHRP-2+GHRH in the same normal younger and the same normal older women. Three μg/kg sc GHRP-2 (last panel) was administered to these subjects, with the exception that there were only 5 of 7 younger female subjects.

The GH response to GHRP-2 and GHRH as well as the marked synergistic release of GH induced by these combined peptides was greater in younger than in older men and women (Figs. 10 and 11) *(68)*. GHRP-2 consistently released more GH than maximal

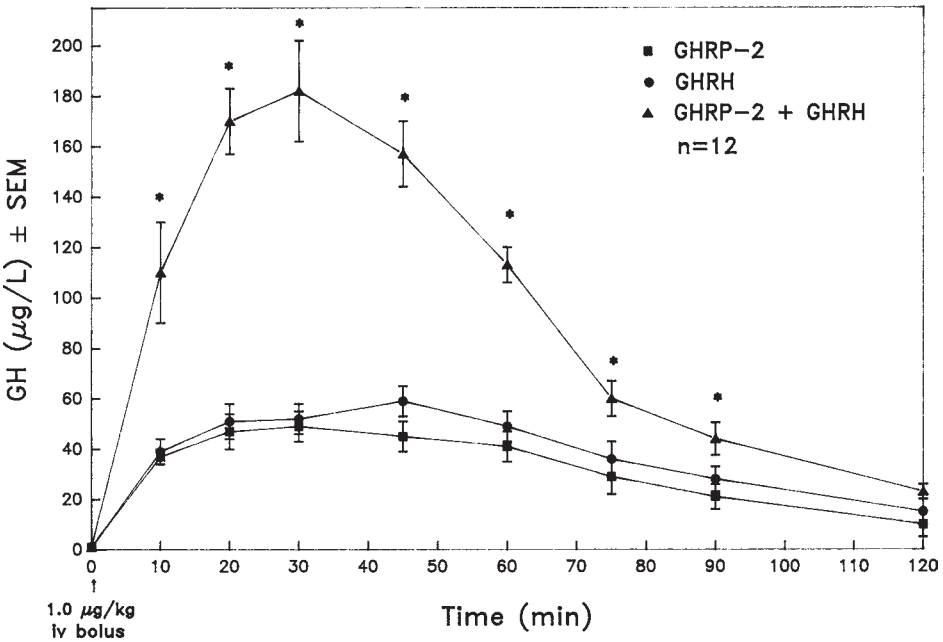


Fig. 12. GH responses over time with administration of iv GHRH (1 μg/kg), GHRP-2 (1 μg/kg), and GHRH+GHRP-2 (each at a dose of 1 μg/kg) in children with GH insufficiency. Mean ± SEM. Reproduced with permission from ref. *69*.

dosages of GHRH even in the older subjects, indicating the pituitary capacity to release GH is not the reason the GHRH GH response is lower in older subjects.

GHRPs are very effective in children. When Pihoker et al. *(69)* acutely administered GHRP-2+GHRH to short-statured children with various degrees of GH deficiency, GH was synergistically released (Fig. 12). In these children, GHRP-2 alone very effectively released GH. Three separate chronic studies by Laron et al. *(70,71)*, Pihoker et al. *(69,72)* and Mericq et al. *(73)* have been performed with hexarelin or GHRP-2 administered intranasally or subcutaneously to short-statured children with partial GH deficiency. In each study, the height velocity was increased by 2.5–3 cm/yr. The results recorded in Fig. 13, obtained by Pihoker et al., indicate that after 6 mo of intranasal GHRP-2 administration 2–3 times/d, the GH response was not desensitized and tended to be increased or up-regulated.

Since 1993, Casanueva and Dieguez et al. *(74)* have performed a series of important studies with GHRP-6 in patients with obesity, Cushings syndrome, and hypothalamic-pituitary disconnections. In obesity, GHRP released a remarkable amount of GH, especially when GHRP-6+GHRH was administered. The GH response to GHRP-6 alone and together with GHRH was markedly decreased in Cushing's syndrome. In patients with a hypothalamic-pituitary disconnection, GHRH released a normal amount of GH and GHRP-6 a lesser amount. These results indicate new dimensions in the secretion of GH and eventually understanding them in more detail will reveal new insight into the physiological and pathophysiological secretion of GH in humans. Also, other studies reveal that GHRP releases GH from pituitary tumors of acromegalic patients in vitro and in vivo *(28,41,75,76)*.

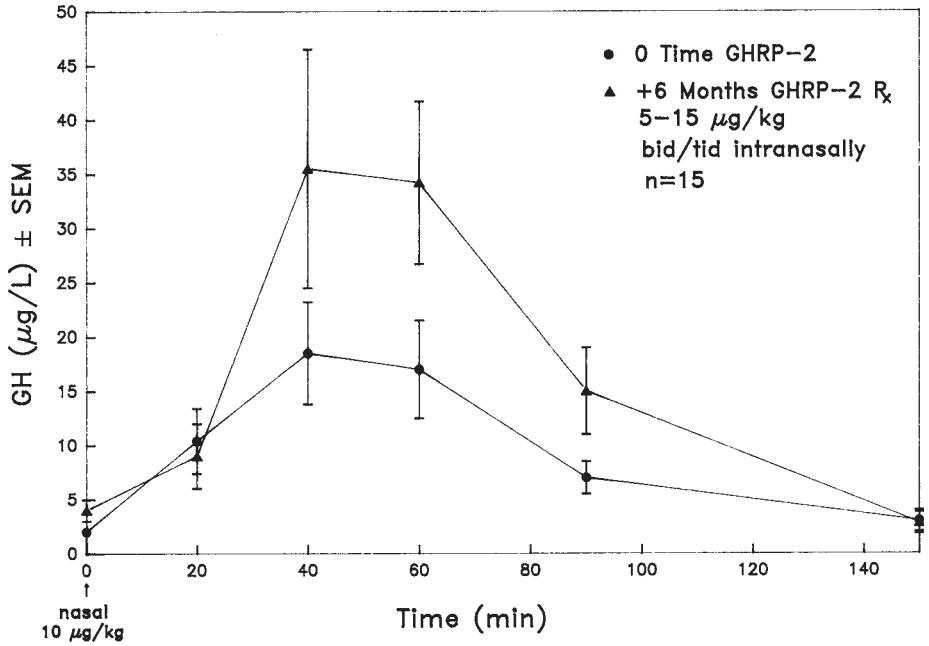


Fig. 13. GH responses to an acute intranasal dose of GHRP-2, 1 μg/kg on initial testing and 6 mo after daily GHRP-2 administration in children with GH insufficiency. Initial testing, $n = 12$, + 6 mo, $n = 9$. Mean ± SEM. Reproduced with permission from ref. *72*.

Results of the GH responses to GHRP-2 or GHRP-2+GHRH during chronic administration to normal older subjects every other day for 60 d are recorded in Figs. 14 and 15 *(77)*. The acute GH response to GHRP-2, GHRH, or GHRP-2+GHRH was not differentially influenced by GHRP-2 alone or GHRP-2+GHRH administered chronically. Neither GHRP-2 nor GHRP-2+GHRH desensitized or up-regulated the acute GH response to GHRP-2 or GHRH. In these studies, serum IGF-I levels remained unchanged during chronic administration of GHRP-2 as well as GHRP-2+GHRH.

Although demonstration of a decreased GH response to 1 μg/kg iv bolus GHRH is essential for understanding the pathophysiology and for making the diagnosis of pathological secretion of GH in older men and women, the GHRH response alone is considered insufficient for these two purposes. Almost all normal elderly men and women have considerably lower GH responses to 1 μg/kg GHRH than normal younger men and women and therefore, utilization of this criteria alone would tend to include all normal elderly subjects. The results of age-dependency of GH release are recorded in Figs. 10 and 11 for GHRP-2 with and without GHRH *(68)* and Fig. 16 for GHRP-1 with and without GHRH after iv bolus administration of the peptides *(78)*. Furthermore, the GHRH approach alone would not distinguish a low response due to excess secretion or action of SRIF.

Particularly needed is a new approach based on a better understanding of the pathophysiology in order to distinguish the decreased secretion of GH associated with aging *per se* from a pathological decreased secretion of GH due to a possible specific hormonal deficiency. As unlikely and illogical as this may seem at first, the putative GHRP-like hormone and GHRP-2 appear intimately and perhaps fundamentally related to the pathophysiology and to the diagnosis of the pathological decreased GH secretion in the elderly.

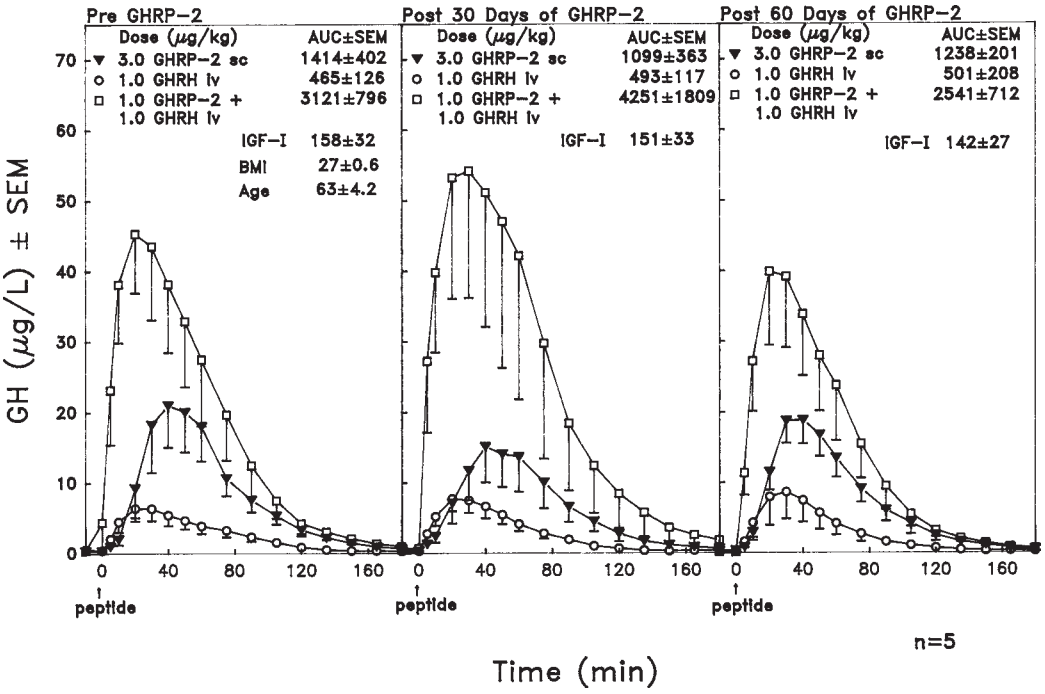


Fig. 14. Effect of 3 μg/kg GHRP-2 sc every other day in the AM for 60 d in normal older adults. The GH responses to iv bolus GHRP-2, GHRH, and GHRP-2+GHRH were the same before treatment, at +30 d, and at +60 d. The IGF-I mean levels did not change.

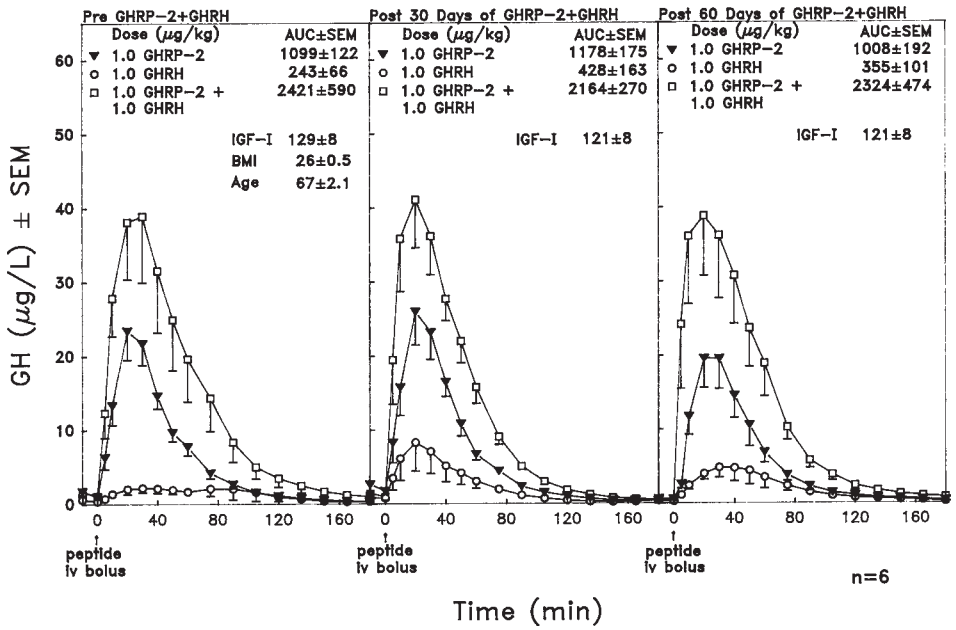


Fig. 15. Effect of 1 μg/kg GHRP-2+GHRH every other day in the AM for 60 d in normal older adults. The GH responses to iv bolus GHRP-2, GHRH, and GHRP-2+GHRH were the same before treatment, at +30 d, and at +60 d. The IGF-I mean levels before and after treatment were unchanged.

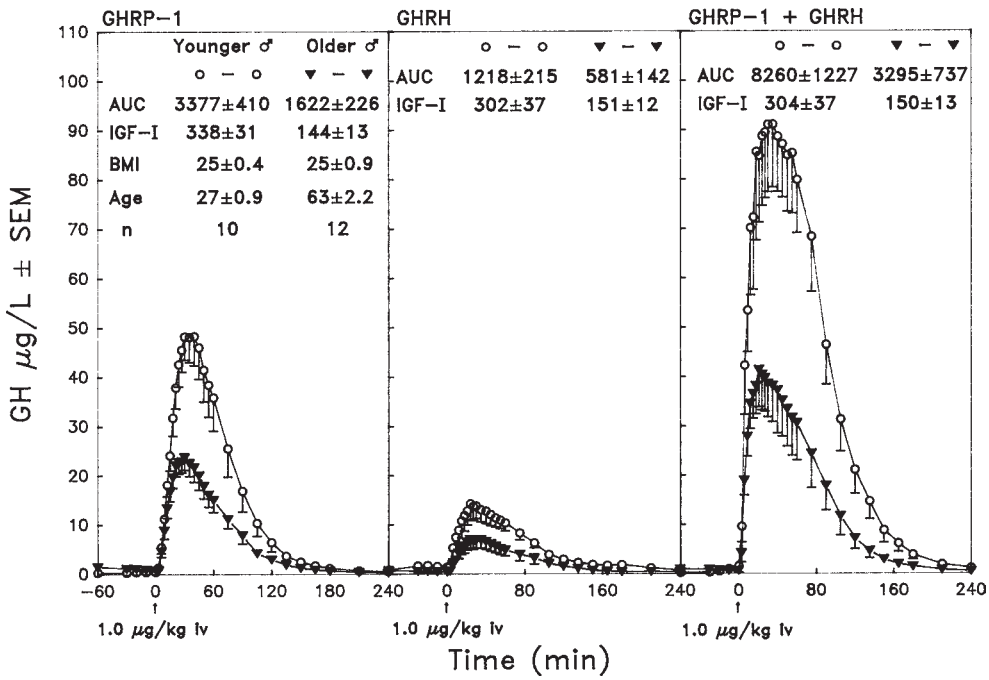


Fig. 16. Comparative mean GH responses to GHRP-1, GHRH and GHRP-1+GHRH in normal younger and older men. Values are the mean ± SEM. Reproduced with permission from ref. *27*.

Bercu and Walker *(79)* have performed a series of studies in animals and humans in order to understand the pathophysiology of decreased GH secretion that occurs during aging and also to develop an approach to diagnose this endocrine abnormality(s).

In order to understand this pathophysiology and to develop a way to distinguish the decreased GH secretion due to normal aging from that due to a pathological abnormality in older men and women, our approach has been to assess and establish the clinical value of a dual linked index of GH release designated a quantitative GH release index and a qualitative GH release index. Our hypothesis is that the pathophysiology of the pathological decreased GH secretion in older men and women is due to a deficiency of the putative hypothalamic GHRP-like hormone rather than a primary deficiency of GHRH or an excess of SRIF. The basic finding that has led to this hypothesis is that the pituitary action of 1 μg/kg GHRH on GH release is quantitatively impaired and that this impairment is reversed by iv bolus 0.1 μg/kg GHRP-2 + 1 μg/kg GHRH *(80,81)*. An example of these GH responses in a normal older woman is recorded in Table 7. Also in Fig. 17 is recorded the results of an acute GH response of this same older woman before and during twice daily 0.1 μg/kg sc GHRP-2 chronically for 30 d. Noteworthy is that 0.1 μg/kg GHRP-2 consistently and dramatically reversed the markedly impaired GH responses of 1 μg/kg GHRH on d 0, 15, and 30. This supports that the impaired action of GHRH on GH release is basically and primarily a hypothalamic rather than a pituitary pathological abnormality.

Whether the impaired GHRH GH release is reversed by a low dose of GHRP-2 can be qualitatively decided in an all or none way as being positive or negative. A qualitative positive index would be when the ratio of the peak GH release of the combined peptides (0.1+1 μg/kg) is at least threefold greater than that of the arithmetic sum of the individual

Table 7
Effect of Chronic GHRP-2

	Dose μg/kg iv bolus[a]	*Peak GH μg/L*	*AUC GH μg/L × 4 h*
GHRH	1.0	2.7	183
GHRP-2	0.1[a]	0.7	221
GHRH+GHRP-2	1.0 + 0.1	44.1	2436
GHRP-2	1.0	47.6	2540

[a]sc.
GF-I, 85 μg/L.
BMI, 21.4.
GHRP-2, 0.1 μg/kg sc administered to a 66-yr-old female 2×/d for 30 d.

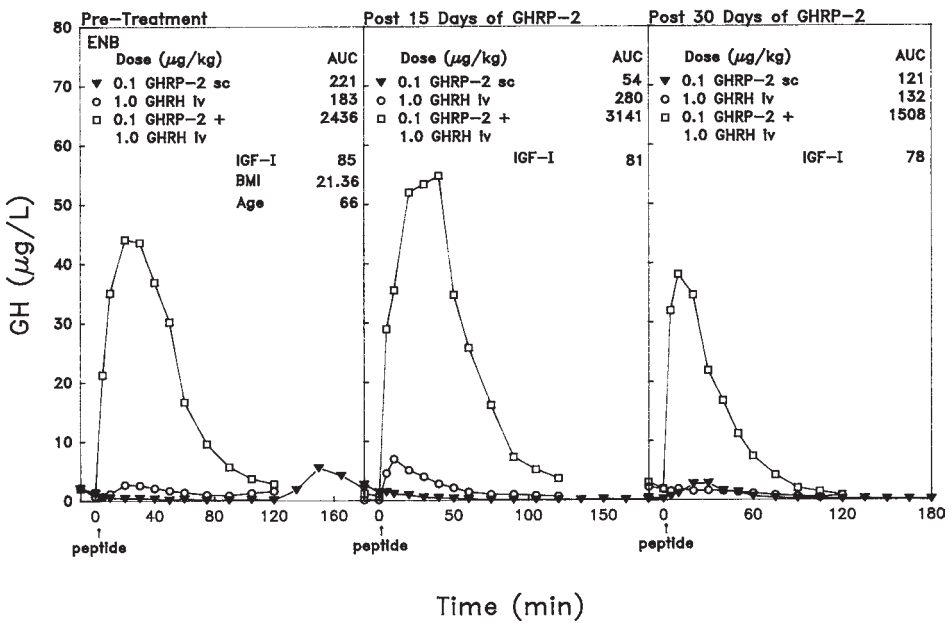


Fig. 17. Effect of 0.1 μg/kg GHRP-2 administered sc 2×/d for 30 d in a 66-yr-old female. Subject tested before treatment, at +15 d, and at +30 d after treatment. There was no change in the IGF-I levels.

peptides. The GH response to 1 μg/kg GHRP-2 is considered to impart more insight into the pathophysiology and more insight into the pituitary capacity to release GH. Without endogenous GHRH secretion GHRP-2 does not release GH and thus the GH release induced by GHRP-2 indicates the secretion of endogenous GHRH. Although still impaired in comparison to younger adults, 1 μg/kg GHRP-2 releases considerably more GH than 1 μg/kg GHRH in older normal adults and thus, this indicates more about the capacity of the pituitary to release GH. However, because 1+1 μg/kg GHRP-2+GHRH or 10 μg/kg sc GHRP-2 releases much larger amounts of GH than 1 μg/kg GHRP-2 alone in normal young men, eventually one of these two approaches may be considered more optimal to assess the maximal capacity of the pituitary to release GH.

In Table 8 are the results of the acute iv bolus GH responses to 1 μg/kg GHRP-1, GHRH and the combined peptides in normal older men and women *(78)*. The results of these 19

Table 8
Comparison of GH Responses (Peak GH and AUC × 4 H) in the Elderly to GHRP-1, GHRH(1-44)NH_2, and GHRP-1+GHRH[a,b]

			GHRP-1 μg/L ± SEM		*GHRH-1 μg/L ± SEM*		*GHRP-1+GHRH μg/L ± SEM*	
Age (yr)	*BMI[c]*	*IGF-I μg/L*	*Peak GH*	*AUC × 4 h*	*Peak GH*	*AUC × 4 h*	*Peak GH*	*AUC × 4 h*
No synergism 2♀, 5♂								
64.3 ± 1.9	27.9 ± 1.4	136.3 ± 13.3	18.7 ± 2.6	1067.0 ± 166.0	2.5 ± 0.7	201.0 ± 44.0	23.6 ± 2.4	1349.0 ± 182.0
Synergism 5♀, 1♂								
65.5 ± 2.9	27.9 ± 2.0	104.6 ± 12.0	13.6 ± 1.8	746.0 ± 105.0	3.9 ± 0.9	373.0 ± 65.0	40.0 ± 5.4	2539.0 ± 424.0
High synergism 3♀, 3♂								
60.5 ± 2.3	23.6 ± 1.1	150.6 ± 22.8	24.8 ± 4.8	1522.0 ± 376.0	13.6 ± 2.5	980.0 ± 199.0	79.6 ± 9.3	6367.0 ± 845.0

[a]Reproduced with permission from ref. *78*.
[b]Mean ± SEM.
[c]Body Mass Index.

subjects are grouped according to whether the synergistic GH response of the combined peptides was absent, normal or high. In all three groups, the peak GH responses to GHRP-1 were nearly the same (18.7 ± 2.6, 13.6 ± 1.8, 24.8 ± 4.8), but for GHRH were different in the third group (2.5 ± 0.7, 3.9 ± 0.9, 13.6 ± 2.5). The results of the mean GH AUC paralleled the peak GH responses. Apparent is that the GH response to 1 μg/kg GHRH was markedly impaired in the first two groups and in the second group, 1 μg/kg GHRP-1 reversed the impaired GH response to 1 μg/kg GHRH by synergistically releasing GH. It is assumed that synergism in the first group was not induced because of the limited capacity of the pituitary to release GH. From our later GHRP-2 studies, it could be postulated that if a lower 0.1 μg/kg GHRP-1 dose had been administered in combination with the maximal 1 μg/kg dose of GHRH, a synergistic release of GH would have been elicited in all three groups. What is seemingly so fundamentally important is that the action of GHRH on the pituitary is markedly impaired and this impairment can be uniquely reversed by administering low dose GHRP + high dose GHRH.

Presumably the variable capacity of the pituitary to release GH will depend on the duration and severity of the putative GHRP-like hormone deficiency as well as the amount of endogenous GHRH and SRIF being secreted. The high sensitivity of the GHRP-2 effect on the reversal of the impaired GH releasing action of GHRH is against a primary decreased function of the somatotroph *per se* or a primary excess secretion or action of SRIF as the immediate cause of the pathological decreased GH secretion in older men and women. Envisioned is that when endogenous GHRH is secreted in greater amounts or SRIF is secreted in smaller amounts, 0.1 μg/kg GHRP-2 will be more effective in enhancing the GHRH GH response and thus the effect of low dose GHRP-2 will be an indicator of endogenous GHRH secretion and also SRIF secretion.

To what degree the pituitary capacity to release GH will parallel and determine the type and efficacy of the neuroendocrine therapeutic approach will require special evaluation. The secretory status of endogenous GHRH, SRIF, and the putative GHRP-like hormone as well as the pituitary somatotrophs, alone and collectively, probably will significantly dictate the type and design of neuroendocrine therapeutic approach.

At present, if the quantitative GH release index is abnormally low, i.e., the GHRH peak GH response is <6 μg/L to 1 μg/kg iv bolus GHRH and the qualitative GH release index is threefold or greater, i.e., synergistic release of GH is induced by 0.1+1 μg/kg iv bolus GHRP-2+GHRH, the subject's decreased GH secretion would be considered to be pathological possibly due to a deficiency of the hypothalamic GHRP-like hormone.

Major results which have evolved so far from our studies on the pathophysiology of pathological GH deficiency in elderly men and women are the following:

1. Impaired pituitary GH response to a maximal 1 μg/kg dosage of GHRH;
2. Increased GH release to 0.1 μg/kg low dose GHRP-2 + high dose GHRH;
3. Relatively high GH response to 1 μg/kg GHRP-2.

Major conclusions about elderly subjects are:

1. GH release induced by 1 μg/kg GHRP-2 alone indicates endogenous GHRH is being secreted because without endogenous GHRH secretion GHRP-2 does not release GH;
2. GHRH pituitary action on GH release is impaired, which is necessary but not a specific indicator of the pathological decreased GH secretion;
3. Impaired pituitary action of GHRH is mainly due to a secondary hypothalamic abnormality rather than a primary pituitary abnormality, because a maximal GHRH dose releases a subnormal amount of GH;

4. Low-dose GHRP-2 reverses the high-dose, GHRH-impaired pituitary GH response, indicating this occurs via a hypothalamic action of GHRP-2 rather than a pituitary action, which does not involve release of endogenous GHRH;
5. High sensitivity of GHRP-2 in reversing the impaired pituitary action of GHRH is against increased release or action of SRIF as the reason GH secretion is decreased;
6. Reversal of the GHRH-impaired pituitary action by GHRP-2 is considered to be mediated via the hypothalamic action of GHRP-2 to release U-factor (unknown factor) rather than to release GHRH or to inhibit SRIF;
7. Low-dose GHRP-2 has such a unique effect on the GHRH pituitary action that the pathological decreased secretion of GH in some older men and women may result from a deficiency of the putative hypothalamic GHRP-like hormone.

In conclusion, it is postulated that a putative GHRP-like system probably does exist and is involved in the physiological regulation of GH secretion. In addition, because of the unique actions of GHRP on GH release, it is likely to be valuable clinically both diagnostically and therapeutically.

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3 The Design of Peptidomimetic Growth Hormone Secretagogues

Arthur A. Patchett, PhD,
and Matthew J. Wyvratt, PhD

Contents

INTRODUCTION

Discovery of the Growth Hormone Releasing Peptides (GHRPs)

In 1977, C.Y. Bowers and his coworkers at Tulane University reported a series of synthetic peptide analogs of Leu- and Met-enkephalins that specifically released growth hormone from the pituitary but possessed no opioid activity *(1)*. However, this pioneering work was overshadowed a few years later by the discovery of an endogenous peptide hormone—growth hormone releasing hormone (GHRH)—as one of two hypothalamic peptides, in addition to the inhibitory peptide hormone somatostatin, known to regulate the release of GH from the pituitary *(2–4)*.

Bowers continued to explore the structure–activity relationships of his early synthetic growth hormone releasing peptides (generally referred to as GHRPs). The hexapeptide GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) emerged as an early benchmark and was shown to be an extremely potent and safe GH secretagogue (GHS) in animals and in humans *(5–9)*. Interestingly, GHRP-6 was shown not only to release GH from the pituitary via a mechanism distinct from the natural regulator GHRH, but also in fact acted synergistically with GHRH to release GH. Whereas GHRH activates protein kinase A via cAMP accumulation, GHRP-6 and its peptidomimetics (vide infra) activates phospho-

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lipase C to liberate the second messengers IP_3 and diacylglycerol. The two pathways converge to release GH from an influx of Ca^{+2} ions through L-type channels *(10–12)*. Recently, the human receptor for GHRP-6 has been identified in the pituitary and hypothalamus and shown to be a unique G-protein coupled receptor with little homology with other known receptors, including the GHRH receptor *(13,14)*. The natural ligand for this new orphan receptor has not yet been identified, but it undoubtedly plays an important role in the regulation of GH.

The availability of recombinant human growth hormone (rhGH) in the mid-1980s led to many clinical investigations of its potential applications *(15,16)*. In addition to the treatment of GH deficient children and adults, rhGH exhibited beneficial effects in the treatment of patients with burns, bone fractures, and Turner's syndrome. Recently, rhGH has shown promise in reversing the catabolic effects of glucocorticoids, chemotherapy, and AIDS and in improving body composition in elderly individuals *(17–20)*. This explosion in potential clinical applications for GH stimulated further research on the GHRP's and their peptidomimetics. More potent analogs of GHRP-6 have been described (Fig. 1) and their clinical evaluation are currently underway *(7,21–24)*. More recently, cyclic peptides and modified tri- to pentapeptides based on the GHRPs were reported by McDowell et al. *(25)* to exhibit potent GH releasing activity. Although low oral bioavailability (<1%) has been reported for all the GHRP's to date, they have clearly established that a relatively small molecule (MW <1000 kDa), administrated orally, can stimulate the release of endogenous GH and thus may offer a practical alternative to subcutaneous treatment with costly rhGH.

DISCOVERY OF THE BENZOLACTAM SECRETAGOGUES

Directed Screening Approach

With the renewed interest in potential clinical applications of GH, Merck researchers in 1988 became interested in discovering an orally active nonpeptidyl mimic/peptidomimetic of GHRP-6. Extensive structure-activity relationships for GHRP-6 had already been published *(5,8,9)*. Aromatic residues were favored at positions 2, 4, and 5 and a basic amino terminus was important for GH releasing activity. In addition, preliminary evidence at Merck suggested that the GHRP-6 receptor (hereafter referred to as the GHS receptor) may be G-protein linked. Based on this information, compounds from the Merck Sample Collection were selected for screening in a GH releasing rat pituitary cell culture assay *(26)*. Data from this assay are presented in this chapter as EC_{50}'s - the dose required for half maximal GH release. From this effort, benzolactam **1** *(10,27)* was discovered and shown to release GH in a dose-dependent and specific manner with an $EC_{50} = 3\ \mu M$. Notwithstanding its modest potency in this assay (cf. GHRP-6, $EC_{50} = 10$ n*M*), it was a remarkable achievement considering the rarity in 1988 of nonpeptide mimics of peptide agonists. The carboxylic acid moiety in **1** was initially replaced by a tetrazole, a well established carboxylic acid bioisostere in many angiotensin II antagonists, to give the more potent racemic analog **2** (EC_{50} = 120 n*M*). Resolution of racemic **2** identified the R-enantiomer **3** (L-692,429) as the biologically active isomer (EC_{50} = 60 n*M*) (Fig. 2.)

Benzolactam **3** exhibited little or no activity at 10 μ*M* in over 50 other receptor binding assays, except for modest activity ($IC_{50} = 6\ \mu M$) as an angiotensin II antagonist. Molecu-

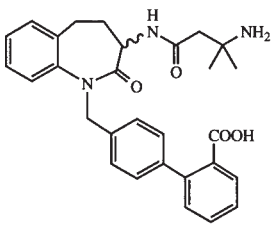
GHRP-6 His-*D*-Trp-Ala-Trp-*D*-Phe-Lys-NH_2

GHRP-1 Ala-His-*D*-β-Nal-Ala-Trp-*D*-Phe-Lys-NH_2

GHRP-2 *D*-Ala-*D*-β-Nal-Ala-Trp-*D*-Phe-Lys-NH_2

Hexarelin His-*D*-2-MeTrp-Ala-Trp-*D*-Phe-Lys-NH_2

Fig. 1. Selected growth hormone-releasing peptides (GHRPs).



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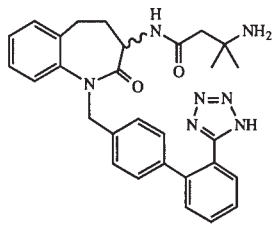

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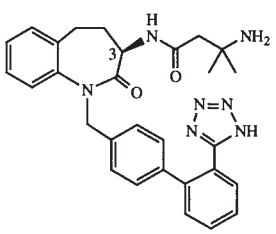
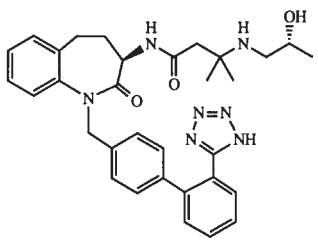


3
L-692,429



4
L-692,585

Fig. 2. Benzolactam growth hormone secretagogue lead structures.

lar modeling of **3** and GHRP-6 places the benzolactam ring and its C-3 chiral center onto the D-Trp residue and its α-carbon in GHRP-6, respectively. The basic amine side-chain in **3** occupies the same region as the N-terminal amino group in the hexapeptide *(27)*. DeVita has recently published cyclic analogs of **3** that exhibit potent GH releasing activity and thus, lends support for a bent conformation for GHRP-6 and the close proximity of the side-chain amine and the biphenyltetrazole in the bioactive conformation of **3** *(28)*. Mechanistically, **3** is identical to GHRP-6 in vitro. Rat pituitary cells maximally stimulated by **3** are unaffected when treated with GHRP-6 (and vice versa) but remain responsive to GHRH treatment. The hexapeptide antagonist His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2 blocks the GH releasing properties of GHRP-6 and **3**. Subsequently, **3** was shown to have a $K_i = 63$ n*M* compared to 6 n*M* for GHRP-6 in a rat pituitary membrane receptor binding assay *(29)*.

Clinical Evaluation of L-692,429

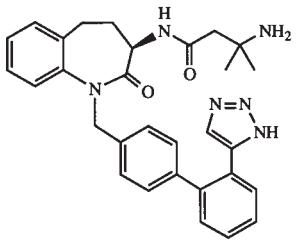
L-692,429 (3) was shown to release endogenous GH in rats, pigs, sheep, dogs, and rhesus monkeys when administered intravenously. In dogs the release of GH was shown to be dose-dependent with a minimum effective dose of 0.1 mg/kg *(30)*. L692,429 had little effect on other hormones except for slight elevation in cortisol. Unfortunately, **3** showed poor oral efficacy in dogs (>30 mg/kg) owing to poor oral bioavailability (2%) *(31)*.

Even though excellent clinical efficacy with GHRP-6 had been demonstrated, L-692,429 was tested intravenously in humans in order to validate our peptidomimetic approach to GH release in humans. In healthy young males L692,429 ($t_{1/2}$ = 3.8 h) was found to release GH in a dose-dependent fashion with a minimum effective dose of 0.2 mg/kg *(32)*. As observed with all the GHRPs, there were small transient increases in cortisol and prolactin after L-692,429 administration. No significant changes in other pituitary hormones or changes in insulin-like growth factor (IGF)-1, glucose, or insulin levels were observed. L-692,429 was well tolerated with only a transient flushing or warm sensation being reported. In healthy elderly (71± 5 yr) subjects L-692,429 has been reported to release GH, although the response is somewhat less than in healthy young men *(33)*. L-692,429 has also been shown to partially reverse glucocorticoid suppression of GH secretion and may therefore be useful in reversing the catabolic effects of prednisolone *(34)*.

Structure-Activity Relationships

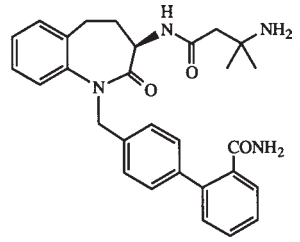
The validation of L-692,429 as a peptidomimetic of GHRP-6 in humans and its excellent safety profile prompted a major program at Merck to discover a more potent analog of L-692,429 with good oral bioavailability for development as an oral GH secretagogue. To address these issues of potency and oral bioavailability, an extensive investigation of the structure-activity relationships for **3** was undertaken. This effort has been reviewed recently *(35–37)* and only the key results will be discussed herein. The basic amino group in **3**, as with the GHRPs, is critical for GH releasing activity. Modifications of this amino group afforded the 2(R)-hydroxypropyl analog **4** (L-692,585) that was 20-fold more potent than **3** in releasing GH in the rat pituitary cell assay (EC_{50} = 3 n*M*) *(38,39)*. In the rat GHS receptor binding assay *(29)* **4** exhibited a K_i = 0.8 n*M*, which is 60-fold more potent than **3** (K_i = 63 n*M*) (Fig. 2). This analog represented a benchmark since it was the first peptidomimetic that was more potent than the hexapeptide GHRP-6 (EC_{50} = 10 n*M*) in the rat pituitary cell assay. In dogs it was shown to be active at intravenous doses as low as 5 μg/kg: 20-fold more potent than benzolactam **3** and twofold more potent than GHRP-6 *(40)*. Unfortunately, the bioavailability of **4** in rats and dogs was not improved over **3**.

The zwitterionic character of both **3** and **4** most likely contributes to its poor absorption in animals. Since the basic amine is critical for GH releasing activity, much of the early medicinal chemistry on the benzolactam lead focused on removing the negatively charged tetrazole. Because the GHRPs did not require a negatively charged group for potent GH releasing activity, functionalities capable of forming hydrogen bonds with the GHS receptor were investigated as replacements for the tetrazole in the benzolactam lead (Fig. 3). Neutral heterocycles (e.g., triazole analog **5**), carboxamides (e.g., **6**), and ureas (e.g., **7**) were all found to be excellent neutral surrogates for the negatively charged



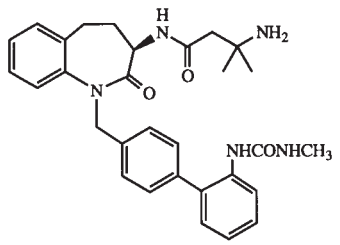
5

EC_{50} = 40 nM



6

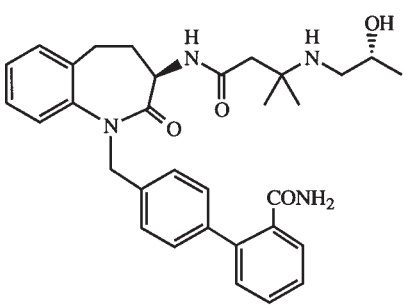
EC_{50} = 80 nM



7

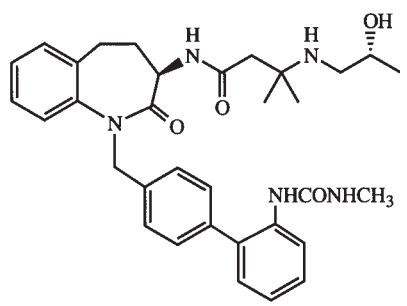
EC_{50} = 50 nM

Fig. 3. Neutral 2'-biphenyl tetrazole replacements.



8

EC_{50} = 4 nM

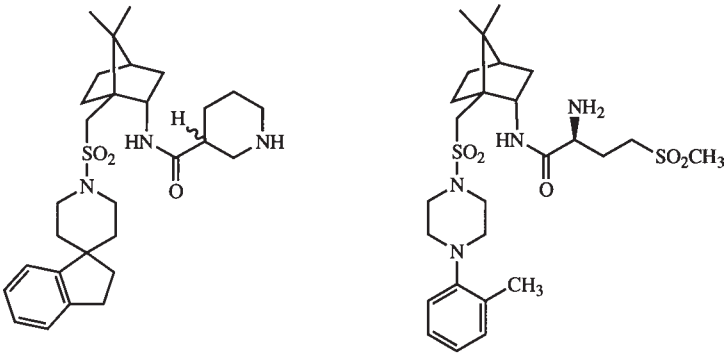


9

EC_{50} = 3 nM

Fig. 4. 2'-Biphenyl surrogates with potency enhancing 2(R)-hydroxypropylamino side-chains.

tetrazole, thus confirming the hydrogen bonding role for the 2'-substituent in this lead *(37,41,42)*. Combined with the 2(R)-hydroxy side-chain, these neutral surrogates afforded very potent analogs (e.g. **8** and **9**) as expected (Fig. 4). However, in spite of these profound structural and physico-chemical changes to these molecules, an improvement in oral bioavailability was not forthcoming. This seemingly unsolvable problem with the benzolactam lead prompted Merck researchers to continue screening for new GH secretagogue structures.



10 **11**

Fig. 5. Camphorsulfonamide growth hormone secretagogues.

THE PRIVILEGED STRUCTURE APPROACH

The Spiroindanylpiperidine Lead From Screening

The discovery of the benzolactams demonstrated that potent nonpeptide GH secretagogue agonists could be discovered in the 500–600 Kda molecular weight range. This was quite a breakthrough. Although there were many nonpeptide antagonists known at the time, the only precedent for non-peptide agonists were the opioid peptide mimetics including morphine and the analgesic benzodiazepine tifluadom. Compound screening continued after the benzolactam discovery in an effort to find different core structures that might more easily be converted to an orally active drug. There was precedent for additional leads in numerous structural variants of morphine especially since GHRP-6 was itself derived from enkephalin.

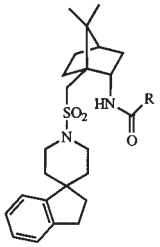
Indeed another lead, compound **10**, was found by directed screening. This camphorsulfonamide originated in a program from which eventually came orally active oxytocin antagonists such as compound **11** *(43)* (Fig. 5).

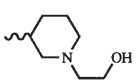
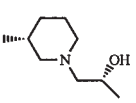
In an effort to enhance the potency and specificity of this new lead (EC_{50} = 0.30 μ*M* (GH secretagogue); IC_{50} = 0.068 μ*M* (oxytocin antagonist), analogs containing a tolylpiperazine or a spiroindanylpiperidine were synthesized. Both series were comparably active and some of the latter type are shown in Table 1 *(44)*.

Like the lead compound **10**, the most active of its analogs contains a nipecotic acid part-structure. This was a surprise to us since we hoped the amino acid side-chains that conferred high potency to the benzolactams might do likewise in the camphorsulfonamide series. Even an *N*-2-hydroxypropyl substituent (**17**) that was taken from the highly active benzolactam **4** (L-692,585) did not afford sufficient potency to justify in vivo testing.

Nevertheless, the discovery of this camphorsulfonamide series was important. It demonstrated that GH secretagogue agonist activity need not be limited to a narrowly defined pharmacophore and it contributed to the selection of the spiroindanylpiperidine nucleus for use in a "privileged structure" derivatization project. The term "privileged structures" was introduced by Evans et al. *(45)* to describe core structures that recur frequently in receptor ligands and whose derivatization, they suggested, was a useful way of discovering agonist and antagonist leads. Their design of cholecystokinin (CCK)-A antagonists was based on the "privileged" benzodiazepine core of the natural product CCK-A antago-

Table 1
Camphorsulfonamide Structure–Activity Relationships



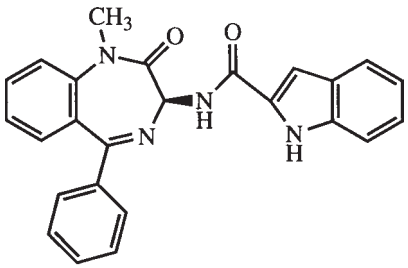
Compound	R	EC_{50} (nM)[a,b]
10		300
12		>1,000
13		140
14		>10,000
15		>10,000
16		300
17		90

[a]Data from ref. *44*.
[b]Data are presented here and in subsequent tables as EC50—the dose required for half maximal GH release from cultured rat pituitary cells as described in ref. *26*.

nist asperlicin *(46)*. They modified and derivatized that core unit culminating in the synthesis of **18** with its remarkable IC_{50} = 0.08 n*M* as a CCK-A receptor antagonist *(47)*. Importantly, the work of Evans et al. *(45)* demonstrated that the privileged structure strategy for biogenic amine antagonists could also be applied to peptide ligands (Fig. 6).

The Privileged Structure Concept

The recognition of conserved structural units in receptor antagonists originated with Ariens et al. *(48)*. They noted the occurrence of a hydrophobic, double-ring motif in many biogenic amine antagonists and suggested that these antagonists bind in "accessory binding sites" close to the "active sites" of receptors. The example of chlorpromazine that has anticholinergic, antihistaminic and α-adrenergic blocking actions was cited by them with implied similarity in the proposed accessory binding sites of these receptors. As a corollary, attaining excellent receptor specificities is often a problem that must be addressed while derivatizing privileged structures.



18

Fig. 6. CCK-A receptor antagonist—devazepide—a privileged structure derivative.

The spiroindanylpiperidine component of **10** was considered by us to be a privileged structure since ligands containing it were also known and subsequently published for the oxytocin *(43)* and sigma receptors *(49)*. And more recently antagonists that incorporate it have been described for the neurokinin (NK)-1 *(50)* and NK-2 *(51)* receptors and in dual NK-1 and NK-2 antagonists *(52)*. In addition agonists of the C_{5a} receptor have been described based on spiropiperidines *(53)*.

Privileged Structure Derivatization with Amino Acids

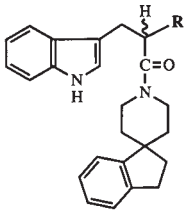
The hypothesis that privileged structures bind near the "active site" of receptors and that they bind to both peptide and nonpeptide receptors was intriguing. It suggested to us that peptide agonists and antagonists might be achieved by appending small peptide units onto privileged structures. Hopefully, there would be some overlap with the peptide agonist binding area. If not, such derivatization could still be worthwhile since amino acid side-chains obviously provide a rich diversity of functionality to interact with proteins. In the case of the spiroindanylpiperidine unit, single capped amino acids were chosen for derivatization in part since it was known from the benzolactams that a large structural unit was not required to produce agonist activity. One of the highlights of this derivatization project was compound **19** (Table 2) whose $EC_{50} = 50$ n*M* in the rat pituitary GHS assay was remarkable especially since it was tested as an unseparated mixture of four diastereomers. In retrospect, this excellent activity was ascribed to the fact that each component of **19** was present in GHS active compounds. The spiroindanylpiperidine came from the screening lead **10**, tryptophan is a key amino acid in the GHRPs and the quinuclidene part-structure was present in an unpublished Merck screening lead. That these modular units are arranged in compound **19** in the proper order, at the proper distances and with acceptable linking groups was quite fortuitous *(54)*. Nor was it predictable that compound **19** would be an agonist since, at the time, the other known spiropiperidines were receptor antagonists.

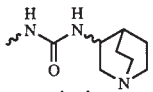
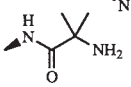
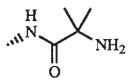
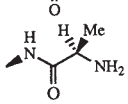
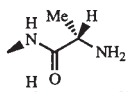
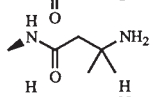
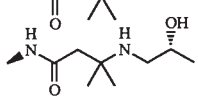
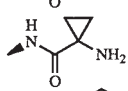
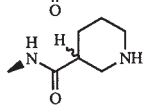
THE DISCOVERY OF ORALLY ACTIVE MK-0677

Structure-Activity Studies Leading to MK-0677

The bioavailability problem had not been solved with **19** since it failed to elevate GH in beagles after oral administration at 5 mg/kg. To address this deficit, attention was

Table 2
Spiroindanylpiperidine Lead–Modification of the Amino Acid Side-Chain



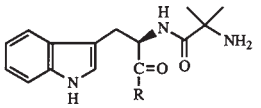
Compound	R	EC_{50} (nM)[a]
19		50
20		14
21		10,000
22		1,000
23		840
24		76
25		2.6
26		2,100
27		90

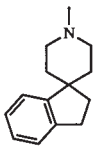
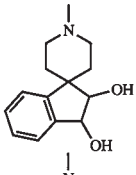
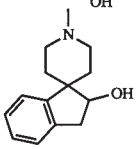
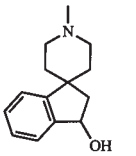
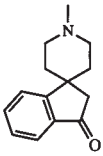
[a]Data from ref. *55*.

focused on the amine side chain in the belief that urea functionality and the strongly basic quinuclidene group might be responsible for poor uptake from the GI tract. Preference for D-tryptophan stereochemistry was established and then we turned to the amino side-chains that were particularly useful with the benzolactams, this time with success, as illustrated in Table 2. Compound **25** containing the potency enhancing 2-hydroxypropyl group of the benzolactam L-692,585 (**4**) had the highest intrinsic potency (EC_{50} = 2.6 n*M*) among these analogs of 19. Nonetheless, the most orally active of these early analogs was compound 20. It produced good GH elevation following an oral dose of 2 mg/kg in dogs despite an EC_{50} of only 14 n*M* in the rat pituitary cell assay *(55)*.

The further characterization of **20** included IC_{50}s >10 μ*M* in twenty-four G-protein linked receptor assays. This specificity was very welcome so early in the project given our categorization of the lead as a privileged structure derivative. Also its bioavailability in rats after iv and po administration was determined to be >40% *(55)*. The lead might

Table 3
Spiroindanylpiperidine Modifications



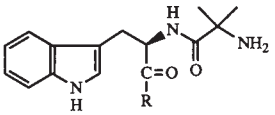
Compound	R	EC_{50} (nM)[a]
20		**14**
28		**30**
29		**65**
30		**0.6**
31		**1.2**

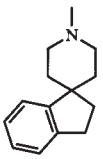
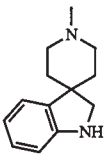
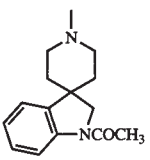
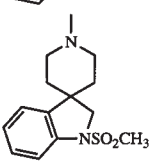
[a]Data from ref. *56*.

have been selected for safety assessment studies but results from ongoing analog syntheses suggested that greater intrinsic activity was possible.

The potency breakthrough was achieved by derivatizing 1'-(t-butoxycarbonyl)-spiro[lH-indene-1,4'-piperidine], which is an intermediate used in the synthesis of 20. Osmylation of the indene double bond afforded a diol that was elaborated to compound **28** whose intrinsic activity was slightly less than the corresponding spiroindane **20** as shown in Table 3 *(56)*. However, hydroboration yielded a 1:1 mixture of alcohols that, after separation, afforded compounds **29** and **30**. The latter with an EC_{50} = 0.6 n*M* in the rat pituitary cell assay is more than 10-fold as potent as compound **20** and the derived ketone analog **31** is nearly as potent. Unfortunately, when these analogs were tested orally in dogs they were only twice as active in elevating GH as compound **20** *(56)*. Apparently they were not as bioavailable as the parent (**20**) possibly the result of carbonyl reduction and conjugation of the alcohol. To investigate the implications of this possibility, other polar substituents with greater metabolic stability were introduced at the indane benzylic position.

Table 4
Spiroindanylpiperidine Optimization



Compound	R	EC_{50} (nM)[a]
20		**14**
32		**7**[b]
33		**4.2**[b]
34		**1.8**

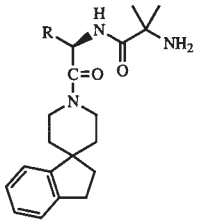
[a]Data from ref. *56*.
[b]Unpublished results.

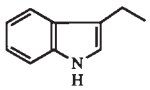
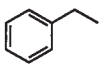
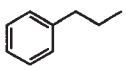
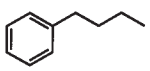
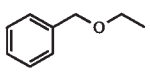
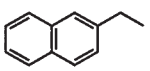
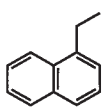
Our initial approach involved introducing and derivatizing an aza group at the indane benzylic position. As summarized in Table 4, the unsubstituted spiroindoline derivative **32** raised rat pituitary cell potency slightly. However, the *N*-acetyl and *N*-methanesulfonyl derivatives **33** and **34** were markedly more potent *(57)*. Other acyl and sulfonyl analogs are active at this position but attention focused on compound **34** (EC_{50} = 1.8 n*M*) with the expectation that metabolic stability and minimal size would favor good oral activity. Nevertheless, despite excellent intrinsic activity, only one of two dogs at both the 0.5 mg/kg and 1.0 mg/kg oral dose levels responded to **34** with good GH elevations *(58)*.

Concurrently, the D-tryptophan component of compound **20** was being replaced with other D-amino acids some of which are shown in Table 5. Napthalene in compounds **39** and **40** was a surprisingly poor replacement for the indole group of compound **20** especially since it is used in GHRP-1 and GHRP-2 as an indole surrogate and in a series of highly active small peptide derivatives described by McDowell et al. *(25)*.

However, the phenylpropyl and benzyloxymethyl compounds **37** and **38**, respectively, retained intrinsic potency quite comparable to that of **20** and even the activity of the phenethyl

Table 5
Aromatic Amino Acid Variations



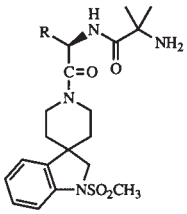
Compound	R	EC_{50} (nM)[a]
20		14
35		250
36		22
37		10
38		17
39		900
40		1,100

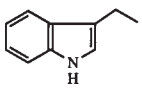
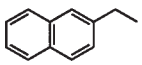
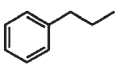
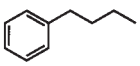
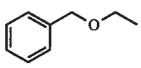
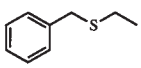
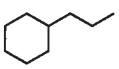
[a]Data from ref. *57*.

analog **36** was only twofold weaker than **20**. The oral potency of **36** in dogs was comparable to **20** but compounds **37** and **38** showed good elevations of growth hormone in oral doses as low as 0.5 mg/kg and thus were approximately fourfold more active orally than compound **20** *(57)*.

These and other indole replacements were tried in the spiroindoline series some of which are shown in Table 6. Activities paralleled those of the corresponding spiroindane derivatives and peaked in the phenylpropyl and benzyloxymethyl analogs **43** and **44**, respectively. β-Napthalene in compound **41** was a poor replacement for indole. The substitution of sulfur for oxygen in compound **45** resulted in a 10-fold loss in cell culture potency apparently indicating a limitation in the optimum length of the amino acid side-chain. The latter need not contain an aromatic residue as indicated by comparable activities of compounds 42 and 46. It was also reported by Patchett et al. *(54)* that the (L)- isomer of compound **38** was only poorly active (EC_{50} = 500 n*M*), which led to the suggestion that this amino acid position might correspond to the 2-D-Trp position of GHRP-6.

Table 6
Discovery of L-163,191 (MK-0677)-Compound 44



Compound	R	EC_{50} (nM)
34		1.8[a]
41		13[b]
42		5.0[b]
43		1.5[c]
44 MK-0677		1.3[a]
45		17[b]
46		7.0[b]

[a]Data from ref. *54*.
[b]Unpublished results
[c]Data from ref. *37*.

The compounds shown in Table 6 were screened in dogs for growth hormone elevation following oral administration. Efficacies were best with compound **43** and compound **44** (L-163,191) and, of these two, the latter seemed to be consistently more potent.

Animal Evaluations of MK-0677

In additional dog studies, compound **44** (L-163,191) was active (a fourfold increase of peak GH over baseline) orally at 0.0675 mg/kg (1:2 responded), at 0.125 mg/kg (6:8 responded) and at 0.25 mg/kg (7:8 responded). Following intravenous administration 4:4 dogs responded at the 0.025 mg/kg level. In a balanced crossover study using eight beagles, compound **44** given orally increased peak GH concentrations in a dose responsive manner with a 5.3-fold increase at 0.25 mg/kg, a 9.0-fold increase at 0.50 mg/kg, and a 15.8-fold increase at 1.0 mg/kg. After a single oral 1 mg/kg dose in three dogs, GH levels remained elevated out to 360 min and insulin-like growth factor (IGF-1) was significantly elevated 30% at 480 min *(59)*.

Because the GH response to compound **44** in pituitary cell culture is rapidly desensitized, chronic in vivo studies were important. When compound **44** was orally administered daily to six beagles for four days at a 1 mg/kg dose, mean GH peak and AUC on day 4 were significantly higher than vehicle treated controls although reduced by 79 and 75%, respectively. GH secretion remained pulsatile throughout the experiment. Importantly, mean IGF-1 levels measured just before dosage on day 4 had increased from the vehicle control level of 50 ± 13.4 ng/mL to 108.8 ± 26.9 ng/mL *(60)*.

The selectivity of L-163,191 *(44)* had been demonstrated earlier in over 50 in vitro assays in which its IC_{50} values exceeded 10 μ*M*. These included receptors for ligands known to affect GH release such as acetyl choline, galanin, somatostatin, met-enkephalin, and clonidene *(14)*. It was also shown that growth hormone releasing hormone (GHRH) would not displace [^{35}S] L-163,191 from its receptor *(29)*. The in vivo selectivity of L-163,191 was examined in dogs *(59)*. When it was given as a single 0.25 mg/kg intravenous dose to eight beagles, the GH mean peak level was increased 20.4-fold while cortisol levels were elevated from 2.6 ± 0.2 μg/dL for the saline control to 6.2 ± 0.5 μg/dL. This increase was not unexpected since cortisol elevations had been seen with GHRP-6 *(6)* and with the benzolactam L-692,429 (**3**) in dogs *(30)* and in humans although within normal ranges *(32,33)*. Also, insulin and glucose levels were slightly elevated in dogs with a 1 mg/kg oral dose of L-163,191 and there were no significant changes in luteinizing hormone, prolactin or thyroxine levels *(59)*. Thus, with the exception of moderate cortisol elevation, the other hormonal and metabolic parameters were not significantly changed by L-163,191 in dogs at 1 mg/kg oral dose.

Pharmacokinetics

A precise bioavailability figure for L-163,191 (**44**) in dogs was not possible owing to nonlinear kinetics, however, it is estimated to be >60% *(54,61)*. In rats the oral bioavailability was dose dependent in the range 6–22% and the terminal half-life was a relatively short 1.8 h at an iv dose of 0.5 mg/kg *(61)*. However, the rate of elimination of L-163,191 was much slower in dogs and its volume of distribution was lower resulting in a terminal half-life of between 4–6 h in this species.

Oral Properties of MK-0677 in the Clinic

Based on the indications of potency, duration of action, oral bioavailability, and selectivity, which are summarized above, compound **44** (L-163,191) as its crystalline mesylate salt was selected for safety assessment studies. Subsequently it entered clinical testing and was given the designation MK-0677 as a potential product candidate. In confirmation of the animal data, MK-0677 was found to raise IGF-1 in man following oral administration. The first published account was by Copinischi et al. *(62)* who treated nine healthy young men daily for seven days in a crossover comparison of placebo and 5- and 25-mg doses of MK-0677. IGF-1 levels were increased in a dose dependent manner without detectable elevations of GH. Nor was any evidence observed of induced hypercortisolism. Chapman et al. *(63)* reported results shortly thereafter of a study in which 32 healthy elderly men and women received placebo, or 2, 10, or 25 mg MK-0677 orally, once daily for two separate study periods of 14 and 28 days. Dose-dependent increases in GH and IGF-1 were observed. Remarkably, the dose of 25 mg/day of MK-0677 in most of these subjects brought serum IGF-1 levels into the range seen in young adults. In this study also plasma and urinary cortisol levels were similar in all groups.

MK-0677 AS A MIMETIC OF GHRP-6

Cell Culture Studies

The evidence that MK-0677 is a functional mimetic of GHRP-6 is extensive and has been summarized by Patchett et al. *(37,54)* and Smith et al. *(14)*. Neither increase cAMP in pituitary somatotrophs, although they synergize with GHRH to increase its GH secretion and cAMP elevation.Pituitary cell cultures become rapidly desensitized to both GHRP-6 and MK-0677 and cells desensitized to one are desensitized to the other. However, the MK-0677 desensitized cells have not been depleted of GH since they remain responsive to GHRH. Furthermore, the GH secretagogue activity of MK-0677 can be antagonized by the GHRP-6 antagonist His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2 MK-0677 and GHRP-6 share a common second messenger pathway in that both activate protein kinase C, produce a depolarization of somatotroph membranes and elevate intracellular Ca^{2+} levels. Neither alone affects the release of ACTH from pituitary cells. In summary, GHRP-6 and MK-0677 in all parameters tested behave functionally the same in rat pituitary cell culture.

Receptor Studies with [^{35}S] MK-0677

Early attempts to identify a specific receptor for the GHRPs by the Merck group and by others *(64,65)* made use of [^{3}H] and [^{125}I]-labeled ligands derived from GHRP-6. Success was limited by their relatively low specific activity and high nonspecific binding nor, in the experience of the Merck group, did the secretagogue activity of compounds correlate well with their potency in displacing labeled ligand. With discovery of MK-0677 and the demonstration of its high selectivity in respect to other receptors, a renewed effort was made to produce from it a radioligand of high specific activity. [^{125}I] could not be used in the benzyloxy-para-position of MK-0677 since considerable loss of intrinsic secretagogue potency would ensue. Nor could the Bolton-Hunter reagent be used since conjugation of the MK-0677 amino group would lead to complete loss of bioactivity. Instead Dean et al. *(66)* developed a synthesis of methane [^{35}S]-sulfonyl chloride and utilized this reagent in the preparation of [^{35}S]-MK-0677 in greater than 99% radiochemical purity with specific activities ranging from 700 to 1100 Ci/mmol. They were thus able to achieve high specific activity in a potent and selective radioligand with relatively low lipophilicity (log P = 3.0).

With the availability of [^{35}S]-MK-0677, Pong et al. *(29)* identified a saturable, high-affinity binding site in porcine and rat anterior pituitary membranes. Its K_D of 161 ± 11 pM in rat pituitary membranes closely corresponded to a K_i of 240 pM determined by the displacement of the radioligand by unlabeled MK-0677. This K_i for receptor binding is slightly lower than its EC_{50} of 1.3 nM for GH secretion in the rat pituitary cell. Corresponding data for GHRP-6 (K_i = 6 nM) were in line with its potency in the cell culture assay (EC_{50} = 10 nM). The specific binding of [^{35}S]-MK-0677 was Mg^{2+} dependent and inhibited by the GTPγS, which is consistent with the receptor being G-protein linked. Importantly, double reciprocal plot analysis of saturation isotherms for the [^{35}S] MK-0677 binding demonstrated that GHRP-6 inhibition could be overcome by increasing concentrations of [^{35}S]-MK-0677. These data suggest that the two secretagogues interact competitively at the same receptor and further confirm the peptidomimetic nature of MK-0677.

Receptor Cloning and Mutagenesis

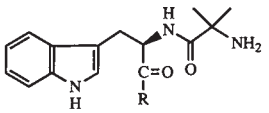
Cloning of the MK-0677 receptor was achieved by Howard et al. *(13)* using cRNA pools derived from a swine pituitary cDNA library. Expression of the receptor in *Xenopus* oocytes was detected by measuring MK-0677 induced Ca^{2+} elevation. Given the low level of receptor expression in the pituitary, high sensitivity was required. It was obtained by co-injecting cRNA for the bioluminescent Ca^{2+} sensitive protein aequorin along with cRNA for the G-protein α-subunit $G_{\alpha 11}$. The initially cloned nucleotide sequence was used to obtain full length swine and human GHS receptor cDNAs. They encode polypeptides of 366 amino acids with seven transmembrane domains and with approx 93% identity comparing the swine and human receptors. They are novel receptors whose sequences are closest to those of neurotensin and TRH with approx 35% and 29% identity, respectively. Ligand binding K_i's were determined in displacement assays using [^{35}S] MK-0677 bound to transiently transfected COS-7 cells and were in general agreement with ligand potencies in the rat pituitary cell assay: MK-0677 (K_i = 0.1 n*M*), GHRP-6 (K_i = 1.9 n*M*) and GHRP-2 (K_i = 0.21 n*M*) *(13,29)*.

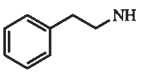
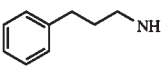
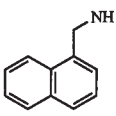
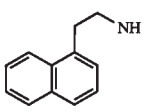
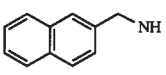
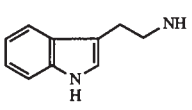
A functional receptor assay was established in HEK 293 cells based on Ca^{2+} elevation measured by aequorin bioluminescence. The assay, in which both MK-0677 and GHRP-6 are active, was used in mutagenesis studies to acquire some understanding of the receptor's essential functionality. An important structural feature of GH secretagogues is their basic amine. Presumably when a secretagogue binds, its amino group makes an electrostatic interaction with a negatively charged residue in the receptor. Attention focused on Glu^{124} in TM3 since it is in the approximate location of Asp^{113} in the β-adrenergic receptor *(67)* and of Asp^{122} in the somatostatin type 2 receptor *(68)*. In both instances these acidic residues, which are critical for receptor activation, are proposed to be amine binding sites. In fact, when the E124→Q124 mutant GHS human receptor was expressed in HEK 293 cells, both MK-0677 and GHRP-6 at 100 n*M* did not activate it as determined by the aequorin assay *(69)*. The inference that E^{124} is an amine binding site in the GHS receptor and that this interaction is important for the activity of GHRP-6 and MK-0677 is a reasonable possibility assuming the E124→Q124 mutation did not cause a conformational change in the receptor.

Tripeptide Analogs and A Pharmacophore Model

The evidence summarized above strongly supports the designation of MK-0677 as a mimetic of GHRP-6 because they are functionally equivalent, they bind competitively to the same receptor, and this binding responds similarly to E124→Q124 mutation. Furthermore, an analog of GHRP-6 with aminoisobutyric acid substituted for its N-terminal histidine is a highly active secretagogue *(70)*. Even if one assumes binding correspondence near Glu^{124}, the overlap, if any, of their other pharmacophore groups is not certain. Ambiguities in defining the active conformation of GHRP-6 also limit definitive comparisons with MK-0677. However, the Merck group *(27)* hypothesized in general agreement with Momany et al. *(9)* and Momany *(71)* that GHRP-6 has a bent conformation when bound to the receptor. Using the published conformation of Schoen et al. *(27)* and superimposing the N-termini of GHRP-6, and MK-0677, we hypothesized that the spiroindoline of MK-0677 and the indole group of Trp^4 in GHRP-6 may share the same binding site on the receptor. To test this hypothesis, some of the compounds in Tables 7 and 8 were synthesized by Yang et al. *(72)*. Reasonable

Table 7
Spiropiperidine Replacements

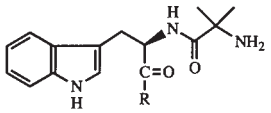


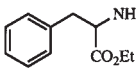
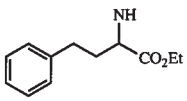
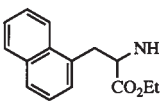
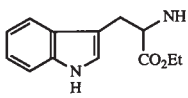
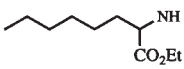
Compound	R	EC_{50} (nM)[a]
47		Inactive
48		390
49		Inactive
50		860
51		85
52		57

[a]Data from ref. *72*.

potency was observed with the β-napthylmethylamide **51** (EC_{50} = 85 n*M*) and the indolylethylamide **52** (EC_{50} = 57 n*M*). However, most strikingly, the D-amino acid derivatives **54** (EC_{50} = 3 n*M*) and **56** (EC_{50} = 6 n*M*) are more active in our assay than GHRP-6 (EC_{50} = 10 n*M*). In contrast, the hydrophobic, aliphatic amino acid **57** was poorly active (EC_{50} = 555 n*M*) and the absence of an amino acid side-chain in **58** led to even less activity (EC_{50} = 1060 n*M*). This SAR study achieved for us a reduction of the GHRP-6 structure to the tripeptide level with retention of greater activity than GHRP-6 in the (D)-isomers of compounds **54** and **56**. A similar achievement based on conformationally restricted analogs and molecular modeling studies also brought McDowell et al. *(25)* to the conclusion that the minimum pharmacophore required for the expression of GHRP type activity is only a basic amino group and two aromatic amino acids. The fact that we were able to convert MK-0677 to a highly active peptide of the same size strongly supports a peptide-peptidomimetic relationship at this tripeptide level.

Table 8
Tripeptide Growth Hormone Secretagogues



Compound	R	Stereochemistry	EC_{50} (nM)[a]
53		D L	240 350
54		D L	3 50
55		D	40
56		D L	6 35
57		DL	555
58		—	1,060

[a]Data from ref. *72*.

Peptide and peptidomimetic agonist ligands need not bind to receptors in the same way *(73)*. However, if the tripeptides and MK-0677 do bind with correspondence of their amino and aromatic residues, then, in this instance, the privileged structure seems to be simply a conformationally rigid moiety which is able to share the binding site of an aromatic amino acid of the tripeptide. Ariens' concept of an accessory binding site as applied to biogenic amine antagonists would then not be necessary nor apply to these peptidomimetic secretagogue agonists.

CONCLUSIONS

The GHRPs were discovered by Cyril Y. Bowers and his colleagues in the late 1970s. Through Dr. Bowers' dedicated efforts, their potency and in vivo properties were perfected culminating in clinical demonstrations of sustained growth hormone release. Efficacy as measured by growth improvements in GH-deficient children has been demonstrated and other possible uses are being studied. It is remarkable that these secreta-

gogues were developed without knowledge of the yet unidentified natural hormone which they presumably mimic.

In its discovery phases, the research that ultimately produced MK-0677 drew heavily upon the structure activity studies that led to GHRP-6, GHRP-2, GHRP-1, and especially benzolactam L-692,429. The work at Merck illustrates the value of privileged structure derivatization and directed screening in the design of peptidomimetics. Nonpeptide selections for screening and for exploratory synthesis reflected the essential structural features of the GHRPs and, remarkably, agonist activity was found in leads whose sizes are considerably less than the GHRPs. At the time only small molecule agonists were known of the opiate peptides.

Even with small molecule peptidomimetic leads, potency, selectivity, and good oral bioavailability were only achieved through the efforts of many chemists, biologists, and drug metabolism specialists. Clinical studies have been undertaken to determine if MK-0677 and other GH secretagogues will make a contribution to medicine. Regardless of that outcome, the potency and selectivity of [^{35}S]-MK-0677 played an important role in the identification and cloning of the GHS receptor. The awaited next step in the GHRP story is the identification of the putative natural hormone. When that is achieved, our knowledge of the regulatory controls of pituitary growth hormone secretion will have reached an additional level of understanding.

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4 Molecular Characterization of Growth Hormone Secretagogue Receptors

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Contents

INTRODUCTION

The synthetic hexapeptide growth hormone releasing peptide 6 (GHRP-6) mediates growth hormone (GH) release from primary pituitary cells through a distinct mechanism from that controlled by growth hormone releasing hormone (GHRH) or somatostatin *(1–3)*. Biochemical and pharmacological evidence supports the notion that GHRP-6 and the nonpeptide growth hormone secretagogs (GHSs) act through the same receptor. Numerous attempts to characterize the GHRP or GHS receptors (GHS-Rs) biochemically were frustrated by a low GHS-R abundance. The development of procedures for high-specific-activity [^{35}S] radiolabeling of the nonpeptide GHS MK-0677 in conjunction with

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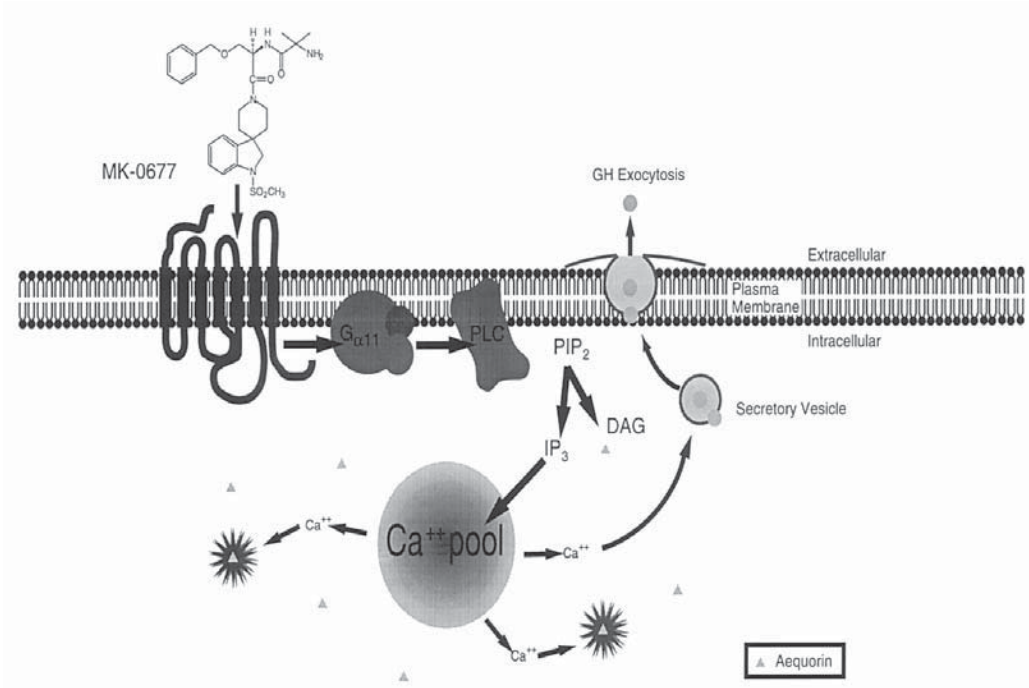


Fig. 1. Expression cloning rationale. Schematic representation of GHS-R coupling to $G_{\alpha 11}$ and PLC leading to intracellular Ca^{2+} release, which can be measured with the photoprotein aequorin.

improved receptor preparation procedures led to the identification of a GHS-R binding site *(4,5)*. The GHS-R bound [^{35}S]-MK-0677 with high affinity, and the rank order of potency of diverse peptide and nonpeptide ligands for [^{35}S]-MK-0677 displacement correlated with their in vivo GH secretory activity. Based on its binding characteristics the authors assumed that the GHS-R was a G protein-coupled receptor (GPC-R) found in low abundance in the anterior pituitary and hypothalamus. This data facilitated the development of a strategy to clone the GHS-R (Fig. 1). The assay for identification of the GHS-R relied on the knowledge that GHS-R activation leads to G protein-mediated activation of phosphoinositol-specific phospholipase C (PI-PLC) and subsequent calcium mobilization.

OOCYTE EXPRESSION CLONING

cDNAs encoding several low abundance cell membrane receptors have been isolated by functional expression either in *Xenopus* oocytes or in mammalian cells using specific assays to detect receptor-ligand interactions. These assays varied from radioligand binding to detection of intracellular calcium mobilization or secretion of a particular hormone *(6,7)*. Cloning of the GHS-R was hampered by the relative paucity of biochemical information on the receptor protein, because of its low abundance (6 fmol/mg membrane protein), and the requirement to use primary pituitary tissue as a source for mRNA or protein since cell lines expressing the receptor were lacking. GHS-R cloning required the development of a sensitive and robust high-throughput screening assay. The ability to functionally express the GHS-R in *Xenopus* oocytes, injected with swine pituitary poly

$(A)^+$ RNA, was shown by the detection of a rapidly activating Ca^{2+}-dependent chloride current in response to MK-0677 administration. Because only a small fraction of the *Xenopus* frogs tested (4 out of 50) had oocytes that gave positive responses, the authors determined whether the expression of a requisite G protein α subunit was limiting in some batches of *Xenopus* oocytes resulting in inefficient receptor-effector coupling.

G PROTEIN ADMINISTRATION RESTORES GHS-R COUPLING IN *XENOPUS* OOCYTES

The expression of several receptors in heterologous cells is increased by the coexpression of specific G_α subunits *(8–13)*. To test whether G protein addition could increase the reliability of GHS-R expression the authors developed a *Xenopus* oocyte expression assay that incorporated the jellyfish photoprotein aequorin, which in the presence of calcium and the cofactor coelenterazine is chemi-luminescent. In these experiments aequorin protein is injected 2–3 d following poly $(A)^+$ mRNA or library pool cRNA *(12,13)* injection into *Xenopus* eggs. The authors opted to coinject aequorin mRNA with pituitary poly $(A)^+$ mRNA into oocytes. The use of aequorin mRNA obviated the need for a second injection resulting in lower background responses (20 vs 80 cps) and higher throughput. In addition, the aequorin mRNA provided a translational control for each oocyte. Coinjection of swine poly $(A)^+$ mRNA with aequorin mRNA gave background light responses (30 cps) to MK-0677 when applied at a concentration of 1 μ*M*. However, when $G_{\alpha 11}$ cRNA was also coinjected (1:12 ratio [w/w] to poly $(A)^+$ mRNA), robust light emission (~1000 cps) was evoked by 1 μ*M* MK-0677 (Fig. 2 and Table 1). MK-0677-induced bioluminescence is selectively dependent on $G_{\alpha 11}$ when expressed concurrently with swine poly $(A)^+$ mRNA. MK-0677-stimulated bioluminescence could not be observed in the absence of poly $(A)^+$ mRNA or when poly $(A)^+$ mRNA was coinjected with six other individual G_α subunits given singly or in combination (PTX-sensitive: $G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha o}$; PTX-insensitive: $G_{\alpha q}$, $G_{\alpha 13}$, $G_{\alpha 16}$) (Table 1). Positive responses could be recorded using either aequorin protein or aequorin mRNA (Fig. 2). Expression appeared maximal at 36–48 h postinjection, can be detected in as little as 18, and is attenuated by 72 h.

A frequently reported pitfall of oocyte expression systems is their inherent variability in expression of heterologous genes. Therefore, the authors tested oocytes from six different *Xenopus* frogs for their ability to express the GHS-R from the same batch of swine pituitary poly $(A)^+$ mRNA. As shown in Table 2, all six frogs gave positive responses in almost all the eggs injected, dependent on the coexpression of $G_{\alpha 11}$. The magnitude of the bioluminescent response varied considerably, but did not prevent the assignment of a positive signal. To confirm and extend their initial observations, the authors evaluated the response of swine pituitary poly $(A)^+$-injected oocytes to challenges with lower concentrations of MK-0677 and GHSs of diverse chemical structures, including peptides (GHRP-6, GHRP-2) and benzolactam GHSs (L-692,429 and its inactive enantiomer L-692,428). Bioluminescent responses could be observed for concentrations of MK-0677 as low as 1 n*M* (data not shown) whereas other bioactive GHSs elicited positive responses as well (Table 3). Additional tissues thought to contain GHS-Rs either by direct radioligand binding or by virtue of their in vivo biological response to GHSs were also evaluated. Poly $(A)^+$ mRNA from human pituitary and rat hypothalamus and pituitary gave positive response to MK-0677, again strictly dependent on $G_{\alpha 11}$ expression (data not shown).

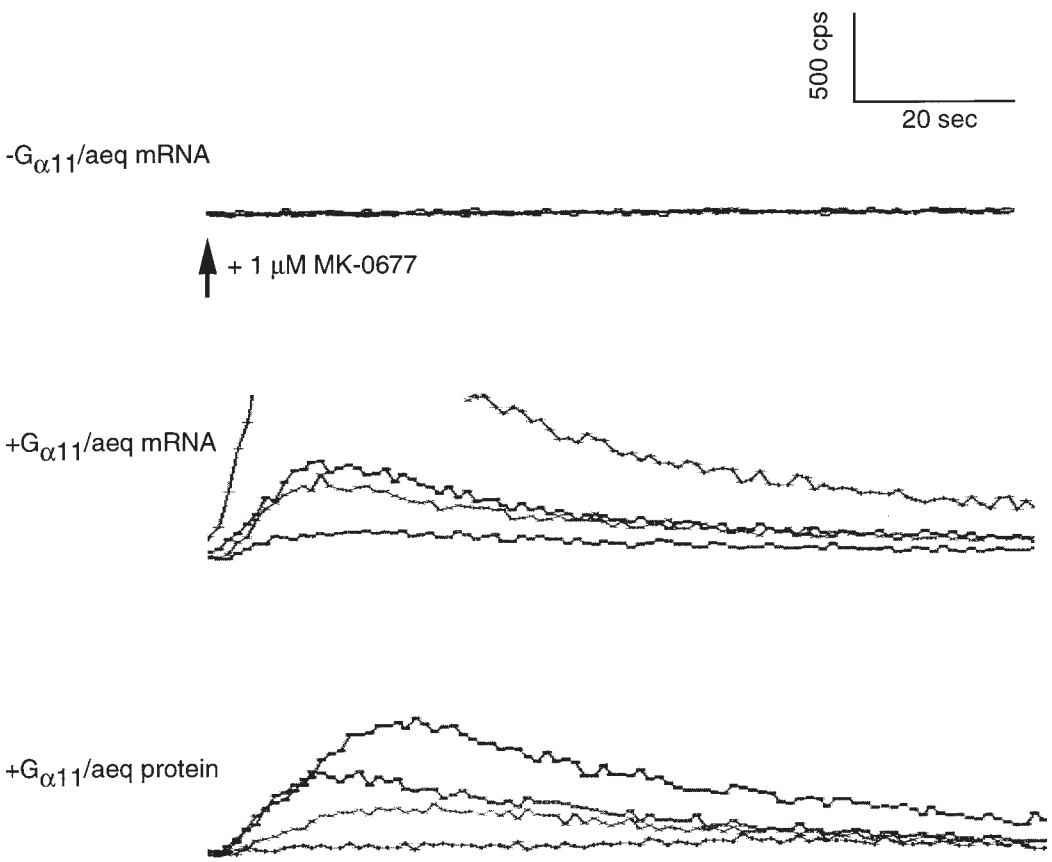


Fig. 2. $G_{\alpha 11}$-dependent detection of the swine pituitary GHS-R in *Xenopus* oocytes: comparison of aequorin mRNA and aequorin protein coinjection. *Xenopus* oocytes were injected with swine pituitary poly $(A)^+$ mRNA, $G_{\alpha 11}$ cRNA, and aequorin mRNA or aequorin protein. Following a 36-h incubation, the *Xenopus* oocytes were challenged with 1 μ*M* MK-0677.

Table 1
Detection of Swine Pituitary GHS-R: Dependence on $G_{\alpha 11}$ Co-Expression[a]

G_a Subunit	*Poly(A)$^+$*	*Bioluminescence (cps; 4 individual oocytes)*
No addition	+	50, 58, 66, 66
G_{11}	–	58, 58, 58, 66
G_{11} (aequorin mRNA)	+	1842, 525, 191, 608
G_{11} (aequorin protein)	+	200, 375, 558, 858
G_q	+	58, 58, 66, 83
G_o	+	66, 66, 58, 66
G_{i1}	+	66,58, 66, 58
G_{i3}	+	58, 58, 66, 58
G_{13}	+	58, 58, 50, 50

[a]Swine poly $(A)^+$ mRNA was injected into *Xenopus* oocytes, with G protein cRNA addition and aequorin cRNA or aequorin protein coinjection. The response to 1 μ*M* MK-0677 was recorded 48 h postinjection.

Table 2
Expression of the Swine Pituitary GHS-R in Oocytes from 6 Different Frogs[a]

Frog	$G_{\alpha 11}$	*# Responding*	*Bioluminescence (cps;3–4 individual oocytes)*
1	–	0/3	50, 50, 54
1	+	3/3	3206, 661, 488
2	–	0/3	72, 88, 133
2	+	3/3	300, 2478, 811
3	–	0/3	55, 55, 50
3	+	3/3	777, 222, 489
4	–	2/3	500, 127, 177
4	+	3/3	166, 516, 177
5/6	+	4/4	2867; 883; 2725; 113,000
5/6	+	4/4	160,500; 209,100; 10,920; 1800

[a]Oocytes from several different frogs (numbered 1–6) are compared. Injections were performed with swine pituitary poly $(A)^+$ mRNA and G protein subunit cRNA (rows marked "+" in the column $G_{\alpha 11}$). Bioluminescence was recorded 36 h postinjection in response to 1 μ*M* MK-0677.

Table 3
Pharmacological Characterization of the Swine Pituitary GHS-R in Oocytes

Ligand	$G_{\alpha 11}$	*# Responding*	*Bioluminescence (cps; 2–3 individual oocytes)*
MK-0677 @ 100 n*M*	–	0/3	72, 72, 61
	+	2/3	111, 555, 155
MK-0677 @ 10 n*M*	–	0/3	72, 72, 72
	+	2/3	527, 94, 77
GHRP-6 @ 5 μ*M*	–	0/3	55, 61, 72
	+	2/3	172, 161, 77
L-692,429 @ 5 μ*M*	–	0/3	61, 61, 72
	+	3/3	577, 116, 166
L-692,428 @ 5 μ*M*	–	0/3	55, 72, 72
	+	0/3	88, 77, 72

[a]Swine pituitary poly $(A)^+$ mRNA was injected into *Xenopus* oocytes with aequorin cRNA. Various ligands were used 36 h postinjection to evaluate the specificity and sensitivity of the oocyte assay.

GHS-R CLONING

The finding that the expression of the GHS-R was fully dependent on the addition of a single G protein subunit was unexpected since in previously published work the addition of a G protein subunit modulated an already existing activity. GHS-R expression could now be restored in oocytes obtained from >90% of the *Xenopus* frogs, suggesting that these oocytes did not contain sufficient quantities of G_α subunits to support GHS-R expression. This *Xenopus* GHS-R expression assay was utilized to screen pools of in vitro transcribed cRNAs derived from a swine pituitary cDNA library.

The authors evaluated the sensitivity of the assay by testing pools of cDNAs with a complexity of 10,000–20,000 cDNAs/pool for the presence of a GnRH-R- or TRH-R-derived signal (cloned receptors titrated in background cRNA). These receptors had been cloned

earlier from tumor-derived poly $(A)^+$ mRNA, which was enriched (~100-fold more receptor than native tissue) for the receptor of interest: GnRH, αT3-1 gonadotroph; thyrotropin-releasing hormone (TRH), Tt T mouse pituitary thyrotropic tumor) *(14,15)*. Robust responses to TRH and GnRH could indeed be observed in the majority of eggs injected with a complex mixture of 10,000–20,000 individual cRNAs.

Screening for the presence of a cDNA that encoded the GHS-R in a swine pituitary cDNA library was initiated in pools with a complexity of 10,000 cRNAs. Following the evaluation of about 2×10^6 cRNAs, pool S10–20 gave a modest but reproducible bioluminescent response to challenge by MK-0677 (1 μ*M*). As shown in Fig. 3, isolation of a pure clone (7-3) resulted from the subfractionation of this pool of 10,000 cRNAs. Clone 7-3 conferred MK-0677-evoked bioluminescence in the aequorin assay, a large inward chloride current in oocytes (Fig. 3, bottom right panel), and high affinity binding of [^{35}S]-MK-0677 to the GHS-R expressed in mammalian COS-7 cells (*see* Pharmological Characterization).

GHS-R GENE STRUCTURE

Determination of the nucleotide sequence of clone 7-3 revealed that it encoded a GPC-R with seven transmembrane α helical domains (7-TM) (Fig. 4). However, the GHS-R gene was truncated at its amino terminus by 13 amino acids *(16)*. Using clone 7-3 as a hybridization probe, additional GHS-R cDNA and genomic clones were obtained from swine, human, rat, and mouse cDNA and genomic DNA libraries *(17)*.

Two types of GHS-R cDNAs were isolated. Type 1a encoded a 7-TM GPC-R with binding and functional properties expected of a receptor for GHSs. The deduced amino acid sequence of the GHS-R highlights features in common with other GPC-Rs, which include conserved cysteine residues in the first two extracellular loops and several potential sites for co/posttranslational modifications (N-linked glycosylation and phosphorylation) and most importantly, the GPC-R signature aromatic triplet sequence (E/DRY) found immediately after TM-3 in the second intracellular loop *(18)*.

Type 1b GHS-R cDNA represents an inactive, C-terminally truncated GPC-R that encoded only five predicted TM domains. The deduced amino acid sequence of type 1a and 1b cDNAs was identical up to leucine-265 (the second amino acid of TM-6) with the type 1b cDNA nucleotide sequence diverging and extending for an additional 24 amino acids.

The type 1a and 1b cDNAs are derived from a single gene by alternative mRNA processing. A genomic clone encoding the human and mouse GHS-R gene was isolated and a partial nucleotide sequence determined (Fig. 5). The human GHS-R gene is divided into two exons by a single intron of ~2 kb in length. Determination of the nucleotide sequence for the proposed human exon–intron boundaries and the complete intron of the human gene confirmed that the intron divides the ORF into an amino-terminal segment ending at leucine-265 (encompassing the extracellular domain, TM-1 through TM-5, and the three intra- and first two extracellular loops) and a carboxyl-terminal segment encoding TM-6, the third extracellular loop, TM-7, and the C-terminal intracellular domain. The position of the intron is highly conserved among rat, human, and swine GHS-R genes. Type 1a cDNA encodes the complete 7-TM GHS-R and results from a splicing event that removes the intron. With type 1b cDNA, the intron is not removed and the reading frame extends into the intron. cDNA analysis indicates that an alternative poly $(A)^+$ addition site is used, which is presumably located in the intron. As a result, the

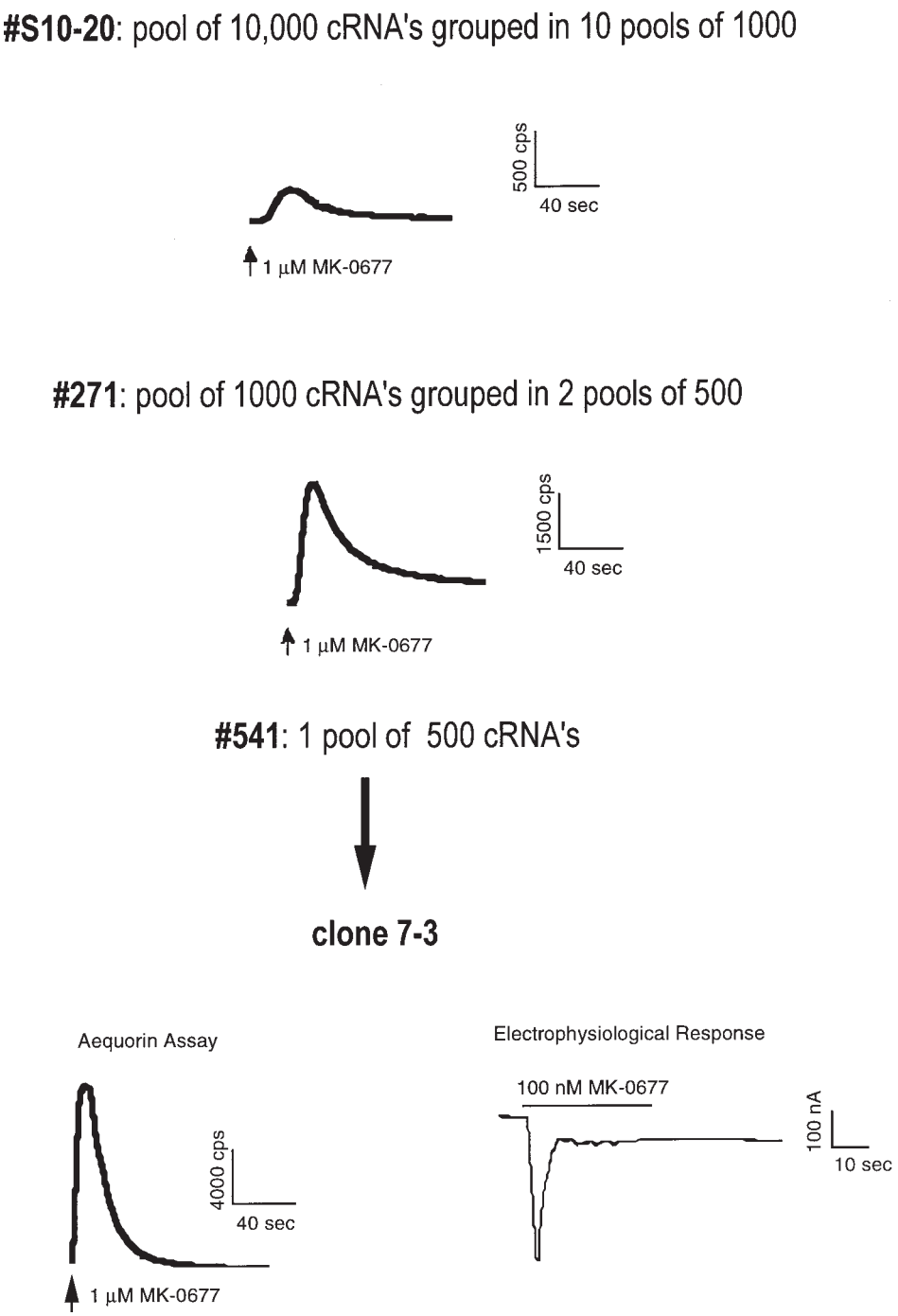


Fig. 3. Identification of swine pituitary cRNA library pool S10–20 and fractionation leading to isolation of a single GHS-R cDNA clone. A GHS-R response could be identified in *Xenopus* oocytes injected with cRNA pool S10–20 (complexity 10,000 cRNAs; aequorin chemiluminescence). This pool was broken down, resulting in pool 271, which contained 1000 individual cRNAs. Pool 541 (500 cRNAs) was subsequently broken down resulting in the isolation of clone 7-3 (aequorin luminescence, **left**; Ca^{2+} activate chloride current in *Xenopus* oocytes, **right**).

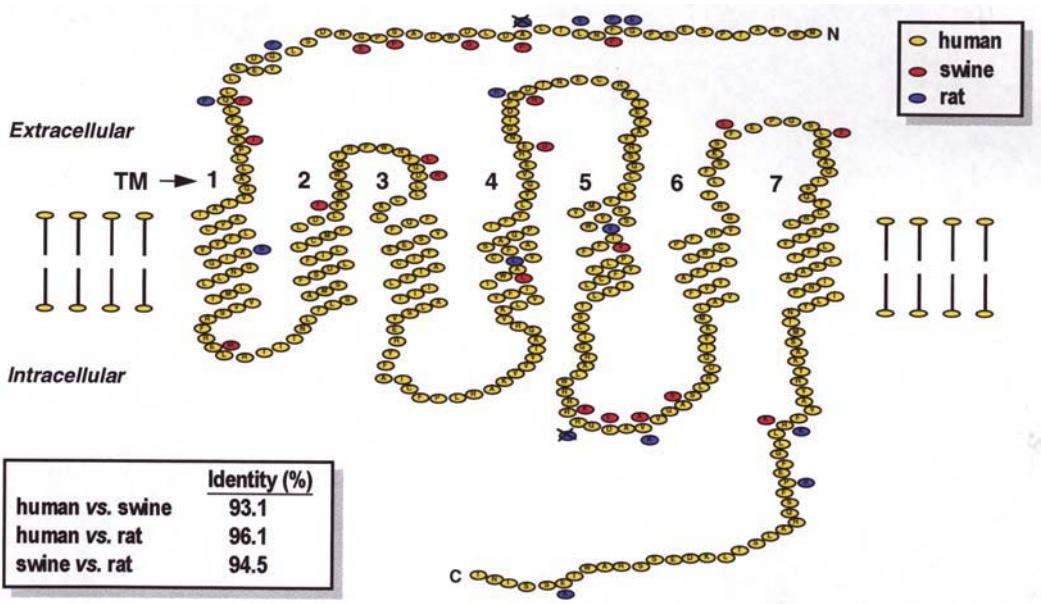


Fig. 4. Deduced amino acid sequences of GHS-Rs from swine, human, and rat. Schematic representation of the human swine and rat GHS-R (*see* inset at top right corner) as a 7 TM GPC-R. Individual amino acid residues are shown (single letter amino acid code). Transmembrane domains are numbered. The inset at the bottom left reveals the overall amino acid identities of the GHS-Rs from human swine and rat.

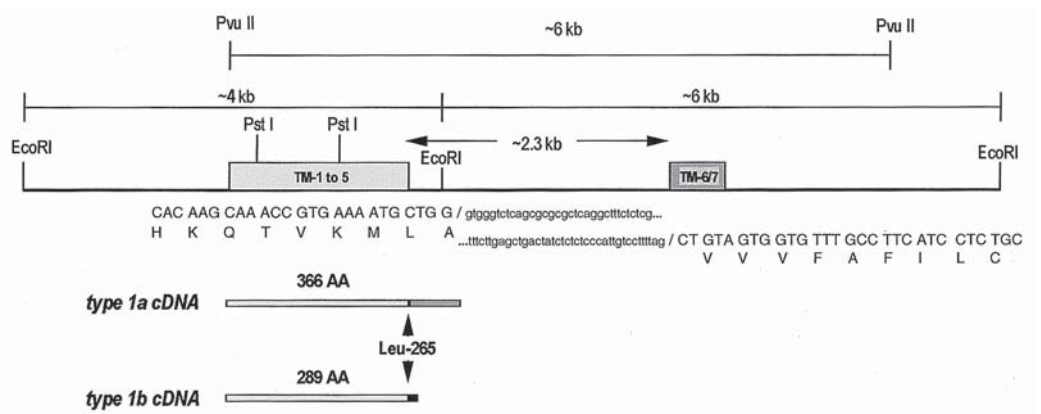


Fig. 5. The human GHS-R gene: physical map and nucleotide sequence of its exon–intron boundaries. The open box represents the coding sequence of TM1–5, the shaded box the coding sequence of TM6 and 7. The single intron of the GHS-R is outlined by the thin line separating the coding exons. Sizes of the restriction enzyme fragments (in kb) are indicated above the physical map. The nucleotide sequence at the exon–intron (upper and lower case, respectively) boundaries is shown just below the physical map. The structure of the type 1a and type 1b cDNAs, which diverge at amino acid 265, is shown below the physical map.

human and swine type 1b cDNA contain a short, 24-amino acid open reading frame fused to leucine-265, which is conserved in humans and swine.

PHARMACOLOGICAL CHARACTERIZATION

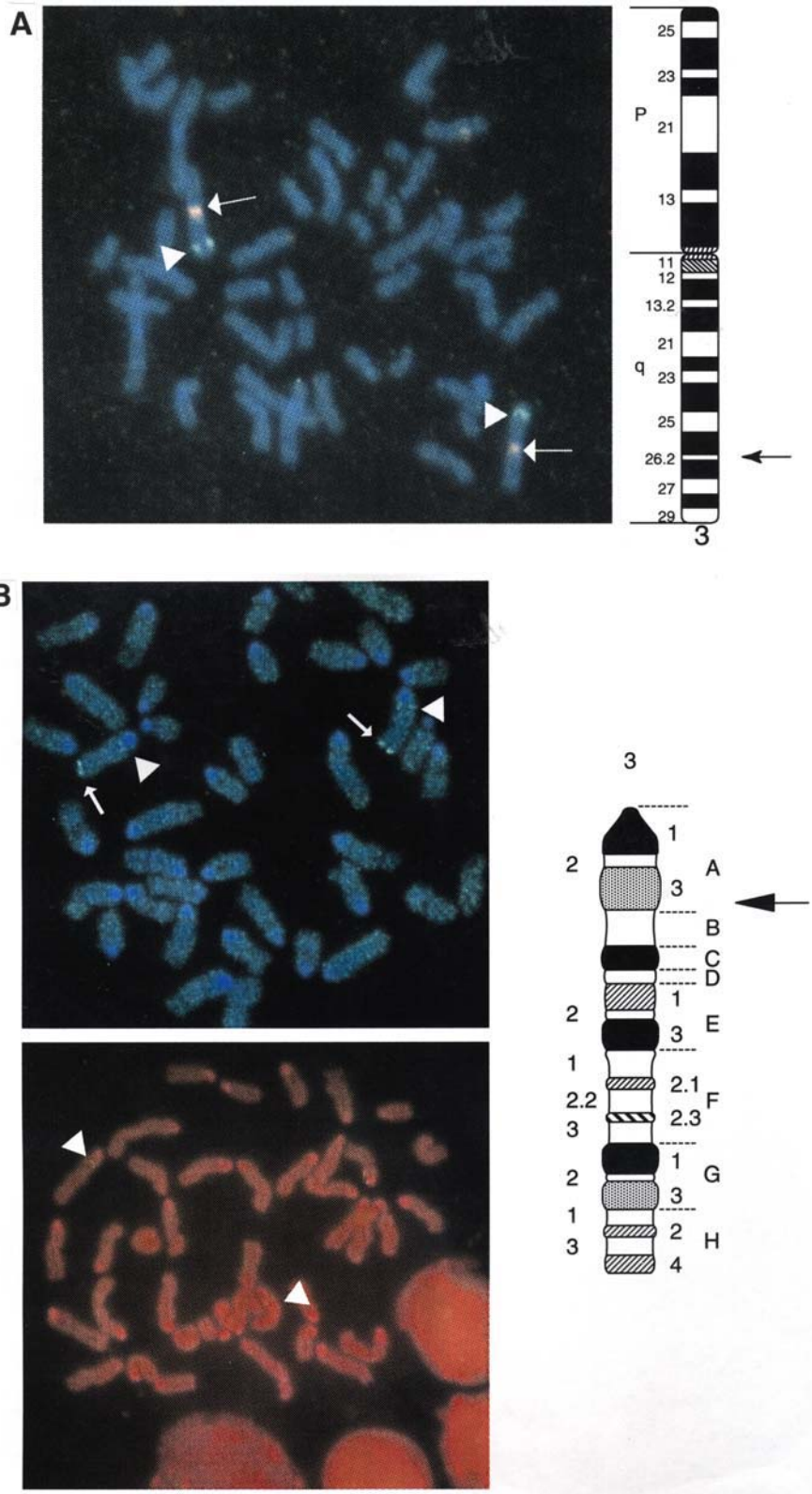
The pharmacological properties of type 1a and type 1b receptors were investigated using the aequorin and electrophysiological functional oocyte assays. In addition, GHS-R expression in COS-7 cells was characterized by [^{35}S]-MK-0677 binding and by an aequorin assay conducted in transfected mammalian cells. Swine and human type 1a cRNAs expressed in oocytes responded to concentrations of MK-0677 ranging from 1 μ*M* to as low as 0.1 n*M* with an ED_{50} of ~5 n*M*. The amino-terminal truncated forms of the type 1a receptor (swine clone 7-3 and human 1146) were ~10-fold less active than their full-length counterparts (data not shown). Peptidyl and nonpeptidyl bioactive GHSs were active in a similar rank order of potency as observed for the native pituitary GHS-R. Type 1b cRNAs failed to give a response when injected into *Xenopus* oocytes or transfected into mammalian cells.

Binding experiments using [^{35}S]-MK-0677 on crude cell membranes prepared from COS-7 transfectants confirmed that the type 1a cDNA, but not the type 1b cDNA, confers high-affinity, saturable, and specific binding (K_D = 0.3 n*M*; B_{max} = 0.2 pmol/mg cell membrane protein) of MK-0677 to a single class of noninteracting binding sites. Displacement of the radioligand by a variety of GHSs on the type 1a GHS-R was in strict correlation with their GH secretory activity. IC_{50}s for MK-0677 and the peptide GHSs, GHRP-6, and GHRP-2 were 0.1, 1.9, and 0.21 n*M*, respectively. Other peptides, such as GHRH, CRF, GnRH, galanin, neurotensin, and neuromedin B, failed to show significant inhibition.

The functional significance of the truncated type 1b receptor cDNA is unknown. The type 1b GHS-R cDNA encodes the complete intracellular second and third loop responsible for G protein binding. Therefore, the type 1b cDNAs may be functional in the appropriate context. Naturally occurring examples of other truncated GPC-R mRNAs are rare and their function has not yet been elucidated. It is of interest to note that coexpression of artificially generated truncated muscarinic M2 or M3 receptors in COS-7 cells (TM1–5 and TM-6–7 expressed from two separate cDNAs) allows a functional muscarinic receptor to be reconstituted with ligand binding properties (and PI hydrolysis) as in the intact receptor *(19)*. A similar study with mutant V2 vasopressin receptors was also recently reported *(20)*. Given these results it is possible that a gene may exist that can restore functional activity of the type 1b GHS-R cDNA, encoding additional transmembrane domains. Alternatively, the predicted 24 C-terminal amino acids of the type 1b cDNA might encode a new TM-6 (H6) domain. The predicted amino-acid sequence is hydrophobic and has similarity to helix 2 (H2) of the GHS-R. Helix 2 normally contacts H3, H6, H7, and H1. In the classical receptor folding model, H1 is positioned separate from the other six domains (TM2–7) and, therefore, may be open to additional contacts. One possible model for the type 1b GHS-R cDNA is that it indeed contains six transmembrane domains, H6 being similar to H2, allowing H6 to utilize contacts with H1 to restore a functional ligand-binding domain in this truncated GPC-R cDNA.

GHS-R CHROMOSOMAL LOCATION

Fluorescence *in situ* hybridization was used to identify the chromosomal location of the human and murine GHS-R genes (Fig. 6). To assure the accuracy of the assignment, two distinct clonal isolates from a human PAC library encoding the GHS-R were utilized for the *in situ* hybridization. Location of the human GHS-R gene relied on the analysis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of 80 metaphase cells for each clone, with ~ 75% of the cells exhibiting specific labeling to both sister chromatids of chromosome 3. Measurement of 10 specifically labeled chromosomes demonstrated that the positive signals correspond to region 3q26.2 at 74% distance from the centromere of the long arm of chromosome 3 (3q). Genes whose defects can result in GH deficiencies did not map to this region. However, this location is in the vicinity of the possible map position for the Brachmann-de Lange syndrome, which is characterized by prenatal and postnatal growth deficiencies, with developmental delay and dysmorphic features *(21)*. The latter mapping data is based on chromosome duplication and translocation mutants, which always included region 3q26. However, recently a cell line with a translocation was identified, indicating that the defect could be telomeric to 3q26 (interval 3q26.31–q27.3). Given the possible proximity for the presumed Brachmann-de Lange location and the GHS-R gene, it will be of interest to determine whether Brachmann-de Lange patients have GHS-R gene deficiencies. The mouse GHS-R localized to the telomere of chromosome 3, band A3.

GHS-R EXPRESSION

The expression patterns of the type 1a and type 1b GHS-Rs were studied by ribonuclease protection analysis in human and rat tissues and by *in situ* hybridization histochemistry in rhesus hypothalamus and rat brain and pituitary. Functional assessment of sucrose gradient-fractionated poly $(A)^+$ mRNA from swine pituitary gave a single peak of GHS-R activity in the size range 1.6–2.3 kb (Fig. 7). However, attempts at detecting GHS-R mRNA by Northern blotting analysis have been unsuccessful, even though control mRNAs for other GPC-Rs could easily be detected. The authors attribute the difficulty in detecting GHS-R mRNA by Northern blotting analysis to its low abundance and potential size heterogeneity. TRH and GnRH receptors were also readily detected functionally. PCR amplification of the swine pituitary GHS-R cDNA sequences from among 11 pools of an unamplified pituitary cDNA library (110,000 individual cDNAs/pool) resulted in GHS-R cDNA identification in only 4 of 11 pools. Therefore, receptor cDNA abundance is most likely <1 in roughly 300,000. The more sensitive techniques of RNase protection and *in situ* hybridization proved more revealing. Table 4 summarizes the GHS-R expression data obtained from several species. GHS-R expression could be confirmed in multiple hypothalamic nuclei and the pituitary, as well as in other brain regions (e.g., hippocampus) and the pancreas. GHS-R transcripts have not been detected in numerous other tissues using RNase protection, including stomach, liver, heart, fetal brain, testis, thymus, adrenal gland, uterus, spinal cord, bone marrow, thyroid, and lung.

Fig. 6. Chromosomal location of the human and murine GHS-R gene. **(A)** Fluorescent *in situ* hybridization mapping for determination of the chromosomal location of the human GHS-R gene. Arrows highlight the location of the GHS-R gene. Arrowheads highlight the location of a chromosome 3 specific marker. A schematic Giemsa chromosome 3 banding pattern (ideogram) is shown on the right. The human GHS-R gene maps to chromosome 3Q26.2. **(B)** Fluorescent *in situ* hybridization mapping of the position of the mouse GHS-R gene. Arrows highlight the location of the GHS-R gene. Arrowheads highlight the location of a chromosome 3 specific marker. A schematic Giemsa mouse chromosome 3 banding pattern (ideogram) is shown on the right. The mouse GHS-R gene maps to chromosome 3A3.

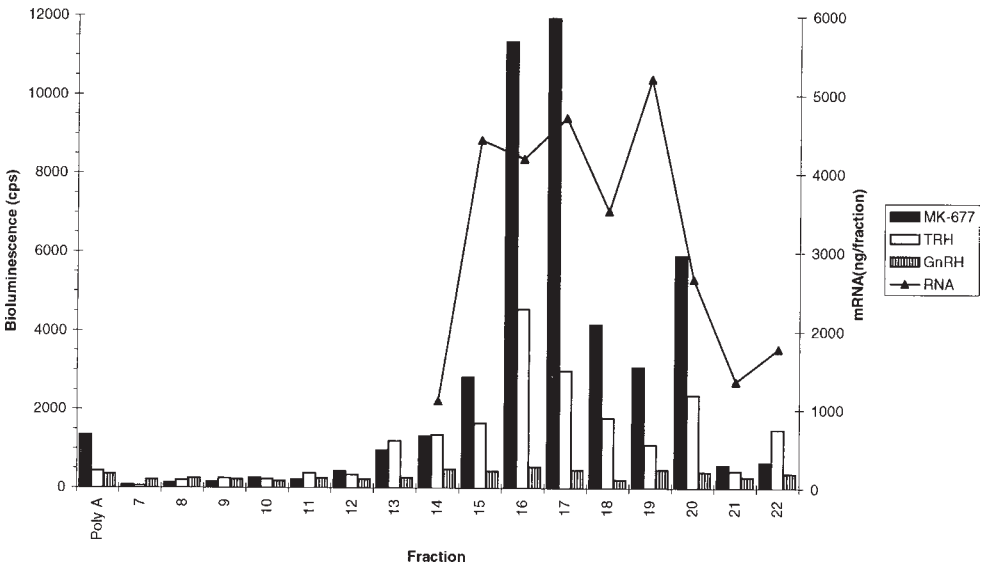

Fig. 7. Determination of the approximate size of GHS-R mRNA by sucrose gradient fractionation of swine pituitary poly $(A)^+$ mRNA. Swine poly $(A)^+$ mRNA was size fractionated by sucrose gradient density fractionation. Aliquots from individual fractions were taken and tested in *Xenopus* oocytes for the presence of GHS-R mRNA. The histogram outlines the aequorin bioluminescence readout of GHS-R mRNA-derived signals (the ligand used to evoke a response was 1 μ*M* MK-0677). TRH and GnRH responses were also evaluated as controls.

Table 4
Summary of the Distribution of GHS-R Expression in Different Tissues[a]

Species	*RNase protection*	In situ *hybridization*	*Radioligand binding*
Human	pituitary subthalamic nuclei hippocampus pancreas (weak)	ND	ND
Rat	pituitary hypothalamus	pituitary multiple hypothalamic nuclei hippocampus (dentate gyrus, CA2 and 3) substantia nigra	pituitary hypothalamus
Swine	ND	–	pituitary
Rhesus monkey	ND	arcuate-ventromedial and infundibular hypothalamus	ND

[a]Analysis of serial coronal rat brain sections by *in situ* hybridization gave a detailed outline of CNS GHS-R expression patterns.

GHS-R: RELATIONSHIP TO OTHER GPC-RS

Sequence alignments performed at the nucleic acid and protein level show that the GHS-R cDNAs are highly related among different species (Figs. 4, 8). Protein database

searches revealed that the GHS-R shares only limited amino sequence identity to other protein sequences. This observation suggests that the GHS-R may be the first member of a new subfamily of GPC-Rs within the rhodopsin/Family I group. Identity is centered in the transmembrane regions of the rhodopsin/Family I group of GPC-Rs, with ~35 and 29% overall identity with rat or human receptors for neurotensin (NT) and TRH, respectively. A dendrogram of the GHS-R and other GPC-Rs indicates its relatively isolated position: most closely positioned adjacent to the NT-R branch, but grouped separately (Fig. 8).

GHS-R RESIDUES INVOLVED IN LIGAND BINDING

The GHS-R sequence (Fig. 4) provides a context for determining the amino acid residues central to ligand binding and receptor activation. Based on current knowledge of the GHS-R and the predicted structures of the GHSs, several amino acid residues in TM 3, 5, and 6 are likely to be involved in ligand binding, mediating the agonist activity of GHRP-6 and the nonpeptide GHSs. An important feature of GHS agonists is the presence of a basic N^+ *(22–25)*. Based on conservation between swine and human GHS-R and the growing body of evidence implicating the transmembrane helices and extracellular loops 3 and 4 in ligand binding in other GPC-Rs, nine acidic sites in the GHS-R stand out as potential candidates for stabilizing the positive charge *(26–28)*. A preliminary 3D model of the swine GHS-R suggests that E_{124} in H3 is disposed similarly to D_{113} of the β adrenergic receptor and an acidic amino acid residue in the equivalent position in the somatostatin type 2 (sst2) receptor. Other candidate sites are arrayed in the extracellular loops ($D_{196, 201, 204}$; $E_{194, 207, 212, 291, 299}$). Site-directed mutagenesis experiments will discriminate which negatively charged amino acid residues are important for GHS agonism.

FUTURE DIRECTIONS

The relatively isolated position of the GHS-R in the dendrogram suggests that GPC-Rs may exist that constitute closely related GHS-R family members. Their isolation and functional analysis may shed light on the relevance of the relationship between the GHS-R and the neurotensin receptor (NT-R). Targeted disruption of the GHS-R will hopefully aid in defining the normal physiological role of the GHS-R. Finally, the identification of the natural ligand for the GHS-R will allow the dissection of the molecular mechanisms by which this receptor and its ligand participate in the control of pulsatile GH release in humans and it will further facilitate the assessment of the clinical role of the GHSs in human and animal health.

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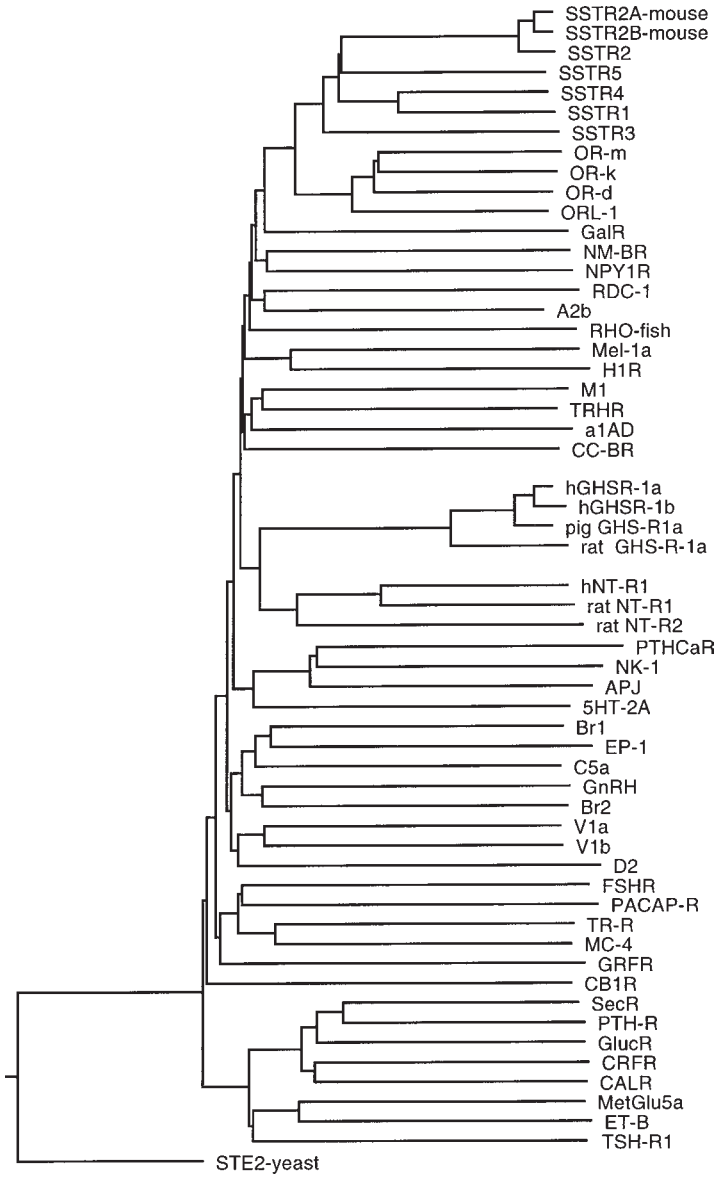

Fig. 8. Dendrogram alignment of the GHS-R and other GPC-Rs. Database searches (Genbank 92, EMBL 43, Swiss-Prot 31, PIR 45, dEST [GBest 92], Prosite 12), sequence alignments and analysis of the GHS-R nucleotide, and protein sequences were carried out using the GCG Sequence Analysis Software Package (Madison, WI; PileUp, peptide structure and motif programs). The amino acid sequence of representative members (56 sequences) for all known classes (Families I–IV and pheromone) of GPC-Rs were used to construct the dendrogram using the clustal method (PAM-250, gap and length penalty =10).

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5 Central Actions of Peptide and Nonpeptide Growth Hormone Secretagogues

Suzanne L. Dickson, PhD, and Gareth Leng, PhD

Contents

INTRODUCTION

For some time now, the authors have been investigating the neuroendocrine events leading to increased growth hormone (GH) secretion following administration of GH secretagogues, including GH-releasing peptide (GHRP-6). It is now well established that these compounds act both at the pituitary *(1–5)* and within the central nervous system (CNS) *(6,7)*. The recent cloning of the GHRP-6 receptor has paved the way for the localization of the receptors at both pituitary and hypothalamic sites *(8)*.

In most species, GH is secreted in a highly pulsatile pattern that is believed to reflect a balanced alternation in the output of two neuroendocrine systems, the GH-releasing hormone (GHRH) neurons and the inhibitory somatostatin neurons. The effects of GH secretagogue administration on the pattern of GH secretion are complex and may be mediated, at least in part, by modification of the output of the central GHRH-somatostatin pulse generator. However, to date, no endogenous ligand for GH secretagogues has been

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identified, and hence, it is not clear whether such a ligand participates in the normal physiological control of pulsatile GH secretion. In conscious male rats, a GHRP-6 infusion causes an initial GH peak followed by a sustained elevation of plasma GH concentrations during which pulses occur, but the normal three hourly pulsatile rhythm is disrupted *(9)*. Similar responses have been observed in pigs infused with the nonpeptide secretagogue L-700,653 *(10)* and in guinea pigs infused with L-692,585 *(11)*. Here we will review what is known about the central site and mechanism of action of the GH secretagogues to consider how an endogenous GH secretagogue ligand might influence the GHRH-somatostatin pulse generator network; and to consider the physiological circumstances in which such a ligand might be released will be reviewed.

ACTIVATION OF CELLS IN THE ARCUATE NUCLEUS FOLLOWING GH SECRETAGOGUE ADMINISTRATION

In 1993 it was shown that an iv injection of GHRP-6 causes activation of cells in the rat hypothalamic arcuate nucleus as reflected by increased electrical activation in a subpopulation of neurosecretory neurons (Fig. 1) and an increased expression of Fos-immunoreactivity in a subpopulation of cells in this region (Fig. 2) *(6)*. Similar central activation follows administration of related, nonpeptide mimetics *(7)*. The arcuate nucleus is the only hypothalamic region to show such a response, though the authors now know that Fos expression is also induced by GHRP-6 in some neurons in the area postrema and of the neighboring nucleus tractus solitarii *(12)*. Fos is the protein product of the immediate early gene (IEG) c-*fos*, and is thought to be involved as a transcription factor linking electrical activity to changes in gene expression. Fos is expressed in many neuronal systems following activation. In the magnocellular neurosecretory system regulating neurohypophysial hormone secretion, Fos expression has been extensively characterized *(13–15)*, and is established to be, for some systems at least, a reliable and sensitive indicator of neuronal activation in a very wide range of physiological and experimental circumstances. In oxytocin neurons, c-*fos* mRNA is induced within 10 min following stimuli that increase neuronal activity by a mean of only about 1 spike/s *(15)*.

The GHRP-6-induced activation of Fos expression in arcuate neurons is the consequence of a direct central action since injection of low doses of GHRP-6 (0.1 μg) into the third ventricle induces a selective Fos expression similar in distribution and equivalent in extent to that induced by an iv injection of 50 μg of this compound *(7)*. Thus, either GHRP-6 penetrates the blood–brain barrier readily following systemic administration, or its primary central site of action is at specialized brain sites where the blood–brain barrier is relatively permeable. Although the median eminence is known to be one such specialized site, the arcuate nucleus itself is not. In the hypothalamus as at the pituitary, GHRH does not act in the same way as GHRP-6, and central administration of GHRH does not induce Fos expression in the arcuate nucleus.

IDENTIFICATION OF THE ARCUATE CELLS ACTIVATED BY GH SECRETAGOGUES

That the central action of the GH secretagogues includes increased GHRH release was first suggested by the observation that, in the rat, the administration of GHRH antiserum attenuates the GH response to GHRP-6 *(9)*. Direct measurement of GHRH release into the portal blood of sheep has confirmed that GHRH is released following GH secreta-

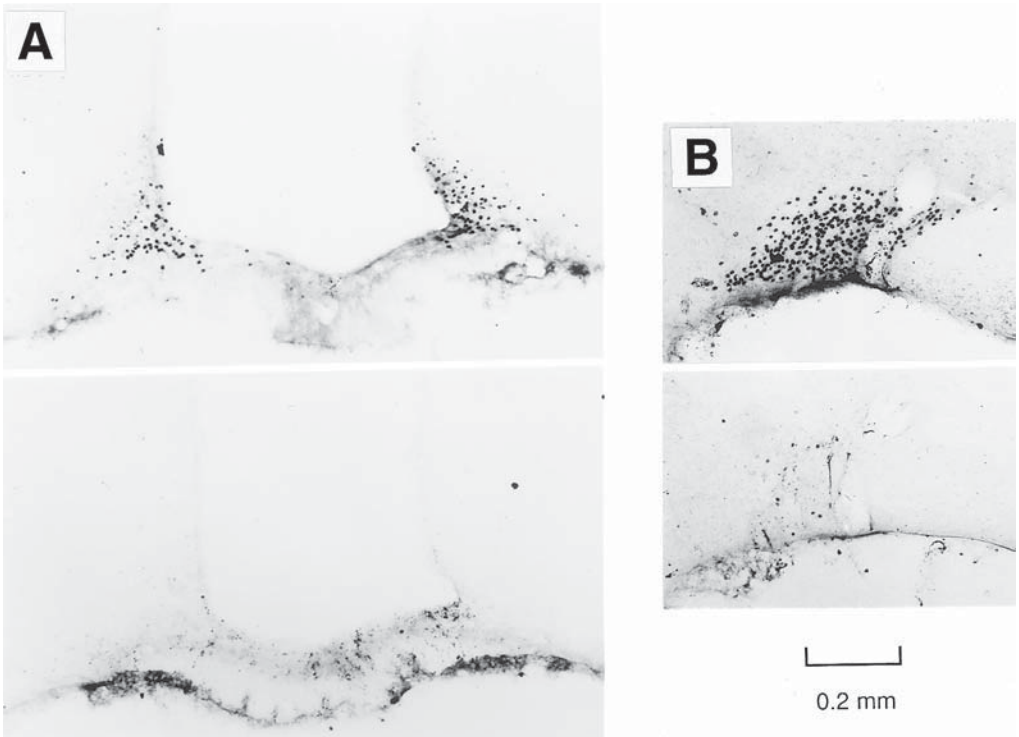

Fig. 1. (A) Fos-like immunoreactivity in the rat hypothalamic arcuate nucleus following an iv injection of either 100 μg GHRP-6 in isotonic saline **(Top)**, or an equal volume of isotonic saline vehicle **(Bottom)**. **(B)** Fos-like immunoreactivity in the supraoptic nucleus of the hypothalamus following injection of either hypertonic saline (top; 1.8 mL/kg body weight of 1.5 *M* NaCl; ip) or 100 μg GHRP-6 (bottom; iv injection, dissolved in isotonic saline). Reproduced from ref. *6* with permission.

gogue administration *(16)*. In the authors' electrophysiological studies they demonstrated that GHRP-6 activates a subpopulation of putative neurosecretory neurons in the arcuate nucleus, identified as projecting to the median eminence, some of which fulfilled multiple criteria for identification as GHRH neurons (Fig. 2) *(6,17)*. This appears to be a direct action of GHRP-6 at the arcuate nucleus since arcuate neurons respond to GHRP-6 and related secretagogues in hypothalamic slice preparations where they are disconnected from all but closely adjacent inputs *(18,19)*.

Studies in the authors' laboratory and others have recently confirmed that the GH secretagogues activate GHRH neurons. In the arcuate nucleus, c-*fos* mRNA is induced in a high proportion of GHRH mRNA-containing neurons following injection of GHRP-6 *(20)* or the peptide GH secretagogue KP-2 *(21)*, though it is also induced in some other subpopulations including, most notably, a proportion of the neurons in the arcuate that express mRNA for neuropeptide Y *(20)*. Currently, it is not known whether activation of neuropeptide Y neurons participates in the neuroendocrine events leading to increased GH secretion following GH secretagogue administration. Since arcuate NPY neurons have been implicated in the regulation of feeding behavior *(22)*, it will be interesting to discover whether activation of this population by GHRP-6 explains the GH secretagogue-induced feeding response *(23)*.

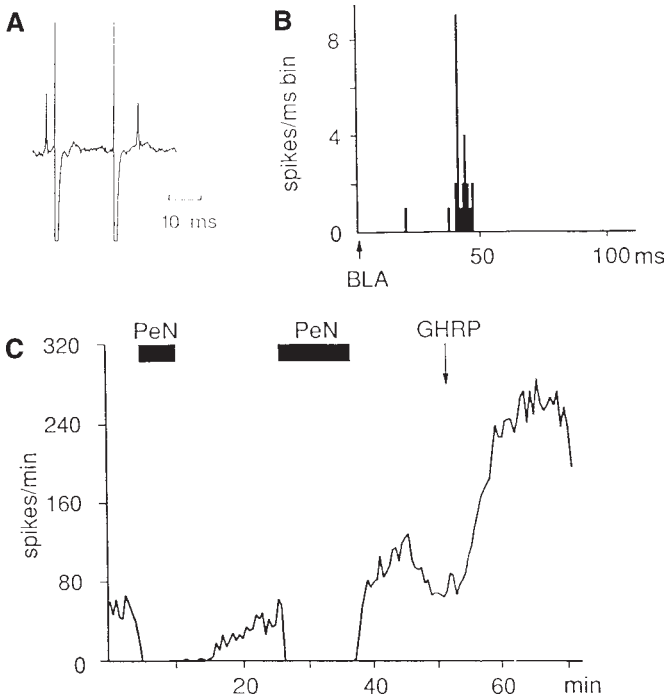

Fig. 2. Extracellular recordings *in vivo* from a single arcuate neuron that **(A)** is antidromically identified as projecting to the median eminence, as demonstrated by the collision of an orthodromic action potential with a antidromic spike, **(B)** is trans-synaptically excited by electrical stimulation of the basolateral amygdala (BLA), as illustrated in the poststimulus time histogram, and **(C)** is inhibited during electrical stimulation of the periventricular nucleus (PeN). Following a 10-min period of stimulation, the cell displayed a rebound hyperactivation. Intravenous injection of 100 μg GHRP elicited a large increase in the firing rate of the neuron.

Studies employing systemic administration of the retrograde tracer, Fluorogold, indicate that as many as 68–82% of the cells excited by GHRP-6 project outside the blood–brain barrier *(24)*. This finding probably implies that most of the arcuate cells activated by GHRP-6 project to the median eminence and, hence, are likely to be neurosecretory neurons involved directly in the regulation of pituitary function. Conversely, most non-neurosecretory neurons are not activated by GHRP-6, although some have displayed inhibitory responses in the authors' electrophysiological studies in vivo *(7)* and in vitro *(18,19)*; in particular, the POMC and the somatostatin neurons do not appear to express c-*fos* mRNA following GHRP-6 injection *(20)*, nor indeed do the periventricular somatostatin neurons. However, it remains possible that GHRP-6 activates arcuate neurons not directly, but indirectly via inhibition of inhibitory interneurons in the arcuate nucleus.

Although not all of the cells activated by GHRP-6 are GHRH cells, the neurosecretory identity of most of the cells activated indicates the possibility that other releasing factors, perhaps including an endogenous ligand for the GH secretagogue receptor, are released at the median eminence in response to GHRP-6 and participate in the regulation of pituitary GH release. These characteristics of GHRP-6, that it is a selective secretagogue for GH, acting at highly selective receptors expressed specifically at both hypothalamic and pituitary sites *(8)* to stimulate GHRH *(16)* and GH release *(1,2)*, respectively, strongly

suggest that GHRP-6 is mimicking an unknown endogenous ligand whose physiological role is specifically concerned with GH secretion. In what physiological circumstances, and through which pathway, might such a ligand act?

PHYSIOLOGICAL STIMULI FOR GH RELEASE: IN SEARCH OF AN ENDOGENOUS LIGAND FOR GH SECRETAGOGUES

It seems possible that an endogenous GH secretagogue ligand could be synthesized in some discrete population of neurons within the CNS, possibly even synthesized in neuroendocrine cells controlling GH secretion. However, it is equally possible that it is produced peripherally in response to physiological stimuli. Under what physiological circumstances are plasma levels of GH higher than normal, possibly reflecting the release of an endogenous GH secretagogue ligand? Interestingly, the endocrine response to GHRP-6 is in some respects similar to that observed in response to exercise. In humans, infusion of GHRP-6 results in an initial rapid increase in plasma levels of GH, followed by long-lasting elevated fluctuations *(25)*; this profile is in marked contrast to the profile of GH release in response to GHRH infusion, which elicits a rapidly desensitizing response. However, the pituitary response to GHRP-6 desensitizes even more rapidly than that to GHRH, hence the profile of the in vivo actions of GHRP-6 cannot be accounted for from its pituitary actions alone; thus, the long duration of the response in vivo probably reflects the establishment of a new dynamic equilibrium in the hypothalamic output, involving episodic fluctuations in the output of hypothalamic-releasing factors. During prolonged exercise there is similarly a rise in plasma levels of GH that is maintained throughout the exercise period unlike the expected consequences of a sustained exposure to GHRH. Some of the more potent GHRP-6 mimetics have slight effects to increase plasma concentrations of cortisol (resulting from elevated adrenocorticotropin [ACTH] secretion) *(26)*. These secretagogue effects on cortisol are within the normal physiological range and, perhaps significantly, also mimic the cortisol response to exercise. Thus GHRP-6 and related secretagogues may mimic the actions of an endogenous ligand released in response to acute exercise.

Exercise is a potent stimulator of GH secretion in most species, and this has been studied extensively in humans. The magnitude of the GH response depends on several factors, including the intensity and duration of acute exercise, the muscle mass used during exercise, and the degree of training. It appears that a threshold of exercise intensity must be exceeded before any increase in plasma concentration of GH is detected, and this threshold appears to coincide with the threshold for plasma lactate detection *(27)*. Lactate is the end product of anaerobic glucose metabolism; it accumulates in (and is released from) muscles during strenuous exercise. Luger and colleagues *(28)* demonstrated that, in young men, iv infusion of lactate resulted in a substantial elevation in plasma GH concentration, but since the lactate threshold is higher in trained subjects than in untrained subjects, potentiation of GH secretion in trained subjects cannot be attributed to differences in lactate production alone. Little is known about the central regulation of exercise-induced GH secretion and most studies have been directed toward resolving the question of whether increased plasma concentrations of β-endorphin (resulting from exercise) mediate the increase in GH secretion. The opiate antagonist naloxone inhibits exercise-induced GH release in highly trained athletes *(29)*, suggesting a stimulatory effect of β-endorphin on GH responsiveness. Other studies have implicated central cholinergic pathways in exercise-induced GH release since muscarinic antagonists block this GH response *(30)*.

Food deprivation induces a dramatic alteration in GH secretion in all species studied. In rats, food deprivation inhibits pulsatile GH secretion, and refeeding results initially in low-amplitude pulses (at a higher frequency than the endogenous rhythm) giving way to normal 3 h high-amplitude pulses within 6–8 h *(31)*. A variety of nutrients modulate GH secretion including free fatty acids and amino acids *(32)*. The effects of refeeding are mimicked by iv injection of amino acids, but not by iv glucose, whereas iv injection of lipid abolishes pulsatile GH secretion *(31)*. Food deprivation results in a dramatic reduction in GHRH gene transcription in rats, an effect that can be reversed with refeeding of dietary protein *(33)*; in humans, by contrast, fasting stimulates GH secretion. Thus, exercise and diet have a major physiological influence on the hypothalamic regulation of GH secretion, but the pathways involved in these influences are poorly understood.

PHYSIOLOGICAL EVIDENCE IN SUPPORT OF THE EXISTENCE OF MULTIPLE GH SECRETAGOGUE RECEPTORS OR RECEPTOR SUBTYPES

To date, only one receptor for the GH secretagogues has been identified that is present at both pituitary and hypothalamic sites *(8)*. With the large number of peptide and nonpeptide GH secretagogues now available for study, it is emerging that these compounds have subtle differences in action, raising the possibility that multiple GH secretagogue receptors or receptor subtypes may exist.

The electrical activation of arcuate neurons by GHRP-6 has a number of distinctive characteristics; most particularly the activation is extremely long lasting. Typical excitation is sustained for over 1 h following a bolus administration at a just-suprathreshold dose, and typically excitation reaches its peak intensity only after a latency of 5–10 min; this response appears to be even later for nonpeptide agonists than for GHRP-6, although the degree of electrical activation of responsive cells appears similar. It is unclear whether this reflects slower penetration of the blood–brain barrier by the nonpeptide secretagogues or a different mode of action. Moreover, the nonpeptide secretagogues MK-0677 (unpublished observation) and L-692,585 *(7)* appear to be slightly, but consistently, less effective than GHRP-6 for inducing Fos protein expression in the arcuate nucleus. It may be that these nonpeptide GH secretagogues are more selective than GHRP-6 in their central action or that they are simply less potent. Certainly, comparing the potencies of these secretagogues at the pituitary level for stimulating GH secretion, there is no reason to suppose that the nonpeptide GH secretagogues are less potent than GHRP-6 *(34)*. Moreover, the magnitude of electrical activation of responsive cells in the arcuate nucleus appears very similar whether the activation is induced by GHRP-6 or by a nonpeptide GH secretagogue. Thus, it is possible that GHRP-6 is more effective than the nonpeptide secretagogues in inducing Fos protein expression by binding to more than one receptor or receptor subtype in the hypothalamus.

Other evidence in support of the existence of multiple GH secretagogue receptors is suggested from studies in dwarf *lit/lit* mice, a GH-deficient mutant resulting from a point mutation in the extracellular binding domain of the GHRH receptor *(35)*. The pituitaries of these mice appear to be unresponsive not only to GHRH *(36)*, but also to GHRP-6 *(37)*, suggesting that the GH secretagogue receptor may be dependent on the presence of a functionally intact GHRH receptor. Although this may be true at the pituitary level, the authors demonstrated that the hypothalamic response to GHRP-6 (in this case, the induc-

tion of Fos protein) remains intact *(38)*. Taken together, these observations seem to suggest that the presence of functional GHRP-6 receptors in the pituitary depends on the existence of a functional GHRH receptor, whereas the presence of functional GHRP-6 receptors in the hypothalamus is independent of the presence of functional GHRH receptors.

The effects of Hexarelin pretreatment (3–10 d) on subsequent GH responses to GH secretagogues are very different in neonatal vs young adult rats: Hexarelin enhanced GH secretion in neonates and inhibited GH secretion in young adult rats *(39)*. One possible explanation of this finding is that in the early postnatal period a different subtype of GH secretagogue receptor is expressed in the pituitary or that both the neonatal and adult subtype are expressed together.

INTERCOMMUNICATION BETWEEN SOMATOSTATIN NEURONS AND GHRH NEURONS AND THE CENTRAL ACTIONS OF GH SECRETAGOGUES

Classically, the secretion of GH is controlled by the GHRH neurons of the arcuate nucleus and periventricular somatostatin neurons, which stimulate and inhibit GH secretion, respectively. Immunoneutralization of somatostatin enhances GHRH-evoked GH release *(40)*, but does not enhance GH release evoked by GH secretagogues *(2)*, suggesting that GH secretagogues may have a dual action to stimulate endogenous GHRH release and suppress endogenous somatostatin release. In male rats, a GHRP-6 infusion has been shown to disrupt the cyclic responsiveness in GH release following regular injections of GHRH *(41)*; since this cyclic responsiveness has been attributed to cyclic release of somatostatin, it seems likely that the GH secretagogues disrupt the cyclic release of somatostatin.

In the rat, the secretion of GH is sexually dimorphic: in males the pulses of GH are larger, less frequent, and arise from lower interpulse baseline compared with females *(42,43)*. Androgens play an important role for maintaining the low baseline GH levels and for controlling GH pulse height *(44)*. The sexually dimorphic patterns appear to derive from dimorphic behavior of the somatostatin neurons, possibly reflecting the dimorphic expression of androgen receptors by these neurons *(45)*. It appears probable that, in the male rat, GHRH and somatostatin are released alternately to produce alternate peaks and troughs of GH release, whereas in the female somatostatin is released more continuously. Interestingly, in the male rat, prolonged infusion of somatostatin leads to a sustained inhibition of GH release, followed by a dramatic rebound secretion of GH after the end of somatostatin infusion *(46)*. Although this rebound is partly generated at the level of the pituitary, it also appears to reflect a large rebound secretion of GHRH. Similar rebound secretion of GHRH follows electrical stimulation of the periventricular nucleus *(47)*. The periventricular nucleus appears to provide a direct inhibitory projection to neurons in the arcuate nucleus *(17)*, and 55–60% of the GHRH neurons visualized either by immunocytochemistry *(48)* or *in situ* hybridization *(49)* express somatostatin receptors. Hence, it is possible that the reciprocity in the hypothalamic output of GHRH and somatostatin during spontaneous pulsatile GH secretion reflects neuronal interactions between the GHRH and somatostatin cells. The authors' electrophysiological studies in vivo support this hypothesis, since neurosecretory neurons in the arcuate nucleus that were excited by GHRP-6 or nonpeptide secretagogues

were also inhibited during electrical stimulation of the periventricular nucleus *(6)*, and such secretagogue-responsive neurosecretory cells are also inhibited following iv injection of somatostatin or Sandostatin (a long-acting somatostatin analog). By contrast, cells that are not responsive to secretagogues are mainly unaffected by somatostatin injections. Furthermore, icv injection of Sandostatin suppresses the GH response following iv injection of the GH secretagogues *(50)*. In addition, the central actions of GH secretagogues to induce expression of Fos in the arcuate nucleus can be attenuated by systemic or central administration of Sandostatin *(51)*. Thus, it would appear that a subpopulation of the arcuate cells activated by GH secretagogues are also inhibited by central somatostatin action.

Suppression by Sandostatin of the GH secretagogue-induced increase in the expression of Fos in the arcuate nucleus is likely to be mediated by a direct central action of this peptide since injection of a very low dose of Sandostatin (2 μg) was as effective as iv injection of 100 μg *(51)*. Indeed, this also suggests that Sandostatin is able to gain access to central sites when administered by the iv route.

When considering the inhibitory effects of systemic administration of Sandostatin on GH secretagogue-induced GH release, it is difficult to distinguish between the inhibitory actions at the level of the pituitary and central actions. At the pituitary, somatostatin suppresses both spontaneous GH release *(52)* and release induced either by GHRH *(53)* or by GH secretagogues *(1,2)*. However, the suppression of GH release is likely to reflect, at least in part, a central action, since Sandostatin inhibits GH secretagogue-induced GH release when administered intracerebroventricularly *(50)*. Although it seems likely that Sandostatin acts via somatostatin receptors on GHRH neurons, it is also possible that it acts via an afferent pathway to these cells. Indeed, it is not possible to determine whether the cells that are the direct target for the action of the secretagogues are also the direct target for the action of Sandostatin.

Nonetheless, the interaction between the central effects of GH secretagogues and somatostatin suggests that, although many of the arcuate neurons activated by the secretagogues are not GHRH cells, they are nonetheless likely to be intimately involved in the regulation of GH secretion. One possibility is that these are interneurons linking the population of GHRH neurons to provide the necessary co-ordination needed for generating pulsatile discharge. Another possibility is that they are activated by inputs from GHRH cells and provide the missing link in a reciprocal influence of GHRH neurons on the periventricular somatostatin neurons. As yet nothing is known about the projections of these neurons other than their deduced projection to the median eminence, and establishing their cellular connectivity is likely to be a prerequisite to understanding their function.

The GH secretagogues have thus fortuitously provided a potentially important tool to dissect the neuronal circuitry underlying the GH pulse generator. It has yet to be established that their actions are more than serendipitous pharmacology, but the selective expression of receptors on pituitary somatotrophs and in the hypothalamus, and the specific actions within the hypothalamus on GHRH cells and other cells that are sensitive to somatostatin, strongly suggest the existence of an endogenous ligand. This pattern of receptor distribution suggests that this ligand is likely to be either present in neuroendocrine neurons projecting to the median eminence, or else is produced peripherally, but has access to sites within the blood–brain barrier. Either of these alternatives will open a fresh chapter in our understanding of the physiological regulation of GH secretion.

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6 Pharmacology of Growth Hormone-Releasing Hormone and Its Peptide Analogs

David H. Coy, PhD

Contents

INTRODUCTION

When the isolation (from human pancreatic growth hormone [GH]-secreting tumors) and structures of several forms of growth hormone-releasing hormone (GHRH) were first reported *(1,2)*, much surprise was generated by the size of the peptide since all previously sequenced hypothalamic hormones were made up of relatively short amino-acid sequences. Also surprising at the time was the high degree of amino-acid sequence homology between GHRH and members of the quite extensive vasoactive intestinal polypeptide (VIP)/glucagon family of peptides (Fig. 1) all of which were of gastrointestinal or pancreatic origin. There is clearly a common evolutionary pathway, presumably owing to gene duplication, which has resulted in two major branches of this family: GHRH/PACAP, VIP/PHI, and secretin on one hand and glucagon/glucagon-like polypeptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) on the other and these aspects have been reviewed recently *(3)*. As with other known hypothalamic hormones, with the exception of somatostatin, GHRH is highly specific having demonstrable potent biological activity on GH release from the pituitary. However, there are indications that GHRH might also play a peripheral role, for instance in fetal/placental development, reproduction, and immune function *(3,4)*. Indeed, GHRH immunoreactivity has been found not only in the hypothalamus and pituitary but also in pancreas, kidney,

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Fig. 1. Amino-acid sequences of peptides in the same family as GHRH. Vertical lines indicate where sequence shortening can be effected with little loss of potency. Basic residues are emphasized in order to highlight there different spacing from peptide to peptide—this may influence their receptor specificity *(see text)*.

duodenum, lung, testis, ovary, adrenal, heart, and brain *(5,6)*. Although the biological responses, if any, of GHRH at these tissues are far from being fully characterized, the peptide does stimulate pancreatic exocrine secretion in vitro and in vivo *(7,8)* and weakly interacts with receptors for other GI peptides, particularly VIP *(9,10)*. There is also evidence of GHRH has effects on secretion of other peptides from several cell lines, for instance, stimulation of neurotensin and calcitonin from rat C cells *(11)*. These additional interactions and activities have to be kept in mind during the design of highly potent peptide analogs of GHRH in case unwanted side effects are inadvertently enhanced.

Because a fair amount of structure-activity work had already been performed on the older members of the series, particularly VIP and secretin, it was clear that some previously successful analog design approaches could probably be applied to GHRH. Indeed, it was found *(2,12)* almost immediately that the 40 or 44 amino-acid chain of GHRH (Fig. 1) could be shortened from the C-terminus until it was similar in length to VIP, secretin, and glucagon. The shortest, fully potent fragment appeared to be GHRH(1–29) *(12)* and the amidated form of this peptide has provided the basic structure for the vast majority of structure-activity studies that have been reported, largely because synthetic difficulties associated with peptides of this size are minimized. Another useful guide to structural features responsible for receptor binding and/or activation has also been derived from sequence comparisons among animal species. There are reports describing the GHRH structures in rat *(13)*, cow *(14)*, pig *(15)*, sheep *(16)*, and goat *(16)* and these are shown in Table 1. The greatest sequence difference exists between human and rat GHRH, which has His rather than Tyr in position 1 and a Ser for Asn replacement in positions 8. However, the functional results of this are not great and it is apparent that the high homology between species in the core 1–29 region reflects its importance for high biological potencies in all members of this peptide family.

There is currently much interest in the therapeutic use of agents that increase GH levels in hormone-deficient situations such as short stature children and elderly populations. Clinical studies *(17)* with growth hormone-releasing factor (GHRF)(1–40) and (1–29) are perhaps relatively disappointing *(18)* compared to therapy with GH itself, owing to

Table 1
Amino-Acid Sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly
B															
C															
D													Ile		
E															
F	His							Ser				Arg	Ile		

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln
B															
C													Asn		
D													Asn		
E													Asn		
F			Tyr						His	Glu			Asn		

	31	32	33	34	35	36	37	38	39	40	41	42	43	44
A	Gln	Gly	Glu	Ser	Asn	Gln	Glu	Arg	Gly	Ala	Arg	Ala	Arg	Leu-NH2
B				Arg				Gln			Lys	Val		
C				Arg				Gln			Lys	Val		
D				Arg				Gln			Lys	Val		
E				Arg				Gln			Lys	Val		
F				Arg				Gln	Arg	Ser		Phe	Asn	OH

[a]Amino-acid sequences of A, hpGHRF(1–44)NH_2; B, porcine GHRH; C, bovine GHRH; D, bovine GHRH; E, caprine GHRH; F, rat GHRH.

the poor GH responses achieved with the natural peptides. Indeed, the first-phase plasma disappearance rate of GHRH(1–40) measured by RIA in men is only in the region of 8 min *(19)*. There have been a number of studies aimed at elucidating the metabolism of GHRH using both the natural sequences and stabilized synthetic analogs sequences. The latter will be discussed in subsequent analog sections of this chapter. Interestingly, the primary cleavage point of all the GHRHs in human plasma is at the 2–3 peptide bond, which is hydrolysed by a dipeptidylpeptidase IV enzyme *(20)*. After removal of the N-terminal dipeptide, little further N-terminal degradation from the 3 position onwards seems to occur. Tryptic degradation at the 11–12 and, depending on the total length of the peptide, at position 12–13 was also noted *(20)*. Similarly, in pig plasma the half-life of GHRH(1–29)NH_2 was around 13 min *(21)* with GHRH(3–29)NH_2 being the major metabolite. Evidence of deamidation of the Asn residue in position 8 of GHRH by incubation in aqueous solution at neutral pH has also been reported *(22)*. Overall then, there is an excellent medicinal rationale for developing enzyme-resistant, high-affinity GHRH analogs.

PHYSICO-CHEMICAL STUDIES ON THE GHRH SEQUENCE(S)

Successful analog design approaches to be used on peptides the size of the GHRHs can be aided enormously by an examination of solution conformation, usually by circular dichroism measurements and 2D nuclear magnetic resonance (NMR) studies. It can be

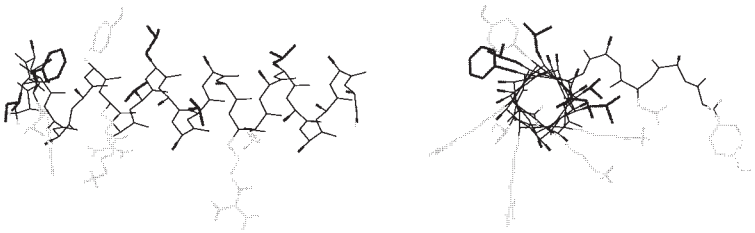

Fig. 2. Molecular modeling of [D-Ala^2, Ala$^{8,9,15,16,18,22,24–28}$]GHRH(1-28)NH$_2$ (JF-01-40; Table 2) in its projected amphiphilic α-helical state from residues 6-29. The hydrophilic side-chains, consisting of the basic Arg and Lys residues and Tyr residues, are shown in a lighter shade and the hydrophobic residues in the dark shade. Note how they align respectively on two surfaces of the molecule (left panel, amino terminus at the left of the picture). This is particularly evident from the end-on view of the molecule (right panel).

quite hard or impossible to infer a receptor-bound conformation from solution data, however, in the case of GHRH the tremendous tendency of virtually the whole sequence to adopt an α-helical conformation points strongly towards this also holding true when the peptide is complexed to the receptor. Thus, although in water alone there is limited helicity, this increases to 80–90% in 50% aqueous alcohol *(23,24)* and 65–70% in the presence of phospholipid liposome *(23)*. Helical tendencies of the whole chain were also indicated from two-dimensional proton NMR data *(25,26)*. Furthermore, the α-helix formed appears to be of the amphiphilic type *(27,28)*, in that it has two distinct surfaces on which either hydrophilic and hydrophobic amino-acid side-chains cluster. A molecular model of this effect can be seen in Fig. 2, in which a helically-stabilized, Ala-substituted analog (to be discussed in detail in a later section) was assembled and energy-minimized using the SYBYL program. More sophisticated computer molecular modeling, taking into account solution NMR NOE distance data and restrained molecular dynamics simulation, pointed to a structure having helices from position 6–13 and 16–29, but a more flexible short β-strand from the N-terminus to position 5 *(29)*. In general, this is in agreement with the other data already discussed. This N-terminal flexibility has been investigated by analog studies in which increased conformational restriction is introduced, for instance, by the use of D-amino acids.

A more simplistic approach to computer conformation prediction—Chou-Fasman sequence analysis *(30)*—has also been useful for delineating helical, β-sheet, and folding motifs in peptides of this size and was used by us initially to provide design information. The results of this technique applied to GHRH(1–29)NH$_2$ are shown in the top panel of Fig. 3 and, again, the high probability of the 16–29 sequence being helical is indicated. This method, however, did imply some structural ambiguity in the 8–11 region, which was an impetus to early analog design strategies aimed at probing this region also.

GENERAL STRUCTURE-ACTIVITY RELATIONSHIPS IN GHRH

It is clear from a comparison of GHRH with other members of the series that its active center comprises the N-terminal hexapeptide region. Early studies also quickly ascertained that the C-terminus could be shortened *(2,12)* only up to position 29 with retention of almost full potency. Reduction of chain length beyond Arg^{29} yields analogs with full intrinsic activity down to position 20 and full loss of activity beyond that point *(31,32)*.

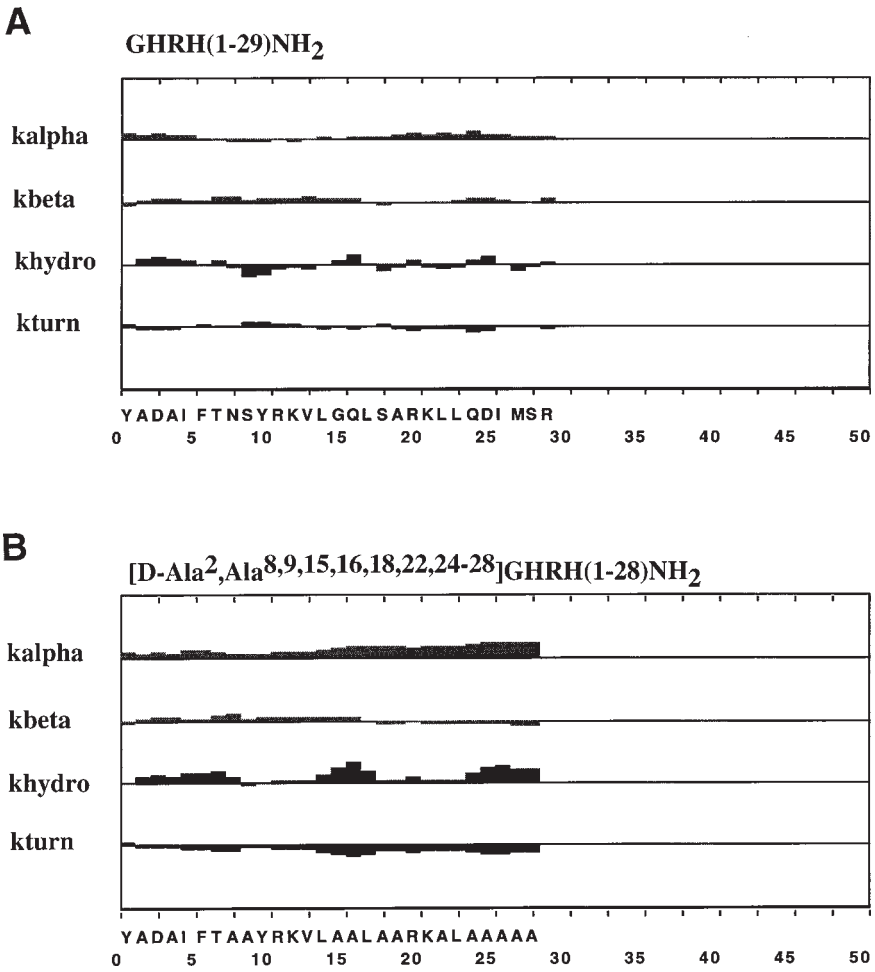

Fig. 3. Chou-Fasman conformational analysis of GHRH(1-29)NH_2 (top panel) and [D-Ala^2, $Ala^{8,9,15,16,18,22,24-28}$]GHRH(1–28)$NH_2$ (JF-01-40; Table 2) (bottom panel) showing the resulting increase in helical probability along the sequence of this potent analog. Hydrophobicity analysis demonstrates how the hydrophobic domaines of the analog are accentuated relative to GHRH(1–29) and are separated by the basic, hydrophilic Arg-Lys sequences.

The demonstration *(33)* that GHRH(1–29)NH_2 is just as potent as GHRF(1–40) in both normal subjects and patients with growth hormone deficiency (GHD) confirmed that this was an excellent sequence choice both for subsequent synthetic and biological studies.

Based on conformational analysis of the GHRH sequence described in the previous section, two of the first studies *(34,35)* undertook the individual replacement of the first 8 amino acids in GHRH(1–29)NH_2 with D-amino acids. The resulting analogs were bioassayed for stimulatory effects on GH release in the anesthetized rat and, surprisingly, the D-Tyr^1, D-Ala^2, D-Asp^3, D-Ala^4, and D-Asn^8-analogs were all far more potent than the parent peptide. In fact the D-Ala^2-GHRH analog was up to 50 times more potent than the native sequence and this was attributed to increased receptor affinity and perhaps the stabilization of secondary structural features at the N-terminus. It was not until later when the same analogs were tested in in vitro systems *(36)* and found to have only modest

increases in potency that it became clear that the high in vivo potency was primarily owing to inhibition of dipeptidase activity. Several of these D-amino acid-substituted analogs were also much more potent in cows *(37)* as in the rat. Unfortunately, this was not found to be the case in humans where D-Ala^2-GHRH(1–29)NH_2 is only about two times more potent than GHRH(1–29)NH_2 itself *(38,39)*, and Ac-D-Tyr^1,D-Ala^{2}GHRH(1–29)NH_2 about equipotent *(40)*, thus suggesting major differences between metabolism and clearance rates of these molecules among various species. The N-acetylated version of GHRH(1–29)NH_2 was also about 10 times more potent in the rat *(34)*, and the N-terminus of GHRH was not at all sensitive to acylation or alkylation. A report *(41)* that both the *N*-acetyl and D-Tyr^1 analogs of GHRH(1–40) had extremely low in vitro biological potency appears to be in error. The D-amino-acid substitution strategy was continued further down the peptide chain to give a complete "scan" of the chain *(42)*. It was found that D-amino acid substitutions in positions 10, 25, 27, 28, and 29 resulted in complete retention of in vitro potency. N-terminal conformation at positions 7–8, 8–9, and 9–10 (where potential folding is indicated by Chou-Fasman analysis (*see* Fig. 3), was further probed by incorporation of a rigid bicyclic β-turn dipeptide unit at these positions *(43)*. Although all analogs were full agonists, there extremely low potency provides some evidence against folding at these points, although the side chains of the amino acids involved were also drastically altered. The susceptibility of GHRH to dipeptidylpeptidase enzyme was also addressed by the substitution of amino acids other than Ala in position 2 *(44)*. Substitution with either Ser, Thr, or Gly resulted in much enhanced stability; however, undesirable effects on receptor affinity appeared to result.

Attempts to modify/stabilize the actual peptide bond CONH units between several N-terminal amino acids via incorporation of the CH_2NH group *(45)* drastically reduced antagonists potencies, although the 9–10 replacement analog was a receptor antagonist at rat pituitary cells.

There are, of course, numerous other portions of GHRH susceptible to proteolysis, particularly the tryptic cleavage sites provided by the two pairs of basic amino acids (Fig. 1). We carried out the modification of the epsilon-amino groups of the two Lys residues by reductive alkylation with various ketones and aldehydes *(36)*, and were surprised that potencies were readily retained even in the presence of bulky substitutions, including long alkyl chains and cyclohexyl groups. This suggested that these residues are not in close proximity to the receptor in the bound state. 2,3-dihydroxyisopropylation of the Lys residues *(46)* either together or individually gave analogs with much superior in vivo properties. Derivatization of either the amino terminus, each of the two Lys residues and Asp in positions 8 and 25 with polyethylene glycol chains was also investigated *(47)*. The amino terminus, Lys^{21}, and Asp^{25} could be acylated with these long polymer chains with little loss of potency and presumably binding affinity in vitro. The in vivo activity of these analogs was not investigated, however.

There have also been attempts to prevent proteolysis of GHRH(1–29) from its C-terminus. C-terminal alkylamide analogs were reported to have good in vivo potency *(48)*. The C-terminal Arg residue was replaced with agmantine (the des-carboxy derivative of Arg) in many analogs containing D-amino acids at the N-terminus and blocked N-termini *(49)*. As with most N-terminally stabilized-analogs, these were up to 70 times more potent in vivo in the rat; however, no in vitro or binding data were presented. Because even D-Ala^2-GHRH(1–29)NH_2 displays comparable levels of potency to these analogs in rats, it seems unlikely that they would be as potent in humans, since D-Ala^2-GRH(1–29)NH_2 is not.

α-HELICALLY-STABILIZED GHRH ANALOGS

As we have seen, the accumulated evidence is very strong for almost the whole GHRH(1–29) sequence existing as an amphiphilic α-helix in its receptor-bound state. There are two ambiguous regions emanating from the computer prediction and NMR data—the areas around Gly in position 15 and the 7–10 sequence. Replacement of Gly^{15} with Ala or α-aminoisobutyric acid (Aib) should increase α-helicity (Ala and Aib being, in that order, the best amino acids for helix enhancement) and indeed around fivefold-increases in in vitro GH releasing potency have been reported for many Ala^{15}- and Aib^{15}-analogs. These results have been reviewed recently *(50)*. A similar Ala-substitution strategy was applied to positions 8 and 9 *(51)*. All Ala^8, Ala^9, and $Ala^{8,9}$-analogs exhibited *(51)* increased in vitro potency thus finally ruling out the presence of a β-turn in this region. Also, another consequence of a helical conformation is that the Asn^8 and Lys^{12} side-chains should be in close proximity and, indeed, these positions can be covalently linked through Asp and Lys side-chains with complete retention of potency *(52)*.

A more extensive Ala and Aib-substitution study *(52)* utilizing GHRH(1–29)NH_2 was aimed at probing both the functionality of each amino acid side chain and α-helical propensity along the chain and yielded much useful information. It was found that the substitution of Ala in positions 8,9,15,22, and 24 resulted in significant increases in in vitro GH-releasing potencies and that substitution in position 16, 18, 19, 24, 25, and 26 resulted in complete retention of potency. Thus, an amazing number of more complex hydrophobic or hydrophilic amino acid side chains could be replaced with the simple methyl group side chain of Ala. Aib substitution in positions 8 and 22 resulted in increased potencies and conserved potencies when present in positions 9, 16, 18, 24, 25, and 28. We found *(53)*, quite unexpectedly, that simultaneous substitution of Ala at all those positions that had produced enhanced or conserved potency resulted in no loss of potency. In fact, [D-Ala^2, $Ala^{8,9,15,16,18,22,24–28}$]GHRH(1–29)$NH_2$ (NC-9-45; Table 2), has 48% Ala content and most of its functional amino acids replaced with Ala and yet was still 1.9 times more potent than the parent peptide. Incorporation of Aib in favorable positions 8, 18, and 24 *(53)* to give [D-Ala^2, $Aib^{8,18,24}$, $Ala^{9,15,16,22,25–28}$]GHRH(1–29)NH_2 (NC-9-96; Table 2) resulted in a 2.6-fold increase in GH-releasing potency.

Chou-Fasman secondary structure and hydrophobicity calculations were performed *(54)* on one of the new high-Ala content analogs (JF-01-40) in comparison with GHRH(1–29) itself and the results are shown in the lower panel of Fig. 3. The new analog, not surprisingly given the number of Ala residues present, is predicted to adopt an α-helical conformation with high probability throughout the whole length of the chain (lower panel, Fig. 3). The calculated hydrophobicity profile (Fig. 2, lower panel) is also interesting to compare to GHRH(1–29) itself. The three hydrophobic and two hydrophilic domains (the latter essentially constituting the pair of -Arg-Lys- sequences) within the chain are now highlighted and might very well comprise the recognition sites for GHRH binding to its receptor. It is also tempting to conclude that the pockets of high hydrophobicity variably separated by periodic basic amino acids (*see* highlighted residues in the sequences in Fig. 1) are responsible for the binding specificity of the various members of this series of related peptides.

The stabilized helix also permits removal of C-terminal residues with retention of high potency. With the GHRH(1–29)NH_2 sequence itself, removal of two and three C-terminal amino acids results in a 10-fold and 100-fold loss of potency, respectively (Table 2).

Table 2
Structure and GH-Releasing Potencies Relative to GHRH(1–29)NH_2 on Rat Pituitary Cells of High Ala/Aib-Content GHRH Analogs with Shortened Chain Lengths

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*GH Potency*	*Code #*
Y	*A*	*D*	*A*	*I*	*F*	*T*	*N*	*S*	*Y*	*R*	*K*	*V*	*L*	*G*	*Q*	*L*	*S*	*A*	*R*	*K*	*L*	*L*	*Q*	*D*	*I*	*M*	*S*	*R-NH_2*	*1.0*	*GRH(1–29)NH_2*
																											–	–	*0.10*	*NC-10-56R*
																										L	–	–	*0.109*	*DC-19-16*
																										–	–	–	*0.0022*	*NC-10-54*
	ᴅA						A	A						A												A		DR	4.9	BIM 28011
	ᴅA						A	A						A	A		A				A		A	A	A	A	A		1.9	NC-9-45
	ᴅA						A	A						A	A		A			A	A		A	A	A	A	A		0.65	NC-9-97
	ᴅA						Aib	A						A	A		Aib				A		Aib	A	A	A	A		2.6	NC-9-96
	ᴅA						A	A						A	A		A				A		A	A	A	A	A	A	0.66	NC-10-28-1
	ᴅA						A	A						A	A		A				A		A	A	A	A	A	–	1.24	JF-01.40
	ᴅA						A	Aib						A	A		Aib				A		Aib	A	A	A	–	–	1.24	NC-10.65
	ᴅA						A	Aib						A	A		Aib				A		Aib	A	A	–	–	–	0.08	NC-10-68
	ᴅA						A	Aib						A	A		Aib				A		A	–	–	–	–	–	0.20	NC-10-60R
	ᴅA						A	Aib						A	A		Aib				A		A	–	–	–	–	–	0.06	NC-10-63

In the Ala/Aib substituted configuration, however, removal of two amino acids results in little loss of potency, because NC-10-65 (Table 2) is still 1.24 times as potent as GHRH(1–29)NH_2. Even removal of three, four, and five amino acids results in potencies far greater than GHRH(1–26)NH_2 (NC-10-54; Table 2).

RECEPTOR ANTAGONISTS OF GHRH

The first antagonist of GHRH was found *(55)* by accident during an evaluation of N-terminally modified analogs for binding and activation of the rat pituitary GHRH receptor. Although D-Ala^2-GHRH(1–29)NH_2 is, as we have discussed, a potent agonist, Ac-Tyr^1,D-Arg^2-GHRH(-29)NH_2 was found to be devoid of GHRH agonist activity and a moderately good antagonist, thus providing additional proof that this part of the GHRH chain comprises the active center of the molecule. As mentioned, another weaker antagonist was produced by introduction of a reduce dipeptide bond into the 9–10 sequence of GHRH(1–29)NH_2 *(45)*.

The antagonist potency of the D-Arg^2-analogs has since been improved upon by the use of combined additional alterations to the peptide chain, such as Ala^{15} *(56)*, $Ala^{8,9}$ *(51)*, and agmantine incorporation at the C-terminus *(57)*. Ac-Tyr^1,D-Arg^2-GHRH(-29)NH_2 was quite effective in blocking pulsatile basal GH secretion and somatic growth in rats *(58)* and could block the effects of GHRH at both the hypothalamic and pituitary level after micro-injection *(59)*. Similarly, high doses of the same analog were able to block GH release in normal human subjects, although its inhibitory potency was relatively weak *(60)*. Constant infusion of an agmantine-containing GHRH antagonist was reported *(61)* to be able to slow the growth of human osteosarcomas transplanted in nude mice and to lower IGF-1 levels in the treated animals.

Interestingly, introduction of D-Phe in position 2 of a GHRH analog, Ac-Tyr^1,D-Phe^2-GHRH(1–29)NH_2, created a VIP receptor antagonist *(62)* with weak GHRH agonist activity. This analog cannot be considered the best choice of VIP antagonist because it has quite weak binding and partial VIP agonist activity in some biological systems *(63)*.

CONCLUSIONS

The secondary structure of the GHRH sequence(s) has now been well-elucidated and appears to consist of an amphiphilic α-helical conformation along the whole chain. Advantage has been taken of this to synthesize a great number of more potent analogs in which the helix is stabilized by various means. One method employs the use of Ala/Aib substitution for many of the amino acids in the chain, including several with functional side chains. In addition, this design approach allows for significant chain shortening to be carried out on the C-terminus of GHRH(1–29)NH_2 with good retention of in vitro potency. This results in structures that are much simpler than the parent peptide and which may be of some use in molecular modeling of the peptide with its receptor. These simplified analogs are also considerably easier and more cost efficient to prepare on a large scale. Also, it is expected that these design approaches will be of similar value when applied to other members of this family of peptides.

Although a number of analogs exist with significantly improved potency in the rat, those that have been carried through to human studies have so far produced disappointing results. However, as more and more proteolytic sites are stabilized along the sequence, it might be expected that significantly more potent and longer-acting analogs will even-

tually prove useful clinically. Quite potent GHRH receptor antagonists now exist; however, a clinical utility for this type of compound remains to be demonstrated, given the efficacy of somatostatin analogs in blocking GH release. With respect to somatostatin, the recent discovery *(64)* of potent type 2 receptor antagonists could have profound clinical consequences, because they might be used for GH stimulation either alone or in combination with GH, GHRH, or GHRP. This is because GH tone is generally under strong negative control by endogenous somatostatin and somatostatin release can actually be stimulated by these agents.

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7 Somatostatin Receptor Subtypes and Regulation of GH Secretion

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Contents

ABSTRACT

Somatostatin (SRIF) is a 14 amino-acid-containing peptide primarily expressed in the hypothalamus. It is a major physiological regulator of growth hormone (GH) secretion and is critical in maintaining the pulsatile release of GH. SRIF induces its biological effects by interacting with membrane-associated receptors, of which a family of five have recently been cloned. The cloned receptor subtype referred to as sstr2 may have an important role in mediating the inhibitory effects of SRIF on GH secretion. This is suggested from pharmacological studies showing that a large series of SRIF analogs, including the clinically used peptides octreotide and lantreotide, had a similar rank order of affinities for binding to sstr2 and to inhibit GH secretion. Stimulation of sstr2 may lead to inhibition of Ca^{2+} influx into somatotrophs to reduce GH secretion. Structural analysis of the cloned sstr2 has revealed binding domains of the receptor that may be useful in the development of antagonists and nonpeptide agonists at this receptor, which could have clinical uses in the regulation of GH secretion and other biological functions of SRIF.

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INTRODUCTION

SRIF is an important regulator of GH secretion from the anterior pituitary *(1)*. The effects of SRIF are mediated directly at the level of the pituitary and are also mediated via feedback loops in the hypothalamus. The peptide has other physiological actions. It is secreted from delta cells of the pancreas to inhibit insulin and glucagon release and has a role in regulating amylase and gastric acid secretion. SRIF is also a neuromodulator in the brain involved in regulating locomotor activity and cognitive functions.

In addition to SRIF, a larger precursor, somatostatin-28 (SRIF 28) is also expressed in the body and like SRIF can regulate hormone and neurotransmitter release. Pharmacological studies have even suggested that SRIF and SRIF 28 may have some different primary biological roles; since SRIF 28 was reported to be more potent than SRIF in controlling insulin release whereas it was equipotent with SRIF in control glucagon secretion *(2)*. Furthermore, SRIF 28 is the predominant somatostatin peptide expressed in the gastrointestinal tract, suggesting a primary role of the peptide in controlling gastric-acid secretion. In contrast, SRIF is primarily expressed in the hypothalamus and may subserve a more essential role than SRIF 28 in regulating GH secretion.

Recently, a third somatostatin-like peptide was identified, cortistatin *(3)*. This peptide has a somewhat similar structure to SRIF, but is derived from a different prohormone. This peptide is able to induce some similar actions as SRIF and can bind to the same receptors as SRIF. However, it is also able to induce distinct actions from SRIF, in particular effects on slow-wave sleep. This latter finding suggests that cortistatin may be able to interact with a different receptor than SRIF.

SRIF RECEPTOR SUBTYPES

SRIF induces its biological actions by interacting with membrane-associated receptors. A number of studies had suggested that subtypes of SRIF receptors are expressed in the body *(1)*. Studies on the pancreas suggested that SRIF 28 was much more potent than SRIF in blocking insulin secretion, whereas the peptides had similar potencies in controlling glucagon release *(2)*, indicating that different receptors may be involved in mediating SRIF peptide effects on pancreatic hormone secretions. Receptor binding studies suggested that radiolabeled SRIF analogs bound to multiple sites in different tissue preparations *(4)*. Synthetic peptides such as octreotide and MK 678 bound potently to one site and not the other. Furthermore, one binding site was sensitive to guanine triphosphate (GTP) analogs and Na^+ whereas the binding of SRIF to the other site was not. In addition, antibodies made against native SRIF receptors revealed heterogeneities in the size of SRIF receptors, which are likely to be owing to the antibodies being raised against different receptor subtypes *(5)*.

Cloning of SRIF Receptors

The cloning of a family of SRIF receptors confirmed that subtypes of these receptors exist and are expressed in the body *(1,6)*. Bell and his associates *(7,8)* cloned the first three SRIF receptors and named them sstr1, sstr2, and sstr3. Berelowitz and his associates *(9)* cloned the fourth receptor and O'Carroll *(10)* cloned the fifth receptor. The five

receptors have approx 40% amino-acid sequence similarity, with highest similarity in the transmembrane spanning regions *(1,6)*. They are dissimilar in amino-acid sequence from any other receptors except the opiate receptors *(11)*. Interestingly, some SRIF analogs, such as octreotide, bind potently to opiate receptors *(12)* and the selective mu opiate antagonist CTOP was designed based on the structure of octreotide *(12)*, suggesting that the structural similarities of these two families of receptors may afford some functional similarities.

The genes of the five receptors are localized to different chromosomes *(1)*. Both RNA analysis and *in situ* hybridization studies have shown that the mRNA for the different receptors have distinct but overlapping distributions *(1,6)*. Most notably, all five receptor mRNAs are expressed in the anterior pituitary, although at different levels.

The pharmacological properties of each receptor have been characterized following their expression in different tumor cell lines. All five receptors bind SRIF and SRIF 28 with high affinities *(14,15)*. Only sstr5 shows a preferential affinity for SRIF 28 over SRIF. Both sstr1 and sstr4 have low affinity for most synthetic analogs of SRIF. This similarity is consistent with the unusually high amino-acid sequence similarity of these two receptor subtypes *(1)*. No subtype-selective ligand has been identified for sstr4. However recent studies have identified the peptide des-AA^{1,2,5}-[DTrp^{8}, IAMP^{9}]SRIF as a selective ligand for sstr1 *(16)*. The peptide binds to sstr1 with an affinity in the 5–10 n*M* range, whereas it interacts with the other cloned receptors with much lower affinities. As a result, des-AA^{1,2,5}-[DTrp^{8},IAMP^{9}]SRIF and its tyrosine analog, which can be iodinated, may be useful for detecting expression of sstr1 in tissues and for activating this receptor to determine its biological functions.

Several different SRIF analogs were identified that selectively bind to sstr2. The octapeptide NC8-12 and the hexapeptide BIM 23027 have over 100-fold higher affinity for sstr2 than the other receptors and can be used to selectively detect and activate this receptor *(14,15)*. The analogs octreotide and MK 678 also bind potently to sstr2, although they do have some crossreactivity with sstr3 and sstr5.

No selective analogs have been agreed upon for sstr3, but the linear peptide BIM 23052 has been reported to have some selectivity for rat sstr5 *(14,15)* and functional studies by Coy and his associates *(17,18)* suggest that this peptide can be used to identify selective functions of rat sstr5. This peptide does not show selectivity for human sstr5. In fact, rat and human sstr5 show considerable species variations in amino acid sequence and major differences in ligand selectivities *(19)*.

FUNCTIONS OF SRIF RECEPTOR SUBTYPES

Development of subtype selective ligands is essential for identifying the location of these receptors which is critical in gaining insights to their functions. Analysis of mRNA localization has its limitations because mRNA detection is not always a true reflection of receptor expression. This is most clearly shown in the case of sstr3, whose mRNA is highly expressed in the adult cerebellum, yet very little if any SRIF receptor binding is detectable in the cerebellum *(20)*.

Recently, antibodies have been developed against some of the SRIF receptor subtypes, in particular sstr2, and have been useful in detecting expression of this receptor by immunoblotting *(21,22)*. Development of antibodies and ligands for the other receptor should also facilitate their localizations.

Pharmacological studies have suggested that sstr2 is involved in mediating the inhibition of GH release by SRIF *(14,15)*. The rank order of affinities of a large series of SRIF analogs to bind to cloned mouse sstr2 was similar to their rank order of potencies to inhibit GH release from rat pituitary cells in culture. In contrast, there was no clear correspondence between binding to the other receptors and inhibition of GH release. sstr2 mRNA and protein, as detected by immunoblotting are expressed in the anterior pituitary *(1,20,21)* consistent with the role of this receptor in controlling growth hormone secretion.

Several analogs with high affinity for sstr2, such as octreotide, are very effective in blocking GH release in human and rodents both from normal pituitaries as well as pituitary tumors. These analogs also bind to sstr5 *(15)*, a receptor that is also likely to be expressed in anterior pituitary. However, the rank order of potencies of SRIF analogs to bind to rat sstr5 was not similar to their potencies to inhibit GH release. Furthermore, many SRIF analogs, such as octreotide, have relatively low affinity for human sstr5 *(19)*, suggesting that their main mechanism of action in blocking GH secretion is via sstr2. Finally, sstr5 has been shown to exhibit higher affinity for SRIF 28 than SRIF. In contrast, SRIF 28 and SRIF are similar in potency in blocking GH secretion *(14)*. These findings suggest that sstr2 is likely to be an important receptor in the control of GH secretion.

CELLULAR MECHANISMS OF ACTION OF SRIF RECEPTOR SUBTYPES

The cellular mechanisms by which sstr2 may mediate the inhibition of GH release are not established. However, the cellular responses linked to this receptor have been identified. sstr2 couples to adenylyl cyclase and mediates inhibition of cAMP formation induced by SRIF *(1,23)*. The cloned sstr2 has been reported to couple to voltage-sensitive Ca^{2+} channels and mediate inhibition of Ca^{2+} influx *(24)*. Recent studies have also shown that sstr2 selective ligands can inhibit an L-type Ca^{2+} channel in AtT-20 cells to block Ca^{2+} influx *(25)*. In these cells, some sstr2 analogs, such as BIM 23027 and MK 678 potentiate a K^+ current via an inwardly rectifying K^+ channel *(26)*. However, the pharmacology of this effect is not consistent with a role of sstr2, because octapeptide analogs that are selective for sstr2, such as NC8-12 and octreotide, have no effect on this current. Furthermore, the ability of SRIF to potentiate this K^+ current is blocked by the peptide developed by Coy and his associates *(17,18)*, referred to as the somatostatin antagonist. This peptide has no affinity for sstr2 *(14)* indicating that the SRIF receptor linked to the K^+ current in AtT-20 cells is a receptor that has not been cloned. These studies suggest that either inhibition of cAMP accumulation or Ca^{2+} influx are the major mechanisms by which sstr2 is likely to mediate inhibitory effects of SRIF on GH release.

SRIF receptors are linked to these diverse cellular effector systems via G proteins *(1,27)*. Biochemical studies have revealed that SRIF receptors in brain and AtT-20 cells are capable of coupling to G_{ia1}, G_{ia3}, and G_{oa} *(27–31)*. Functional studies have revealed that these different G proteins can link SRIF receptors to different cellular effector systems. G_{ia1} was reported to couple SRIF receptors to adenylyl cyclase *(32)*. G_{ia3} has been shown to link SRIF receptors to K^+ channels *(33)* and G_{oa} mediates SRIF's inhibition of Ca^{2+} currents *(34)*. By acting through different G proteins, SRIF can independently regulate each effector system.

SRIF RECEPTOR DESENSITIZATION

sstr2 has been reported to desensitize following continuous agonist pretreatment *(23)*. Pretreatment of cells expressing sstr2 with agonist has been reported to reduce high-affinity agonist binding to sstr2 and reduce the ability of SRIF to inhibit cAMP accumulation. The enzyme beta-adrenergic kinase (BARK) has been proposed to be involved in SRIF receptor desensitization *(35)* and sstr2 becomes phosphorylated when stimulated with agonist *(36)*. However, recent mutagenesis studies suggest that a more complex mechanism may exist for sstr2 desensitization *(37)*.

To test the role of the carboxy terminus of sstr2 in desensitization, this domain was excised from the receptor *(37)*. The truncated receptor was expressed in CHO cells, had similar pharmacological properties as the wild-type receptor and coupled to G proteins and adenylyl cyclase. Furthermore, it desensitized, suggesting that the carboxy terminus is not essential for agonist regulation *(37)*.

The third intracellular loop of sstr2 has been proposed to have a role in G-protein coupling and has several serine residues at positions 244 and 245 which could serve as phosphate acceptors. Mutation of serine 244 to a tryptophan did not affect agonist regulation of the receptor nor did mutation of serine 245 to an alanine. This finding suggests that phosphorylation of these residues is not essential for the receptor to desensitize.

However, most importantly, mutation of serine 245 to a glutamine made sstr2 much more resistent to agonist regulation. This result was unexpected and indicates that the region around this serine has a role in the regulating the receptor. Conceivably, the positive charge of the glutamine may affect the interaction of sstr2 with cellular factors involved in receptor desensitization.

In addition to desensitizing, sstr2 is rapidly internalized when bound to agonist *(38)*. sstr2A transfected in COS-7 cells bound fluorescent labeled SRIF analogs and internalized within an hour of incubation at 37°C. There is no information on whether similar molecular events are involved in internalizing and desensitizing sstr2. Furthermore, it is not clear whether the internalization is physiologically relevant, since the studies were conducted on transfected cells overexpressing the receptor.

Although sstr2 can be regulated, SRIF inhibition of GH release does not desensitize. This suggests that either sstr2 does not desensitize under physiological conditions or somatotrophs expressing sstr2 lack critical factors needed for desensitization. Although sstr2 coupling to adenylyl cyclase desensitizes, its link to Ca^{2+} channels is maintained following prolonged agonist stimulation *(25)*. If the inhibition of Ca^{2+} conductance and influx by SRIF is directly linked to the inhibition of GH release by SRIF, the maintenance of SRIF's control of GH secretion is likely to be owing to the resistance of sstr2/Ca^{2+} coupling to desensitize. Alternatively, if sstr2 desensitizes, the continued ability of SRIF to inhibit GH secretion may be owing to other receptor subtypes that do not desensitize to SRIF, compensating for sstr2 desensitization and maintaining SRIF control of GH secretion. In this regard, sstr1, is relatively resistent to SRIF regulation *(39)*. Agonist pretreatment does not affect high-affinity agonist binding to sstr1, and this receptor is slow to internalize *(38)*. sstr1 mRNA is expressed in pituitary *(1,20)* and could link up to GH secretion if sstr2 were desensitized and compensate for the loss of sstr2 responsivity.

sstr2 may also be involved in mediating antiproliferative effects of SRIF analogs in the treatment of cancer *(40–42)*. Octreotide, which is used to treatment pituitary ade-

nomas and other tumors, potently stimulates sstr2. Buscail et al. *(40,41)* reported that sstr2 stimulation can lead to an inhibition of cell proliferation. sstr2 was found to couple to a tyrosine phosphatase and activation of the phosphatase parallels the reduction in growth of the cells transfected with sstr2. The coupling of sstr2 to the tyrosine phosphatase is mediated by G proteins and is yet another cellular response coupled to this receptor subtype. Whether SRIF's ability to inhibit cell proliferation desensitizes is not known, although in general SRIF analogs have not proven to be extremely effective as anticancer agents.

STRUCTURE-FUNCTION ANALYSIS OF SSTR2

Presently there are major limitations of SRIF receptor pharmacology. The lack of antagonists specific for each receptor subtype has greatly limited studies designed to identify the selective functions of SRIF receptors. Furthermore, no nonpeptide SRIF ligands are commonly available. As a result, SRIF analogs as therapeutic agents is limited. Conceivably, with the availability of the cloned receptors, pharmaceutical companies may advance in the development of these agents.

Structure-function analysis of the ligand binding domains of the receptors may aid in development of new SRIF ligands. Recently, several studies have focussed on ligand binding determinants of sstr2. Fitzpatrick and Vandlen *(43)* showed that the second and third extracellular loops of sstr2 are critical for the binding of MK 678 and Kaupmann et al. *(44)* reported that several amino acids in the vicinity of these loops were essential for the binding of octreotide to the receptor. Liapakis et al. *(45)* reported that a four amino-acid sequence phenylalanine-aspartate-phenylalanine-valine (FDFV) at the interphase of the third extracellular loop and transmembrane seven of sstr2 was involved in the binding of octapeptide and hexapeptides to sstr2. A phenylalanine at residue 294 was most essential for the binding of octapeptides, because this residue inserted into a corresponding region of sstr1 to create the mutant $sstr1_{S305F}$ conferred onto sstr1 the ability to bind octapeptides.

Hexapeptides, such as MK 678, did not bind to $sstr1_{S305F}$, suggesting that this smaller SRIF analog had different requirements for binding than octapeptides. MK 678 has a tyrosine adjacent to the tryptophan and lysine residues needed for binding. Hexapeptide analogs with a phenylalanine at this position bound to $sstr1_{S305F}$, indicating that the phenylalanine of the peptide was critical for the ability of the peptide to interact with the phenylalanine at residue 294 of sstr1. Conceivably, the phenylalanine allows for hydrophobic interactions to occur with the phenylalanine of the receptor, whereas the added hydroxyl group of MK 678s tyrosine serves to repel the peptide from the phenylalanine of the receptor. This structural information reveals both critical determinants of the peptides and receptor needed for binding, which could be useful in the design of new sstr2 select ligands.

The mutagenesis studies also revealed determinants for binding to sstr1 *(45)*. The peptide des-$AA^{1,2,5}$-[$DTrp^{8}$,$IAMP^{9}$]SRIF binds selectively to sstr1 and does not bind to sstr2. The peptide bound to a chimeric receptor consisting of sstr2 with the second extracellular loop of sstr1, suggesting that this region of sstr1 is essential for the binding of this synthetic analog. Thus, the binding domains of selective agonists at sstr1 and sstr2 are at vastly different regions of the receptor.

CONCLUSIONS

A major physiological function of SRIF is to inhibit GH release. SRIF inhibits GH release by activating the receptor subtype sstr2 to inhibit Ca^{2+} conductance and Ca^{2+} influx in somatotrophs. This coupling is maintained following continued activation of the receptor, which may explain the resistance of SRIF's inhibition of GH to desensitization. Synthetic peptide analogs of SRIF, such as octreotide and MK 678, are effective inhibitors of GH release. Development of nonpeptide analogs could serve as better drugs than the peptides and be effective inhibitors of hyper GH release, as occurs in acromegaly. Development of sstr2 antagonists could be useful to facilitate GH release, which could prove useful in the treatment of aging and diseases in which muscle mass is lost. Structure function analysis of sstr2 has revealed selective domains of the receptor critical for agonist binding. Such information will be useful in the design and development of new SRIF drugs.

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8 Mathematical Modeling of the GH Release Axis

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Contents

INTRODUCTION

Although the hypothalamus functions as a temporal pattern generator of growth hormone releasing factor (GRF), the pituitary plays major dynamic roles in amplifying and modulating this pattern. First, in response to continued application of GRF, the pituitary somatotrophs rapidly desensitize and this process is important in shaping the pulsatile profile of growth hormone (GH) secretion. Second, the pituitary can amplify a pulsatile output of GRF from the hypothalamus. This may seem unnecessary given the possibility of generating a larger signal directly from the hypothalamus; however, the amplification is nonlinear (the dose-response curve of the pituitary to releasing factors is sigmoidal when plotted on a semilog scale). The nonlinearity is partly a result of short-term desensitization at the pituitary, which contributes to terminating secretory episodes, leading to a stereotyping of both pulse amplitude and duration, as well as ensuring a minimum interpulse interval. Furthermore, the pituitary coordinates a number of inputs from the hypothalamus and elsewhere.

The secretion of growth hormone in the rat is sexually dimorphic. In both sexes secretion is pulsatile, but in males the pulses are larger, less frequent, and arise from a lower interpulse baseline than in females *(1)*. That pulsatile GH secretion fuels faster growth has been demonstrated both in animals deficient in growth hormone and in animals experimentally deprived of hypothalamic GRF *(2–4)*. In the male rat, pulses occur

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at intervals of about 3 h, and since the growth-promoting effects of pulsatile GH administration appear to saturate at about nine pulses per day, the physiological pattern of growth hormone secretion appears to be optimally efficient. Pulses of growth hormone secretion derive from episodic secretion of GRF, but growth hormone secretion is also regulated by the secretion of somatostatin *(5–8)*.

Somatostatin (SRIF) inhibits the secretion of growth hormone, and although acting at a separate receptor, acts as a functional GRF antagonist in that in the presence of somatostatin the growth-hormone releasing ability of GRF is attenuated; both the GRF receptor and the somatostatin receptor are G protein-linked receptors, and activation of these receptors produces opposing effects upon intracellular calcium and cAMP levels and on calcium entry, in particular, through L-type channels. The sexually dimorphic patterns of growth hormone secretion in the rat appear to derive from sexually dimorphic behavior of the somatostatin neurons, possibly reflecting the sexually dimorphic expression of androgen receptors by these neurons *(9)*. In the male rat, GRF and somatostatin are probably released alternately to produce peaks and troughs of growth hormone release, respectively, whereas in the female, somatostatin is released more continuously. However, inferring the nature of the hypothalamic signals from the pituitary response is not simple because the pituitary responsiveness to releasing factors is variable. Variability arises from the interactions of the hypothalamic factors with each other from desensitization of the pituitary during sustained exposure to probably either factor, from actions of the hypothalamic factors on the synthesis of growth hormone, and in the case of somatostatin, a dramatic "off" effect when somatostatin is removed, reflected by an increase of basal release in the absence of GRF and a sensitization to GRF *(10,11)*.

Application of artificial growth hormone secretagogues also promotes GH release in a similar dose-dependent manner by different receptors *(12)*, and there is evidence in vivo of synergism between the two stimulants in the sense that GH release is in total greater when GRF and secretagogue are applied together than the sum of the releases when they are applied separately.

MODELING THE PITUITARY RELEASE OF GROWTH HORMONE

For the growth hormone system, the pituitary modifies the hypothalamic output and any exogenously applied stimulation with hormone releasing peptides to such an extent that, if the authors wish to infer the output of the hypothalamus from the observed profile of GH in the circulation, then as a first step they must establish a mathematical model that adequately reflects the behavior of the pituitary. A preliminary model has been based *(13)* on the Law of Mass Action applied to reversible binding of GRF and/or secretagogue to its receptors (Fig. 1). For the present the general discussion will be phrased just in terms of GRF. However, in general, analogous arguments hold for the actions of GHRP and other artificial secretagogues at the pituitary when these are applied in isolation, so the following model framework can be extended naturally to encompass these additional factors. How to model the pituitary response to simultaneous application of GRF and artificial secretagogues is beyond the scope of this chapter.

GRF acts via G protein-coupled receptors and the transduction pathway involves activation of adenylate cyclase and protein kinase A, elevated levels of cAMP, and calcium entry through voltage-gated channels and calcium-entry-dependent exocytosis. Somatostatin acts at a separate G protein-coupled receptor to oppose the GRF-induced

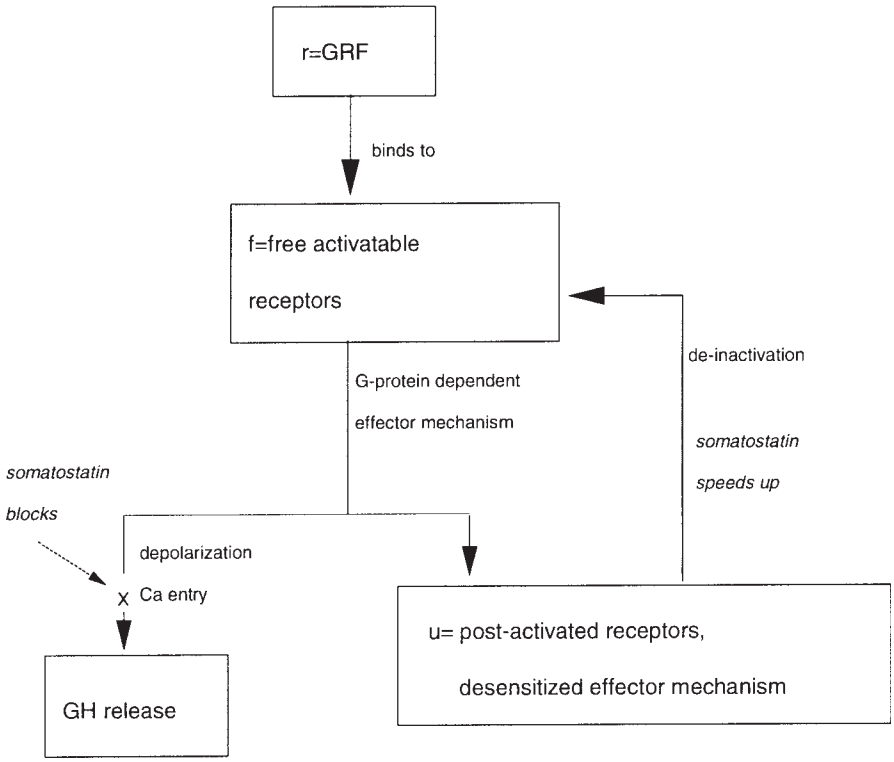

Fig. 1. A schematic form of the model.

transduction pathway, reducing the intracellular concentration of cAMP, and also induces hyperpolarization by increasing potassium conductance. Therefore, in the authors' model, the release rate of growth hormone is directly proportional to the rate of binding of GRF, but inversely proportional to the concentration of SRIF, resulting in pulses of growth hormone in the absence of SRIF and much smaller pulses in its presence. The model assumes proportionality between receptor binding and the release signal, which is probably a function of localized intracellular calcium concentration arising from calcium entry via L-type channels, which are modulated by second messenger pathways.

The Mathematical Model

GRF (R, concentration r) binds reversibly to receptors (F, density f) on the pituitary somatotroph:

$$R + F \underset{k_b}{\overset{k_1}{\rightleftharpoons}} U$$

The rate of the forward reaction is k_1. The rate of backward reaction depends on the concentration of SRIF, s, being $k_b = k_2 + k_3\,\phi(s)$, where $\phi(s) = 1 / (1 + \exp[-(s - s_0)/\delta_0])$ is a sigmoidal function of s, rising from zero to unity over a range of concentrations of SRIF of $s_0 \pm 3\delta_0$. The authors thus get the equations

$$\frac{df}{dt} = -k_1 (r + r_0) f + \left[k_2 + k_3 \phi(s)\right] u \tag{1}$$

$$\frac{du}{dt} = k_1 (r + r_0) f - \left[k_2 + k_3 \phi(s)\right] u \tag{2}$$

where u reflects the number of postactivated, desensitized GRF receptors, r is the concentration of GRF in the extracellular space, and r_0 reflects constitutive activation of the GH release mechanism. The step in the sigmoidal function, $\phi(s)$, can be made more or less steep by reducing or increasing δ_0. If k_3 is positive, the rate of the backward reaction is greater in the presence of somatostatin. The rate of release of growth hormone is proportional to the rate of binding (the constant of proportionality being a function of s) and given by

$$Rel(t) = [k_4 + k_5(1 - \phi[s])][r + r_0] f \tag{3}$$

Thus, in the presence of very high levels of somatostatin, the rate of release is $k_4(r + r_0)f$, and in the absence of SRIF it is $(k_4 + k_5)(r + r_0)f$. To simulate the model we need a differential equation for s,

$$\frac{ds}{dt} = I_s - k_7 s \tag{4}$$

where I_s is the rate of release (of infusion) of SRIF, and k_7 its decay rate. The dynamics of SRIF concentration are separate from the other dynamical variables: SRIF affects the dynamics of the interaction between GRF and its receptors, and growth hormone release, but there are no effects of these on SRIF release or decay. A further differential equation in r could be added

$$\frac{dr}{dt} = I_r - k_1 (r + r_0) f - k_6 r$$

where k_6 is the relative rate of decay of GRF in the absence of binding, and I_r is the input of GRF. However, other factors frequently have a substantial effect on r and it is simpler for the purposes of initial modeling to assume that external factors maintain the level of r either as a constant infusion or a pulsatile pattern of stimulation. Understanding the model may be easier if SRIF is thought of as acting as a switch that changes the behavior of the somatotroph when present at suprathreshold concentration (substantially above s_0). In the absence of SRIF successive pulses of GRF desensitize the somatotroph as the receptor-effector mechanism is progressively inactivated. Somatostatin resensitizes the somatotroph by speeding up the recovery of the receptor-effector mechanism to the free, available state. The variable u, being proportional to the number of postactivated, desensitized GRF receptors, can be thought of as an index of the extent of desensitization of the release system. If the further assumption is made that the total number of receptors is constant (i.e., in the short term, receptors are neither created nor destroyed), and the total concentration of sensitized receptors is fT, then $u = fT - f$ can be written, and, therefore, f can equally well be regarded as a complementary index of sensitivity: the ability of the somatotrophs to respond to stimulation by GRF.

Analytical Solution of the Model

One particular advantage of the very simple model formulation outlined above is that for periods when r and s are constant, the differential equations can be solved to give

explicit expressions for the extent of desensitization and for the rate of GH release. Making the substitution $u = f_T - f$ (where f_T is the total concentration of free receptors in the completely sensitized state, i.e., the maximal concentration of free receptors) and $k_b = k_2 + k_3\phi(s)$ in Eq. (1), the result is

$$\begin{aligned}\frac{df}{dt} &= -k_1(r + r_0)f + k_b(f_T - f)\\ &= k_b f_T - [k_1(r + r_0) + k_b]f\\ &= k_b f_T - Kf\end{aligned}$$

where $K = k_1(r + r_0) + k_b$. The equilibrium values of f, f^*, is given by solving $df/dt = 0$, obtaining

$$f^* = \frac{k_b f_T}{K} = \frac{k_b f_T}{k_1(r + r_0) + k_b} = \frac{[k_2 + k_3\phi(s)]f_T}{k_1(r + r_0) + k_2 + k_3\phi(s)} \tag{5}$$

Thus, f^* is an irreducible minimum sensitivity below which the system will not fall. This is a dynamic equilibrium or minimum, though, since it depends on both r and s and will therefore change as they do. Rewriting the differential equation in terms of f^*, the result is

$$\frac{df}{dt} = -K(f - f^*) \tag{6}$$

with solution

$$f(t) = f^* + (f_0 - f^*)e^{-Kt} \tag{7}$$

assuming that the initial concentration of free receptors at time $t = 0$ is f_0. Thus, after a change in the r,s environment, the excess sensitivity of the system over the new equilibrium declines exponentially with time, and the relative rate of decline is $K = k_1(r + r_0) + k_b$, which equals $k_1(r + r_0) + k_2 + k_3\phi(s)$.

Therefore, the release rate is given by

$$\rho(r,s,f) = [k_4 + k_5(1 - \phi[s])](r + r_0)f \tag{8}$$

$k_r = k_4 + k_5(1 - \phi[s])$ is defined to be the rate constant for release, and therefore $\rho(r,s,f) = k_r(r + r_0)f$. We can therefore calculate the cumulative amount released as the integral of this, obtaining

$$P(r,s,t) = k_r(r + r_0)[f^*t + (f_0 - f^*)(1 - e^{-Kt})/K] \tag{9}$$

if the initial free receptor concentration is f_0, These expressions enable theoretical dose/response curves for any concentrations of r, s to be obtained. These can then be fitted to dose/response curve data, first of all as a test of the model, and second to estimate the model parameters.

MODEL PROPERTIES

In vitro and in vivo the somatotrophs display a striking desensitization to GRF, and as discussed in the previous section, the authors' model also exhibits this property (Fig. 2). Desensitization might occur at many stages between ligand binding to the receptors and

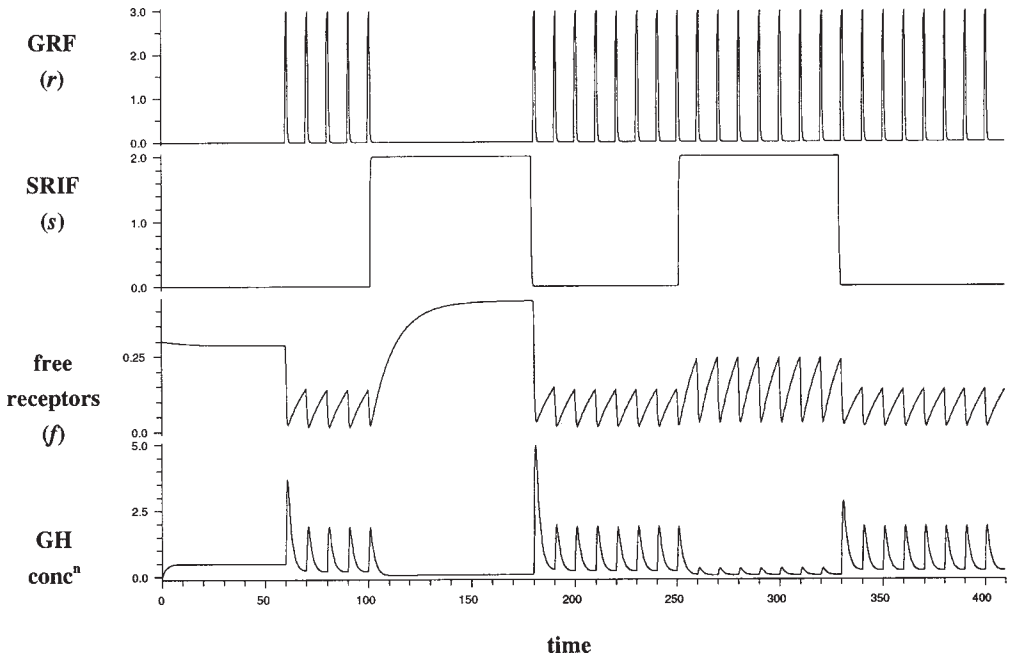

Fig. 2. Model simulation showing pulsatile release of growth hormone as a consequence of pulsatile application of GRF; desensitization to repeated pulses of GRF; a complete resensitization as a result of infusion of somatostatin in the absence of pulses of GRF; almost complete inhibition of release as a result of somatostatin infusion even though the GRF pulses continue; a partial resensitization as a result of this somatostatin infusion as pulses of GRF continue. Parameters used were $k_1 = 1$, $k_2 = 0.002$, $k_3 = 0.036$, $k_4 = 1.5$, $k_5 = 30$, $k_6 = 5$, $k_7 = 5$, $k_8 = 0.5$, $r_0 = 0.05$, $s_0 = 0$, $\delta_0 = 0.05$.

exocytosis; however, desensitization to GRF is not accompanied by desensitization to other secretagogues like GHRP6, which act via different G protein-coupled receptors and different intracellular second messenger pathways, but which also result in exocytosis via L-type channel gated calcium entry. Thus desensitization does not reflect depletion of a readily releasable pool of granules, nor inactivation of the calcium channels. In the authors' model the GRF receptor mechanism becomes transiently inactivated during sustained exposure to GRF, hence the model displays dose- and interval-dependent desensitization of growth hormone release in response to regular pulses of GRF (Figs. 3, 4). In the presence of a sufficiently high concentration of SRIF, both release of growth hormone and desensitization of the GRF receptor mechanism are less (Fig. 2); it would appear that the bound GRF receptor or the subsequent effector mechanism is transiently inactivated only if it is first functionally activated; this is equivalent to postulating a postactivation latent phase at any stage subsequent to receptor activation. A model incorporating this behavior responds to infusions of GRF with a dose-dependent release of growth hormone and desensitization of the pituitary, whereas coinfusion of SRIF results in inhibition of secretion followed by a rebound hypersecretion (Fig. 5); an "off" effect will occur in the absence of SRIF if some constitutive activation of the secretory pathway is postulated, which may be equivalent to assuming a nonzero resting level of cAMP. Interestingly, if pulses of SRIF are imposed on a background of constant GRF, the pulses result in a paradoxical dose-dependent stimulation of growth hormone release.

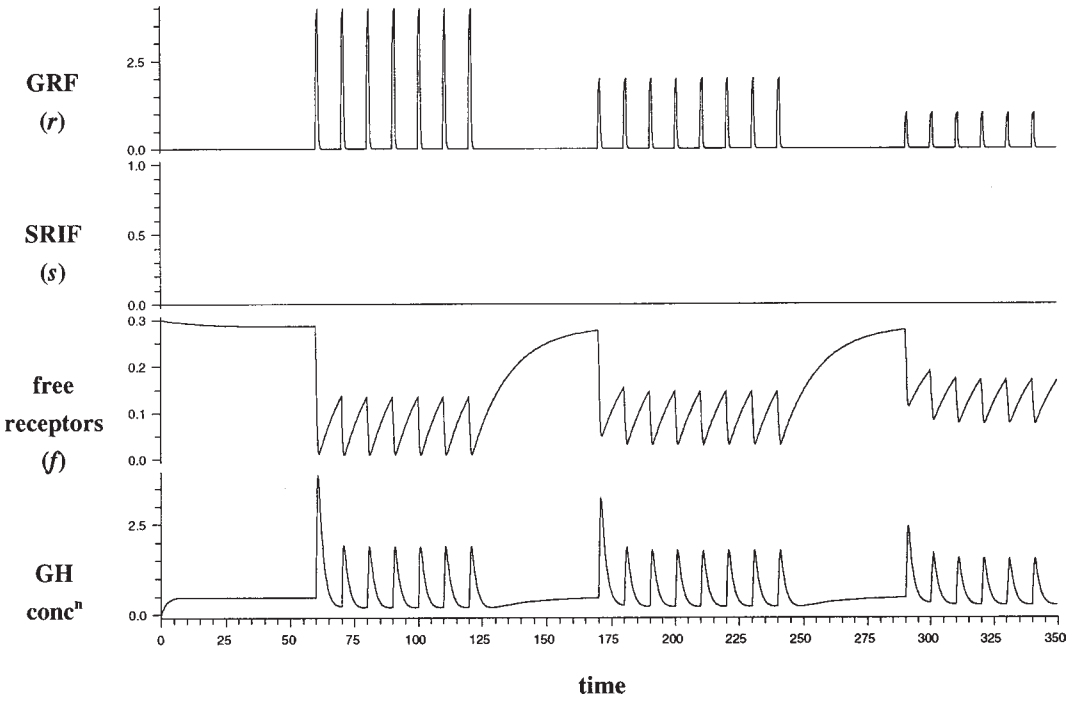

Fig. 3. Model simulation showing concentration-dependent desensitization to pulses of GRF.

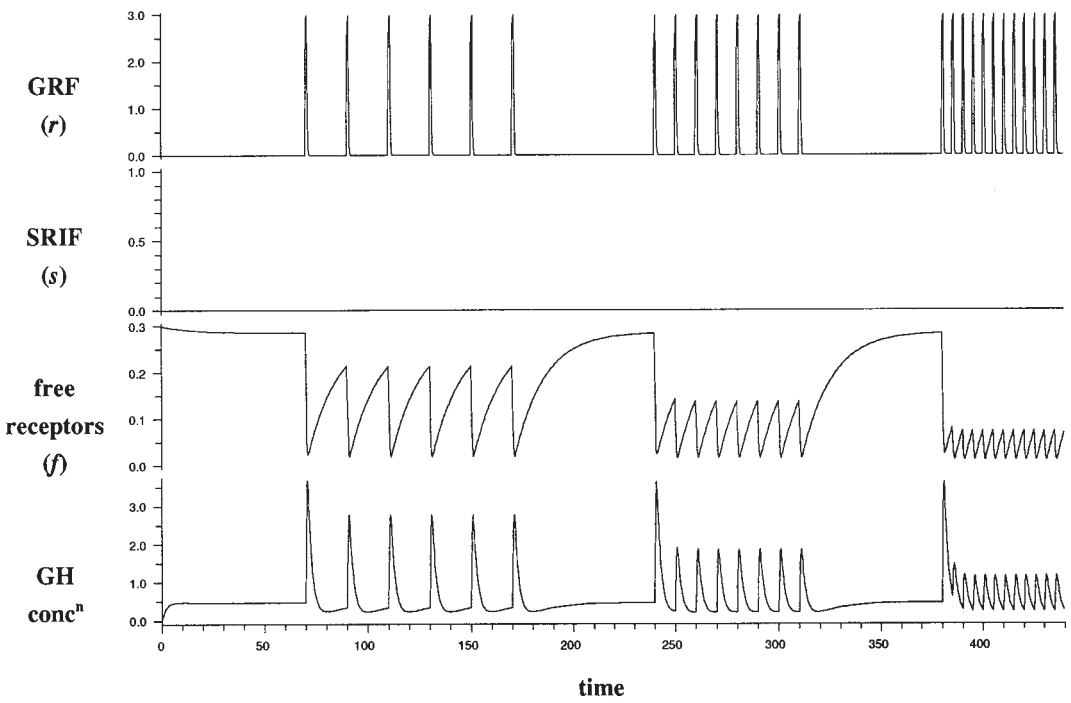

Fig. 4. Model simulation showing interval-dependent desensitization to pulses of GRF.

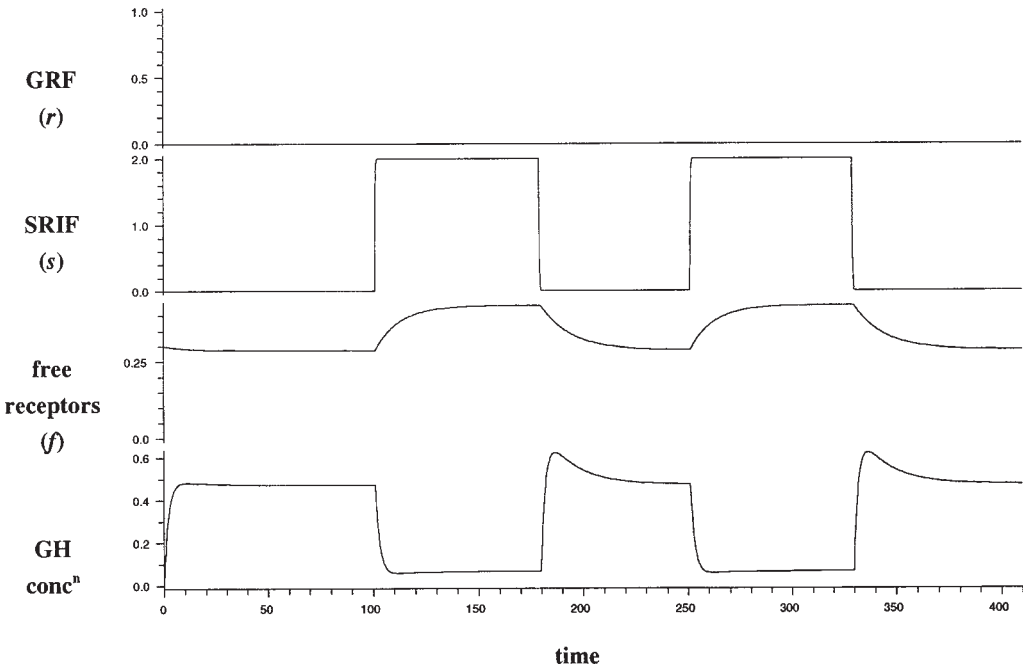

Fig. 5. Model simulation showing rebound after somatostatin application, indicating the presence of constitutive activation of the GH release pathway, which partially desensitizes in the absence of somatostatin, and after application of somatostatin, resensitizes.

FITTING AND EMPIRICAL TESTING OF THE MODEL

There is a very wide range of experimental data, from in vitro and in vivo experiments that could be used to test the model and to estimate parameters not directly measurable. Presented here are the results of such empirical tests for two sets of in vitro data.

Infusions of GHRP

In a perifusion experiment, somatotroph cells were infused with GHRP6 at a constant 100 n*M* concentration and GH concentrations measured in the peritusate as displayed in Fig. 6. By substituting for f in the expression given for ρ, and assuming the initial concentration of free receptors is f_0, the following equation is obtained

$$\rho(r,s,f) = k_r(r + r_0)[f^* + (f_0 - f^*)e^{-Kt}] = A + Be^{-Kt} \tag{10}$$

The amount of GH, h, in the perifusate is given by the differential equation

$$dh/dt = \rho - \lambda h \tag{11}$$

where λ is the perifusion rate. Solving this differential equation the following result is obtained

$$h = \frac{A}{\lambda} + \frac{B}{\lambda - K}e^{-Kt} - \left(\frac{A}{\lambda} + \frac{B}{\lambda - K}\right)e^{-\lambda t} \tag{12}$$

This nonlinear model was fitted by least squares; the estimated curve is plotted in Fig. 6. Also plotted is the release rate of GH from the somatotrophs that results in this fitted curve.

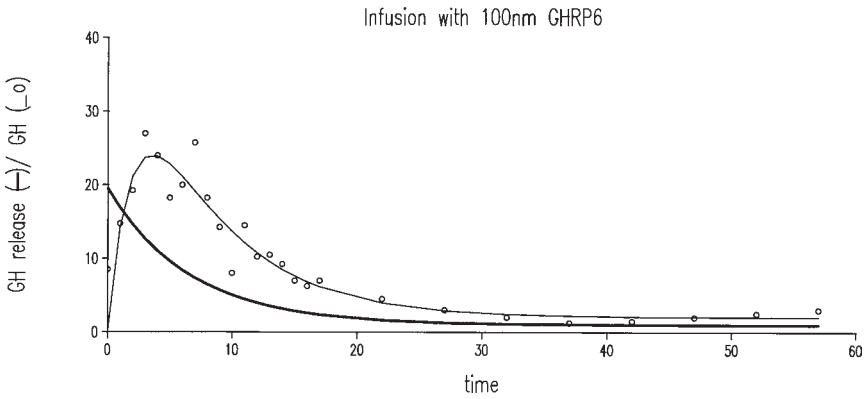

Fig. 6. Using the model to interpret real data. The open symbols show measured GH release from isolated, dispersed somatotrophs in vitro during an infusion with 100 nm GHRP6 for 60 min (from time 0). The model assumes that desensitization is a smooth, continuous process, directly associated with the process of receptor activation that leads to GH release, but this is not readily apparent from the observed data. Indeed the measured data at first sight appear consistent with the interpretation that there are successive phases of sensitization and desensitization, or with the interpretation that there is a lag before the onset of desensitization. The authors have used the model described in the text to generate the best model fit to the observed data, and this fit is indicated by the thin line. The thick line denotes the rate of release of GH, which would result in the fitted GH concentration curve, confirming that the observed data are indeed consistent with rapid, smooth desensitization of the GH release mechanism.

This fitted curve only fixes A, B, K, and λ. The estimates and their standard errors are $A = 0.98$ (se 0.59), $B = 18.5$ (se 3.5), $K = 0.15$ (se 0.05), and $\lambda = 0.49$ (se 0.18). From these figures, under certain assumptions, can be estimated k_1, k_b, and k_r (in the absence of SRIF) and equilibrium levels of free receptor. Furthermore, within the limitations of the data, the model fits adequately.

GRF Dose-Response Curves in the Presence and Absence of Somatostatin

Brazeau et al. *(14)* obtained dose-response curves for a range of doses of native hpGRF-44 in the presence of (1) zero SRIF, (2) 1 n*M* SRIF-14, or (3) 1 n*M* SRIF-28 (Fig. 7). It is assumed that the pituitary cells are initially desensitized to constitutive activation (equivalent to a level of GRF of r_0), it can be seen, by applying Eq. (5), that the initial concentration of free receptors is $f_0 = k_b f_T / (k_1 r_0 + k_b)$. Expression (9) can then be rewritten

$$P(r) = \frac{k_r k_b (r + r_0) f_T}{k_0 + k_1 r} \left[t + \left(\frac{1}{k_0} - \frac{1}{k_0 + k_1 r} \right) \left(1 - e^{-(K_0 + k_1 r)t} \right) \right] \tag{13}$$

where $K_o = k_1 r_0 + k_b$. This was fitted by nonlinear least squares to these three sets of data allowing different SRIF concentration-dependent parameters, k_r, k_b, but all remaining parameters the same, resulting in the fitted curves also plotted on Fig. 7. Where the true value of a parameter is close to zero it sometimes happens that the least squares estimate for a specific data set is negative and this happened in this case for k_b at zero dose of SRIF. In order for other parameters dependent on it to make sense it was necessary to constrain the estimate of k_b to be positive. However, the fit changed very little as did the other parameters except k_r for zero SRIF (which was inversely proportional to k_b) for various

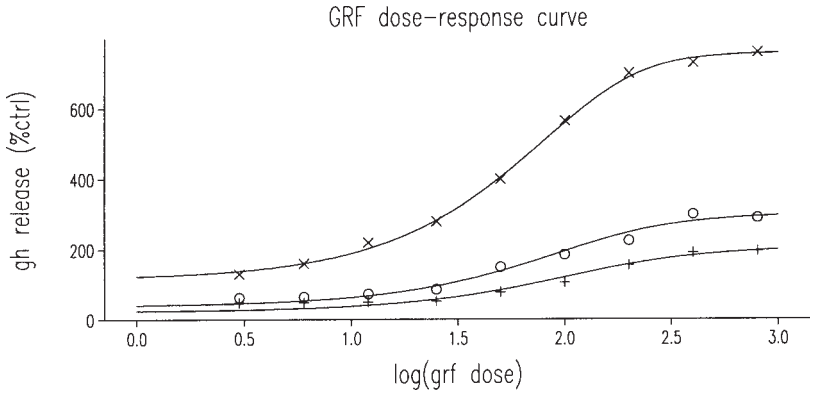

Fig. 7. Scaling the model parameters to real data. To use the model for quantitative rather than qualitative predictions it is necessary to set the parameters of the model appropriately, and then to show that the model thus adjusted provides a good predictor of data collected independently in comparable conditions. The symbols indicate measured GRF dose-response curves for GH release from isolated somatotrophs, using data taken from ref. *14*. GH released (as percentage of control) is plotted against $\log_{10}$ (hpGRF-44 dose) when given alone (symbol X), with 1 n*M* SS-14 (O), and with 1 n*M* SS-28 (+). The curves show fits of the model to these data; best fits have been found using nonlinear least squares (*see* Eq. 13).

lower limits to k_b close to zero. The estimates were as follows: zero SRIF, $k_b = 10^{-3}$, $k_r = 1.53 \times 10^3$; 1 n*M* SRIF-14, $k_b = 0.25$, $k_r = 4.9$; 1 n*M* SRIF-28, $k_b = 0.45$, $k_r = 2.4$. The common estimates of k_l and r_0 for the three sets were $k_l = 0.013$, $r_0 = 13.6$. Again the model predictions fitted well and the parameters fitted separately for each set show the orderings expected a priori.

DISCUSSION

Predicting GRF/GHRP from GH Output

Given the knowledge of the state of desensitization of the somatotrophs (indicated by f_0) at the beginning of the experiment, and the somatostatin levels (assumed constant for present purposes at s_0) throughout the experiment, Eq. (10) can in principle be used to determine the GRF/secretagogue stimulation, which would produce any given pattern of GH output as follows (assuming only one stimulating compound is present). GH released in interval $(t, t + \Delta t)$ is approximately given by

$$H(t,t+\Delta t) = \int_t^{t+\Delta t} \rho(r,s,f)dt = \int_t^{t+\Delta t} [k_4 + k_5(1 - \phi[s_0])](r + r_0)[f^* + (f_0 - f^*)e^{-Kt}]dt$$

$$= [k_4 + k_5(1 - \phi[s_0])](r + r_0)(f_0 - f^*)e^{-Kt}(1 - e^{-K\Delta t}) / K$$

If Δt is sufficiently small for r to be assumed approximately constant during the interval, and if the other parameters are known for the particular application, then this equation can be solved for r, and Eq. (7) used to obtain the change in f. The process can be repeated (making appropriate adjustments to f_0 each time) for each subsequent Δt. In this way the temporal profile of GRF can be built up sequentially. This simple estimation method is dependent on the parameters determining the somatotroph resensitization rate being

known quite precisely. Otherwise, errors would accumulate because of f_0 being updated incorrectly at each stage.

Value of Simplicity in a Pituitary Model

Much more complex models of this system could be constructed, and for some stages, many detailed models already exist. For example, there are a number of models describing intracellular calcium fluctuations in response to agonists applied in the extracellular environment *(15,16)*. Models are also available of the exocytosis of hormone or neurotransmitter in response to a rise in intracellular calcium *(17)*. Therefore, it would be possible, to assemble these models into an overall model describing somatotroph growth hormone output as a function of GRF or artificial secretagogue stimulation. This would still leave the inhibitory input of SRIF and its interaction with GRF to be included. However, the resulting complete model would be very complex. Goldbeter *(18)* discusses a four differential equation model for the response of pituitary cells to luteinizing hormone-releasing hormone, which is essentially a more complex version of the authors' model here (without any somatostatin terms). The extra variables are desensitized releasing hormone receptors in their free and bound states. The authors began their modeling studies assuming that at least three differential equations (including a desensitized bound receptor state) would be necessary, but found that just two closely related differential equations (in f and u) were all that was needed to explain the experimental behavior. Somatostatin appears to exert its effect on the system only when present, and apart from its resensitizing effect on the receptors, little residual memory of its presence remains. Therefore, no further differential equations are needed to cater for its effects.

As well as its likely greater robustness, the particular value of simplicity in our present model is threefold. First, it is a relatively simple matter to determine the model's properties requiring only straightforward analytical techniques. Second, the model can be much more easily tested against experimental data and parameters estimated. Finally, as indicated in the previous section, it can be used to infer the pattern of input of GRF/GHRP from observations of the pattern of growth hormone output from the pituitary if the somatostatin concentrations are known, or to infer the input patterns of GRF and somatostatin if neither are known, but the nature of their interdependence is.

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9

Activation of the Human Growth Hormone Receptor

Structure and Function of the Ligand-Receptor Complex

Kenneth H. Pearce, PhD and James A. Wells, PhD

Contents

INTRODUCTION

The hematopoietic family of cytokines and receptors, to which human growth hormone (hGH) and its receptor (hGHR) belong, are a set of hormone-receptor pairs that are classified on the basis of having similar three-dimensional topologies, as well as similar functional characteristics *(1–3)*. The complex between hGH and the hGHR is one of the best understood among the ligand-receptor pairs of the hematopoietic cytokine family (for other reviews *see* refs. *4* and *5*). A number of the ligands in this family (e.g, hGH, prolactin, erythropoietin, thrombopoietin) transduce signals through their receptors via a sequential dimerization mechanism. In this process, a single hormone molecule can bind to the extracellular domain of two receptors. Here, the manner by which hGH recognizes and binds to its receptor and the molecular aspects whereby hGH transduces its cell proliferative signal through the membrane are reviewed in more detail.

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STRUCTURAL CHARACTERISTICS OF THE HORMONE AND ITS RECEPTOR

Structure of Human Growth Hormone

Although little sequence identity exists among members of the hematopoietic cytokine family, most have either been shown or predicted to be four-helical bundles *(3)*. The four-helix bundle structural motif was first described with the crystal structure for the porcine growth hormone *(6)*. In the case of hGH, the four-helical bundle topology was first observed in the crystal structure of the hGH-hGHR complex *(7)*. Hormones belonging to this group can be subdivided into two general classes called short-chain and long-chain. hGH is classified as a long-chain helical cytokine because of the length of each of the helices (between 21 and 30 amino acids). All members of this cytokine group are characterized by an antiparallel up-up-down-down arrangement of the helices. This helical organization requires rather long extended loops between helices 1 and 2 and helices 3 and 4 (Fig. 1). In hGH, helices 1 and 4 are longer (26 and 30 residues) than helices 2 and 3 (21 and 23 residues).

Overall, the four-helical bundle topology of hGH makes for a very compact and stable molecule. Most of the inner core of the four-helix bundle consists of hydrophobic residues. hGH contains two disulfide bonds. One disulfide connects the first crossover loop to helix 4 via a C53 to C165 linkage. The other disulfide bridge (C182 to C189) connects the C-terminus to the end of helix 4. Additionally, hGH contains three prominent, yet short, minihelical segments. Two of these minihelical segments, between residues 38–47 and 64–70 in the connecting segment between helix 1 and 2, play significant roles in receptor binding. In uncomplexed hGH, significant portions of helices 1, 3, and 4 are exposed to solvent *(7,8)*. Most of the residues on helix 2 are either buried in the core or are covered by the helix 3 to helix 4 connecting loop. The importance of each of the solvent exposed helical surfaces in binding the hGHR will become evident with further discussion.

Structure of the Growth Hormone Receptor Extracellular Domain

The hGHR belongs to the hematopoietic receptor superfamily *(1)*; other members of this family include the receptors for prolactin, interleukins-2, -3, -4, -6, and -7, granulocyte-macrophage colony-stimulating factor, erythropoietin, thrombopoietin, and interferons-α, and -γ. Receptor members of this superfamily generally contain a rather large extracellular domain (about 200–400 amino acids), a single transmembrane helix, and an intracellular domain. Even though little overall sequence similarity exists between members of the family, there is very strong conservation of secondary structural elements in an extracellular domain commonly referred to as the cytokine receptor homology (CRH) domain *(1)*. The extracellular part of many of the cytokine receptors contains two CRH domains and other domains of similar topography. However, the extracellular region of the hGHR receptor consists of only a single CRH domain. The nonmembrane anchored hGH-binding protein (hGHbp), which occurs naturally in serum, can be isolated *(9)*. This soluble protein consists of the entire extracellular region and binds to hGH with an affinity nearly identical to that of the full-length receptor expressed on cells *(10,11)*.

For the hGHR, the fold of the extracellular region (about 240 amino acids) has been shown by crystallography to be a two-domain structure (Fig. 2) *(7)*. These two distinct domains of the extracellular region are each classified as a fibronectin type III fold, a fold that is similar to the immunoglobulin constant domain. Each domain consists of seven

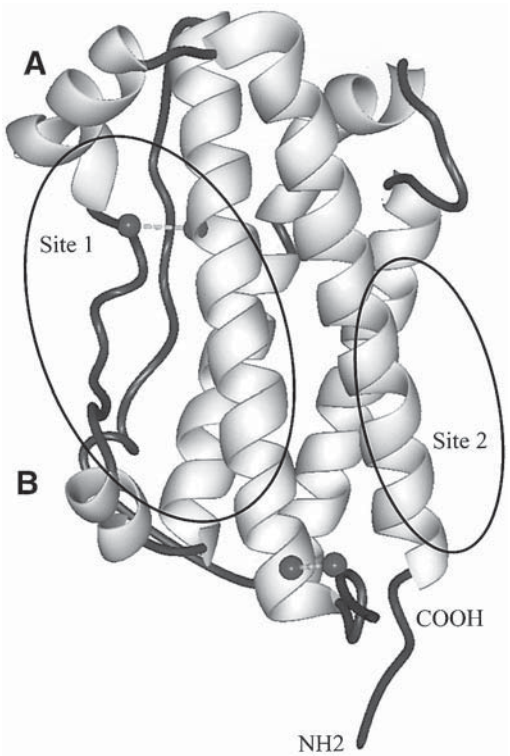

Fig. 1. Ribbon diagram of the structure of hGH. The coordinates used for making this model were taken from ref. *1*. Each of the four helices are labeled. The helices are arranged with an up-up-down-down topology and the two long loops between helices 1–2 and 3–4 are labeled. Two of the three minihelical segments are labeled as minihelices **(A)** and **(B)**. Cysteine residues C53, C165, C182, and C189 are shown as spheres.

antiparallel β-strands that are organized to make two sheets; one sheet consists of three strands and the other sheet contains four strands. The two sheets are arranged to make a β-sandwich. The middle of the sandwich is made of mostly hydrophobic residues that are weakly conserved among other superfamily members. The N-terminal domain of the hGHR contains three cysteine bridges (C38–C48, C83–C94, C108–C122), which are buried between the two β-sheets. The C-terminal domain of the hGHR contains no disulfide bonds. The two domains are connected by a short four-residue linker segment.

Although the structure of the free hGH receptor is unknown, the structures of several ligand-receptor complexes show that the receptor has few intradomain contacts *(7,12)* (M. Ultsch and A. M. de Vos, unpublished results). In these complexes the two fibronectin type III domains are oriented in space approximately perpendicular to one another. This arrangement displays the loops connecting the β-strands to bind hGH in the complex; residues throughout six of these loops govern most of the hormone binding.

Although total sequence similarity is rather low among receptors in this superfamily, there are some prominently conserved amino acids throughout the structure. In the hGHR, these residues include four of the six cysteines in the N-terminal domain, several hydrophobic side-chains, such as W50 and W157, and two consecutive proline residues near the four-amino-acid linker segment. Additionally, proximal to the membrane-spanning

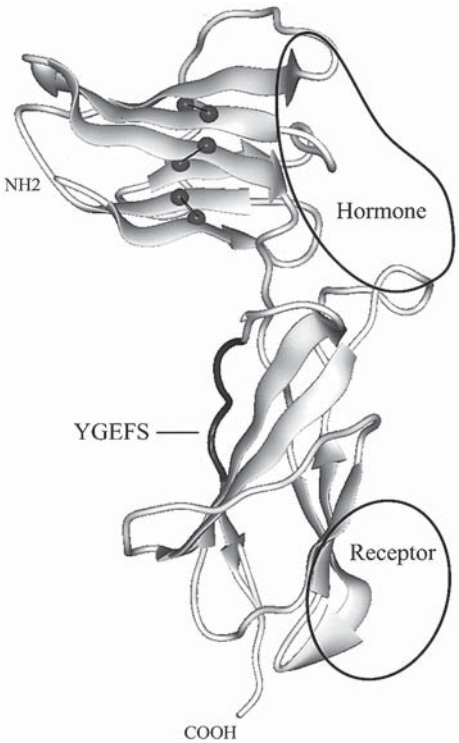

Fig. 2. Ribbon diagram of the structure of the extracellular domain of hGHR. The model is based on X-ray coordinates from ref. *1*. The location of the consensus sequence YDEFS (WSXWS box) is indicated with an arrow. Cysteines in the N-terminal fibronectin type III domain (C38–C48, C83–C94, C108–C122) are shown as spheres.

region, most receptors in this family contain a stretch of amino acids (WXSWS) that may possibly play a structural role. For the hGHR, this sequence is YDEFS and the crystal structure of the hGH-hGHR complex shows that these side-chains are not likely to have an important role in ligand binding *(1)*. The precise functional importance of the WSXWS box remains somewhat of a controversy. In general, all of these conserved residues are likely to be important for maintaining the structural arrangement and global fold of the hGHR (and other receptors in the family), whereas the less conserved loop segments between the β-strands are mostly involved in determining ligand-binding specificity.

BINDING OF HGH TO ITS RECEPTOR AND THE MECHANISM FOR TRANSMEMBRANE SIGNALING BY THE COMPLEX

For many years the mechanism whereby hGH transduces signal via its receptor through the membrane remained a mystery. One of the most crucial and yet surprising findings in this respect was the discovery that one hGH molecule can bind two molecules of the hGHR. Both functional *(13)* and crystallographic *(1,14)* data were used to demonstrate the stoichiometry of binding.

Mutagenesis data in conjunction with titration calorimetry, gel filtration chromatography, and a fluorescence-quenching assay were used to show that one hGH molecule can bind two receptors and that hGH has two distinct receptor-binding sites *(13)*. The stoichi-

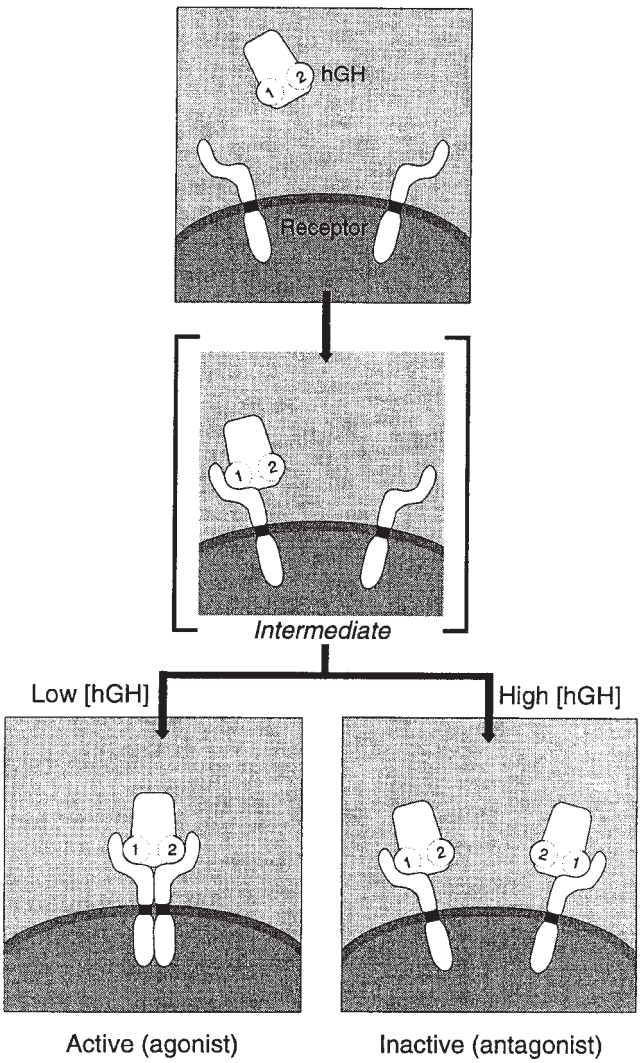

Fig. 3. Sequential dimerization mechanism for the binding of hGH to its cell surface receptor (taken from ref. *15* with permission). hGH has two receptor-binding sites labeled as Site 1 and Site 2. At low concentrations (<1 n*M*), hGH first binds to a receptor through its high-affinity site (Site 1). Then, through a two-dimensional diffusion process, this 1:1 complex binds to a second free receptor. This 1:2 complex triggers signal transduction. At very high concentrations of hGH, receptors can be saturated in 1:1 complexes and self-antagonism occurs.

ometry of binding was further supported by analysis of components found in crystals of hGH and the extracellular domain of hGHR *(14)*. The two receptor-binding sites on hGH are referred to as Site 1 and Site 2. Through the use of mutagenesis data it was demonstrated that receptor binding occurs sequentially (Fig. 3) *(13,15)*; hGH first associates with an hGHR to form a 1:1 complex through Site 1, the high affinity site. This complex is then capable of binding a second receptor through Site 2 of hGH. Significant hGHR-hGHR contacts also contribute to the stability of the ternary complex. No evidence exists for the formation of a 1:1 complex through Site 2.

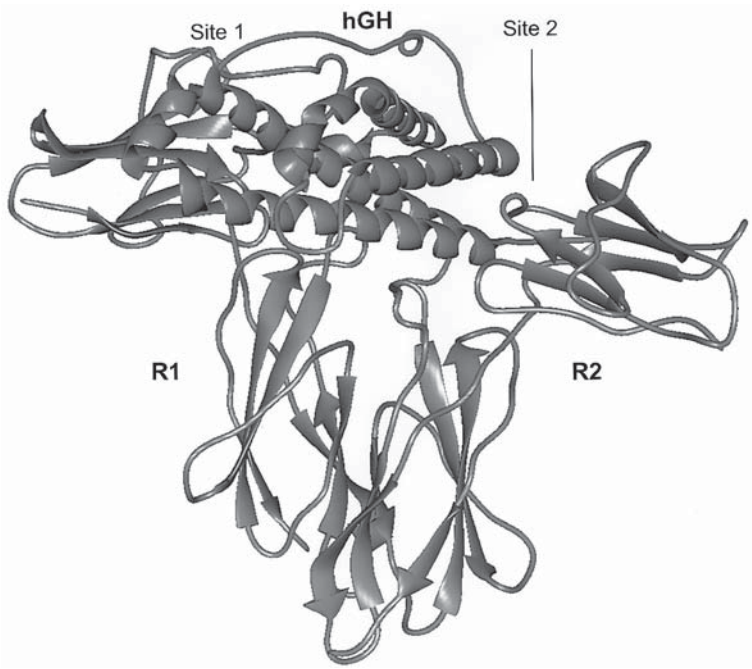

Fig. 4. The crystal structure of the 1:2 complex of hGH with its receptor (modified from ref. *1*). The two receptor binding sites on hGH are shown as shaded areas and are labeled as Site 1 and Site 2. Approximately 1300 $Å^2$ of hGH is buried at the Site 1 receptor interface, whereas only 900 $Å^2$ of hGH is buried between the hormone and receptor at Site 2. Additionally, the contact surface between the C-terminal domains of each receptor is shown. This receptor–receptor interface constitutes about 500 $Å^2$ of buried surface area at Site 2.

This mechanism of action is further supported by the crystal structure of the 1:2 complex of hGH and the extracellular domain of its receptor (Fig. 4) *(1)*. Site 1 on hGH has a much larger receptor-binding site than Site 2. Approximately 1300$Å^2$ of hGH surface area is buried at the Site 1 interface whereas the interface between the hormone and receptor at Site 2 consists of only about 900$Å^2$. Furthermore, the crystal structure shows that about 500$Å^2$ of area is buried at the interface between the C-terminal domains of the receptors. The relative sizes of these interfaces helps to rationalize the sequential dimerization mechanism. Because the entire Site 2 interface consists of both hGH-hGHR and hGHR-hGHR contacts, binding at Site 1 is likely a prerequisite for formation of the 1:2 complex. In addition, the affinity between the two soluble extracellular domains in the absence of hGH is too weak to be measured *(13,16)*.

Little evidence exists for a large conformational change in either hGH or the hGHR on association at Site 1. The structure of an unbound mutant hGH shows essentially the same global structure as the receptor-bound wild-type hormone *(8)*. Some minor structural changes have been seen in one of the minihelices in a recent structure of the free hormone *(17)*. The structure of the free receptor has not been solved yet; however, little structural change is seen in the bound receptors. The structure of the 1:1 complex overlaps very well with that of the 1:2 complex *(17a)*. Additionally, the two receptors in the 1:2 complex superimpose with each other, including the relative orientation of the N-terminal and C-terminal domains.

The hGH-binding site on the receptor consists of residues from both the N-terminal and C-terminal domains around the hinge region. The first 30 amino acids of the N-terminal domain are not visible in the 1:2 structure and are not important for hGH-binding *(17a)*. The residues on the receptor that make contact with hGH are predominantly found in the loops that are near the two-domain linker segment. Interestingly, nearly the identical set of residues on each receptor are used to bind two very distinct sets of residues on hGH (Site 1 and Site 2). This demonstrates the remarkable recognition flexibility designed into the hormone-binding site of the receptor.

Generally, the residues on hGH that are important for Site 1 binding reside on the two minihelices between helix 1 and helix 2, and along the solvent exposed face of helix 4. This binding site on hGH is slightly concave *(1)*. Side-chains on Site 2 of hGH that are important for interaction with the hGHR reside on the N-terminal region on one side of helix 1, and along the exposed face of helix 3. Compared to Site 1 on hGH, the second receptor-binding site is relatively flat. Interestingly, the hGH residues that are functionally important in Site 1 are completely different in nature than those involved at Site 2 *(13)*.

Many experimental studies support the notion that the 1:2 complex is the transmembrane signaling species. First, some anti-hGHR monoclonal antibodies (MAbs) are capable of acting as agonists for signal transduction in a cell-based assay *(18)*. Second, chimeric receptors consisting of the extracellular region of the hGHR and the transmembrane/intracellular domains of the granulocyte colony-stimulating factor receptor (G-CSFR) are fully capable of transmitting a G-CSF-like signal in response to hGH *(15,19)*. These studies support the model that receptor dimerization at the extracellular domain simply serves the purpose of bringing intracellular domains within close proximity. Furthermore, one particular site-directed mutant (G120R) that destroys Site 2 binding, but does not interfere with Site 1 binding, does not confer growth-promoting activity *(15,20,21)*. This Site 2 mutant can also act as an hGH antagonist by locking up receptors in 1:1 complexes *(15,22)*. Similarly, when very high concentrations of hGH are used in a cell-based assay, self-antagonism is observed *(18,23)*. This demonstrates that when hGH receptors are fully occupied in 1:1 complexes at Site 1, no signal can be transmitted.

MUTATIONAL ANALYSIS OF THE HORMONE-RECEPTOR INTERACTION

Alanine-Scanning Mutagenesis of hGH

One of the key techniques used for deciphering the mechanism of action of hGH was a high-resolution analysis of the binding event by alanine-scanning mutagenesis *(13,24,25)*. Initially, single mutations were made at all side-chains (62 in total) between residues 2–19, 54–74, and 167–191. Binding analysis of each of these hGH mutants showed that only about 12 residues gave a fourfold lower binding affinity for the hGHR compared to wild-type hGH. When a similar set of mutants was tested for the ability to dimerize the hGHR using a fluorescence-quenching assay, one subset of mutations affected binding of the 1:1 complex (Site 1), whereas a different set of mutations had no effect on 1:1 binding, but did have an effect on receptor dimerization (Site 2).

To further investigate the role of individual residues in the 1:1 hGH-hGHR binding event, equilibrium and kinetic data were obtained using surface plasmon resonance on a Pharmacia BIAcore device *(25)*. To study Site 1 exclusively, a receptor mutant (S237C) was coupled to the sensor chip in a manner that prevents dimerization on hGH

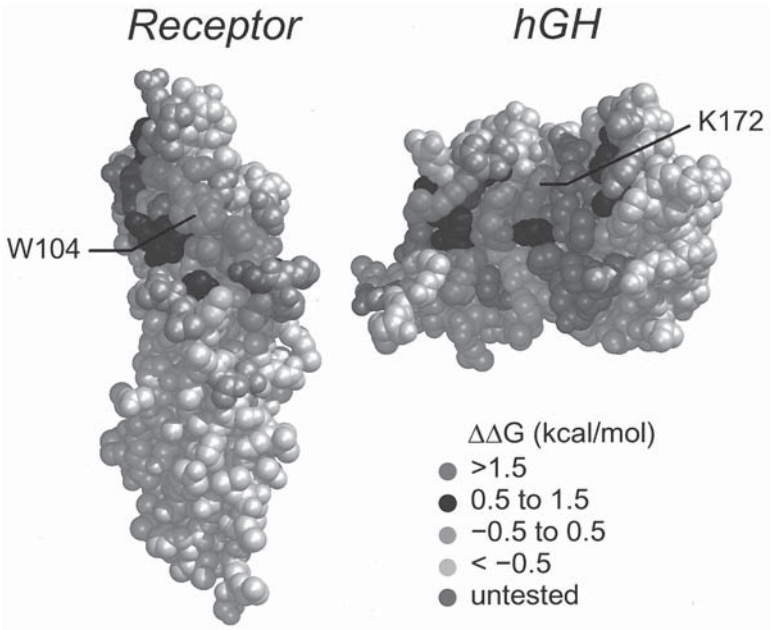

Fig. 5. Space filling model of the structure of the 1:1 complex of hGH and the hGHR. (Figure reproduced from ref. *27* with permission; see ref. *27* for color figure.) The two molecules are separated to show the energetic contribution to binding of individual residues. hGH is shown with residues at the Site 1 interface shaded in darker gray. One of the more important residues, K172, is labeled. The hGHR interface is shown with all Site 1 contact residues shaded in darker gray. W104, which is labeled, is one of the two most important residues on hGHR for binding hGH.

binding. Using this technique, dissociation and association rates ($\approx 2 \times 10^{-3}$ s^{-1} and $\approx 3 \times 10^{5}$ $M^{-1}s^{-1}$, respectively for wild-type hGH) were determined for alanine mutations at all residues in the receptor interface. This study showed that only about 25% of the residues on hGH, which are known from the crystal structure to be in contact with the hGHR, are actually important for binding (Fig. 5). These residues (predominantly L45, R64, K172, T175, F176, and R178) cluster at the center of the receptor contact interface or the "structural epitope;" collectively, these energetically important residues are referred to as the "functional epitope." Mostly, mutations at these residues cause a faster dissociation rate, which means that these residues function to stabilize the bound complex by slowing hGH dissociation.

Because many of the affinity-inert contact residues are polar or charged, it is possible that these side-chains are important for maintaining specificity and solubility. Interestingly, mutation of some of these affinity-inert residues has been shown to have large yet compensating effects on the enthalpy and entropy of binding *(12)*.

Alanine-Scanning Mutagenesis of the hGHR

Based on fluorescence-quenching experiments, it was originally speculated that a tryptophan residue in the extracellular domain of hGHR was involved in hGH binding *(26)*. Additionally, mutation of W104 resulted in a very large decrease in binding affinity for hGH (about 2500-fold) *(26)*. Further insight into the importance of W104 was gained with the crystal structure of the hGH-hGHR complex *(1)*. In Site 1, this residue is highly

buried at the core of the hGH-hGHR interface and makes van der Waals contact with several residues of hGH (K168, T175, and K172). A thorough alanine-scanning investigation of all of the Site 1 contact residues (30 side-chains) showed that a hydrophobic cluster involving W104 and W169 accounts for most of the free energy of binding (Fig. 5) *(27)*. Importantly, it was also shown that the functionally significant residues on the hGHR are in direct contact with those on hGH.

The receptor also contains a centralized "functional epitope" surrounded by a number of hydrophilic residues that are generally less important for binding. Once again, it has been suggested that the nonfunctional, charged residues surrounding the very hydrophobic binding site are probably responsible for promoting solubility and specificity *(27)*. In fact, it has been demonstrated that a single arginine residue (R43) in the extracellular domain of the hGHR contributes significantly to species specificity for growth hormone binding *(28)*. Taken together, both of these mutational studies (on hGH and hGHR) clearly demonstrate that only a small subset of contact residues are important for modulating the energetics of binding.

INTRACELLULAR EVENTS TRANSMITTED FOLLOWING FORMATION OF THE HGH(HGHR)$_2$ COMPLEX

In contrast to the detailed understanding of the extracellular binding events, relatively little is understood about intracellular processes following receptor dimerization. One of the key issues yet to be addressed concerns how receptor dimerization changes the structure of the intracellular domain so that it is capable of binding protein kinases and/or transcriptional elements.

The cytoplasmic region of the hGHR is 350 residues in length; presently, little is known about the structural organization of this domain. Very little overall homology is found between intracellular domains for receptors in the hematopoietic superfamily. Since no structural information is available, it remains to be seen whether common folding motifs exist in the intracellular domains of these receptors. However, most receptors in the extended family do have a proline-rich segment (box 1; residues 276–286 in hGHR) found about 10 amino acids from the transmembrane-spanning helix *(29,30)* and another short conserved stretch of amino acids (box 2; residues 325–339 in hGHR) further in sequence from the membrane *(31)* (Fig. 6). The intracellular domain of the hGHR shows no sequence homology to known protein kinase domains, however, intracellular tyrosine kinase activity associates noncovalently with ligand binding and receptor dimerization *(32,33)*.

A number of studies using various cell lines have shown that hGH stimulates tyrosine phosphorylation of the hGHR intracellular domain, the JAK2 intracellular protein kinase, and several STAT proteins *(34–36)*. It has also been demonstrated that JAK2 directly interacts with the hGHR *(37)*. Following hGH-induced receptor dimerization, JAK2 rapidly phosphorylates itself as well as specific tyrosine residues on the receptor and on STAT (Fig. 6). STATs 1, 3, and 5 have all been implicated in the growth hormone signaling pathway *(38,39)*. Even though the intracellular domain of the hGHR is also phosphorylated, mutation of the phosphotyrosine residues does not affect proliferative signaling or JAK2/STAT activation *(40)*.

The molecular basis for the activation process and the interaction between the intracellular domain and downstream signaling molecules, such as JAK2 and STAT, is pres-

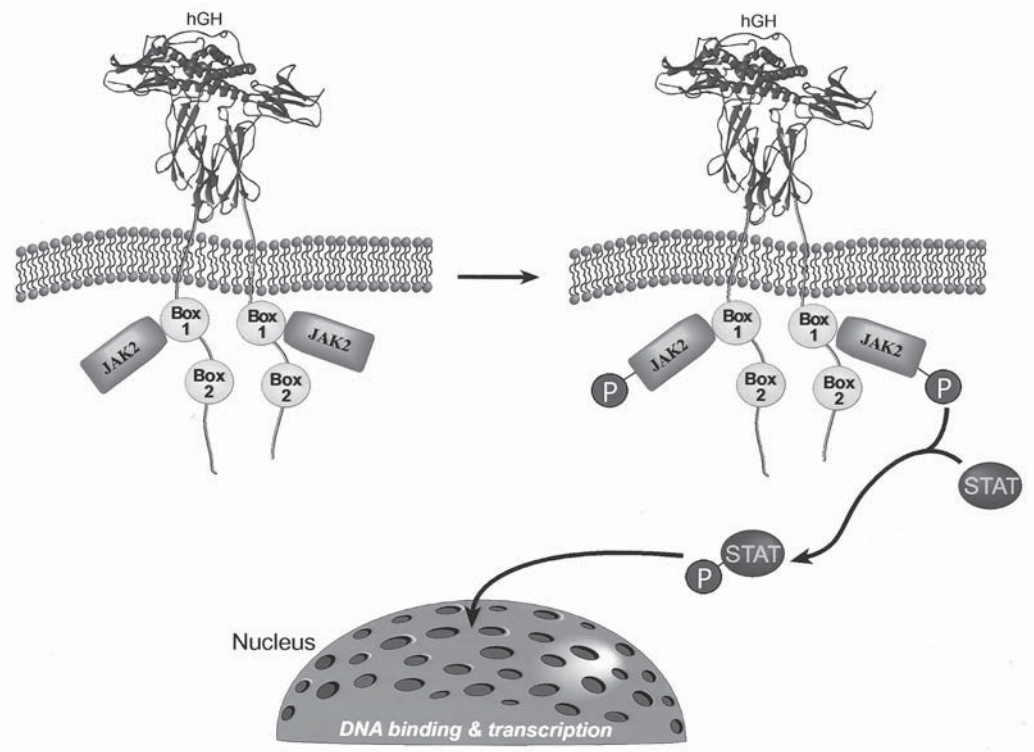

Fig. 6. Model for the activation of cytosolic components following hGH-induced receptor dimerization. Shown in the intracellular domain of the two hGHRs are the location of the consensus sequences referred to as box 1 and box 2. This model shows the association and activation of the JAK2 tyrosine kinase following receptor dimerization *(37)*. This complex in turn can activate via phosphorylation the DNA transcription protein, STAT (p91). The phosphorylated and dimerized STAT protein translocates to the nucleus and binds to DNA elements that are responsible for gene transcription

ently not well understood. A limited number of mutagenesis and functional studies have revealed only a fraction of the information required for complete description of the activation process. First, it has been shown that both a proliferative response and JAK2/STAT phosphorylation can be induced by hGH on a truncated version of the hGHR, which contains only the first 54 residues of the intracellular domain *(31,41)*. In the intracellular domain of the hGHR, a short stretch of approx 25 amino acids adjacent to the membrane is absolutely required for hGH-induced activity *(42)*. This region contains the box 1 consensus sequence. Mutation of proline residues and a lysine within or near the conserved box 1 region causes loss of hGH-induced responses *(41–43)*. Of course, improved understanding of the roles of individual residues in the hGHR intracellular domain will come with structures of relevant molecular complexes.

CONCLUSIONS

The information potential about hormone action that can be obtained from the combined use of functional and structural studies is enormous. Significant progress has been made toward understanding the binding events and signal transduction mechanism of

hGH through its cell-surface receptor. It has been shown that hGH can dimerize its receptor and that this process is a sequential one. hGH first binds a single receptor molecule in a 1:1 complex, which in turn creates a binding site for the association of a second receptor. The second receptor makes molecular contacts with both hGH and the first bound receptor. For the 1:1 complex, the observation that the energetics of binding are governed by only a few centralized contact residues suggests that the design of small molecule agonists or antagonists may be possible. Such molecules could essentially mimic binding at a large protein–protein interface, but in the context of a compact small molecule.

Obviously, a key step in understanding hormonal molecular recognition is obtaining a three-dimensional structure of hormone-receptor complexes. Such information is crucial for guiding mutagenesis studies and, furthermore, for construction of second generation molecules through the use of phage display *(44)*.

The gap in our understanding between extracellular events and cell activation, growth, and differentiation will undoubtedly be shortened in the years to come. Even though great progress has been made toward identifying the cytosolic ligands for the activated hGHR, information regarding the specific molecular complexes formed after receptor dimerization is lacking. The precise manner whereby ligand-induced dimerization on the outside of the cell stabilizes the interaction between receptor intracellular domains and cytosolic kinases remains to be understood. In all, understanding the molecular detail of such signaling pathways, from extracellular to intracellular events, is of critical importance for deciphering how cells communicate, proliferate, and differentiate. Molecular-level understanding of these events can potentially allow for the design of clinically relevant agonists and antagonists.

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10 The Central Nervous System as a Direct Target for Growth Hormone Action

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Contents

INTRODUCTION

To stimulate postnatal body growth in a coordinated fashion, growth hormone (GH) acts directly or indirectly on virtually every tissue in the body. Furthermore, GH has metabolic actions that are important in many species long after major statural growth has been accomplished. Although the actions of GH were long thought to be mediated entirely via the generation of hepatic insulin-like growth factor-1 (IGF-1), it is now clear that GH

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also has direct effects in many tissues acting in concert with locally generated IGF-1 (and probably many other growth factors) in addition to IGF-1 from the circulation. Although the brain is not usually considered an obvious target tissue for GH there is increasing circumstantial and direct evidence to support this idea. In this short chapter the authors address three principle questions:

1. Are there specific functional receptors for GH in the central nervous system (CNS)?
2. How does GH reach these receptors in adequate amounts? and
3. Do these receptors mediate physiological effects of GH within the CNS?

Some data from new experimental approaches that have recently been used to address some of these issues will be reviewed.

GROWTH HORMONE RECEPTORS IN THE CNS

Early studies with radiolabeled GH suggested the presence of specific binding sites in CNS, but these were of low abundance and principally identified in hypothalamic regions and choroid plexus although a much wider distribution has been claimed *(1)*. These studies have not been universally confirmed and are fraught with technical difficulties. A major concern is the specificity of binding because homologous ligands have rarely been used. For example, human GH (hGH) often gives the strongest binding in rat tissues, but its specificity is unclear since hGH also binds to and activates several rodent prolactin receptors. Much the same problems faced the demonstration of GH receptors (GHRs) in peripheral tissues with the exception of the liver. High resolution autoradiography has recently been used to confirm such sites in choroid plexus *(2,3)* and iodinated homologous ligands have been used in some studies, suggesting that specific GHRs may indeed be present in the CNS.

The successful purification of GHRs made possible the development of specific antibodies to measure GHR proteins *(4)*. These have been useful to demonstrate GHRs in peripheral target tissues where GHRs are of low abundance (e.g., within the epiphyseal growth plate). Immunocytochemistry has also been used to confirm that GHRs are indeed expressed in the brain, particularly in fetal and young animals *(5)*, and show a widespread distribution including regions of the CNS not obviously expressing GHR in later life. Similar immunohistochemical studies demonstrated GHR expression in human fetal brain tissue *(6)*. One potential complication is that GHR can also give rise to a GH binding protein (GHBP) either by proteolytic cleavage of the full-length receptor or as a translation product of an alternatively spliced mRNA in rodents *(7,8)*. Because of this, antibody localization studies employing an epitope directed against the extracellular domain of the GHR will also recognize GHBP moieties able to bind GH, but lacking an intracellular domain able to transduce a signal. This problem can be overcome by development of antibodies directed against intracellular portions of the GHR, or to a peptide sequence present only in the hydrophilic tail of the alternate GHBP splice product in rodents *(8,9)*. More recent methodological advances with enhanced immunocytochemical staining have been applied with specific GHR or GHBP antisera in other areas of low GHR density, such as the rat epiphyseal growth plate, and can also be used to visualize GHRs in the CNS (E. F. Gevers, personal communication). However, the presence of GHBP, rather than full-length GHRs, may not be without function. Similar observations of truncated forms of the leptin receptor in choroid plexus have implicated extracellular domains as possible vectors for carrier-mediated transport of leptin from the circulation to the CNS *(10,11)*,

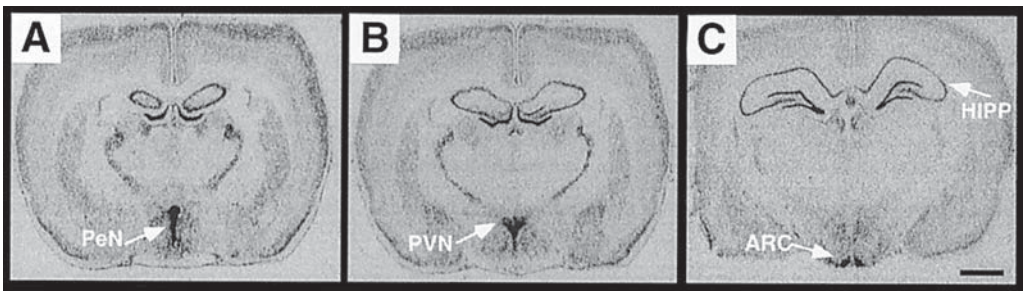

Fig. 1. *In situ* hybridization histochemistry for GH receptor mRNA in coronal brain sections through **(A)** periventricular nucleus, **(B)** paraventricular nucleus, and **(C)** arcuate nucleus. Note the intense specific hybridization signal in the hippocampus in addition to the hypothalamic nuclei indicated by arrows. Scale bar represents 1 mm.

so GHBP-like binding sites in the choroid plexus could conceivably serve a similar function for GH.

The purification of the GHR led rapidly to cloning of the GHR *(12)* and nucleotide probes based on these sequences could then be used to confirm the presence of transcripts coding for GHRs and to determine the sites of expression of the GHR gene in the CNS *(13–15)*. GHR and GHBP gene expression is detectable in the rat CNS throughout life, from as early as embryonic d 15 *(16)*, and in the whole embryo as early as d 12 *(17)*, and perhaps much earlier *(see below)*. It can be concluded that the GHR gene is indeed expressed in the brain and translated into protein. Although it remains necessary to demonstrate the presence of intracellular signaling pathways functionally coupled to GHR activation in sites where GHR protein is located, these are found in most cell types. Therefore, it can reasonably be concluded that functional GHRs are indeed expressed in the CNS in both fetal and adult life.

WHERE ARE GHRS EXPRESSED?

Recent *in situ* hybridization studies have provided information on the specific distribution of GHR gene expression. In most species so far investigated, the main neural sites of expression of GHR in the CNS of adult animals are in the hypothalamus and hippocampus with the majority of studies carried out in the rat (Fig. 1). Within the hypothalamus, there is increasing evidence that GHRs are involved in a short loop feedback regulating GH secretion. Initial studies from Burton et al. *(18)* localized GHR expression to periventricular nucleus (PeN) somatostatin (SRIF) neurons, consistent with a feedback loop increasing SRIF expression and release in the face of high GH expression. GHR transcripts were also found in the arcuate nucleus (ARC) consistent with the idea that GH feedback inhibits growth hormone releasing hormone (GHRH) expression. Although ARC GHR expression is present in a small number of GHRH-containing neurons *(19)*, the majority of ARC GHR-expressing cells also express neuropeptide-Y (NPY) *(20)*. Thus, GH feedback inhibition of GHRH may be indirect, perhaps via changes in activity of NPY cells *(see below)*. Other hypothalamic structures, such as paraventricular nucleus (PVN) also express GHRs, but their function here is less obviously related to the direct neuroendocrine control of the GH axis. Similar considerations apply to extrahypothalamic sites of GHR expression, prominent among which is the hippocampus (Fig. 1). The

function of GHR expression in this region is not so clearly understood, but is consistent with a role for GH in consolidation of memory *(see below)*.

Although *in situ* hybridization is a powerful technique, it is important to recall its limitations. It describes mRNA located in the site of transcription—there is no guarantee that the RNA will always be translated into functional protein, when it will be translated, or where such GHR protein itself is finally located. As a plasma membrane receptor, GHR may be transported considerable distances from the sites of synthesis either to dendrites or to axon terminals. Conversely, its final target may not be the cell surface; some GHR protein may be targeted intracellularly or even to the nuclei of the cells in which it is expressed, perhaps to signal there *(21)*. For these reasons it will be interesting to determine the intracellular location of GHR proteins in specific regions of the CNS at a higher resolution and to evaluate the physiological significance of the observation that, in addition to neuronal cells, some GHRs may be expressed in glial, microvascular, or other nonneural elements in the CNS *(22)*.

A second important problem is the heterogeneity of GHR transcripts in these tissues. Almost all *in situ* hybridization studies have been carried out with probes directed against exons for the coding region of GHRs. However, it has become clear that there are a variety of upstream 5' untranslated regions (UTRs) contributing alternative first exons to both GHR and GHBP transcripts in all species that have been examined. In the liver, these 5' UTRs are regulated and are much more closely correlated with changes in GHR or GHBP protein than probes against the common coding exons that do not distinguish these 5' UTRs *(23,24)*. Little is yet known about the 5' UTRs in brain GHR transcripts other than the fact that the liver-specific rat GHR_1 exon (V2 in the terminology of Domene et al.) is not detectable in brain *(25)*, whereas the 5' UTR identified as V4 by Domene et al. *(25)* is prominently expressed in brain tissue. Mapping the relative distributions of transcripts containing these alternate 5' UTRs in the CNS may give further insights into the regulation of GHR expression in the brain because they may underlie the differential effects of peripheral steroid hormones on brain vs hepatic GHR expression, or the opposite changes in GHR expression in brain and liver seen from fetal life to adulthood.

Some information has recently been reported for the genomic organization of these upstream UTRs in the human *(26)*. Zou et al. *(26)* have found that V1,V4,V7, and V8 UTRs are all located in close proximity, upstream of the hGHR gene and that there may be other UTRs in addition. Practical benefits could flow from further studies of these 5' UTRs in humans. For instance, specific analyses for mutations in the coding exons of the human GHR have identified possible causes of partial GH insensitivity in some short individuals *(27)*. A similar approach, but one that looks for mutations in the upstream UTR exons, may prove equally fruitful to identify deletions or mutations in individual 5' UTRs that could theoretically give rise to tissue-specific GH insensitivity. There is very little information on the genomic arrangements of the 5' UTRs in other species to date.

DEVELOPMENTAL REGULATION OF CNS GHR EXPRESSION

It is now clear that GHR gene expression is strongly developmentally regulated, but in a pattern different from in the liver. GHR expression in the CNS is much more widespread in fetal life, is maximal in early postnatal life, and diminishes in CNS tissue in adulthood (Fig. 2) *(5)* in contrast to many peripheral organs that increase their GHR expression postnatally and maintain a higher postnatal expression than CNS during

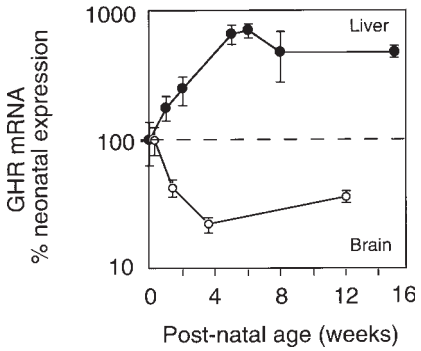

Fig. 2. Ontogeny of GH receptor mRNA expression in the rat liver and brain. RNA levels were quantified by solution hybridization and are redrawn from *(13)* liver, and *(22)* brain, shown here as percentage change from birth. Note the postnatal decline in GH receptor expression in brain compared to its increase in liver. Y-axis values are shown on a log scale.

adulthood *(13,28)*. Therefore, it is reasonable to assume that GHR signals may be involved in early events in tissue proliferation and development because there is evidence for early expression in embryonic stem cells *(29)*. This raises the question of what the source of ligand might be (embryonic vs extraembryonic). GH may also be produced in very early embryos or reach them later from a maternal or placental source, but one may also have to consider the possibility that other GH-related ligands may signal via the embryonic receptor, or even that some form of basal signaling might be constitutive in the absence of ligand and autonomously controlled by varying the level of GHR expression itself.

The functional role of fetal GHR expression is far from clear. It seems unlikely that GHR signaling has a major effect on intrauterine growth since Laron dwarves that lack functional GHRs have relatively minor growth impairments at birth. Furthermore, the IGF-1 link, so well established in later life, seems less obvious during fetal life with nutritional influences of IGF-1 of more importance. Nevertheless, GH secretion is high during fetal life particularly around term. Experimental studies in sheep have shown this to be regulated by hypothalamic control mechanisms, such as GHRH and SRIF, although some GH feedback mechanisms do not seem to be operative in fetal and neonatal sheep *(30)*. Recent multiple sampling studies in premature infants confirm the marked pulsatility of GH secretion several weeks before full term *(31)*. Although some of the GH secreted in neonates may not be intact, biologically active GH, the role of such high GH secretory activity in the face of high GHR expression if the GHRs are not functionally coupled to growth promotion remains unclear. Although the evidence that GH is necessary for statural growth in fetal life is rather unconvincing this does not rule out the possibility that GHRs are coupled to metabolic processes and could be involved with normal tissue proliferation and development in such tissues as skin, gut, and brain. How CNS effects of GH relate to the inability of most CNS neural cells to proliferate after birth is unclear, but the fetal GH/GHR axis has been implicated in the protective responses of the fetus to maternal under nutrition that may have adverse consequences in later life *(32)*. GHR activation may be poorly coupled to IGF-1 generation in the CNS, but that does not mean that all its effects are direct. By analogy, it may be that some effects of GH in the CNS are exerted indirectly via the regulation of one or more of the newly described tissue-specific growth and survival factors for CNS glial and neuronal cells.

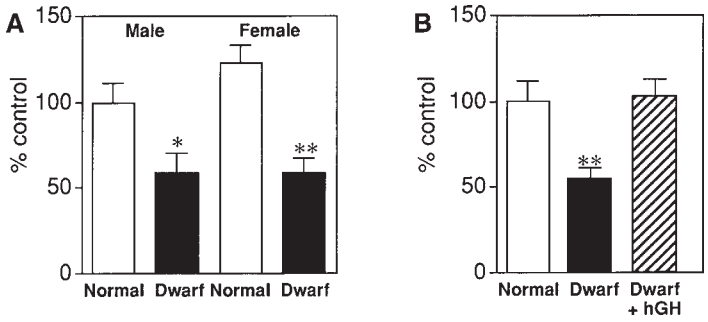

Fig. 3. GH receptor transcripts in the arcuate nucleus of the rat brain in adult age-matched normal and *dw/dw* GH-deficient dwarf rats. GHR expression receptor was identified by *in situ* hybridization using full-length riboprobes. Measurements are expressed as a percentage of normal. **(A)** is a comparison of GH receptor gene expression in normal vs dwarf rats. **(B)** Infusion of hGH at 200 μg/d for 6 d in dwarf rat normalized the reduction in GHR. $*p < 0.05$, $**p < 0.01$ compared to normal. (Redrawn from ref. *40*).

GH/GHR AUTOREGULATION

In peripheral tissues, particularly liver, GH appears to regulate the abundance of its own receptors. Acutely, GH downregulates GH binding sites in the liver *(33)*. Chronic treatment increases hepatic GH binding *(34)*, although this requires continuous rather than intermittent GH replacement *(35)*. It is not clear whether GHRs in the CNS are sensitive to different patterns of GH quite separately from their effects on growth. Differential sensitivity to different aspects of the GH secretory pattern has been clearly shown for hepatic receptors in GH-deficient rats *(36)*, but it is doubtful whether such pattern sensitivities can be maintained to targets that lie behind a blood–brain barrier, which would certainly dampen, if not prevent, CNS exposure to rapid fluctuations in plasma GH. However, conversely, it may be that only the high plasma concentrations attained by secretory pulses of GH will enable sufficient GH to traverse these barriers to stimulate GHRs in some regions of the CNS.

Following hypophysectomy, GHR expression varies in a tissue-specific manner. For example, GHR transcripts have been shown to increase in muscle *(37)*, increase or remain unchanged in liver *(13,37)*, and decrease in the ovary *(38)* and adipose tissue *(37)*. Central GHR expression is also sensitive to regulation by GH. Hypothalamic GHR expression decreases in hypophysectomy or in dwarf rats with specific GH deficiency, and is restored by treating them with exogenous GH (Fig. 3) *(39,40)*.

Most recent measurements have focused on hypothalamic abundance of GHR RNA. The aspect of these studies that always needs to be kept in mind is the mechanism of changing mRNA abundance. The authors and others assume they relate mostly to changes in GHR gene transcription, but particularly in the case of steroid regulation *(41)* they could also reflect changes in stabilization of mRNAs with no change in transcription rate. Although both mechanisms could reasonably be expected to lead to more translation product, this is by no means certain. Even if GH treatment can be shown to be associated with changes in transcription of GHR gene expression, these effects could also be secondary to changes in the activity of SRIF cells in PeN or NPY cells in ARC since the peptide products of these cells are also profoundly regulated by GH feedback. Nevertheless, the authors' working hypothesis is that the autoregulation of GHRs is part of the

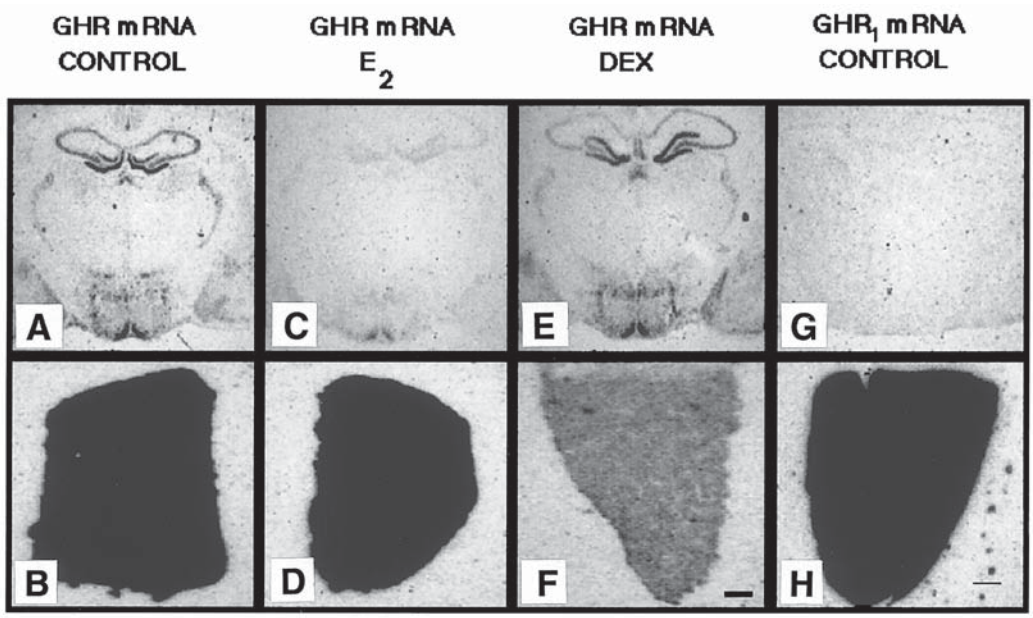

Fig. 4. *In situ* hybridization histochemistry for GH receptor mRNA in brain and liver of normal rats **(A,B)**, rats given estradiol for 14 d, **(C,D)**, or given dexamethasone 14 d **(E,F)**. Note the reduction in signal intensity with estradiol in brain, but not liver, and the reduction in GH receptor with dexamethasone in liver, but not brain. *In situ* hybridization with a probe specific for transcripts containing the GHR_1 5'UTR shows prominent expression in liver **(G)**, but no expression in brain *(H)*. Redrawn from ref. *47*.

autofeedback loop by which GH regulates its own secretion and action, both peripherally and in the CNS *(40,42)*.

GONADAL STEROIDS

In addition to the well-documented sexual dimorphism in GH secretory pattern in the rat, hepatic GH binding is also different between the sexes with females having two- to threefold more binding than males *(43)*. Levels of circulating GHBP are also higher in female than male rats *(24,44,45)* although total GHR mRNA levels are not markedly different between the sexes, within the CNS *(40)*, or in liver *(13)*. Nevertheless, there is evidence that the sexual dimorphism also extends to GH feedback because the pituitary responsiveness to GHRH during hGH infusions is markedly different in males and females *(46)*. The authors have shown that GHR expression in the rat CNS is sensitive to regulation by peripheral steroids and that CNS and hepatic expression of GHR are differentially regulated by the same treatments *(47)*. For example, the induction of hepatic GHR expression by estradiol is known to involve the transcription of an alternative 5' untranslated first exon, GHR_1 *(23)*. GHR_1, although readily detectable in the liver, could not be detected in the CNS (Fig. 4). Estradiol treatment, which stimulates hepatic GHR expression, significantly reduced ARC GHR mRNA levels (Fig. 4). Furthermore, dexamethasone treatment, which profoundly suppressed hepatic GHR transcripts, had no effect on their abundance in the CNS (Fig. 4) (although a decrease has recently been reported *[48]*), with a fourfold higher dose of dexamethasone).

Although this differential effect of dexamethasone could be explained by other factors, such as different glucocorticoid receptor expression in liver and brain, it may also be profitable to reinvestigate the effects of these hormones using 5'-specific probes for the other brain variant UTRs, because these could show a similar differential sensitivity for glucocorticoids as they do for estrogen.

OTHER POTENTIAL REGULATORS

A major regulator of peripheral GHR expression is nutrition, particularly protein malnutrition, which causes a profound fall in GHR expression, GHR binding, and associated GH insensitivity *(49,50)*. A similar situation exists with diabetes *(51)*. So far there is little information about central GHR expression under different planes of nutrition. It may be difficult to interpret studies in the rat in this regard, since this species shows a marked reduction in GH secretion with undernutrition, unlike most other species, which show a rise. The latter might follow from a reduction in central GHR feedback, but could equally well be ascribed to the lack of feedback from IGF-1 under conditions of malnutrition. IGF-1 itself could affect GHR expression, although the effects on peripheral IGF-1 feedback on GH expression are not marked *(52)* unless coadministered with IGF-2 *(53)*.

GHRs are also regulated by thyroid hormones. The growth-promoting effects of exogenously administered GH are blunted in hypothyroid rats, hepatic GH binding sites are reduced in hypothyroidism, and elevated binding is observed in hyperthyroid rats *(54)*. The authors have recently observed reduced GHR mRNA expression in the ARC in response to thyroidectomy, an effect that is rapidly restored by thyroid hormone replacement (P. A. Bennett, unpublished observations).

HOW DOES GROWTH HORMONE GAIN ACCESS TO THE CNS?

The association of changes in exogenous or endogenous GH with altered CNS GHR gene expression does imply a central action of GH. Although the most economical hypothesis is that GH directly regulates itself via its own receptors, it is always difficult to exclude indirect effects of GH, either on whole body growth or on metabolism causing secondary changes via IGF-1. This can be partially overcome by demonstrating that direct administration of GH at doses below those causing peripheral changes still exerts central effects, but this then raises the question of how circulating GH can gain access to brain GHRs.

There is some evidence that GH can enter cerebral spinal fluid (CSF), at least at high secretory rates *(55)*, and that exogenous GH can affect CSF endorphin levels *(56)*. As mentioned earlier, GH might enter the CNS by receptor-mediated or GHBP-mediated transport in the choroid plexus as is suggested for prolactin *(57)*. The choroid plexus has been shown to contain GHR binding sites in humans *(58)* and in the rat, GHR immunoreactivity *(5)* and GHR gene transcripts *(59)* have been identified in this region. Alternatively, GH could reach some areas of the hypothalamus by retrograde transport from the pituitary up the pituitary stalk *(60)*. Studies carried out on the ability of iodinated GH to cross the blood–brain barrier have mainly demonstrated low levels of GH uptake *(61–63)* apart from one earlier study in rat brain *(64)*, although this could change if the blood–brain barrier is compromised, either from disturbances in the pituitary stalk vasculature secondary to tumor growth or to other effects on brain capillary permeability *(65)*.

The possibility of an extrapituitary central source of GH cannot be completely excluded because GH transcripts as well as GH immunoreactivity have been detected in the rat brain *(62,66)*. Although the existence and regulation of such central GH is still controversial, placental expression of GH variants is well established *(67)*, lymphoid derived cells have been shown to produce GH *(68)*, and recent studies also implicate mammary tissue as an alternative source in several species, including human *(69,70)*. Again the presence of GH-like material in the CNS is not widely accepted and may not correspond to authentic pituitary GH, which could explain discrepant results with antibodies of different specificities. Irrespective of its source, the important question in this context is whether GH or GH-like molecules are present at the right sites and in sufficient amounts with the ability to activate or inhibit GHRs; this still remains to be established. On the other hand, the amounts of GH that would need to reach the CNS need not be large. Animal models bearing an hGH transgene with expression in the CNS show suppressed endogenous GHRH and GH expression and dominant dwarfism *(71–73)* with a net decrease in peripheral circulating GH, despite very small amounts of GH locally produced from such transgenes in the CNS.

PHYSIOLOGICAL EFFECTS OF GH IN THE CNS: GH FEEDBACK

The best case for a physiological effect of GH in the CNS is inhibition of its own release, as part of an autofeedback circuit *(74)*. Experiments in chronically cannulated rats (using GHs from other species to permit measurement of endogenous rat GH) have repeatedly confirmed that GH inhibits its own secretion *(75–78)* rapidly blocking spontaneous pulsatility (Fig. 5). Indirect evidence in humans also supports the notion of GH autofeedback *(79,80)*, but it is more difficult to interpret since it is obviously difficult to distinguish exogenous and endogenous GH in humans.

Carlsson et al. used continuous infusions of hGH in conscious rats to compare the effects on spontaneous GH secretion, and found equivalent inhibition in both males and females *(46)*. However, this blockade was readily overcome by GHRH injections in females, but not in males, suggesting that the relative effects on GHRH inhibition and SRIF stimulation might differ in the sexes. These studies did not address the involvement of GH feedback in setting the spontaneous pulse rhythm, but when pulsatile GH was given to conscious male rats, a resetting of the spontaneous pulse generator to the exogenous GH feedback rhythm was evident *(81)*. This could reflect "driving" a regular SRIF rhythm in this experiment. Although this is an artificial situation, it is possible that it indicates an involvement of GH in setting an oscillation in SRIF, and the fall in this sets the timing of subsequent GH pulses. Infusion of hGH blocks the rebound secretion of GH following cessation of SRIF infusion in female rats *(78)* and it would be interesting to see if a "male" type rhythm of GH release could be established in female rats by trains of hGH pulses as it can by SRIF withdrawal.

MECHANISM OF FEEDBACK

Although expression of GHRs can be detected in the pituitary gland *(82)*, there is little evidence in favor of a direct effect of GH to inhibit its own release at the pituitary *(83,84)*, but rather that it acts in the hypothalamus to target the peptidergic systems controlling GH release *(76,85,86)*. This may also have implication for the time-scale of GH feedback. Although acute effects on GH release may be easy to demonstrate, the more physiologi-

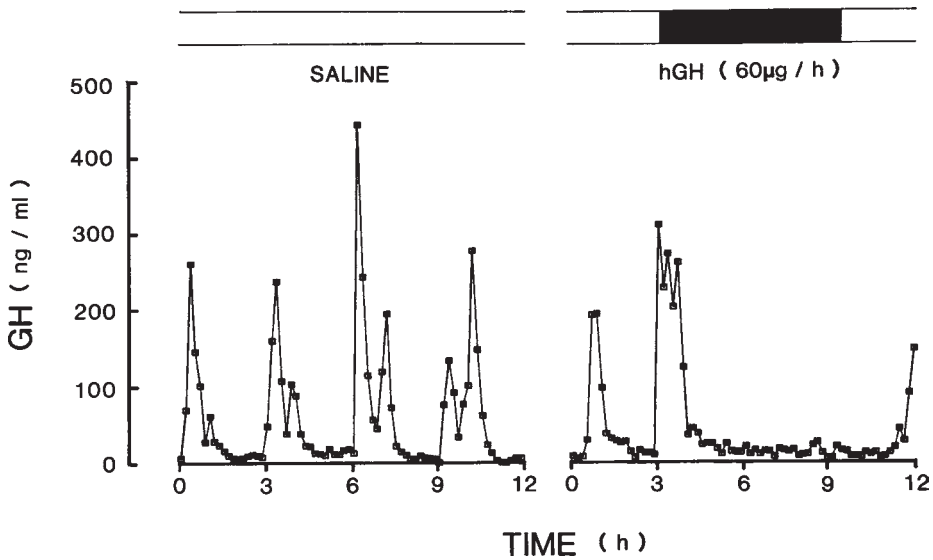

Fig. 5. Effects of iv infusions of human GH on spontaneous GH secretion in a conscious male rat. **(Left)**: saline infusion. **(Right)**: hGH infusion at 60 μg/h for 6 h (dark bar). Serial blood samples were withdrawn automatically and assayed for rGH. From ref. *135*.

cally relevant timescale may be much more prolonged since the GH feedback on pulsatile GH release is maintained with time *(86)*. In the case of GH deficiency, whether partial or total, specific or nonspecific, hypothalamic GHRH expression is increased *(87)*, whereas GH treatment reverses these changes *(40)*. Conversely, hypophysectomy reduces SRIF expression in the hypothalamus, whereas excess GH stimulates hypothalamic SRIF synthesis and release *(76,88)*. Changes in GHRH activity may be responsible for altering the hypothalamic drive to maintain the somatotroph proliferation so that the main physiological role of GH feedback on GHRH is to regulate the GH secretory reserve over a much longer timescale. Since these changes in GHRH and SRIF can be readily observed with GH, but not with IGF-1 alone *(52)*, and both these cells either express GHRs themselves or are in close proximity to cells that do so, it is likely that these provides sites of direct feedback for GH and not secondary to peripheral IGF-1 generation *(86)*, although local generation of IGF-1 in response to GH action in the CNS cannot be ruled out.

Other evidence for an effect of GH is provided by studies showing that *c-Fos* expression in some specific brain regions is also stimulated by exogenous GH *(89–91)*. Although GHRH expression is markedly increased in GH deficiency, this may be mediated indirectly via lack of GH feedback on NPY cells. Chronic administration of NPY icv markedly reduces pituitary and plasma GH, whereas intraventricular injection of NPY antiserum causes a significant increase in plasma GH *(92,93)*. Chan et al. showed that NPY expression is GH sensitive and may be the primary target for GH feedback in the ARC *(94)*. The authors have also found that ARC NPY mRNA is reduced in GH-deficient *dw/dw* dwarf rats, and this deficit is corrected by GH administration *(95)*. Therefore, it is easy to construct a hypothesis whereby GHRs exert negative feedback on GHRH-induced GH release via ARC NPY cells, whereas the effects on SRIF could most simply be explained via direct stimulatory effects on PeN SRIF-positive and GHR-positive cells. However, ARC NPY expression of GHR may not be confined to the control of GH. For

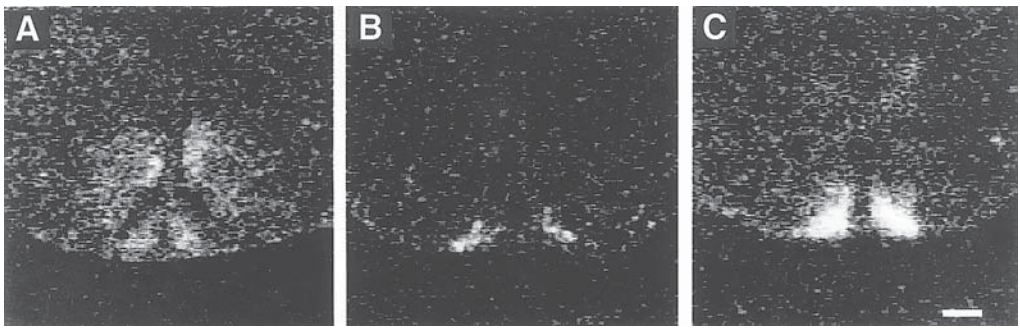

Fig. 6. Distribution of GHS-R, GHRH, and NPY transcripts in *dw/dw* rats. Sections were hybridized for **(A)** GHS-R, **(B)** GHRH, and **(C)** NPY expression and are shown in dark-field. Note the ventral expression of GHS-R in ARC compared to the more lateral expression in GHRH, and the lack of NPY expression in VMN, which shows prominent GHS-R expression. Scale bar is 1 mm. From ref. *99*.

example, NPY is implicated in regulation of food intake, so GH action on these cells might be involved in coordinating food intake drive with activity in the GH/IGF-1 axis affecting food utilization, rather than being primarily involved in growth regulation.

GHR expression is also found in extrahypothalamic areas and in other hypothalamic nuclei associated with peptides, which may or may not be directly involved in GH control. For example, CRF inhibits GH secretion whereas a CRF antagonist increases it *(96)*. It is not clear whether CRF normally participates in the regulation of GH, but if so it could be regulated by GH feedback, which might then explain the GHR expression in PVN (Fig. 1), the primary site of synthesis of hypothalamic CRF. GH secretagogues may also have a role in central GH control. The recently identified GH secretagogue receptor (GHS-R) is expressed in the hypothalamus *(97,98)*, and the authors have recently demonstrated that the GHS-R gene is expressed within the ARC in areas that overlap with the expression of NPY and GHRH (Fig. 6). It has been suggested that this receptor and its putative ligand may have a role in regulating central GH actions, because the expression of this receptor is positively correlated with GHRH, inversely correlated with NPY, and negatively regulated by GH *(99)*. GH secretagogues activate neuronal firing and *c-Fos* expression in the hypothalamus *(100)* in a cell population that also includes GHR-expressing cell *(101)*, but the more widespread central physiology of this new receptor and its relationship to GH feedback remains to be explored.

OTHER ACTION OF GH IN THE CNS

Although largely overlooked, GH has a number of neurotrophic actions (stimulating neuronal and glial proliferation, increasing myelination, and increasing brain size), whereas GH deficiency is associated with deficits in brain development (*see* ref. *1* for review). GH deficiency, and more especially GH treatment, are associated with a variety of changes in the major central neurotransmitters, their biosynthetic enzymes, or their receptors *(102–106)*, but a physiological role for endogenous GH, acting directly on these systems, has not yet been established. As mentioned above, the expression of GHRs in the hippocampus is consistent with a role in learning and memory, and some effects of GH have been reported in ameliorating impairments in CNS function in GH-deficient

animal models *(106,107)*. The classic hippocampal electrophysiological paradigm for learning and memory is long-term potentiation (LTP), but preliminary results suggest that no obvious defects are seen in the ability to induce LTP in GH-deficient dwarf rats (M. L. Errington and I. C. A. F. Robinson, unpublished). This does not exclude the possibility of local GH effects, or that these animals might show some deficits when tested in behavioral paradigms of learning and memory. Further experiments of this type are clearly warranted in view of the increasing evidence from clinical studies that GH administration to GH-deficient adults may be associated with positive effects on mood and mental performance *(56,108)*. Although it is always difficult to exclude secondary effects of GH treatment (increased muscle strength, loss of fat, increased energy), direct effects of GH on the metabolism or protein synthesis of neural tissue have been demonstrated *(109,110)*. Thus, the presence of central GHRs in human brain tissue certainly encourage the speculation that some of the beneficial effects of GH treatment, assessed by psychological testing, may be direct *(111)*.

NEW EXPERIMENTAL APPROACHES TO STUDY GH EFFECTS IN THE CNS

An insight into many aspects of normal physiology has been obtained from the study of mutations in animals. Originally, such animal models were generated by recognizing spontaneous mutations, but the ability to manipulate the genome makes it possible to introduce specific genetic mutations to advance our understanding of the underlying mechanisms involving GH feedback in the CNS.

There are various animal models of GH deficiency in rodents that can be used as an alternative to hypophysectomy to avoid the loss of all the other pituitary hormones. In particular, a number of spontaneous mutations in mice have proved extremely useful in studying the GH axis, and the mutations responsible for some of these defects have been characterized. The Snell *(dw/dw)* and Jackson *(dwj/dwj)* dwarf mice have mutations of the transcription factor Pit-1 *(112)*, which causes profound deficiency in GH as well as in PRL and TSH. Most recently, the mutation in the Ames dwarf has also been identified, as a transcription factor Prop1 *(113)*, acting earlier than Pit-1 in pituitary differentiation, but still affecting the same cell types. In addition, the defect in GH production in the little *(lit/lit)* mouse has now been shown to be caused by a defective GHRH receptor *(114)*. There are also rat models that have arisen spontaneously, such as the spontaneous dwarf rat *(dr/dr)*, which has a mutation in the GH gene itself causing premature termination of the protein sequence *(115)*. Another dwarf rat *(dw/dw)* is homozygous recessive for a specific GH-deficient dwarf phenotype *(116)* associated with a defect in signal transduction although the mutation has not yet been identified *(117,118)*. Where examined, all these rodent mutations causing GH deficiency are associated with increases in GHRH expression, and to a lesser extent, decreases in PeN SRIF expression as a secondary consequence of GH deficiency, some of which may be reversed with GH excess *(119,120)*.

On the other hand, there are conditions in humans in which GH secretion is intact or even increased, but the GH is ineffective because of mutations in the GHR resulting in Laron-type dwarfism. The high spontaneous GH secretion in this condition is probably caused by a combination of lack of GH and IGF-1 feedback. IGF-1 replacement in these individuals suppresses GH secretion *(121)* just as it does in normal individuals subjected to fasting-induced increases in GH secretion *(122)*. A mouse model with the GHR gene

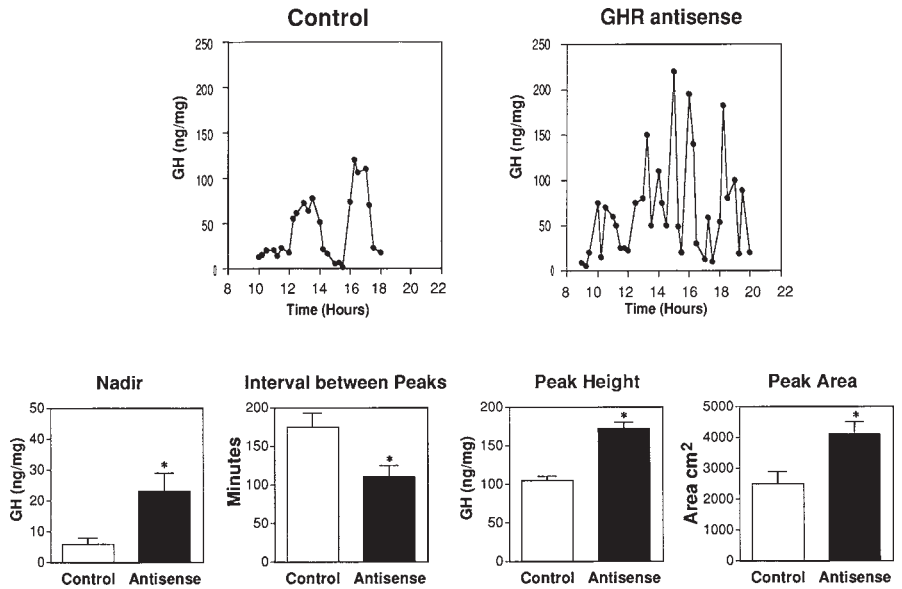

Fig. 7. GH secretory patterns obtained during an 8-h sampling period from a normal rat **(A)** or a rat infused intracerebroventricularly with antisense oligonucleotides against the rat GHR at 2 nmol/h **(B)**. Antisense GHR treatment increased basal, peak height and peak area of GH secretion and reduced the interval between GH secretory peaks compared with control animals. Redrawn from ref. *3*.

knockout has only recently been announced *(123)*. Although not yet characterized, it will be extremely interesting to study the hypothalamic changes in this animal and their reversibility with IGF-1 treatment so that the effects of IGF feedback on GHRH and SRIF may be evaluated separately from effects of GH.

Another approach to GH insensitivity that has recently been exploited is to use antisense oligonucleotides against the GHR to reduce the effectiveness of direct feedback at the hypothalamus *(3)*. The effects of central administration of antisense GHR oligonucleotides were readily evident in the profile of GH secretion (Fig. 7). The antisense oligonucleotides reduced GHR expression, presumably reducing GH feedback, thereby inducing a secondary increase in release of GHRH and/or reduction in SRIF. The effect was specific for GHRs with no effect on GH when antisense against PRL receptors was used. The resultant increased GH peak amplitude through levels, and the number of GH peaks compared to controls provide further direct evidence implicating a role of CNS GHRs in GH autofeedback control *(3)*.

TRANSGENIC MODELS

The GH axis has been a popular target for transgenic manipulations. A number of transgenic lines of mice have been generated expressing GH or GHRH under the control of heterologous promoter sequences *(124–127)*. The resultant phenotype in the majority of lines is increased growth, again usually associated with increased SRIF and/or decreased GHRH, as would be expected *(120)*. However, transgenesis is not the only way to model in rodents, the type of GH hypersecretion normally seen in humans in acromegaly;

it can be conveniently induced experimentally in rodents using implants of a GH-secreting tumor cell line *(128)*. The effects of such cell implants are comparable to excess exogenous administration of GH with raised SRIF and decreased GHRH mRNA levels, and these changes can be reversed after removal of the GH-secreting tumors *(128)*.

Transgenesis has also been used to induce dwarfism, either by widespread expression of a functional GH antagonist *(129)* or by targeting, directly or indirectly, the somatotrophs. Dwarf mice have been produced by selective or total ablation of GH-expressing cells *(130)*, whereas transgenic dwarf rats have been produced by expressing an antisense GH transgene in the pituitary to suppress endogenous GH synthesis *(131)*. Paradoxically, dwarfism can also be induced by targeted expression of GH itself. This was first noticed in a line of mice that fortuitously expressed hGH in the CNS *(71)*. In contrast to the giantism generated by peripherally overexpressed hGH transgenes, mice with such central hGH expression exhibited a dwarf phenotype caused by local short-circuit feedback on hypothalamic GHRH and SRIF *(71)*. This has prompted the production of another mouse model in which hGH was targeted more specifically to the CNS and peripheral nervous system using the tyrosine hydroxylase promoter *(72)*. As expected, the resultant mouse also had a dwarf phenotype and exhibited similar hypothalamic GHRH suppression *(72)*.

The authors have also exploited this GH-negative feedback mechanism by targeting hGH to the CNS under the control of the GHRH promoter *(73)*. This was achieved in transgenic rats, and the resulting transgenic growth retarded (Tgr) rat also displays the expected raised SRIF and low GHRH expression (Fig. 8). Rats have some advantages over the mouse because their larger body size made it possible to carry out blood sampling to observe the effects of transgenes on pulsatile GH secretion and to study the growth responses to patterns of hormone administration *(132)*. Although the transgenic mice expressed hGH in many areas of the CNS *(71)* and in peripheral tissues *(72)*, the Tgr rat has highly restricted expression of the hGH transgene to GHRH neurons that are sensitive to GH feedback so that transgene activation is itself subject to physiological feedback control by GH. The effect of this GHRH-hGH transgene in Tgr rats is also sexually dimorphic with dwarfism much more marked in males than in females. The reason for this is unknown, but may relate to the sex differences already shown for GH feedback in the rat *(78,133)*.

The authors have recently succeeded in adapting the GC cell implant method for maintaining high GH exposure to these dwarf rat models, in order to study the hypothalamic changes in GHRH and SRIF expression in the same animals as their GH status changes rapidly from dwarfism to acromegaly *(134)*. It is interesting to compare this model of secondary dwarfism (Tgr rats) with that of primary pituitary dwarfism (*dw/dw* rats) since they both have low GH output, but exhibit diametrically opposite hypothalamic GHRH activity (Fig. 8). GC cell implants can usually only be made in normal female rats of the Wistar-Furth strain, whereas both dwarves were raised on an AS background that rejects GC cell implants. The authors overcame this problem in two different ways. GC cells survived in *dw/dw* rats when they were given cyclosporin to prevent rejection. An even simpler solution was possible in the Tgr rats, since the Tgr transgene is dominant. By breeding Tgrs with Wistar/Furth animals, the transgene conferred dwarfism on the F1 progeny whereas these retained sufficient Wistar/Furth background to accommodate GC cell implants. In both cases, rapid growth followed implantation of GC cells. Figure 9 compares the effects of chronic GH exposure from

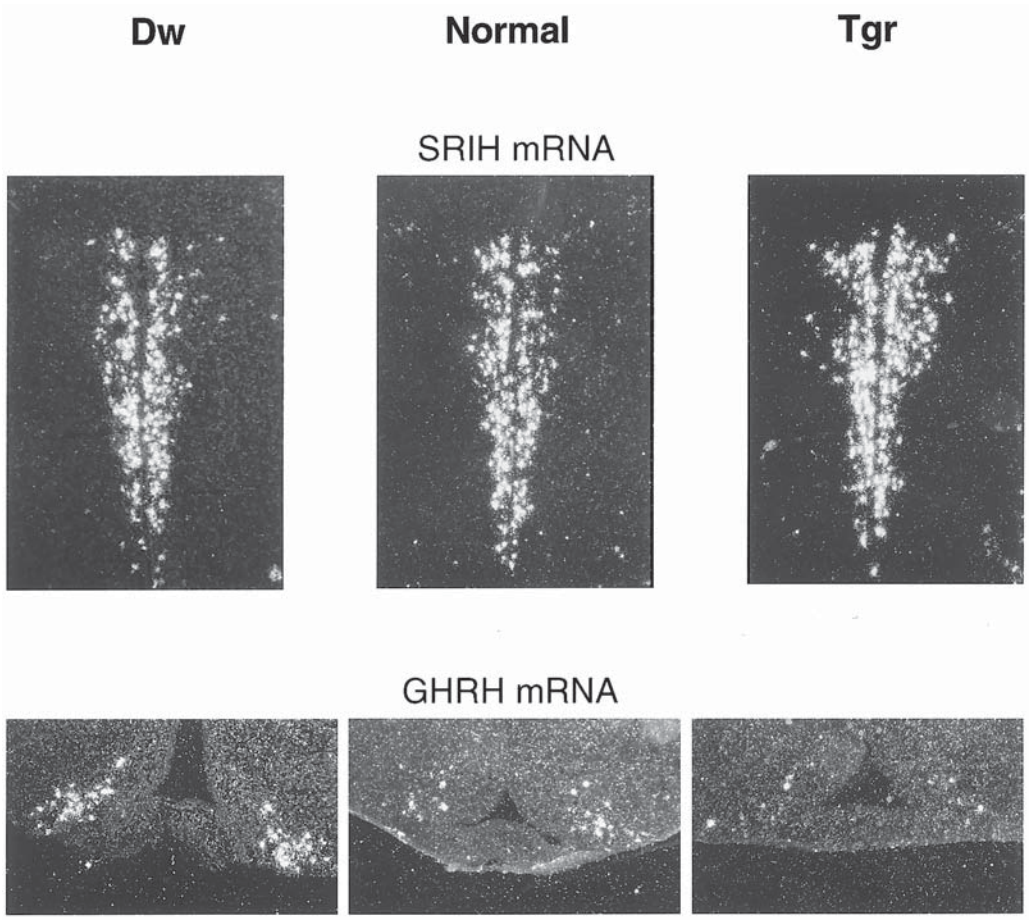

Fig. 8. GHRH and SRIF expression in Dw and Tgr rats. Comparison of the hypothalamic distribution and expression of SRIF **(Top)** and GHRH **(Bottom)** in PeN and ARC respectively, of *dw/dw* dwarf, normal, and transgenic growth-retarded (Tgr) rats (100× magnification). From ref. *134*.

these implants on GHRH expression in the two dwarf models studied by *in situ* hybridization of the ARC. As expected, GHRH expression is high in *dw/dw* rats and was markedly suppressed by GC cell implants. There was a smaller, but significant suppressing effect in the normal animals implanted with GC cells and no effect in the Tgr animals, whose GHRH expression is already maximally suppressed by the local transgene hGH. Thus the effects of central and peripheral GH feedback can be dissociated in these models. Targeting of hGH to other neurons has already been achieved with different neurone-specific promoters (I. C. A. F. Robinson, unpublished observations), and may in the future prove to be a useful means of targeting direct effects of GH in other CNS sites that express GHRs without altering peripheral GH or IGF-1 status, and thereby reveal other potential physiological targets for GH action within the brain.

CONCLUSIONS

It now seems beyond doubt that the brain does represent a physiological target for GH action. GHRs are expressed in a region- and cell-specific fashion, are developmentally

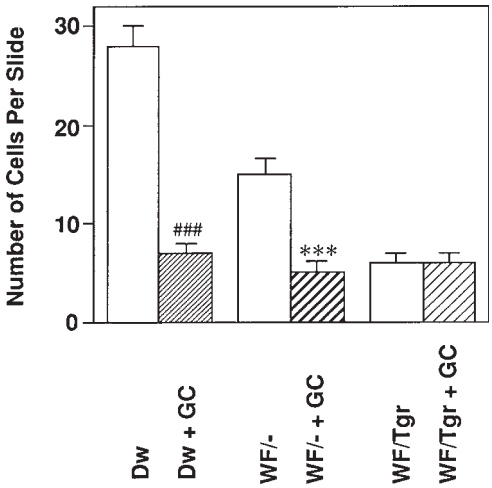

Fig. 9. GH feedback regulation of GHRH in Dw and Tgr rats. Comparison of the effects of chronic GH exposure from GC cell implants on the number of hypothalamic cells expressing GHRH mRNA in dwarf (Dw), normal Wistar-Furth (WF/-), and transgenic growth-retarded (WF/Tgr) rats. The high GHRH in dwarf rats was markedly suppressed by GC implants; a suppression was also evident in normal, but not Tgr rats. ### $p < 0.001$ compared to Dw, ***$p < 0.001$ compared to WF/-. From ref. *134*.

regulated, and are subject to peripheral regulation by GH and other endocrine factors. Hypothalamic GHRs are involved in mediating autofeedback regulation of GH secretion via powerful effects on GHRH and SRIF activity and probably on the GHRP system as well. These actions may be direct or indirect via other hypothalamic systems, such as those expressing NPY. Much less is known about the role of GH in other brain sites, about how GH gains access to these sites, and whether this access is by diffusion or by some form of regulated transport. Brain GHRs are subject to a different developmental and endocrine regulation from those in peripheral tissues. Both GH and GHR are present in fetal life, although what physiological roles they play remains unclear. Beneficial effects of GH therapy on mood in GH-deficient adults imply that GH may affect CNS function in adult life, but further experimental studies are needed to evaluate the importance of these and whether they could be exploited for therapeutic benefit.

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II Clinical Practice

11 Molecular Defects of the Growth Hormone Axis

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Contents

INTRODUCTION

The incidence of defects in growth hormone (GH) synthesis resulting in short stature is between 1:4,000 and 1:10,000 live births *(1,2)*. Most of these cases are sporadic, without a known or proposed molecular defect. Some arise from recognizable cerebral defects (e.g., septo-optic dysplasia, pituitary malformations, or hydrocephalus); others have etiologies that are yet unknown. It has been estimated that only 5–30% of all cases of growth hormone deficiency (GHD) are secondary to heritable molecular causes *(3)*.

In spite of the relative rarity of genetic causes of GH deficiency, the classification of GHD states previously has been based upon mode of inheritance (Table 1). We believe that this system was useful before the era of molecular analysis. However, now that we better understand the various elements that comprise the GH axis, this outline has become outdated, with little clinical relevance or utility. We have chosen therefore to organize our discussion of GHD according to recently described molecular defects. Our objective is to cover those defects resulting from molecular abnormalities affecting each part of the GH axis (Table 2). The term growth hormone axis defect (GHAD) has been selected to refer to any condition of clinical growth failure that results from abnormalities of this axis. We also will address the process of diagnosing GHD by examining current clinical practices and new molecular diagnostic techniques.

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Table 1
Traditional Classification System for Growth Hormone Axis Defects

	Inheritance	*Endogenous GH*	*Response to exogenous GH*	*Described molecular defect*
IA	AR	Absent	Often temporary	Complete *GH-1* deletion
IB	AR	Decreased	Present	Partial *GH-1* deletions, GHRH receptor mutations
II	AD	Decreased	Present	Partial *GH-1* mutations
III	X-linked	Decreased	Present	X-chromosome deletions or duplications

AR, Autosomal recessive; AD, Autosomal dominant.

Table 2
Elements of GH Axis

Hypothalamus
Growth hormone releasing hormone
Somatostatin
? Growth hormone secretagogue
Pituitary
*Growth hormone releasing hormone receptor
*Pituitary transcription factors (*Rpx*, *PROP*, *Pit-1*)
*Growth hormone
? Growth hormone secretagogue receptor
Target tissues
*Growth hormone receptor
*Insulin-like growth factor 1 (IGF-1)
*IGF-1 receptor
IGF-binding proteins

*Indicates defect in this element described in humans.

CLINICAL PRESENTATION OF GHAD

Fetal growth is primarily driven by maternal and nutritional factors, rather than by GH action. Children with GHAD generally are born with normal appearances, birth weights only slightly below average, and birth lengths about one standard deviation below the normal mean *(4)*. This may be related to the decreased GH receptor prevalence found in fetal tissues compared to the number found postnatally. It is likely that insulin and the insulin-like growth factors (IGFs) have a more major role than GH does in prenatal growth *(5)*.

Sometimes, GHAD will first be suspected when there is severe neonatal hypoglycemia or other signs of hypopituitarism (hypothyroidism, small phallus in male babies, or neonatal hepatitis). During early childhood, children with GHAD are detected when they demonstrate short stature and subnormal rates of growth. Often these children will have proportionally small limbs, increased body fat, and a cherubic appearance.

MOLECULAR MECHANISMS AND DEFECTS

Hypothalamus

Growth hormone (GH) synthesis and release are primarily regulated by two hypothalamic peptides: somatostatin, which inhibits GH secretion, and growth hormone-releasing hormone (GHRH), which stimulates its release (Fig. 1). A third endogenous hypothalamic GH stimulating factor has been hypothesized and called growth hormone secretagogue (GHS, also known as growth hormone releasing peptide). GHSs have been synthesized, and are small molecules that promote somatotroph growth hormone release independently of, but synergistically with, GHRH *(6,7)*.

GHRH is produced by hypothalamic neurons located primarily in the arcuate nucleus *(8,9)*. These neurons release GHRH into the hypophyseal-portal circulation, where it passes to the anterior pituitary gland with subsequent binding to somatotrophs. The GHRH gene is present as a single copy *(10)*. Using fluorescent *in situ* hybridization and microsatellite markers, it has been localized to chromosome 20q12 in humans *(11)*.

Hypothalamic Gene Mutations

Growth failure secondary to perturbations in these hypothalamic factors could involve loss of function mutations in the ligands GHRH or GHS or gain of function mutations in somatostatin. Although no molecular abnormalities of GHRH, GHS, or somatostatin have been described to date, it has been hypothesized that functionally GHRH-deficient states account for the majority of cases of childhood GH deficiency *(12)*. Chatelain et al. demonstrated that GHRH agonist treatment can stimulate endogenous GH secretion in 77% of patients with GH insufficiency *(13)*.

Two animal models of GHRH deficiency have been described. In one, administration of monosodium glutamate (MSG) to mice causes a selective loss of arcuate nuclei neurons *(14,15)*. MSG-exposed rodents have impaired growth, obesity, hypogonadism, and hypothyroidism *(16)*. The other animal model of GHRH deficiency is the *Gsh-1* homeobox gene knockout mouse *(17)*. Homeobox genes encode a family of DNA binding proteins, and the *Gsh-1* gene encodes a product necessary for GHRH gene transcription and translation. *Gsh-1* knockout mice have extreme postnatal dwarfism, sexual infantilism, leukopenia, significant perinatal mortality, a shortened life span, and biochemical evidence of GHRH deficiency. Their anterior pituitary glands are one-third normal size and possess decreased numbers of somatotrophs and lactotrophs.

Pituitary and Placenta

The components of the GH axis located at the level of the pituitary gland are the GHRH receptor, the GHS receptor, pituitary transcription factors, and GH (Table 2). The GHRH receptor has been localized to human chromosome 7p15 *(18)*. GHRH receptors on somatotrophs are typical G-protein-coupled receptors containing seven transmembrane-spanning domains with three extracellular and three cytoplasmic loops *(19,20)*. On binding GHRH, the receptor activates a G_s protein with a resultant increase in cAMP and intracellular Ca^{2+} *(21,22)*. Via these intracellular signaling pathways, GH synthesis and release are induced.

Recently the GHS receptor was cloned and also shown to be a G-protein-coupled receptor *(23)*. The GHS receptor, unlike the GHRH receptor, does not activate intracellular cAMP, but appears to act via protein kinase C activation *(24)*. The endogenous ligand for this receptor is unknown.

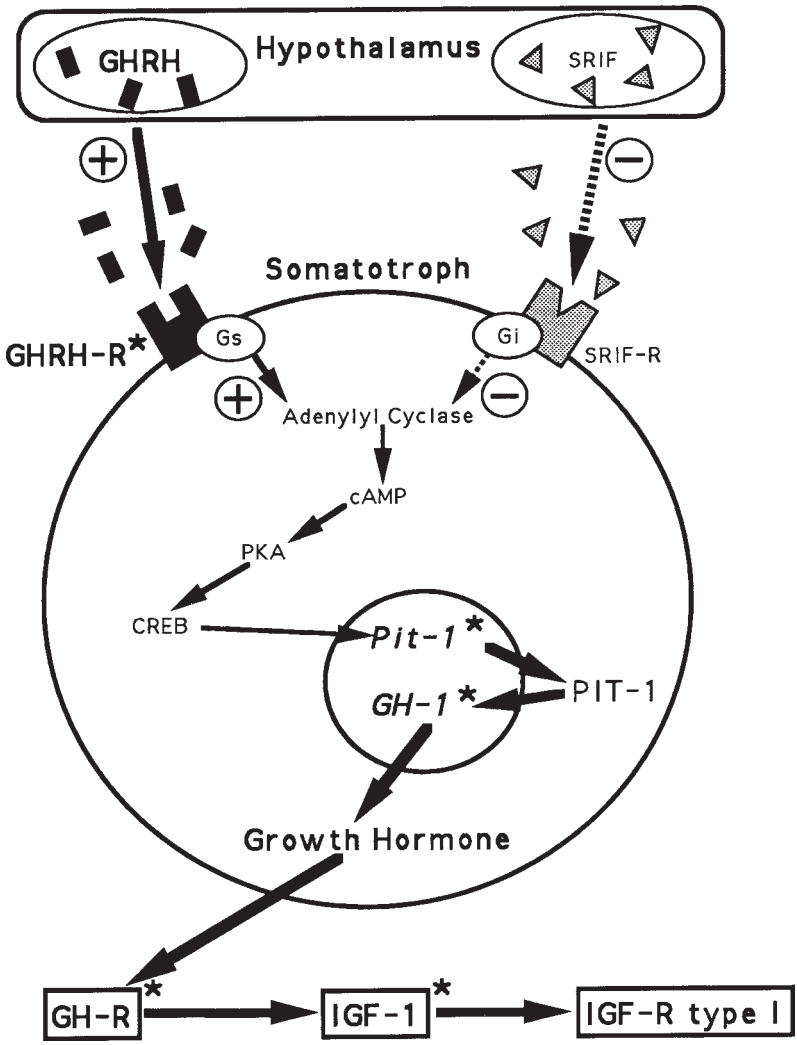

Fig. 1. GH axis. Simplified, schematic representation of the growth hormone axis. Growth hormone (GH) synthesis and release from somatotrophs is predominantly regulated by two hypothalamic hormones, growth hormone releasing hormone (GHRH) and somatostatin (SRIF). GHRH stimulates GH transcription, synthesis, and release via a G_s-protein coupled receptor (GHRH-R). SRIF antagonizes this effect via a G_i-protein coupled receptor (SRIF-R). GHRH activation of the heterotrimeric G_s-protein results in increased cAMP accumulation and activation of protein kinase A (PKA). PKA in turn phosphorylates and activates the cAMP response element binding protein (CREB) that binds to cAMP response elements in the promotor region of the *Pit-1* gene to enhance transcription. Pit-1 augments *GH-1* gene transcription leading to increased growth hormone synthesis. GH, acting via its receptor (GH-R), increases the synthesis and release of insulin-like growth factor 1 (IGF-1), which mediates somatotrophic effects via the IGF type 1 receptor (IGF-R type 1). The regulation of the other pituitary transcription factors such as Rpx and PROP-1 in somatotrophs has not yet been fully characterized. Asterisks denote recognized abnormalities in this axis.

Recent advances in the molecular ontogeny of the pituitary gland have identified a number of nuclear transcription factors necessary for normal anterior pituitary gland and, more specifically, somatotroph development. It is clear that a cascade of transcription factors is involved in differentiation of specific pituitary cell populations.

Early in embryogenesis *Rpx*, (Rathke pouch homeobox, also known as *HESX1*, homeobox gene expressed in ES cells) is expressed in the mouse in visceral endoderm, neural ectoderm, and Rathke's pouch *(25)*. This is a member of the paired-like class of homeobox genes. The human gene for *Rpx* is found at 3p21.2-p21.1 *(26)*.

Other early transcription factor genes important in the formation of thyrotrophs, lactotrophs, and somatotrophs are *LHX3* (lim homeobox 3); *LHX4*; *OTX* (orthodenticle homolog 1); and *PROP-1* (prophet of pit-1) *(27)*. The *LHX* genes are expressed in embryonic and adult mouse pituitaries and appear to be involved in establishing and maintaining differentiated pituitary cells *(28,29)*. *OTX1* is a homeobox-containing gene that may activate transcription of GH, follicle stimulating hormone, luteinizing hormone, and the α-glycoprotein subunit genes *(30)*. PROP-1 is a paired domain protein, whose expression occurs immediately before and in the same tissues as pit-1. PROP-1 function is necessary for *pit-1* expression. In humans, the *PROP-1* gene is found on the distal portion of chromosome 5q *(31)*.

Pit-1 is a 33kd pituitary-specific transcription factor that is necessary for GH, thyroid-stimulating hormone (TSH), and prolactin gene transcriptional activation, and for somatotroph, lactotroph, and thyrotroph establishment *(32)*. The protein is a product of the POU-domain gene family and has three regions: a transcriptional activation domain, a 60 amino acid sequence necessary for high-affinity DNA binding known as the POU homeodomain (POU-HD), and a 76 amino acid highly-conserved region that potentiates POU-HD binding, known as the POU specific domain *(33)*. In humans, the gene encoding pit-1 has been mapped to chromosome 3p11.

GH is a single 191 amino acid polypeptide chain, which is nonglycosylated but contains two disulfide bridges. The *GH-1* gene that codes for GH is part of a 50-kb cluster of five genes that evolved from a series of three sequential gene duplications *(34)*. Located on chromosome 17q22-24, in 5'-3' order these genes are: *GH-1*, chorionic somatomammotropin-like (*CS-L*), *CS-A*, *GH-2*, and *CS-B* (Fig. 2). Except *CS-L*, each gene produces a unique 217 amino acid prohormone that is cleaved to a mature 191 amino acid hormone. *CS-L* was originally categorized as a pseudogene. Subsequent investigations have found that it is translated and undergoes alternative splicing, but the resultant protein products appear to be nonfunctional *(35)*.

The GH-1 gene is expressed in the anterior pituitary and yields a major 22-kb product (GH-N), a minor 20-kb product, and some post-translational variants. The *GH-2* product, known as GH variant (GH-V), only differs from GH-N by 13 amino acids that are distributed along its peptide chain *(36)*. It is expressed as at least four alternatively spliced mRNAs in the placenta and is continuously secreted during the second half of pregnancy *(37,38)*. Placental GH-V accounts for the majority of radioimmunoactive growth hormone detected in the maternal circulation, with maternal pituitary *GH-1* function being suppressed *(39)*. In vitro, it is a potent GH analogue and likely assists during pregnancy in optimizing maternal transfer of nutrients to the fetus.

CS-A and *CS-B* are placentally expressed and encode human chorionic somatomammotropin (hCS, also known as human placental lactogen). HCS is produced in massive amounts by syncytiotrophoblastic cells, but, unlike GH-V, has, on a weight-for-weight basis, only 0.5% the affinity for the GH receptor as GH-N *(40)*. It is similar in structure (85% amino acid homology with two disulfide bonds) to GH-N *(41)*. HCS production is shared by *CS-A* and *CS-B* genes. Deletion of both of these genes is necessary to have hCS deficiency.

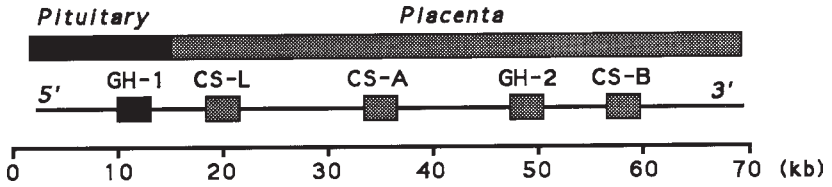

Fig. 2. Map of GH gene family: *GH-1*; *CS-L*; *CS-A*; *GH-2*; *CS-B*. Schematic representation of the GH gene cluster located on chromosome 17q22-24. The tissue where normal transcription occurs is also shown. CS, chorionic somatomammotropin.

Mutations

GHRH Receptor Mutations

Even though isolated GHRH deficiency has not been identified, genetic mutations in pituitary GHRH receptors have been identified i2n humans and mice *(18,20,42)*. The *little (lit/lit)* dwarf mouse has postnatal growth failure and delayed pubertal maturation with biochemical evidence of GHD and high GHRH levels *(20,42)*. Pituitary histology reveals somatotroph hypoplasia with absent secretory granules *(43)*. This condition is inherited in an autosomal recessive manner. These mice have a single nucleotide substitution that produces a missense mutation by changing Asp^{60}→Gly in the GHRH receptor and prevents hypothalamic GHRH binding *(20)*.

In 1996, the first human GHRH receptor defect resulting in profound GHD was described by Wajnrajch et al. *(44)*. They identified an autosomal recessive form of GHD in a consanguineous Indian Moslem kindred. A G→T transversion at position 265 produces a nonsense mutation (Glu^{72}→Stop) in the GHRH receptor. This yields a truncated receptor that lacks membrane spanning regions and a G-protein binding site. In a presumably separate Pakistani kindred, an autosomal recessive form of extreme dwarfism, the Dwarfism of Sindh, is now attributed to an identical mutation in the GHRH receptor gene *(45)*. Patients with GHRH receptor mutations respond well to exogenous GH therapy without antibody formation. Although these loss of function mutations demonstrate the importance of GHRH and its receptor in growth, the current investigative data suggest that GHRH receptor defects will be an uncommon cause of human GHAD *(46)*.

Pituitary Transcription Factor Mutations

To date, human defects in the pituitary transcription factors *Rpx*, PROP-1, and pit1 have been described. These are associated with heritable combined pituitary hormone deficiencies, characterized by GHD in addition to deficiency of one or more of the following: adrenocorticotropic hormone, follicle stimulating hormone, luteinizing hormone, or TSH (Table 3).

Rpx defects in mice cause variable anterior central nervous system (CNS) defects and pituitary dysplasia *(26)*. The spectrum of defects is not unlike those seen in septo-optic dysplasia (SOD). Recently it was confirmed that a homozygous loss of function mutationin *Rpx* results in human familial SOD. The defect is a missense mutation in a highly conserved amino acid arginine 53, converting it to cysteine. Some sporadic cases of SOD have since been confirmed as also having defects in *Rpx (47)*. However, mutations in *Rpx* only account for a small portion of patients with SOD.

Loss of function of PROP-1 results in the Ames dwarfism mouse paradigm *(27)*. Defects of *PROP-1* have now been described in humans with familial multiple pituitary

Table 3
Traditional Classification System for Multiple Pituitary Hormone Deficiencies

	Inheritance	*Endogenous GH*	*Associated deficiencies*	*Molecular defect*
IA	AR	Reduced	LH, FSH, TSH, ± ACTH	*Rpx*, *Prop-1* mutations
IB	AR	Absent/very reduced	Prolactin, TSH	*Prop-1*, *Pit-1* mutations
II	X-linked	Reduced	LH, FSH, TSH	

deficiencies *(48)*. One series examining 10 kindreds and 21 sporadic cases of combined pituitary hormone deficiencies from eight different countries found a *PROP-1* mutation (301 del AG) in 55% of the *PROP-1* alleles from affected families and in 12% of the alleles from sporadic cases *(31)*. The hormonal deficiencies include not only the expected growth hormone, TSH, and prolactin deficiencies, but also gonadotropin deficiency in a number of cases. This finding in humans is surprising because the Ames mouse does not manifest gonadotropin deficiency.

Pit-1 mutations resulting in GHAD were first described in two dwarf mouse mutants *(49)*. These mice have growth failure with intact GH genes *(50)*. Snell mice have a homozygous missense mutation within the POU-HD, which produces a substitution of an invariably conserved tryptophan (Trp^{261}→Cys) *(49)*. Jackson mice have homozygous rearrangements in the *pit-1* gene with a 4kb DNA segment insertion. Both strains of mice have profound pituitary hypoplasia, and lack somatotrophs, lactotrophs, and thyrotrophs.

In 1992, the first *pit-1* mutations that produce pituitary hormone deficiencies in human beings were described *(51,52)*. Since the original descriptions, sporadic, autosomal recessive, and autosomal dominant mutations of *pit-1* have been reported. As of this writing, eight different *pit-1* mutations have been found associated with GH, prolactin, and TSH deficiency. Six of these are recessive; two are dominant-negative mutations. In 1992, Tatsumi et al. described a Japanese girl who had growth failure and severe congenital central hypothyroidism *(51)*. They found a homozygous missense transition (C→T) that converted Arg^{172} to a termination codon, producing a truncated pit-1 protein without a POU-homeodomain. The next homozygous *pit-1* mutation to be described was an Arg^{143}→Gln mutation (the result of an A→G missense mutation) in a Japanese girl with complete growth hormone, prolactin, and TSH deficiency *(53)*. Another *pit-1* missense mutation (Ala^{158}→Pro) has been identified in two Dutch families *(54,55)*. This mutationresults in a pit-1 protein that cannot activate expression of growth hormone and prolactin genes *(55)*. Irie et al. (1995) reported a homozygous Glu^{250}→Stop mutation that results in the loss of the third helix of the POU-HD and clinical GHD *(56)*. Other recessive mutations identified to date are Phe^{135}→Cys and Pro^{239}→Ser *(57,58)*.

Other pit-1 deficiencies are inherited in an autosomal dominant manner. Codon 271 appears to be a hot spot for these mutations *(59)*. A heterozygous C→T mutation resultsin an Arg^{271}→Trp *pit-1* change in patients with GH, prolactin, and eventual TSH deficiency *(52,53,59)*. This may produce a dominant-negative effect via the resultant mutant POU protein that both binds DNA and inhibits transcription *(52)*.

Okamoto et al. (1994) performed a pedigree analysis of multiple members of a family with an Arg^{271}→Trp *pit-1* mutation and found the same heterozygous gene mutation in clinically unaffected family members as in the proband who had GH and prolactin deficiency *(60)*. They found monoallelic *pit-1* transcription (normal gene only, without

mutant gene expression) in the unaffected father, aunts, and grandmother of their index case and skewed biallelic *pit-1* expression (normal > mutant gene) in the patient. They speculated that phenotypic expression of the *pit-1* abnormality was secondary to genomic imprinting that caused biallelic expression in the affected patient. The mutant pit-1 protein exerts a dominant-negative effect on the normal pit-1 protein, thereby neutralizing its activity.

Ohta et al. have described a second heterozygous *pit-1* mutation (Leu^{24}→Pro) in a child with GH and prolactin deficiency *(53)*. This mutation is located in the transcriptional activation domain of *pit-1* and is also assumed to have a dominant-negative effect, although its DNA-binding and transcriptional activation properties have not yet been elucidated.

Growth Hormone Defects

GH-1 Gene Deletions. Some patients with GHAD have complete GHD caused by deletions of the entire *GH-1* gene. These children have the most severe form of inherited GHD. In 1970, Illig et al. identified six patients receiving human pituitary GH therapy who demonstrated growth retardation in infancy with subsequent severe dwarfism, a characteristic facies, and a strong anabolic response to exogenous GH *(61)*. Four of these six children were related to each other. Illig named the syndrome "type A" after the first initial of the family's surname *(62)*. With GH treatment, these children developed high titers of GH antibodies and resultant growth inhibition. Illig hypothesized that these patients became resistant to exogenous GH because their immune systems did not recognize GH as a homologous hormone molecule *(61,63)*. In 1981, Phillips et al. were the first to describe mutations of the *GH-1* gene when they studied these children and found that they had deletions of 6.7 kb of DNA that normally contains the *GH-1* gene *(64)*.

It is now accepted that heterogeneous deletions of both alleles encoding the *GH-1* gene ranging from 6.7 to 45 kb produce complete absence of GH *(65,66)*. The most prevalent mutation is the 6.7 kb deletion. The deletions appear to arise from unequal recombination events owing to meiotic misalignment of wild-type chromosomes. The *GH-1* gene is predisposed to such mutations because it is flanked by long stretches of highly homologous DNA *(67,68)*. Phenotypic heterogeneity is most often found in cases with small gene deletions, but can also be seen in some children with the largest (45-kb) deletions *(69)*.

The prevalence of complete GHD varies between populations; it appears overall that 13–15% of patients with severe GHD (height <–4.5 SD for age and sex) have *GH-1* gene deletions *(70)*. Parks et al. reported that 5 of 13 Oriental Jewish patients with height <–4 SD for age and sex had a *GH-1* gene deletion *(71)*. Mullis et al. examined 78 children with severe GHD from inbred populations of Northern-European, Turkish, and Mediterranean ancestry and found that 10 of them had *GH-1* gene deletions *(3)*. Eight of the 10 had deletions spanning 6.7 kb; two had 7.6 kb deletions. Five of the ten developed antibodies to GH replacement.

Interestingly, despite their total lack of GH production, patients with complete GH gene deletions do not always develop GH antibodies when treated with exogenous GH. Phillips has reported that 82% (14/17) of the patients he examined with *GH-1* deletions developed antibodies during replacement therapy *(72)*. Differential antibody formation appears to be partially explained by the molecular heterogeneity of gene deletions; but individuals within families who share apparently identical deletions can also have discordant antibody formation *(62,73)*. These antibodies may prevent patients from responding to GH treatment, resulting in a type of GH insensitivity, but growth in children being treated

with GH can also be variable even in the face of antibodies. Rivarola et al. described formation of high antibody titers and consequent growth arrest in a child receiving GH whose sibling had similar antibody titers during GH treatment but continued to grow *(74)*. This variable clinical response to GH replacement might be due to factors other than molecular heterogeneity in the gene deletions, including individual responses to different synthetic GH preparations, specific HLA groups, unique immune antibody formation, or production of antibodies with different GH neutralizing capacities *(75)*.

Until recently, unresponsiveness to GH treatment due to antibody formation left no therapeutic alternatives and resulted in extreme adult short stature. Children who develop an immune response to GH therapy that interferes with therapeutic efficacy are candidates for treatment with synthetic IGF-I, although it is not readily available *(76)*.

Multiple GH Family Gene Deletions. There have been two reports of *GH-1* deficiency in combination with other GH family gene deletions *(77,78)*. Goossens et al. found siblings who were homozygous for a 40 kb deletion that eliminated *GH-1*, *GH-2*, *CS-A*, and *CS-B* genes, leaving only *CS-L (77)*. Akinci et al. described a consanguineous Turkish family with children homozygous for a 45 kb deletion encompassing a different four genes (*GH-1*, *CS-L*, *CS-A*, and *GH-2*) *(78)*. The children with these deletions had normal birth weights, but demonstrated subsequent severe growth retardation and hypoglycemia. Their mother, who was heterozygous for the mutation, had normal postpartum lactation. This suggests that placental expression of *CS-L* or *CS-B* alone may be sufficient to sustain a normal pregnancy and prenatal growth, supporting the concept of significant duplication in function of these five genes.

HCS Deficiency (CS-A and CS-B Gene Mutations). Nielsen et al. first detected antenatal hCS deficiency in otherwise normal appearing pregnancies in 1979 during prenatal screening of hCS levels in maternal serum *(79)*. HCS does not appear to be essential for maintenance of pregnancy, fetal growth, or lactation. Some cases of hCS deficiency are total; others are partial. They produce abnormal biochemical phenotypes (with altered maternal IGF-I levels) but no overt disease. In cases of partial hCS deficiency, the amount of hCS produced by the placenta appears to be directly proportional to the number of normal *CS-A* or *CS-B* genes *(80)*.

HCS deficiency results from deletions in the GH and CS gene cluster. Wurzel et al. were the first to describe such a gene mutation when they described a homozygous *CSA*, *GH-2*, and *CS-B* gene deletion responsible for Nielsen's index case of hCS deficiency *(81)*. Both parents and two of the proband's three siblings were heterozygous for the deletion. Since no deletion that encompasses the entire five gene cluster has been reported, it remains possible that any one of the five genes can produce a peptide that performs the essential functions of any missing peptides *in utero*. This is in contrast to the situation postnatally, when, because the placental genes are no longer expressed, mutations in *GH-1* alone produce an abnormal phenotype.

GH Gene Mutations. Partial GHD is attributed to mutations in *GH-1*, which produce a GH molecule that retains some biologic function. Clinically, these individuals are less severely affected than those with *GH-1* gene deletions and complete GHD *(70)*. Patients with partial GHD have low, but detectable levels of GH on provocative stimulation testing. Growth retardation usually has its onset within the first two years of life *(82)*. Children respond well to treatment with GH without developing antibodies. The reported mutations can be autosomal recessive or autosomal dominant.

Cogan et al. described autosomal recessive inheritance of a GHAD when they found a homozygous splice site G→C transversion in intron IV of the *GH-1* gene in a con-

sanguineous Saudi Arabian family *(83)*. This mutation appears to cause a splice deletion of half of exon IV as well as a frameshift within exon V. Amino acid sequences derived from exons IV and V appear to play an important role in targeting the GH peptide into secretory granules. These investigators later identified a G→T transversion at the same location in another family *(70)*. A deletion/frameshift mutation in exon III has also been described in a patient *(84)*. Homozygous nonsense, splicing, and frame-shift mutations can also eliminate biologically active endogenous GH synthesis *(85,86)*.

Cogan et al. have studied a Turkish family with autosomal dominant partial GHD *(83)*; affected members have a heterozygous T→C transition of a *GH-1* intron III donor splice site, which causes skipping of exon III. In 1995, Binder and Ranke demonstrated a de novo G→C splice site mutation of the GH-1 gene that also produced transcriptional exon III skipping and a 17.5 kDa GH protein *(82)*.

In these individuals, presence of one normal *GH-1* allele does not compensate for the presence of the abnormal allele. Mutations appear to produce autosomal dominant expression in a dominant-negative manner *(87,88)*. Their adverse effects result from abnormal allele interference in any step from GH transcription to mRNA splicing, translation, and modification to post-translational protein handling. The degree of growth impairment varies greatly between kindreds and even between affected individuals within the same family.

Binder et al. examined relative expression of mutant and normal *GH-1* allele expression in an individual with autosomal dominant partial GH deficiency and found equivalent amounts of mRNA with and without exon III in peripheral lymphocytes *(88)*. This suggests that the molecular defect does not interfere with mRNA production but instead involves subsequent translation, processing, storage, or secretion. Binder et al. also found identical levels of GH secreted by proband and control lymphocytes, suggesting that the defect is pituitaryspecific. They theorized that these dominant-negative mutations involve pituitary-specific GH dimer formation or GH aggregation within pituitary cell secretory granules *(83,88)*.

Bioinactive GH. Laron et al. described three siblings with clinical features of GH deficiency but with high serum concentrations of immunoreactive GH *(89)*. These children appeared to have growth hormone insensitivity (GHI). Laron et al. initially theorized that most cases of GHI would result from a defect in GH synthesis that produced a GH molecule that was immunoreactive but without biologic activity *(90)*. Recently however, most cases of Laron syndrome have been found to result from growth hormone receptor (GHR) mutations. It took nearly thirty years for the first proven case of bioinactive GH to be described.

In 1996, Takahashi et al. described a child with severe growth retardation and high serum GH levels, elevated GHBP, low IGF-1 levels, and increased GH levels after provocative testing *(91)*. The patient had responded well to exogenous GH therapy. *GH-1* sequencing revealed that the child had an Arg^{77}→Cys mutation. This mutation was inherited from his unaffected father, who produced only wild-type GH. The son was found to express both mutant and wild-type GH. When compared to wild-type GH, the mutant GH had a higher affinity for GHBP, less phosphorylating activity, and an inhibitory or dominant-negative effect on wild-type GH activity. Takahashi et al. have theorized that the cysteine in place of arginine may change the GH molecule configuration by forming a new disulfide bond, resulting in lower bioactivity. A second GH mutation (Asp^{112}→Gly) resulting in a biologically inactive GH was reported by the same group in 1997 *(92)*. This mutant GH is believed to prevent GH receptor dimerization.

GH Deficiency from Other Chromosomal Mutations

Interstitial Xq13.3-Xq21.1 deletions or microduplications of certain Xq regions result in X-linked recessive GH deficiency *(93)*. The phenotype for affected boys is variable. Patients may also have hypogammaglobulinemia, suggesting a contiguous Xq21.3-Xq22 deletion *(94)*. The growth hormone deficiency of Thode-Leonard syndrome (marked short stature, severe mental retardation, unusual facies) has also been shown to be linked to X-chromosome mutations. In 1992, Yokoyama et al. reported growth hormone deficiency and the empty sella syndrome in a child with Thode-Leonard who had a tandem microduplication of Xq13.3→q21.2 *(95)*.

End Organ Targets and Receptors

The GH receptor (GHR) gene has been isolated in humans to the proximal short arm of chromosome 5 (5p13.1-12) *(96)*. The translated product is a 620 amino acid protein encoded by nine exons (numbered, interestingly, 2–10) for the secretion signal (exon 2), extracellular domain (exons 3–7), transmembrane domain (exon 8), and intracellular domain (exons 9–10) *(97)*. The extracellular domain is also found in circulating serum as GH binding protein. The GHR has homology with the prolactin receptor, and it belongs to the cytokine family of receptors that are associated with JAK2, a ligand-activated tyrosine kinase *(98)*. JAK2 phosphorylates both the GHR and the cytoplasmic transcription factors known as STATs *(99)*. After phosphorylation, STATs dimerize and move to the nucleus, where they activate gene transcription.

In most tissues where GH acts, it activates transcription of the IGFs (Fig. 1). The IGFs are a peptide family with diverse metabolic roles that include mediating many of the anabolic and mitogenic actions of GH. IGF-1 is a basic 70 amino acid peptide, while IGF-2 is a slightly acidic 67 amino acid peptide *(100)*. Structurally similar to insulin, they are comprised of A and B chains connected by disulfide bonds *(101)*.

The IGF-1 gene, located on the long arm of chromosome 12, spans 95 kb and contains at least six exons *(102,103)*. Although GH appears to be the primary regulator of IGF-1 gene expression, transcriptional control is complex. It is influenced by nutritional status, GH, hCS, prolactin, glucocorticoids, sex steroids, thyroid hormones and insulin *(104–107)*. The IGF-2 gene is 35 kb in length, contains nine exons, and is located adjacent to the insulin gene on the short arm of chromosome 11 *(102,108)*.

The action of IGFs occurs through two IGF receptors and the insulin receptor. The type 1 IGF receptor gene appears to be the major somatogenic mediator. It is structurally closely related to the insulin receptor and binds both IGF-1 and IGF-2 with high affinity. It is located on the long arm of chromosome 15.

Unlike GH, the IGFs appear to play a major role in prenatal growth. Reece et al. and Verhaeghe et al. found a direct correlation between neonatal weight and cord serum IGF-1 levels *(109,110)*. In 1996, Roth et al. confirmed that cord IGF-1 levels correlate with fetal size even in the macrosomia associated with diabetic pregnancies *(111)*. It has been proposed that fetal IGF release is in part stimulated by growth hormone-like factors produced by the placenta in response to placental GHRH *(111,112)*.

The IGFs in plasma are complexed to carrier proteins with molecular weights of 28–150 kDa. These high-affinity binding proteins (BPs) serve to extend the IGFs serum half-life, to convey IGFs to target cells, and to modulate the IGFs interaction with their receptors. Six distinct human IGFBPs have been cloned and sequenced *(113,114)*; at least four IGFBP-related proteins have also been identified (reviewed in *115*). IGFBP-3 transports over 90% of the circulating IGF in adult serum. In general, IGFBPs modulate the

mitogenic and proliferative actions of IGF, apparently by competing with IGF receptors for IGF peptides and by transporting IGFs to target tissues *(116)*.

Growth Hormone Receptor Defects

As noted above, most patients with Laron syndrome have been found to have GHR defects. This was first indirectly proven in 1984 by Eshet et al., who found that patients with Laron syndrome had a lack of GH binding activity in liver biopsies *(117)*. In 1987, Baumann et al. found that these persons also did not have circulating serum GHBP *(118)*. The first GHR mutation was found by Amselem et al. in 1989 *(119)*. Since then, many other GHR mutations have been described (Table 4) *(120–129)*.

Many of these GHR mutations affect the extracellular domain and therefore manifest with absent or decreased levels of GHBP. Godowski et al. found one such mutation in two patients of Iranian descent, who had deletions of exons 3, 5, and 6 (with retention of exon 4) *(97)*. Berg et al. (1992) found a A→G substitution, which resulted in a new splice site and a truncated exon 6 *(121)*. Another identified GHR mutation is a Phe^{96}→Ser change from a T→C substitution in the extracellular domain *(119)*. This Phe^{96} is evolutionarily conserved in all members of this class of transmembrane receptors. A mutation in this amino acid does not diminish receptor binding, but interferes with intracellular trafficking *(130)*.

Other identified GHR defects do not affect the extracellular domain region, and therefore manifest with normal or even elevated GHBP levels. Screening of children with idiopathic short stature for GHR defects is now underway *(131)*. It has been hypothesized that GHR defects may prove to be a relatively common GHAD, accounting for up to 5% of all cases of idiopathic short stature *(132)*.

Patients with GHI from GHR defects often do not respond well to exogenous GH therapy. Some patients have been treated with IGF-1 *(133–135)*. In 1992, a 7-day trial of IGF-1 therapy in six adults with GHR defects revealed no adverse effects *(136)*. A subsequent recent collaborative study examined IGF-1 therapy over 2 years in five patients with GHI and high basal GH levels and in three patients with complete GHD and growth hormone antibodies *(133)*. The investigators found that with twice-daily subcutaneous IGF-1 treatment, these children initially had a greatly increased growth velocity (from 4.0 cm/yr pre-treatment to 9.3 cm/yr). After the first year, growth rate slowed (to 6.2 cm/yr), but was still significantly greater than pre-IGF-1 treatment. Some patients on high dose (120 μg IGF-1/kg twice daily) treatment developed hypoglycemia; others had selective acceleration of lymphoid and renal tissue growth. It remains to be seen if IGF-1 will cause sustained gain of height velocity without significant attenuation or undesirable side effects.

IGF-Related Defects

Children with primary IGF-1 deficiency have the same phenotype as those with GH gene deletions. A boy with severe prenatal and postnatal growth failure has been described with a homozygous partial IGF-1 gene deletion *(137)*. His growth failure was associated with bilateral sensorineural deafness, delayed motor development, and behavioral difficulties (hyperactivity and short attention span). He did not have a significant delay in bone age or hypoglycemia. An IGFBP-3 level was normal. This case suggests that IGF-1 has a role not only in GH action, but also in CNS development and function.

It is likely that GHAD also can result from post-GHR signal transduction defects (for example JAK2 defects) or from defects in the IGF-1 receptor. Such mutations could produce GH insensitivity with normal to high levels of IGFs or IGFBPs. These have been

Table 4
Described GHR Mutations

Domain	*Ref.*
Extracellular domain	
Cys38→Stop (homozygous)	*(120)*
Arg43→Stop (homozygous)	*(120)*
Glu44→Lys (compound heterozygote)	*(124)*
46 del TT (homozygous, compound heterozygote)	*(123)*
71+1 G→A (compound) heterozygote)	*(123)*
Phe96→Ser (homozygous)	*(119)*
Cys122→Stop (heterozygous)	*(124)*
Pro131→Gln (homozygote)	*(129)*
Arg161→Cys (compound heterozygote)	*(124)*
Codon 180 A→G (homozygous)[a]	*(121)*
189-1 G→T (homozygous)	*(123)*
Arg211→His (heterozygous)	*(124)*
Arg217→Stop (heterozygous?)	*(123)*
Glu224→Asp (heterozygous)	*(124)*
Glu224→Stop (compound heterozygote)	*(127)*
230 del TA or AT (homozygous)	*(123)*
Complex gene deletion of exons 3,5,6	*(97)*
Transmembrane domain	
Exon 8 splice donor site G→C[a] (homozygous)	*(125)*
Intracellular domain	
Codon 310 C deletion/frameshift (compound heterozygote)	*(127)*
Cys422→Phe (heterozygous)	*(122)*
Pro561→Thr (heterozygous)	*(122)*
Exon 9 splice acceptor cite G→C (homozygous)	*(126)*
Intron 9 splice donor site	*(128)*

[a]Does not change encoded amino acid, but changes splice site.

searched for but are yet to be described; it appears that IGF-related mutations will be an infrequent cause of clinical GH deficiency *(138)*. It is possible that mutations that inactivate IGF-1 are lethal.

Some cases of growth failure, however, have been attributed to IGF-1 resistance *(139–141)*. Bierich et al. described a child who was 3 kg and 48 cm at birth, and by the age of 3 had a height 8 standard deviations below the mean *(140)*. Laboratory analysis showed that she had elevated GH levels with elevated IGF-1 levels. Cultured skin fibroblasts had a 50% reduction in IGF-1 binding capacity. Subsequent study by Heath-Monnig et al. in a similar patient showed that the ability of IGF-1 to stimulate fibroblast α-aminoisobutyric acid uptake was markedly diminished compared to control subjects *(141)*. The reported patients have had birth lengths less than the fifth percentile (48, 47.5, and 45 cm), again emphasizing the importance of IGF-1 in fetal growth.

DIAGNOSIS OF GH DEFICIENCY

Classical Evaluation

Diagnosing GHD is not a simple process, but involves multiple laboratory and clinical criteria. Since the late 1950s, when it was first recognized that GH isolated from human

pituitaries would stimulate growth in children with deficient GH secretion, clinicians and clinical scientists have been exploring ways of defining which children would benefit from available GH therapy. The introduction of expensive, commercial, recombinant DNA-derived hGH has only intensified the need to perfect diagnostic tools.

Current practice incorporates a combination of clinical, auxologic, and laboratory criteria to define GHD. This begins with an evaluation of stature, relative to genetic expectations, and growth velocity, calculated from serial height determinations. Children who demonstrate consistently subnormal growth velocities for age are candidates for further screening. This generally begins with exclusion of non-GHAD causes of poor growth and incorporates a thorough history, physical examination, bone age assessment, and laboratory screening as appropriate. The majority of children referred to endocrine clinics for short stature will not have GHD. It is important to separate those children with normal variants of growth, such as constitutional delay of growth or puberty, or intrinsic short stature from those with chronic diseases that may be clinically silent except for their effects on growth.

Laboratory assessment of GH sufficiency is difficult because of the intermittent, pulsatile pattern of GH secretion. Single GH levels cannot predict overall GH pulse amplitude, a value that correlates with GH adequacy. The gold standard for diagnosis of GHD involves the administration of pharmacologic stimuli of GH followed by serial blood sampling. Choosing whom to test should be based primarily on objective criteria. Children who have documented subnormal bone age-adjusted growth velocities, severe delays in skeletal maturation, or obvious predispositions to pituitary dysfunction (including intracranial tumors or other pituitary hormone deficiencies) are deserving of further laboratory evaluations. IGF-1, IGFBP-3, and GHBP levels are commercially available and can be useful adjuncts in diagnosing GHD. However, these tests are also problematic since some children with GHD may have values in the normal range *(142–144)*. Similarly, some children with malnutrition or liver disease may have low levels of these growth factors.

In most pediatric endocrine centers, provocative testing procedures for GH adequacy incorporate two of the following GH stimuli: L-dopa, clonidine, arginine, propranolol, glucagon, insulin induced hypoglycemia, and/or exercise. An inadequate response (currently defined in most centers as peak GH levels of <10 ng/mL) suggests GHD. Unfortunately, even provocative GH testing is not a reliable gold standard: age and sex-specific norms are inadequately determined, GH assays in use are not well-standardized, and the specificity and result reproducibility of GH tests are poor *(145)*. A consensus statement published by 16 endocrinologists at 14 centers in 1994 concluded that careful auxological evaluation, supplemented by assessment of appropriate elements of the GH-IGF axis, provides the best foundation for a rational diagnosis of GHD *(146)*.

New Avenues

Most isolated GHD is sporadic; thus, genetic screening of all cases for gene mutations is not feasible at present. However, new molecular techniques may soon make more widespread surveying possible. Evaluations for inherited GHAD should be considered in any family with a history of consanguinity or a second case of GHAD.

Initial observations of *GH-1* deletions in patients were made using Southern blot analyses *(64)*. PCR techniques provide an easier screening tool as they make it possible to amplify specific sequences from complex genomic samples directly. Vnencak-Jones

et al. in 1990 described a technique where PCR amplification, followed by digestion with the restriction enzymes *Bgl*I, *Hae*II, and *Sma*I, and then visualization of DNA fragments, could identify individuals with a variety of *GH-1* gene deletions *(147)*. This technique is useful for screening, but Southern blot analysis is still necessary in order to obtain the size of the deletion.

PCR-based single-strand conformational polymorphism (SSCP) analysis uses differential electrophoretic mobility to identify subtle conformational differences in single-stranded DNA *(148)*. These differences can be evident even from a change as small as a point mutation resulting in a single base substitution. This technique also allows for monitoring of cosegregation by restriction-length polymorphism (RFLP) analysis.

Dideoxy fingerprinting (ddF) improves SSCP analysis, bringing its accuracy for detecting single base changes to nearly 100% *(149)*. It also allows for amplification of large segments and subsequent screening of smaller regions. Reverse transcription PCR (RT-PCR) allows analysis of mutant mRNA transcripts to determine the effects of the genetic mutations in specific tissues *(150)*. In the future, it is probable that these newer molecular diagnostic techniques will prove useful both in the prenatal and postnatal diagnosis of GHAD *(151)*.

CONCLUSIONS

The regulation of growth by elements of the GH axis is a complex process that we have only begun to understand. We are currently in an exciting era of basic and clinical investigations of growth hormone axis defects. Further evaluation of the genes and physiology involved in the production, secretion, and actions of GH will continue to clarify the molecular basis of other growth hormone axis defects. This, in turn, may allow us to target new diagnostic and therapeutic strategies for affected patients.

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12 Growth Hormone

Relevance to Pediatrics

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CONTENTS

INTRODUCTION

Pituitary human growth hormone (hGH), a 191-amino acid peptide, is responsible for a spectrum of biologic effects including: skeletal growth; nitrogen, sodium and phosphorus retention; calcium excretion, carbohydrate and fat metabolism and stimulation of insulin-like growth factor (IGF-1) production. The location of the hGH gene is in close proximity to the genes for human chorionic somatomammotropin (hCS), within a 50 kilobase region on chromosome 17 *(1)*. Ten percent of pituitary dry weight is GH which is 800-fold greater in quantity than any of the other pituitary hormones *(2)*.

Secretion of GH results from a complex series of events influenced by a multitude of (neuro)modulators, as well as nutritional status and health of the organism, sleep stage, and hormonal milieu. Recombinant DNA technological development has promoted our ability to produce unlimited quantities of this peptide, fostering research into its use as

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a therapeutic agent. Newer areas of clinical investigation in children include: GH therapy in burn victims and short bowel disorders *(3)*; attempts to correct the growth retardation seen in chondrodysplasia syndromes *(4)* and non-GH-deficient short stature *(5)*, continue to keep GH at the forefront of pediatric endocrine research.

This chapter focuses briefly on the complex biochemical events related to dysregulation of GH secretion as seen in a variety of pathophysiologic conditions in pediatrics and our clinical experience with the use of spontaneous GH secretory profiles in a variety of settings. Although our knowledge of the dynamics of GH secretion continues to expand, we still need to search for improved methods of identifying those individuals who will most benefit from the use of exogenous hGH therapy *(5)*.

PULSATILE GH SECRETION AND REGULATORY MECHANISMS AFFECTING GHRH AND SOMATOSTATIN SECRETION

Pulsatile GH secretion is under the control of two hypothalamic peptides, growth hormone-releasing hormone (GHRH) and somatostatin (somatotropin release-inhibiting factor/SRIF). Our current understanding comes from a series of classical experiments which demonstrated that SRIF modulates GH trough levels and is important in the inhibition of GH secretion, whereas GHRH regulates pulsatile GH release from the anterior pituitary gland *(6)*.

The timing of a spontaneous GH surge influences the effect of GHRH on GH secretion. Identical doses of GHRH stimulate a greater GH secretory response when administered during a spontaneous GH secretory episode, a time when SRIF is low, as compared to a GH trough when SRIF secretion is increased *(7)*. A variety of neuromodulators (neurotransmitter, pharmacologic, cytokine, and so forth) capable of disrupting this intrinsic rhythm, enhance GH secretion by inhibiting somatostatin release or by stimulating GHRH release. Neurotransmitters involved in GH secretion include adrenergic, dopaminergic, cholinergic, and serotonergic pathways *(8,9)*.

PHYSIOLOGIC STATES AFFECTING GH SECRETION

Sleep

Secretion of GH coincides with the sleep onset, reaching peak levels within 1 h *(10)*. Further, secretion of GH is associated with slow-wave sleep and is not influenced by rapid-eye movement sleep *(11)*. Sleep appears to not only facilitate, but also to augment GH secretion, with GH pulse frequency nearly twofold greater than during awake hours *(12)*. GHRH-stimulated GH secretion is inhibited following awakening when compared with the undisturbed sleep state, a possible role of SRIF. Sleep-onset, then, appears to promote pituitary GH release via alterations in somatostatin tone *(13)*.

Glucose

Glucose administration acutely inhibits GH secretion, whether in the presence or absence of provocative stimuli *(14,15)*. Infants demonstrate a paradoxical increase in GH concentrations following glucose administration during the first week of life *(16)*. Acute hypoglycemia is a potent stimulus for the release of GH, a counterregulatory hormone *(17–19)*. The GH responses to hypo- and hyperglycemia are mediated through alterations in hypothalamic somatostatin secretion *(7)*.

Amino Acids

Arginine and ornithine are potent secretogogues for GH release. Arginine enhances the GH response to GHRH administration, even in the face of increased SRIF tone. Pyridostigmine does not potentiate the GH response to arginine, suggesting that arginine itself inhibits SRIF release directly *(7)*. Leucine produces only modest increases in plasma GH, whereas valine and isoleucine administration result in equivocal GH responses *(20)*.

Free Fatty Acids

Parenteral lipid formulations that raise serum free fatty acid (FFA) levels are associated with a diminished GH response to GHRH in adult men *(21)*. The inhibitory effect of FFAs on GH secretion is thought to be related to their effects on the lipid bilayer of somatotropes, which in turn may alter stimulatory signals directed towards the somatotrope or inhibit the secretory capacity of the cell *(7)*. FFA-induced inhibition of GH secretion is specific only to GH.

Fasting

Fasting enhances pulsatile GH release in normal adult subjects *(22)*. GH secretion in healthy subjects following a 32–56 h fast increased fivefold in the 24-h endogenous GH production rate compared to subjects on a controlled diet, and twofold in the number of GH secretory bursts and mass of GH secreted per burst. Insulin-like growth factor 1 (IGF-1) concentrations were unchanged. Thus, starvation-induced increases in GH secretion appear to be mediated by the effects of increased GHRH release or reduced SRIF tone *(22)*. Similar results are expected in children.

PATHOPHYSIOLOGIC STATES AFFECTING GH SECRETION APPLICABLE TO CHILDREN

Stress/Illness

Although many of these studies were done in adults, the results are applicable to children. Exercise increases GH levels in normal subjects, an effect inhibited by naloxone, atropine and oral glucose administration *(23)*. Elevated plasma GH levels are seen following acute trauma, major surgery, and electroconvulsive therapy, with mild increases observed following venipuncture *(20)*. Twenty-four-hour GH secretory profiles during severe illness are characterized by higher basal levels of GH and reductions in serum IGF-1, but no differences in mean GH concentration or number of GH pulses *(24)*. The dissociation between GH and IGF-1 is similar to that seen in catabolic states including prolonged fasting, nutritional dwarfing and anorexia nervosa.

Major depression is associated with increased GH secretion and higher 24-h urinary cortisol measurements *(25)*. Differences in cortisol and GH response to cardiac catheterization appears to correlate best with individual coping behavior. Calm, depressed patients show no increase in plasma GH or cortisol, whereas anxious subjects have elevations of both GH and cortisol *(26)*.

GH concentrations are increased in subjects with inflammatory disease or experimental endotoxemia *(27)*. Receptors for the interleukins, or inflammatory cytokines, are found in nearly all endocrine glands, specifically in the hypothalamus, pituitary, adrenal, thyroid, testis/ovary, and islet cells. Through a complex feedback system

unique to each cytokine, GH secretion is influenced via autocrine, paracrine or endocrine mechanisms. Growth retardation, however, is a common feature of most chronic inflammatory diseases, which suggests some interference in GH or IGF action at the level of peripheral tissues.

Nutritional Dwarfing/Anorexia Nervosa

Nutritional dwarfing, an entity characterized by nutritional deprivation, body weight below 90% of ideal, growth retardation and growth failure, is associated with increased serum GH and decreased IGF-1 concentrations. The dissociation between GH and IGF-1 suggests that impaired somatic growth is related to reduced IGF-1 synthesis or action, whereas GH may mediate the metabolic adaptation to starvation through its effects on hepatic glucose production, lipolysis, and nitrogen conservation *(22)*. In a recent study of 16 children with nutritional dwarfing, pubertal subjects had reduced mean 12-h GH concentrations in subjects *(28)*. Spontaneous overnight GH secretion appears to be more sensitive to the effects of chronic undernutrition, and the pubertal subject is at particular risk for impaired GH secretion and potential compromise of final adult height. Despite these clinical findings, GH concentrations in a variety of malnourished states appears variable *(28)*.

Anorexia nervosa, a psychiatric disease characterized by a disordered body image, severely limited caloric intake and body weight well below ideal, has been associated with elevated GH concentrations and a variable response to provocative stimuli *(29)*. Studies using GHRH as a secretogogue demonstrate a variable GH response to food, in a manner similar to what has been observed in obese subjects, a group with unique neuroendocrine dynamics including blunted GH secretion.

Subjects with "fear of obesity," an eating disorder characterized by poor growth and delayed sexual development owing to caloric restriction over fear of becoming obese *(30)*, is not associated with abnormal GH secretion. A spectrum of pituitary responsivity to stimuli was noted in nine subjects, distinct from that observed in anorexia nervosa and related to the degree of individual undernutrition *(30,31)*.

In a study conducted in France *(31)*, vitamin A and GH secretory status in 68 healthy, short prepubertal children was examined. Plasma vitamin A concentrations correlated positively ($r = +0.64$) with plasma GH during the night. Further, a group of 12 children with neurosecretory dysfunction and low vitamin A intake demonstrated significant increases in overnight GH secretion after 3 mo of supplemental vitamin A *(31)*. The role of vitamin A and other fat-soluble vitamins as it relates to GH secretion needs to be further clarified; however, for subjects with cystic fibrosis or other forms of pancreatic insufficiency, this study provides additional support to the critical role nutrition plays in influencing GH secretory dynamics.

Diabetes Mellitus

Debate exists regarding the heights of children and adolescents with newly diagnosed Type 1 diabetes mellitus (IDDM) *(32–34)*, however, it appears that growth deceleration may be seen prior to islet cell failure and overt symptoms of diabetes *(35)*. Further, poor metabolic control of IDDM is associated with chronic elevation of serum GH concentration, growth retardation, and delayed sexual development *(36,37)*. The metabolic effects of the elevated GH concentrations have been implicated as a causative factor in the development of diabetic retinopathy and other microvascular complications *(38)*.

In 52 adolescents with IDDM, GH secretory dynamics and IGF binding proteins (IGFBPs) were assessed during puberty *(39)*. Subjects were divided into two separate groups based on glycemic control. Using overnight GH sampling studies, no significant differences were found in the two groups with respect to total GH secretion, number of GH pulses, or GH peak amplitude. The data were similar to that seen in non-IDDM adolescents *(39)*. Other investigators report diminished ^{125}I-GH binding to the high-affinity GH binding protein (HA-GHBP), associated with higher random, unstimulated serum GH levels. The authors propose the existence of a serum GH inhibitor, which may reduce in vivo and in vitro binding of GH to its binding protein and thus impair its bioactivity *(40)*. IGFBP3, the binding protein most closely correlated with GH secretion, was reduced in IDDM subjects during pubertal stages 3 and 5; no increase in IGFBP3 occurred with advancing age in contrast to controls. IGFBP1, a GH-independent binding protein, is inversely related to insulin concentration. IGFBP1 levels were elevated throughout puberty in diabetic subjects when compared with controls; IGFBP1 was positively correlated with hemoglobin A1C concentration in a subgroup of poorly controlled diabetics (hemoglobin A1C >8.5%), suggesting lower insulin levels. IGFBP1 has been demonstrated to inhibit growth of chicken embryo cartilage *(39)* and suppress IGF-1 bioactivity *(41)*. Elevation of IGFBP1 and depression of IGFBP3 and IGF-1 in IDDM subjects with poor metabolic control may provide one explanation for poor linear growth.

Following a 4-wk period of intensive insulin and dietary intervention to improve glycemic control in six adolescents with poorly controlled IDDM, there was no change in the mean 24-h GH concentration, pulse frequency or amplitude. Serum IGF-I concentrations improved significantly, reflecting improved GH sensitivity and partial correction of a GH resistance-like state *(37)*. Enhanced GH secretion in the face of chronic hyperglycemia and following GHRH administration appears consistent with a state of diminished somatostatin tone in subjects with diabetes *(37)*.

Hypothalamic Lesions

Lesions in the hypothalamus and pituitary stalk affect GH secretion in humans. Magnetic resonance imaging of the central nervous system in subjects with hypopituitarism demonstrate an increased incidence of anterior lobe hypoplasia, attenuation or transection of the pituitary stalk, and formation of an ectopic posterior pituitary lobe at the base of the hypothalamus *(41,42)*. Pituitary stalk transection or total hypophysectomy results in attenuation of the GH response to insulin associated with low or undetectable basal levels of GH *(44)*. The diencephalic syndrome, a disorder frequently associated with lesions of the anterior hypothalamus, is characterized by elevated plasma GH levels and a paradoxical rise in GH secretion following glucose administration, reflecting dysregulation in hypothalamic GH-inhibiting pathways *(45)*.

Cranial Irradiation

The deleterious effects of cranial irradiation on GH secretion are an unfortunate and common morbidity for individuals with a variety of neoplastic and hematologic diseases. The initial studies detailing the hypothalamic-pituitary effects of cranial irradiation exposure were performed with male rhesus monkeys (40 Gray), demonstrating a blunted GH secretory response to insulin hypoglycemia and a decrease in GH pulse frequency and amplitude. Doubling the dose of insulin (0.1–0.2 units/kg) normalized the GH response, suggesting an intact, but altered or "reset" hypothalamic sensitivity for influencing GH secretion (Figs. 1 and 2) *(46)*.

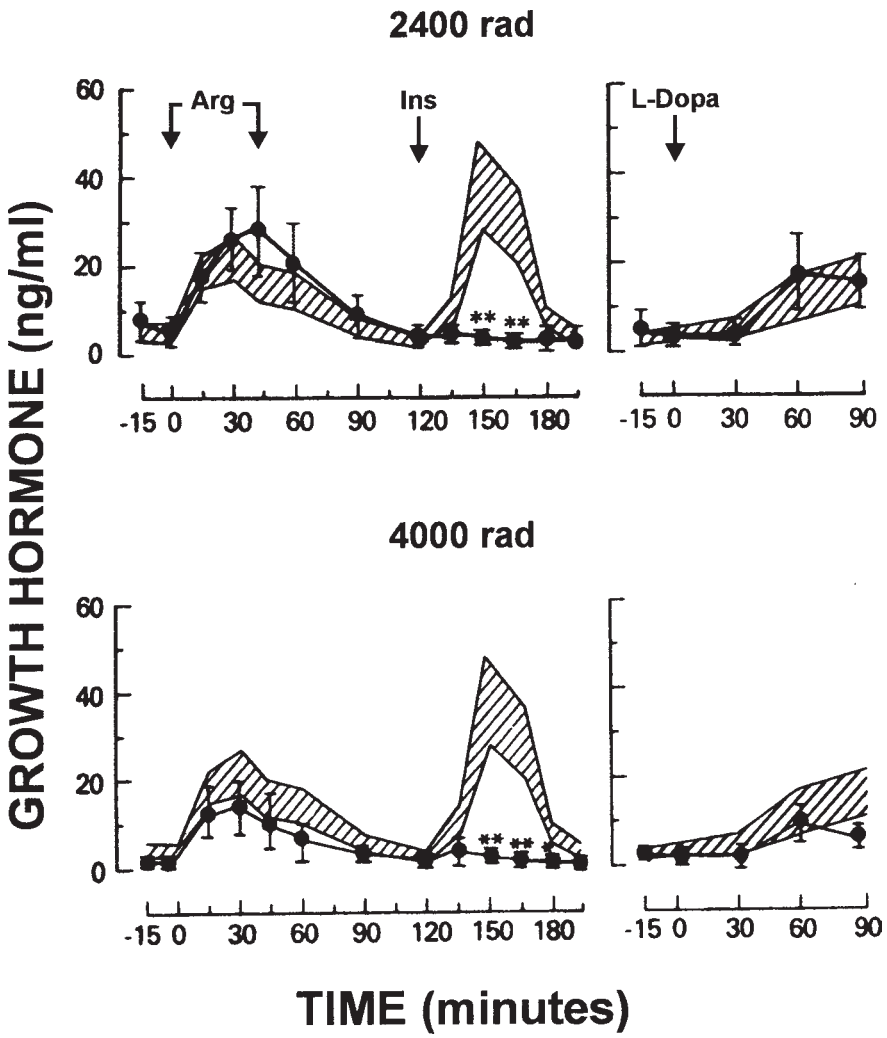

Fig. 1. GH responses to arginine (Arg), insulin (Ins), and L-dopa stimulation 50 wk after cranial irradiation. Primates treated with 2400 rads (open circles, $n = 4$) and 4000 rads (closed circles, $n = 4$) showed a normal response to arginine and L-dopa, but a blunted response to insulin (0.1 U/kg, iv). The shaded area represents the mean ± SEM from 9–13 controls. *, $p < 0.001$. Reproduced with permission from ref. *46*.

Subsequently, cranial irradiation at lower doses (24 Gray [Gy]) has been associated with growth retardation and diminished spontaneous GH secretion in subjects with acute lymphoblastic leukemia (ALL) *(47)*. Twenty-four h sampling of spontaneous GH secretion appears to be a more sensitive means of identifying quantitative and qualitative abnormalities in GH secretion, including reductions in GH pulse amplitude and frequency (Fig. 3). A "normal" GH secretory response to provocation in subjects with a history of cranial irradiation associated with an abnormal 24-h GH study suggests the presence of selective defects in neurotransmitter control of GH secretion *(47)*. The term "neurosecretory dysfunction" was coined to describe subjects with growth retardation and neuroregulatory abnormalities of GH secretion.

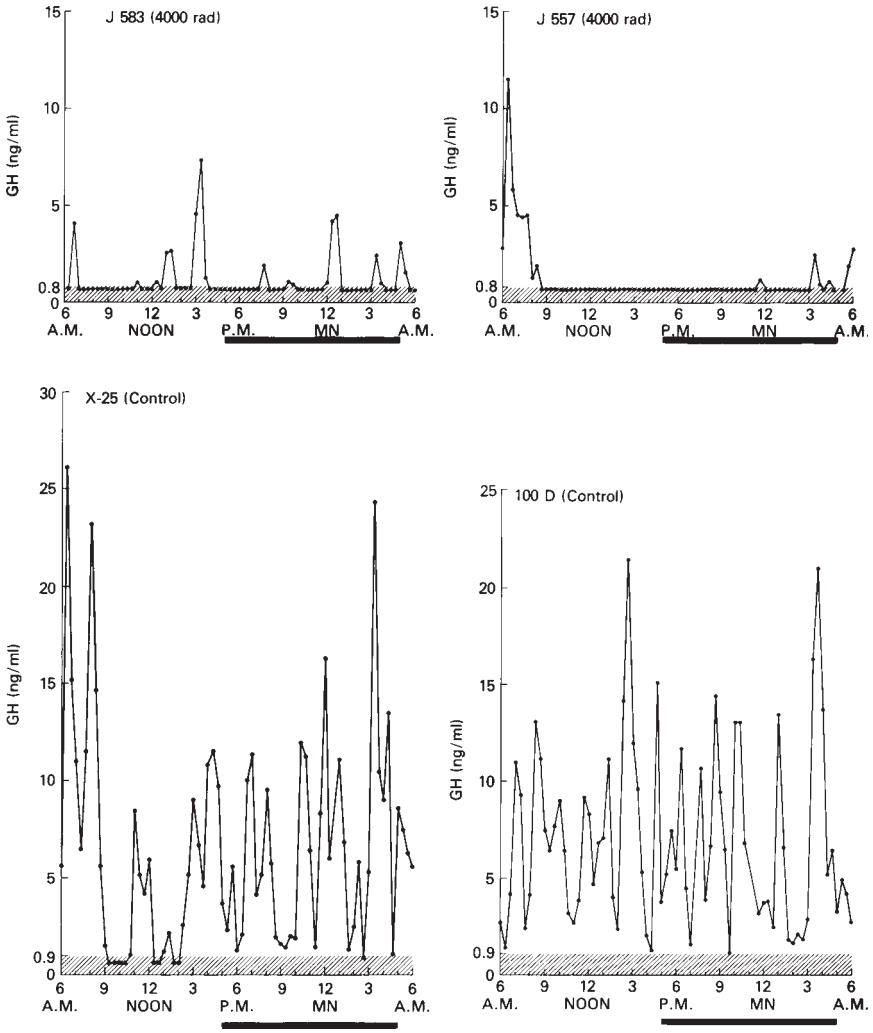

Fig. 2. GH secretory pattern over 24 h in two primates treated with cranial irradiation (4000 rads; top panel) and two normal controls (bottom panel). The study was performed 1 yr after treatment. There was a decrease in the number (frequency) and amplitude of secretory spikes in the animals that received radiation. The shaded area represents the detection limit of the assay. The dark period was from 1700–0500 h (solid bar). Reproduced with permission from ref. *46*.

We also examined 24-h studies of spontaneous GH secretion in a group of children following central nervous system or total body irradiation for acute lymphoblastic leukemia (ALL) and a variety of other central nervous system (CNS) tumors not involving the hypothalamic-pituitary axis. Mean 24-h GH concentrations were significantly reduced (1.8 ± 0.2 vs 3.9 ± 0.3 μg/L, treatment vs controls; $p < 0.001$). Blunting of the peak GH response to a variety of provocative stimuli again suggests widespread neuronal damage affecting neurotransmitter regulation of GH secretion including dopaminergic (levodopa); noradrenergic (clonidine, propranolol); GABAergic (valproic acid); and cholinergic (pyridostigmine) neurons. GH response to a serotonin-like compound (L-tryptophan) was not significantly affected *(9,48)*.

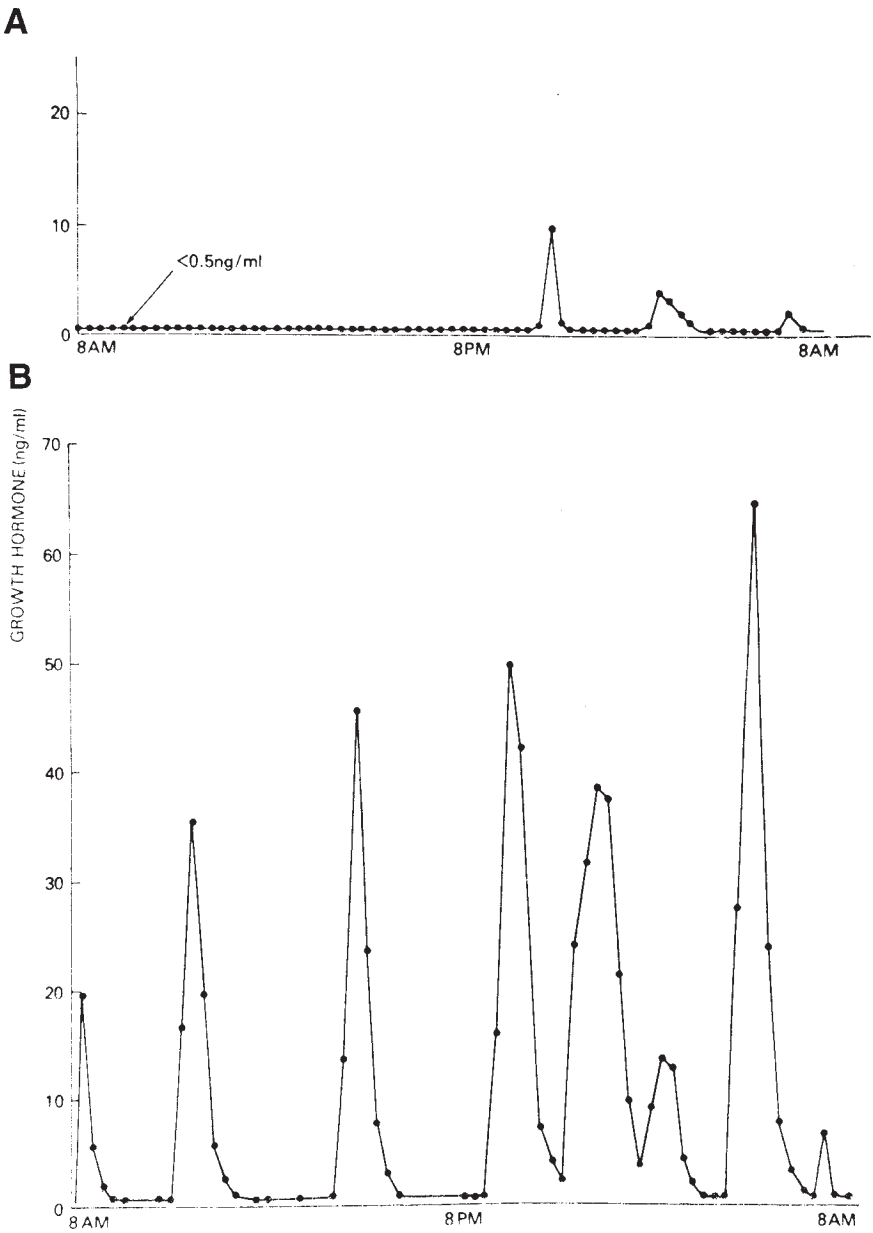

Fig. 3. Spontaneous pulsatile growth hormone secretion in **(A)** a representative patient with acute lymphoblastic leukemia who had received CNS-preventative therapy with 2400 rads cranial radiation and intrathecal methotrexate, and **(B)** a representative normal child. Reproduced with permission from ref. *47*.

A study of 82 children (0.2–18.9 yr; median 4.3 yr) following cranial or craniospinal irradiation with doses between 24–45 Gy for primary brain tumors not involving the hypothalamic-pituitary axis or prophylaxis against central nervous system leukemia identified a GH deficiency in 74% (insulin hypoglycemia). Children receiving doses greater than 30 Gy developed GH deficiency faster, with 100% of subjects GH-deficient

within 3 yr *(49)*. Analysis of GH secretion in 28 children with ALL 4.1–10.6 yr (median 8.2) following treatment with 1800 cGy of prophylactic cranial irradiation identified 64.3% GH-deficient after arginine and levodopa provocation and 81.5% with diminished overnight spontaneous GH secretion. These biochemical abnormalities correlated with magnetic resonance imaging findings that included empty sella in 25% and a reduction in anterior pituitary lobe height correlating with the GH peak response to arginine and the mean overnight GH concentration *(50)*.

Replacement therapy with biosynthetic hGH increases growth velocity in GH-deficient subjects following cranial irradiation, but final height is significantly less than the midparental height. These findings are reflective of the lag time before initiation of hGH therapy and the detrimental effects of irradiation, especially to the growing spine, in young children *(51)*.

Obesity

The GH response to a variety of provocative stimuli (insulin-hypoglycemia, arginine, opiates, glucagon, levodopa and GHRH), is diminished in obese subjects *(52)*. One study evaluating spontaneous GH secretory dynamics in obese men (body mass index [BMI] >42) demonstrated a reduction in endogenous GH half-life relative to control subjects (BMI < 31), a daily production rate of GH 4.1-fold less compared with controls and a threefold decrease in GH secretory burst frequency despite preservation of the GH ultradian rhythm *(52)*. This principle applies to children as well.

Cholinesterase inhibitors, including pyridostigmine, decrease somatostatin tone. Following exogenous pyridostigmine, obese subjects have an increase in the GH secretory response to provocative stimuli, supporting the SRIF hypothesis. These reports suggest the postprandial period is associated with reduced cholinergic tone and a parallel increase in somatostatin tone in normal and obese subjects *(53)*. Sustained weight reduction in man and experimental animals leads to a partial restoration of GH release in obese subjects *(52)*. Short-term very low-calorie diets appear to have no effect on the 24-h GH secretory profile *(54)*.

Other Clinical Settings

We and others have demonstrated GH neurosecretory abnormalities in other clinical settings including renal failure *(55)* and high altitude *(56)*.

HORMONAL REGULATION OF GH SECRETION: PHYSIOLOGY AND PATHOPHYSIOLOGY

Although many of the studies reviewed in this section were done in adults, they can be generalized to children.

Thyroid Hormone

Spontaneous and stimulated GH secretion is markedly attenuated in clinical and biochemical hypothyroidism in humans; this abnormality corrects during thyroid hormone replacement *(7,57,58)*. Thyroid hormone deficiency is associated with reduced hypothalamic GHRH production, leading to GH deficiency and down-regulation of GHRH receptor numbers on pituitary somatotropes *(7,59)*. Thyroid hormone facilitates binding of GHRH to its receptor. GH secretion is reduced in hypothyroid subjects following

pyridostigmine and arginine administration *(60)*, substances known to decrease somatostatin tone *(61,62)*. GH synthesis, rather than secretion, appears to be impaired in hypothyroidism *(59)*.

Hyperthyroid subjects have significant increases in 24-h GH pulse frequency, augmented GH pulse amplitude, and 3.7-fold higher GH production rates, similar to the effects of sex steroids on GH secretion *(63)*. These observations of enhanced GH secretion in hyperthyroidism suggest some alteration in GH neuroregulatory control including increased somatotrope responsiveness to GHRH or a reduction in somatostatin tone, and is independent of IGF concentration *(63)*. On the other hand, other investigators report reductions in the 24-h GH secretory rate in untreated thyrotoxicosis and normalization following antithyroid therapy *(64)*. Further, the GH response to a variety of provocative agents (insulin, clonidine, and GHRH) is impaired *(65)*. Pretreatment with a β-adrenergic receptor blocker (propranolol) corrects and augments the GH response to GHRH and insulin *(65)*. Pyridostigmine and galanin have no effect on GHRH-induced GH secretion in hyperthyroid subjects *(59)*. Thus, it appears that hyperthyroidism may in part be related to altered somatostatin tone; however, the defective GH response to pharmacologic stimuli may be related to a chronic deficiency in GHRH or a direct effect of elevated thyroid hormone on the somatotrope *(59)*.

Thyrotropin-releasing hormone (TRH) stimulates GH secretion in a variety of conditions including acromegaly, anorexia, and depression (*20,66*; *see* ref. *66* for a more complete list). The mechanism for this paradoxical GH response to TRH is unclear, but may also reflect the presence of TRH receptors on pituitary somatotropes or impaired hypothalamic control of GH secretion *(66)*. TRH by itself does not affect GH secretion in humans, yet pretreatment with TRH decreases the mean peak GH response to dopamine, while augmenting peak GH levels when administered after dopamine infusion. Pretreatment with triiodothyronine (T3) blocks this inhibitory effect of TRH *(67)*. Thus, TRH appears to work at a different level when GH concentration is elevated (i.e., acromegaly, following dopamine infusion) compared to physiologic states with lower GH concentration.

Glucocorticoids

Glucocorticoids represent an enigmatic dilemma relative to their effects on GH secretion. In vitro data demonstrate glucocorticoids directly stimulate GH release from the anterior pituitary *(68)*, and regulate pituitary GH gene expression by augmenting GH gene transcription and by an increase in GHRH receptor number *(7)*. In vivo, glucocorticoids appear to blunt endogenous and stimulated GH secretion *(68)*. The proposed mechanisms by which glucocorticoids effect this dual role in the regulation of GH secretion include: 1) facilitating GH secretion by enhancing pituitary somatotrope responsiveness to GHRH by increasing cAMP *(68)* and increasing somatotrope GHRH receptor number *(69)*; and 2) inhibiting GH release by stimulating hypothalamic SRIF release through glucocorticoid-enhanced β-adrenergic receptor responsivity *(7)*.

The in vivo effects of increased glucocorticoid concentrations is to blunt GH secretion in physiologic systems. Through the use of a variety of pharmacologic stimuli, investigators have attempted to identify which agent(s) can reverse glucocorticoid-induced inhibition of GHRH-induced GH secretion. Despite their utility as potent GH secretogogues, dopaminergic agonists (bromocriptine, apomorphine) have no effect on glucocorticoid-inhibition of GH release *(68)*. Short-term (12 h) and long-term (4 d)

exposure to dexamethasone blunts GHRH-induced GH secretion, whereas subjects with Cushings syndrome, and acromegalic individuals receiving steroids, have a blunted GH response to GHRH *(68)*.

Corticosteroid therapy longer than 48 h in duration blunts the effect of insulin on GH secretion, whereas acute administration of glucocorticoids in normal volunteers leads to a normal response. Thus, the duration of exposure to steroids was influential on the subsequent GH response *(68)*. Arginine stimulates GH secretion by inhibiting SRIF release *(70)*. In the presence of glucocorticoids, arginine has had a variable effect on GH secretion, suggesting that either arginine and corticosteroids have a common site of action or arginine-induced GH secretion is unaffected by glucocorticoids *(68)*.

Blunted GHRH-induced GH secretion in Cushing syndrome subjects was not affected by the addition of pyridostigmine, whereas others have demonstrated partial restoration of GH secretion in healthy volunteers receiving dexamethasone and children on chronic glucocorticoid therapy *(68)*. Clonidine appears to partially restore and propranolol augments GHRH-induced GH secretion following dexamethasone administration *(71)*. Glucocorticoid exposure increases β-adrenergic receptor number on SRIF-neurons *(72)*. Propranolol therapy inhibits SRIF release, whereas clonidine, a postsynaptic ($\alpha 2$) receptor agonist is only able to influence a partial GH response in the presence of glucocorticoid *(68)*.

GABAergic (gamma-aminobutyric acid) agonists (baclofen) influence GH release through the dual mechanism of enhanced GHRH tone and inhibition of SRIF *(68)*. Patients suffering from depression associated with hypercortisolism demonstrated blunting of GH response to GHRH following baclofen (a structural analog of GABA) administration *(73)*. Prolonged hypercortisolism appears to blunt this GABAergic mechanism of GH release.

Although there are numerous studies of in vivo glucocorticoid inhibited-GH release, there remain conflicting reports of augmented GH secretion in the presence of steroids. One report demonstrated a change in the pattern of GH secretion (a reversal of the ultradian rhythm), but the total amount of GH secreted was unchanged *(74)*. In another study, there was an increase in GH pulse frequency and mass per GH burst and an overall increase of two and a half times the amount of GH release *(75)*. These stimulatory effects on GH secretion were duration-dependent (short-term) and independent of the dose or the type of steroid used *(76,77)*.

Significantly, from a pediatric perspective, the hallmark of endogenous or exogenous glucocorticoid excess in children is growth failure as a result of inhibition of GH secretion and IGF-1 production, protein catabolism, and the direct effect of glucocorticoid on bone. Glucocorticoids inhibit IGF-1 bioactivity, both following acute and chronic administration of glucocorticoids and in the absence of measurable reductions in IGF-1 concentrations *(78)*. The reduction in IGF-1 activity is a result of increases in circulating IGF inhibitors, a common finding following steroid therapy *(79)*.

A study of nine children with chronic renal failure and short stature (>2 SD below mean for age) treated with recombinant hGH following renal transplantation demonstrated improvement in growth velocity after 12 mo of therapy; however, all subjects were receiving either alternate-day or low-dose glucocorticoid immunosuppression *(80)*. Anecdotal reports of exogenous GH therapy in subjects receiving chronic daily glucocorticoid immunosuppression have not demonstrated improvement in linear growth and this therapy has the theoretical disadvantage of worsening carbohydrate intolerance and antagonizing the therapeutic benefit of glucocorticoid-mediated immunosuppression.

A report of 83 slow-growing subjects receiving corticosteroid therapy for a variety of disorders (post-transplant, inflammatory disease, asthma, other) under the auspices of the National Cooperative Growth Study, revealed a doubling of growth velocity in the first 2 yr of therapy. There were 16 adverse events, but only 6 related to GH therapy (transplant rejection [2], slipped capital femoral epiphysis [1], diabetes mellitus [1], irritability [1], musculoskeletal [1]) *(81)*. Presently, the short-term benefits of exogenous GH therapy must be weighed against the unknown long-term benefits on final height and potential adverse outcomes.

Sex Steroids

Sexual dimorphism of GH secretion is well-recognized in the rat and characterized by high-amplitude GH pulses occurring at precise 3.3-h intervals and low or undetectable GH troughs in males, whereas females exhibit irregular, more frequent, low-amplitude GH pulses with elevated basal GH levels, no distinct troughs, and slower somatic growth relative to male animals *(82)*. 17β-estradiol administration to gonadectomized and sham-operated adult male rats converts the typical male GH secretory profile to a female-like pattern. Previous work in gonadectomized neonatal male rats has demonstrated reduced GH pulse amplitude that was fully restored by testosterone replacement therapy, providing evidence for the importance of androgen in maintaining the GH secretory profile of the male rat *(82)*.

Estradiol-treated rats (sham and gonadectomized) demonstrate a regular pattern of GH responsiveness to GHRH that is typical of what is observed in female rats *(82)*. Loss of this cyclic responsiveness to GHRH is thought to be mediated through alterations in hypothalamic SRIF secretion. The sexual dimorphism of GH secretion in rats, therefore, appears to be related to the temporal pattern of SRIF secretion with females showing a more continuous pattern of SRIF release compared with a more pulsatile pattern in males. The feminizing effects of short-term exposure to estradiol in both sham-operated and gonadectomized rats identifies estradiol as another important modulator of SRIF tone in the neuroregulation of GH secretion and responsible at least in part for the dimorphic nature of GH secretion in the rat *(82)*.

Previous studies of spontaneous GH secretion in humans demonstrates a lack of gender-specific GH secretion until the peripubertal and pubertal periods *(83,84)*, characterized by enhanced GH secretion in response to the opposing effects of testosterone and 17β-estradiol on SRIF release. Testosterone appears to stimulate SRIF release; whether this is related to the direct effects of testosterone or secondary to central aromatization to estradiol is unclear. Spontaneous GH secretion in women is characterized by more frequent GH secretory bursts when compared with men. Trough GH values are slightly lower in men *(7)*.

One recent study demonstrated significant increases in GH release in adult men, 19–24 yr, following pretreatment with increasing doses of pyridostigmine prior to GHRH administration, while adult women failed to respond in a similar fashion to pyridostigmine *(85)*. Previous studies have shown no differences in the GH response to GHRH during the different phases of the menstrual cycle, suggesting that men have higher SRIF tone associated with low cholinergic control of GH. Women appeared to have higher cholinergic tone associated with diminished SRIF levels and a failure of pydridostigmine to augment GHRH-stimulated GH release. Further, women had more pyridostigmine-related side effects presumably related to presence of enhanced cholinergic tone *(85)*. These observations are likely applicable to adolescents.

Studies of spontaneous GH secretion in normal boys and girls show enhanced GH secretion during the pubertal period, characterized by an increase in GH released per secretory event (amplitude), independent of any changes in pulse frequency, duration or GH half-life *(86)*. The change in the GH secretory profile is most evident at night, with a mean nighttime GH concentration highest at testicular volume 10–15 mL in boys *(84)*. Twenty-four-hour spontaneous GH values in late puberty were triple the values seen in prepubertal subjects, and twice the levels seen in adults. The 24-h secretory rates were inversely correlated with BMI and positively correlated with plasma IGF-1 concentrations *(86)*.

Subjects with gonadotropin-dependent and independent forms of precocious puberty also manifest augmented GH secretion. Treatment of central precocious puberty with gonadotropin-releasing hormone (GnRH) agonist therapy reduces mean nighttime GH secretion after 6 mo of gonadal suppression *(87)*; however more recent studies demonstrate spontaneous nocturnal GH secretion is not altered and suggests that decreases in growth velocity associated with GnRH agonist therapy is related to the direct effects of sex steroids on skeletal growth and not to alterations in GH secretion *(88)*. Women using oral contraceptives have greater mean peak GH response to provocative stimuli when compared with women on no medication *(89)*. Estrogen pretreatment prior to GH provocative studies is frequently used to "prime" prepubertal and peripubertal subjects to improve the likelihood that a normal child will have a normal GH response to a variety of stimuli *(20)*.

No differences in mean 12-h GH concentrations was demonstrated between groups of normal-statured males and growth-retarded males with constitutional delay of growth *(90)*. The constitutional growth delay males were significantly shorter, had a greater bone age delay and diminished growth velocity relative to their normal-statured peers. These constitutional growth delay males had a longer secretory burst half-life relative to their normal statured peers, a feature previously described in girls with Turner syndrome *(91)*. Paradoxically, the constitutional growth delay males had a decreased mass of GH released per burst. The authors theorize a deficiency in the amount of GHRH released per GH secretory event further supported by previous work in prepubertal males with constitutional growth delay *(92)*.

Growth Hormone

Consideration of GH feedback is relevant for understanding physiology in children. From a practical therapeutic point of view, exogenous GH readily increases growth velocity. GH is directly and indirectly involved in the feedback control of its own secretion as the hypothalamic neurons that generate the GH pulse are sensitive to the pattern of GH secretion they generate *(93)*. Administration of exogenous GH results in a blunted GH response to clonidine and GHRH in short normal boys *(94)* and to insulin and GHRH in healthy adult men *(95,96)*. GH acutely inhibits its own secretion through a direct effect on the hypothalamus *(97)*, and through increases in IGF-1, which indirectly inhibits GH release at the level of the pituitary and hypothalamus *(7)*.

SRIF release modulates the negative feedback of GH on its own secretion independent of adrenergic and cholinergic mechanisms *(97)*. A single injection of recombinant hGH to adult male rats acutely attenuates GH responsiveness to GHRH and subsequently enhances somatotrope sensitivity to GHRH administration owing in part to increased somatostatin tone *(98)*. Intraventricular injection of GH in anesthetized rats increases SRIF levels in hypothalamic-hypophyseal portal blood, whereas hypophysectomy

decreases hypothalamic SRIF-like immunoreactivity in rats *(7)*. Using a double-label in vitro hybridization technique to identify neurons that co-express SRIF mRNA and GH receptor mRNA, populations of these SRIF/GH receptor mRNA-neurons were identified in the periventricular and paraventricular nuclei of the Sprague-Dawley rat, providing additional support for the direct effects of GH on hypothalamic SRIF neurons in the regulation of its own secretion *(99)*.

GH treatment of GH-deficient children has been associated with hypothyroidism thought to be related to increased SRIF tone and its inhibitory effect on thyroid-stimulating hormone (TSH) secretion. TRH stimulation testing of TSH secretion during formal assessment of the hypothalamic-pituitary GH axis frequently identifies subjects with hypothalamic or pituitary hypothyroidism prior to initiation of GH replacement therapy (B. Bercu, unpublished data). Thus, a direct effect of GH replacement and thyroid dysfunction is unlikely. Along with increases in SRIF, in vitro studies suggest GH inhibits GHRH release in a dose-dependent fashion *(7)* indicating GH autofeedback is under dual control of both hypothalamic peptides.

GH excess, or pituitary gigantism, is an uncommon disorder of childhood, most often associated with isolated pituitary somatotroph adenoma *(100–102)*, McCune Albright syndrome *(103,104)* or less commonly as part of the multiple endocrine neoplasia-type 1 syndrome *(105,106)*. Acromegaly, the adult form has occurred from pancreatic tumors secreting GHRH *(107,108)*. Hormonal secretion from tumor explants in culture obtained from a large mammosomatotroph adenoma in an 8-yr-old boy demonstrated increased adenylate cyclase activity and high levels of adenylate cyclase-stimulatory G protein alpha subunit (G_s alpha). Bromocriptine therapy resulted in reduced adenylate cyclase activity and Pit-1 mRNA expression; G_s alpha levels paradoxically increased, suggesting a beneficial effect of bromocritpine to short circuit G_s alpha-stimulated adenylate cyclase activity via reduction in Pit-1 *(109)*. Gigantism in a 7-yr-old male, and histologic evidence of somatotroph, lactotroph, and mammosomatotroph hyperplasia was associated with hypersecretion of GHRH expands the spectrum of GH hypersecretion *(110)*.

Endogenous GH Secretagogues

In addition to the known hypothalamic hormone, GHRH, we speculate that there is an endogenous ligand for a novel family of GH secretagogues (peptide and nonpeptide) based on the prototypic compound, GH-releasing peptide (GHRP); these compounds which readily release GH in synergy with, and dependent on, available GHRH (as well as the reciprocal relationship). Evidence for development of this hypothesis is reported elsewhere *(111–113)*. Based on these suppositions we have developed a diagnostic test in children and in aging subjects that attempts to discern whether there is a deficiency in endogenous GHRH and/or endogenous ligand for GHRP *(111–113)*. The recent report of a receptor in the pituitary and hypothalamus *(114)* should accelerate the search for an endogenous ligand for the orally active peptide and nonpeptide GH secretagogues.

GH DEFICIENCY AND HYPOPITUITARISM

GH deficiency (GHD) may result from dysfunction at different levels:

1. Higher brain centers: neurotransmitter defects
   a. Lack of stimulators
   b. Excess of inhibitors

2. Hypothalamus: neuropeptide defects
   a. Lack of GHRH and/or theoretical endogenous ligand for GHRPs
   b. Excess of somatostatin
3. Somatotroph
   a. Lack of synthesis of GH
   b. Secretion of bioinactive GH
4. Receptors
   a. Defects in GH receptors
   b. Defects in GHRH receptors
   c. Defects in neurotransmitter receptors

A classification of causes of GHD deficiency and GH resistance is shown in (Table 1).

GHD in the Newborn Period

Hypopituitarism may present in the newborn in a nonspecific fashion. Signs and symptoms include: apnea, cyanosis, pallor, lethargy, jitteriness, and seizures. The differential diagnosis of hypogylcemia includes GHD and hypopituitarism, which could include cortisol deficiency. Prolonged hyperbilirubinemia may be owing to TRH or TSH deficiency, causing hypothyroidism in a neonate with multiple hormone deficiency. Patients with congenital hypopituitarism may have a turbulent neonatal course, generally more characteristic of a full-term infant because of the frequency of neonatal problems in preterm infants. Neonatal glucocorticoid deficiency can present as hyponatremia.

In general, newborns with congenital hypopituitarism have normal birth weights and body proportions. Micropenis, with or without hypoglycemia, may be owing to hypopituitarism. GH deficiency must be considered when there are midline lesions including septo-optic dysplasia (optic nerve hypoplasia and absence of the septum pellucidum).

GHD Presenting During Childhood

GHD/insufficiency and hypopituitarism can present in infancy and childhood in the following clinical settings: hypoglycemia; growth failure (<7 cm/yr prior to age 3 yr, <4.5–5.0 cm/yr from age 3 yr to puberty, <5.5–6.0 cm/yr during pubertal yr); diabetes insipidus; disorders of pubertal development including micropenis and pubertal delay; children with visual, neurological abnormalities and developmental defects; characteristic truncal obesity.

GHD/insufficiency should be considered in children with abnormal linear growth for age, subnormal height (>2 SD below mean for age), delayed bone age, absence of organic disease that could cause growth failure, and normal body proportions. The diagnostic protocols used to detect (GHD) can be classified as either pharmacologic (use of provocative agents) or physiologic (measurement of spontaneous endogenous GH secretion). Standard provocative stimuli include insulin-induced hypoglycemia, arginine, clonidine, levodopa, propranolol, levodopa plus propranolol, and glucagon. Physiologic stimuli include exercise, sleep, diurnal GH secretory profile, IGF1 (previously known as Somatomedin-C), and IGFBP3. The diagnosis of GH deficiency/insufficiency is based on two provocative stimuli with peak GH <10 μg/L or 7 μg/L (using polyclonal GH radioimmunoassay) in the appropriate clinical setting. Our preferred diagnostic test provides comprehensive information at one sitting about the hypothalamic-pituitary endocrine axis as well as assesses GH secretion after two separate stimuli (Table 2). Insulin-induced hypoglycemic challenge should not be used in children with seizure

Table 1
Causes of GH Deficiency or Defective GH Action

Acquired GH Deficiency	*Congenital GH Deficiency*
Idiopathic: Neurosecretory dysfunction CNS tumors: Craniopharyngioma Dysgerminoma Optic glioma Hamartoma Trauma: Perinatal insult: breech deliveries, hypoxemia asphyxia, difficult forceps delivery, intracranial hemorrhage, precipitous or prolonged delivery, twin pregnancy Child abuse Accidental trauma Inflammatory diseases: Viral encephalitis Bacteria, group B streptococcal meningitis, etc. Fungal Granulomatous: tuberculosis, syphilis, sarcoidosis, unknown etiology Autoimmunity: lymphocytic hypophysitis Irradiation: CNS radiation for brain tumors, leukemia Vascular lesions: Aneurysms, pituitary vessels Infarction Hematologic disorders: Hemochromatosis Sickle cell disease Thalassemia Histiocytosis Transient defects in GH secretion or action: Peripuberty (secretion) Primary hypothyroidism (secretion, action) Psychosocial stress (secretion, action) Malnutrition (action) Glucocorticoid excess (?) Drug use	Decreased GH secretion: Idiopathic Hereditary: autosomal recessive, or dominant Embryologic defects: Aplasia, hypoplasia, ectopia Anencephaly, arrhinencephaly Septo-optic dysplasia Midline facial dysplasia Empty sella syndrome Miscellaneous syndromes Biologically inactive GH Neurosecretory dysfunction GH resistance: Laron type dwarfism (GH receptor deficiency) Pygmy type dwarfism (IGF deficiency) IGF-1 Resistance

Table 2
Comprehensive Hypothalamic–Pituitary Testing Performed Over Limited Time Interval (4.5 *h*)[a]

	Combined Insulin Tolerance-TRH-GnRH Arginine Stimulation Study[b]									
Time (min)	*GLU*	*GH*	*PRL*	*LH/FSH*	*TSH*	*T_3RIA*	*F*	*E_2/T/ DHEA-S*[b]	*T_4*	*T_3RU*
–30 min	X	X	X	X	X	X	X	X	X	X
0	X	X	X	X						
Regular insulin 0.05–0.1 U/kg IV TRH 7 μg/kg IV (maximum 500 μg) GnRH 100 μg IV										
+ 10	X	X	X	X						
+ 20	X	X	X	X						
+ 30	X	X	X	X	X					
+ 40	X	X	X	X			X			
+ 60	X	X	X	X	X					
+ 90	X	X	X	X						
+ 120	X	X	X	X	X	X		X		
Arginine 0.5 gm/kg to maximum of 30 grams iv 30 min										
+150		X								
+180		X								
+210		X								
+240		X								

[a]*Water* only after midnight. Insert IV normal saline at "to keep open rate." Then initiate protocol at times noted.

[b]TRH, throtropin-releasing hormone; GnRH, gonadotropin-releasing hormone; GLU, glucose; GH, growth hormone; PRL, prolactin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyrotropin; T_3RIA, total triiodothyronine radioimmunoassay; F, cortisol; E_2, estradiol; T, testosterone; DHEA-S, dehydroepiandrosterone sulfate; T_4, throxine; T_3RU, T_3 resin uptake.

[c]Gender-appropriate gonadal steroid.

[d]Levodopa or clonidine given orally could be substituted for arginine. Levadopa dose = 0.5 g/1.73 m^2. (125 mg if <10 Kg, 250 mg if 10–30 Kg, 500 mg if >30 Kg.) Clonidine dose = 0.1 mg/m^2.

disorder or at a lesser dose in children with "at risk" neurological problems. Twenty-four or 12-h overnight diurnal GH studies should be reserved for the more difficult cases where provocative testings are not discriminatory.

THE CLINICAL UTILITY OF SPONTANEOUS GH SECRETORY PROFILES

Limitations in Defining Pulsatile Hormonal Secretion

A variety of clinical studies assessing the role of various modulators of GH secretion have used the measurement of spontaneous GH secretion over a 24-h period to define GH pulse frequency and GH pulse amplitude in a given subject. Investigators have faced difficulties as they attempted to define the characteristics of pulsatile endocrine signals. These problems include the relatively short half-life of GH which may limit the detection

of low-amplitude or high-frequency GH pulses and assay sensitivity; the use of an immunoassay for measurement when the hormone-specific response depends on the bioactivity of the hormone; the ability to measure only a limited number of data points because continuous measurement of hormones by biosensors is not yet available *(115)*; and a multitude of other factors, including state of nutrition, sleep, age, sex, and exercise, which influence GH secretion.

Mathematical Models

A variety of computer software programs that detect discrete GH peaks, including Pulsar, Ultra, Detect and Cluster analysis, have allowed further refinement of the pulsatile pattern of GH release. Deconvolution analysis overcomes some of the inherent limitations of defining the pulsatile nature of GH hormone secretion by attempting to remove the impact of hormone clearance kinetics on plasma hormone concentrations, thereby exposing the underlying secretory profile *(115,116)*. This mathematical model allows the calculation of hormone pulse amplitude (maximal secretion rate), pulse duration, subject-specific metabolic clearance rates, hormone half-life, the presence and magnitude of any basal ("tonic") hormone secretion, and the number and temporal location of all significant secretory bursts *(115)*. Time series analysis has also been used to characterize GH pulsatility *(117–119)*.

Spontaneous GH Secretory Profiles

Pulsatile release of GH in humans is a reflection of the variability in somatotrope responsivity to hypothalamic GHRH mediated by fluctuating levels of endogenous hypothalamic SRIF. Twenty-four-h studies of spontaneous GH secretion that have demonstrated large GH pulses are actually composed of multiple discreet smaller bursts of GH resolved only with more frequent (30-s) sampling *(120)*. Electroencephalographic (EEG) monitoring during these studies identified a significant correlation of GH secretion and slow-wave sleep. A model of GH secretion has been suggested whereby specific cortical or midbrain events correlating with slow-wave sleep precedes both pituitary GH secretion and the generation of a peripheral GH secretory burst, which is influenced by hypothalamic neurons that increase GHRH release or decrease SRIF secretion (or both), thereby increasing GH secretion by the somatotropes *(11)*.

Twenty-four-hour studies performed in healthy adult subjects using 20-min sampling intervals and an ultrasensitive immunoradiometric assay (IRMA) for GH with a detection limit of 20 ng/L identified an absolute GH nadir of 40 ng/L, well below the sensitivity of most assays *(120)*. The ultrasensitive IRMA allowed the demonstration of an oscillatory rhythm of GH secretion, whereas GH secretion was thought to be episodic in nature *(120)*. In addition, previous estimates of integrated GH (2.50 μg/L in women; 2.33 μg/L in men) may therefore be falsely elevated as a result of overestimation of GH levels below the level of assay sensitivity using standard radioimmunoassays *(120)*. This same principle of the oscillatory rhythm likely also applies to children.

Neurosecretory Dysfunction

The clinical evaluation of infants, children, and adolescents with growth disorders may include provocative tests of GH secretion and 24-h profiles of spontaneous GH release; however, there is currently no gold standard laboratory test for the diagnosis of GH deficiency *(121,122)*. A blunted GH response to known GH secretogogues may help

to identify subjects suspected to be GH-deficient in the clinical setting of growth retardation (<3rd percentile), diminished growth velocity, and delayed bone maturation (bone age); however, no single stimulation test provides adequate specificity. As a result, a minimum of two provocative tests of GH secretion are required to make the diagnosis of GHD.

It had been suspected that a subset of children with clinical features of GHD (diminished growth velocity, delayed bone age) might have abnormalities of GH secretion despite a normal response to provocative GH testing. Subjects with growth retardation who demonstrate biochemical abnormalities in GH secretion, including variable GH peak response to provocative stimuli and abnormalities in spontaneous GH secretion, are at risk for GH neurosecretory dysfunction (GHND), a treatable cause of growth retardation (Fig. 4) *(123,124)*. Newer statistical models have been suggested that make use of 24-h spontaneous GH secretion and IGF-I levels to improve specificity in identifying subjects with disorders of GH secretion *(125)*.

We recently analyzed data collected from 300 24-h studies of spontaneous GH secretion (20-min sampling) in 272 children over a 7-yr period. Control subjects were defined as having a growth velocity standard deviation score (SDS) of ≥ –1.0 and height SDS of ≥ –3.0 of the mean for chronologic age without a recognizable syndrome, cranial irradiation, precocious puberty, or obesity. Subjects were further categorized by diagnosis for comparison, including chronic disease states, chronic renal failure, Noonan syndrome, obesity (BMI >95th percentile for age), precocious puberty, cranial or craniospinal irradiation, and Turner syndrome (Fig. 5) *(48,126)*.

The mean 24-h (0800–0800) and 12-h (2000–0800) GH concentrations in control subjects was 3.8 ± 2.1 μg/L (SD) and 5.6 ± 3.4 μg/L, respectively, using a standard polyclonal radioimmunoassay for GH. Data analyzed by Cluster analysis identified preservation of GH pulsatile secretion and uniformity of GH pulse frequency in all subgroups except for obese subjects. Total spontaneous GH secretion increased in a linear fashion with increasing body mass index in children until the index reached 20–25 and has been confirmed by others *(52)*. Mean 24-h GH concentration correlated positively with the peak GH response to provocation (arginine, insulin, L-DOPA, clonidine) ($r = +0.52$; $p < 0.001$, $n = 245$) and GHRH ($r = +0.35$; $p < 0.001$, $n = 119$) (*48,126*; B. Bercu, unpublished data).

Significant decreases in mean 24-h GH and mean GH peak amplitude were noted in the cranial irradiation and obese subsets; serum IGF-I remained normal in obese subjects, but were decreased in the cranial irradiation and Turner syndrome subjects. A subset of 36 growth retarded, non-GH deficient children demonstrated reduction in mean 24-h GH concentration (4.1 ± 1.9 μg/L; $p = 0.02$), but without significant changes in mean GH peak amplitude *(48,126)*.

Thus, spontaneous GH secretory profiles are a biochemical representation of a series of complex events with significant clinical utility. This large experience reported here demonstrates two things: diminished GH peak amplitude and frequency in obese subjects, whereas cranial irradiation subjects demonstrate decreased GH pulse amplitude; and short, slowly growing, non-GH deficient children have alterations in their spontaneous GH secretory profile relative to controls *(48,126)*.

Abnormal elevation of serum prolactin has been demonstrated in subjects with classical GH deficiency (blunted peak GH response to two or more provocative stimuli and reduced 24-h spontaneous GH secretion), suggesting a disturbance in the normal dopaminergic inhibitory pathways on prolactin secretion *(127)*. Pooled 24-h prolactin samples (equal aliquots from each 20 min sample combined between 0800–0800) and 8-h daytime

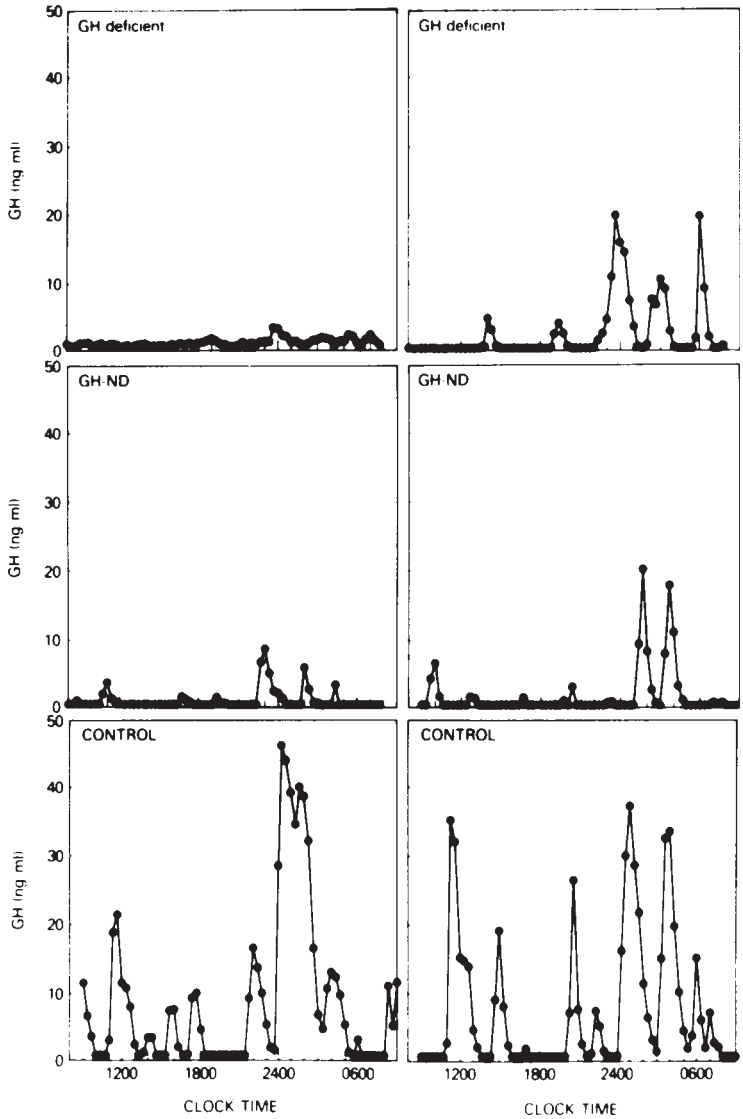

Fig. 4. Representative 24-h GH secretory patterns in GH-deficient, GH neurosecretory dysfunction (GHND), and control subjects. Control subjects in the left and right lower panels are Tanner stage I and IV, respectively. Note that a child with classic GH deficiency (right upper panel) had three pulses higher than 10 ng/mL and two above 20 ng/mL. This child had a mean endogenous 24-h GH concentration less than that of two other children with GHND. By definition, the patients with GHND had two or more normal GH provocative tests (peak ≥10 ng/mL), unlike classic GH-deficient children (two or more GH provocative tests <10 ng/mL). The GHND children had a linear growth response to exogenous GH similar to the classic GH-deficient children. Reproduced with permission from ref. *123*.

pools (0800–1600) were higher in classical GH deficient subjects when compared to control and GH neurosecretory dysfunction subjects and demonstrate a bimodal distribution in the GH deficient group, suggesting variability in the anatomic level of abnormality (i.e., hypothalamic vs pituitary) affecting GH secretion (*48,126*; B. Bercu, unpublished data).

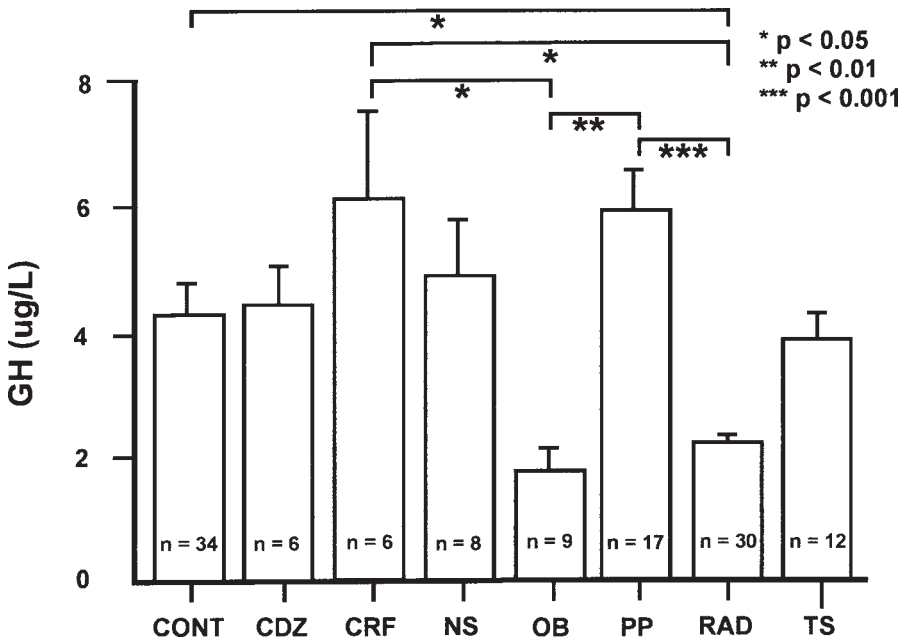

Fig. 5. Mean 24-h GH concentrations in a variety of conditions associated with growth retardation. (CONT, controls; CDZ, chronic disease, including asthma, coeliac disease, and thalassemia; CRF, chronic renal failure; NS, Noonan syndrome; OB, obesity; PP, precocious puberty; RAD, CNS irradiation; TS, Turner syndrome). Reprinted with permission from ref. *141*.

A critical step in the evaluation of growth disorders is the documentation of individual growth velocity, clinically the most useful biologic marker of GH secretion. Thirty-eight children with growth retardation underwent provocative GH testing along with 24-h sampling of spontaneous GH secretion. Children were further divided into three distinct groups based on their GH secretory dynamics and pretreatment height velocity (preHV). Regardless of the individual GH test results, 88% of the children with pretreatment height velocity ≤2 cm/yr, 94% of subjects with a preHV >2 cm/yr but ≤4 cm/yr, and 79% of those with a preHV >4 cm/yr had an increase in height velocity of 2 cm/yr or greater while receiving exogenous recombinant hGH. A significant negative correlation between pre and post GH-therapy growth velocity ($r = -0.67$; $p < 0.001$) supports the conclusion that growth velocity is the more sensitive marker of future response to exogenous growth hormone therapy rather than individual GH secretory status *(48,126,128)*.

REFINEMENTS ON PROVOCATIVE GH SECRETORY TESTING

Provocative GH secretory tests have been used as the gold standard for determining insufficiency in GH secretion. Specifically, two blunted provocative tests are used. This has been controversial as suggested by GH neurosecretory studies and the observations of many clinicians. Recently, somatostatin pretreatment was used to reduce the variability of GHRH stimulation testing *(129)*. This refinement may help because the timing of the GHRH stimulus to the GH pulse influences the magnitude of the response. Somatostatin pretreatment could bring all subjects to the same trough level of GH. It is possible to speculate that this methodology could be applied to all GH-provocative tests.

ETHICAL ISSUES REGARDING TREATMENT OF SHORT-STATURED CHILDREN

There is agreement about the treatment of the short-statured GH deficient child. What is controversial is the use of recombinant hGH in the treatment of the non-GH deficient child. This discussion is separate from the problems inherent in diagnosing GH deficiency as previously reviewed in this chapter. In the United States, Food and Drug Administration-(FDA) approved indications for hGH treatment at the time of preparation of this chapter include: GH deficiency, growth failure associated with chronic renal failure and Turner Syndrome, wasting in AIDS and GH-deficient adults.

The treatment practices in non-GH-deficient, short-statured children by U.S. pediatric endocrinologists were recently reported in the *Journal of the American Medical Association (130)*. Because of the controversial nature of this common practice among pediatric endocrinologists, this article was accompanied by an editorial *(131)*. An arbitrary definition of non-GH-deficient children with short stature would include otherwise healthy children with heights <3 SD below the mean for age, abnormal growth velocity (<25th percentile for bone age), and normal provocative testing with peak GH ≥10 ng/L (using a polyclonal radioimmunoassay). Parental pressure to mitigate short stature in their children is driven by a cultural "heightism" that permeates American society. Taller college graduates make more money, and most (80%) presidents have been the taller candidate *(132)*. Being teased or bullied, having poor self-esteem, feeling athletically incompetent, and being treated as younger than their chronological age by older people are frequent concerns of short children or their parents *(133)*. There are several psychological studies that indicate that short stature *per se* does not result in negative psychological adaptation *(134)*. To date, there are no completed long-term controlled studies of GH therapy in non-GH-deficient children who have been followed to final height. The international uncontrolled observations of this heterogeneous group is mixed with about one-third of the patients achieving final heights greater than predicated heights *(135,136)*. In one study, the authors demonstrated an average mean gain in height of 3–5 cm *(37)*. Data presented at recent scientific meetings report conflicting results, with one study showing no improvement *(138)*, whereas another showed about an 8.5 cm increase *(139)*; both studies compared treated to untreated patients.

CONCLUSIONS

GH secretion is the result of a complex series of interactions occurring both in peripheral tissues and in the central nervous system (CNS). Although our knowledge of these detailed interactions continues to grow, it is interesting to note that this knowledge does not have as much clinical application to the growing organism as one might expect. Despite our knowledge of neurotransmitter control and the impact of specific pharmacologic agents, physiologic and pathophysiologic entities, we still lack a specific gold standard to identify those subjects that may benefit from exogenous GH therapy *(140)*.

The use of overnight and 24-h GH secretory profiles, along with standard GH provocation testing and accepted markers of GH secretory "sufficiency," including IGF-1 and its binding proteins, supplement physical findings and growth velocity. Together, this information allows the clinician an educated guess in selecting subjects for GH therapy, thus enabling us to maneuver through the confusing and complex biochemical events described previously. In this manner, we can deliver this therapeutic modality to those

who can benefit the most. These decisions must be made in the context of the physician's understanding of the psychological and ethical issues in the treatment of these short-statured children.

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13 Growth Hormone Deficiency in Adults

The Rationale for Growth Hormone Replacement

Paul V. Carroll, MD, Emanuel R. Christ, MD, and Peter H. Sönksen, MD, FRCP

CONTENTS

INTRODUCTION

Until the last decade most physicians considered growth hormone (GH) to have no biological relevance following the cessation of linear growth. The first evidence that GH is important throughout adult life came from a report, in which a 35-year-old hypopituitary adult described increased vigor, ambition, and well-being, following GH replacement *(1)*. Recombinant technology resulting in a limitless supply of GH has prompted intensive investigation of the effects of GH in health and disease, and the effects of GH replacement in adults with GH-deficiency has received particular attention.

These studies have led to the identification of a specific constellation of symptoms, signs and investigative findings, which is now recognized as the 'GH-deficiency syndrome'. The main features of the syndrome are listed in Table 1. Subsequent studies have addressed the effects of GH replacement on these features, in the form of both randomized placebo-controlled trials, and smaller open studies. These studies have produced consistent results, demonstrating that adults with GH-deficiency are both psychologi-

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Table 1
The Clinical Features of GH-Deficiency in Adults

Background
Known pituitary pathology ± previous treatment
Full 'conventional' pituitary hormone replacement
Need for GH treatment as a child
Symptoms
Abnormal body composition
reduced lean body mass
increased abdominal adiposity
Reduced strength and exercise capacity
Impaired psychological well-being
depressed mood
reduced vitality and energy
emotional lability
impaired self-control
anxiety
increased social isolation
Signs
Overweight, with predominantly central (abdominal) adiposity
Thin, dry skin; cool peripheries; poor venous access
Reduced muscle strength
Reduction exercise performance
Depressed affect, labile emotions
Investigations
Stimulated GH level below 3 μg/L
Low or low-normal serum IGF-I
Elevated serum lipids, particularly LDL cholesterol
Reduced lean body mass / increased fat mass
Reduced bone mineral density

cally and physically less healthy than their age-matched peers, and that GH replacement results in substantial and sustained benefits. This chapter details the important features resulting from GH-deficiency and summarizes the effects of GH treatment, assessing the rationale for GH replacement in adults with GH-deficiency.

PSYCHOLOGICAL WELL-BEING AND QUALITY OF LIFE

The psychological well-being and quality of life (QoL) of GH-deficient patients and the effects of GH replacement have been addressed in several studies. In the majority, self-perceived well-being, and QoL have been assessed using validated questionnaires, and comparisons have been made with healthy control subjects of similar age, sex and socioeconomic status. Although several instruments have been used in these trials the results have been remarkably consistent.

Decreased psychological well-being has been reported in hypopituitary adults despite replacement of all hormone deficiencies with the exception of GH *(2)*. In studies comparing adults with long standing GH-deficiency with matched controls the patients reported lower openness, less assertiveness, less energy, greater emotional lability, more difficulties with sexual relationships and a greater sense of social isolation *(2,3)*. Evi-

dence from a recent study suggests that the severity of psychological distress correlates positively with the duration of GH-deficiency *(4)*.

McGauley was the first to demonstrate in a double-blind placebo-controlled study that GH replacement (0.5 IU/kg/wk; 25 μg/kg/d) was associated with an improvement in mood and energy levels in GH-deficient adults *(5)*. These findings have been confirmed in subsequent studies *(4,24)*. Whitehead et al. *(6)* examined the effects of GH replacement (0.25 IU/kg/wk; 12.5 μg/kg/d) on well-being of 14 adults with GH-deficiency in a 6-mo double-blind placebo-controlled crossover trial, using the Nottingham Health Profile and the Psychological General Well-Being Schedule. In contrast, no significant changes in psychological well-being were observed, but in this study many patients failed to demonstrate a rise in circulating insulin-like growth factor-1 (IGF-1), indicating that noncompliance may have contributed to this lack of effect.

The direct mechanism behind alterations in perceived QoL remain unknown. Recently GH treatment of GH-deficient adults has been shown to alter levels of vasoactive intestinal polypeptide and the dopamine metabolite, homovanillic acid, as well as elevating β-endorphin levels in cerebrospinal fluid, but whether these changes are responsible for improvement in mood and well-being is not yet known *(7)*. GH, IGF-1, and the IGF-binding proteins may have direct effects on the nervous system. In addition abnormal sleep patterns have been described in GH-deficient adults with a restoration to normal patterns following GH replacement *(8)*.

In summary adults with GH-deficiency report reduced self-perceived psychological well-being compared with matched healthy subjects. GH replacement results in significant improvements in QoL and psychological well-being in patients with long standing GH-deficiency.

BODY COMPOSITION

Many studies have investigated the consequences of GH-deficiency on body composition and the alterations associated with GH replacement. The majority of these have referred to a two-compartment model, consisting of fat mass (FM) and lean body mass (LBM). These studies have employed a wide range of methods to assess body composition *(9)*. Nevertheless, despite the methodological differences the findings from these studies are strikingly similar.

GH-Deficiency and Body Composition

Reduced skeletal muscle mass, the most prominent component of LBM, is an important feature of adult GH-deficiency. Consistently studies have demonstrated reduction in LBM of 7–8% corresponding to approx 4 kg of lean tissue *(10–14)*. This reduction is mirrored by an increase in fat mass, in the region of 7% *(10)* in GH-deficient patients compared with predicted values based on age, sex, and height. This figure has been confirmed by other investigators *(13,15–19)*, using a variety of measurement techniques. The distribution of this excess fat mass has been the focus of a number of studies *(10,13,20,16)* and these have demonstrated the excess fat accumulates in a central (abdominal) distribution, mostly in the visceral component.

Both the radioisotope dilution technique and the bioimpedance (BIA) method indicate that total body water (TBW) is reduced in adult GH-deficiency *(14,17)*. This is mainly due to a reduction in extra-cellular water (ECW, *14,19,21*). Recent studies suggest that reduced plasma volume (PV, *17*) and total blood volume (TBV) contribute to the reduced ECW *(18)*.

The Effects of GH Replacement on Body Composition in Adults with GH-Deficiency

In all reported studies investigating the effects of GH treatment on body composition, an increase was recorded in average LBM, varying from 2–5.5 kg after six months GH replacement *(11–16,20–24)*. Similar findings have been reported from studies in patients with both adult-onset (AO) and childhood-onset (CO) GH-deficiency *(25)*.

In these studies the changes in LBM were associated with reductions in fat mass of approximately 4-6 kg *(11–16,20–24)*. Similarly this reduction occurs equally in both CO and AO GH-deficiency *(25)*. Anthropometric measurements indicate that the most important change occurs in the abdominal region *(10)*, corresponding to reduced visceral fat mass *(16,20)*.

GH has a potent anti-natiuretic effect, most likely mediated through direct effects of GH and/or IGF-1 on renal tubular sodium absorption, in addition to stimulatory effects on the renin-angiotensin system *(26)*. GH replacement results in an increase in TBW *(11,18)*, particularly in ECW within 3–5 d *(26)*. Plasma volume has been shown to increase after three weeks of GH replacement *(17,18)* and in a recent study, GH therapy resulted in a 400-mL increase in TBV after three months of treatment *(18)*.

BONE MINERAL DENSITY AND BONE METABOLISM

Studies of bone mineral density (BMD), despite the use of several techniques have universally demonstrated reduced bone mass at a variety of skeletal sites in patients with childhood-onset *(27,28)*, adult-onset *(29)*, and mixed-onset *(30–33)* GH-deficiency, compared with healthy control subjects. Evidence suggests that adults with GH-deficiency are at increased risk for osteoporotic fractures *(34)*.

Effects of GH Replacement on BMD and Bone Metabolism in GH-Deficiency

Short-term studies (3–6 mo) of GH replacement have failed to demonstrate an increase in bone mass *(13,31–33)*. In several studies reduced BMD has been recorded following six months GH therapy *(32,34)*, but after more than 12 mo treatment increases of 4–10% above baseline have been demonstrated *(30,32)*.

Markers of serum bone formation (osteocalcin, bone alkaline phosphatase, bone Gla protein, carboxyterminal propeptide of type 1 procollagen = PICP) and urinary bone resorption markers (deoxypyridinoline, pyridinoline, crosslinked telopetide of type 1 collagen = ICTP) increased in both short-term and long-term studies indicating an activation of bone remodelling *(13,30–34)*.

In summary, adult GH-deficiency is associated with reduced bone mass as assessed by bone mineral density measurements. The available data provide evidence that GH is an osteoanabolic hormone when given to GH-deficient adults. The findings in most of the trials suggest that GH has a biphasic effect: following an initial predominance of bone resorption, stimulation of bone formation leads to a net gain in bone mass after 12–24 mo of treatment.

CARDIOVASCULAR SYSTEM

Epidemiological data suggest that adults with hypopituitarism have reduced life expectancy compared with healthy controls, with a greater than twofold increase in mortality from cardiovascular disease *(35,36)*. GH-deficiency has been proposed as the variable accounting for this increased mortality, and the hypothesis that long standing GH-deficiency predisposes to the development of premature atherosclerosis. The mecha-

nisms responsible for the increased cardiovascular mortality remain largely unknown, but increased intima-medial thickening, intimal plaque formation *(37)*, and reduced arterial compliance *(38)* in the carotid artery have been demonstrated in hypopituitary adults on conventional replacement therapy.

A study comparing echocardiographic findings in GH-deficient adults with healthy controls demonstrated reduced left ventricular mass and impaired cardiac systolic function in the patients *(15)*. Similar findings were reported from a study using radionuclide scanning *(39)*. In both of these studies treatment with GH for six months normalized these indices, and six months following cessation of therapy, cardiac function had returned to baseline. Six months GH replacement has been shown to increase left ventricular mass (18%), and cardiac output (43%) in GH-deficient adults *(40)*, and studies suggest that these benefits are sustained to three years following commencement and continuation of GH therapy *(23,41)*.

A recent study investigating total blood volume measuring red cell mass and plasma volume has shown that erythropoiesis is impaired in adult GH-deficiency. GH therapy stimulates erythropoiesis (oxygen capacity) and increases plasma volume and total blood volume and may, therefore, contribute to observed increased in exercise performance associated with GH therapy *(18)*.

In summary, adults with GH-deficiency have an increased cardiovascular mortality compared to matched controls. Cardiac function is impaired in GH-deficiency and GH replacement reverses these deficits in studies lasting up to three years.

EXERCISE PERFORMANCE AND MUSCLE STRENGTH

Exercise capacity is dependent on both muscle strength and cardiovascular performance. Several studies have addressed exercise performance in GH-deficient adults using cycle ergometry *(42,43)*. In these studies, values for maximum oxygen uptake were significantly reduced, being on average 72–82% of those predicted for age, sex, and height *(42)*. Maximum oxygen uptake increased significantly *(42)* following six months GH treatment reaching predicted values *(42)*. Evidence suggests that the increased performance is largely attributable to increased muscle mass *(43)* although observed increases in cardiac output, extracellular fluid volume, and red cell mass following GH replacement may all contribute. Thus adults with GH-deficiency have a reduced exercise performance capacity, which can be improved and probably normalized with six months physiological GH replacement.

The decreased LBM of GH-deficiency results in a mild to moderate reduction in muscle strength. Isometric quadriceps force has been shown to be reduced in GH-deficient adults compared with matched normal controls *(43)*. The effects of GH-replacement on muscle strength has been investigated in several studies *(43–45)*. These have demonstrated, an increase in limb girdle force after six months GH treatment, but neither isometric quadriceps force nor quadriceps torque increased significantly in any of the studies. This was despite marked increases in thigh muscle cross-sectional area. Only after more prolonged GH treatment (at least 12 mo) has a significant increase in quadriceps force been demonstrated with a further increase and normalization seen after three years *(23)*. It is likely that the difficulties inherent in measuring muscle strength contribute to the difficulty in demonstrating statistically significant effects when small numbers of patients are studied.

METABOLISM

Energy Expenditure

Adults with long-standing GH-deficiency have reduced whole body resting energy expenditure (REE), with lower values than predicted for age, sex, height, and weight *(46)*. GH replacement in GH-deficiency results in rapid and large increases in REE *(10,47,48)*. Because REE is largely dependant on LBM metabolic activity, much of this increase is attributable to the observed increase in LBM associated with GH replacement. However, when changes in REE are expressed per LBM these rises are still significant, indicating that direct increases in cellular metabolism are responsible for some of the increased REE *(10)*. GH treatment of the GH-deficient adult results in an increase in circulating tri-iodothyronine (T3) levels, both in patients on thyroxine replacement and those with normal thyroid function *(49)*, indicating that GH is a physiological regulator of thyroid function, in particular the peripheral conversion of thyroxine (T4) to T3. This effect on T4 metabolism probably accounts for some but not all of the calorigenic effect of GH. In addition GH replacement has been shown to increase fat oxidation *(47)* and protein synthesis *(48)*. These processes are energy requiring and result in an increase in energy expenditure.

Protein Metabolism

A small number of studies have assessed protein metabolism in GH-deficiency. These have employed stable isotope tracer techniques, and have demonstrated reduced protein flux and synthesis in adults with GH-deficiency compared with normal matched controls *(11,48)*. GH replacement results in considerable increases in protein synthesis in the short term (1–2 mo *[11,48]*), with a return to baseline rates after six months *(11)*, most likely as a result of achieving a new baseline rate of metabolism.

Carbohydrate Metabolism

As described earlier, GH-deficient adults have increased central adiposity. This contributes to the observed hyperinsulinaemia in GH-deficiency, indicating insulin resistance *(10)*. The presence of insulin resistance has been confirmed by studies using hyperinsulinaemic euglycaemic clamp techniques and using Bergman's minimal model *(50–52)*. In addition there is evidence that adults with GH-deficiency have reduced hepatic glycogen stores *(51)*. GH replacement has been demonstrated to further increase insulin resistance over a period of six weeks therapy *(52)* but although hyperinsulinaemia persists, carbohydrate metabolism returns to baseline following 3 mo GH treatment *(50,52)*.

Lipid and Lipoprotein Metabolism

Consistently studies have demonstrated that adults with GH-deficiency have elevated concentrations of total cholesterol, low density lipoprotein-cholesterol (LDL-C) and apolipoprotein B (ApoB) compared with an age and sex matched control population or the predicted range *(25,53,54)*. Characteristically, high density lipoprotein-cholesterol (HDL-C) is reduced and triglyceride (TG) concentrations are elevated compared with healthy controls *(55)*. Therefore GH-deficiency is associated with a lipid profile recognized to be associated with premature atherosclerosis and cardiovascular disease.

Effect of GH Replacement on Plasma Lipids and Lipoproteins in GH-Deficiency

GH replacement results in decreases in total cholesterol *(10,25,54,56–59)*, LDL-C and ApoB *(56)*. In addition GH therapy has been shown to increase HDL-C *(25,60)*, without altering plasma concentrations of triglycerides and apolipoprotein A *(10,56–60)*.

In longer term studies these "favorable" effects on plasma lipid and lipoproteins have been sustained up to 3 years after commencement of GH therapy *(25,58)*. Recent evidence suggests that the observed early decrease in TC (3–6 mo) is transitional and is followed by an increase in HDL-C *(25,61)*.

The major exception to the trend of beneficial effects on cardiovascular risk factors with GH replacement is lipoprotein (a) concentration, a proposed independent risk factor for the development of atherosclerosis and myocardial infarction *(62)*. In studies of the effect of GH treatment, lipoprotein (a) levels rose in four of five studies *(57–60)*. The importance of this observation is not yet known.

Considerable progress has been made in the understanding of the mechanisms responsible for the GH mediated alterations in lipid and lipoprotein metabolism. There is evidence that GH replacement upregulates hepatic expression of the LDL-receptor *(61)* and stable isotope studies suggest that GH has a role in the regulation of apolipoprotein B metabolism *(63)*, a major determinant of lipid metabolism.

REPORTED ADVERSE EFFECTS OF GH REPLACEMENT

Recombinant GH has an identical structure to human growth hormone and is not associated with the risks attributable to human or animal derived products. The majority of 'adverse' effects associated with GH replacement in GH-deficient adults are related to the anti-natriuretic effect of GH and are in reality 'effects' rather adverse effects. The dose used in early studies (approx 0.5 IU/kg/wk *[10,11,20]*) was used to ensure that no effect of GH in the adult was missed and were based on calculated secretion rates in young adults and treatment doses used in the pediatric cases of GHD. It is now clear that GH is more potent than was anticipated and the doses selected (without any gradual escalation of dose as would routinely done with T4 in hypothyroidism) resulted in clinically relevant adverse effects requiring a dose reduction in up to 40% of patients. These doses also resulted in circulating IGF-1 levels in excess of the normal range in a similar proportion of patients confirming that this replacement dose was supra-physiological. More recent studies have employed gradual dose escalation and lower doses and are better tolerated and can be considered more physiological *(12,13,15–18,22)*.

Patients most at risk of adverse effects are elderly, obese, with a greater GH response on provocative testing and the largest IGF-1 rise on GH treatment *(64)*. The most common side effects arise from the physiological action of GH in regulating sodium and water retention which are in fact restoring normal extracellular fluid volume after years of depletion. Weight gain, dependent edema, a sensation of tightness in the hands, or symptoms of carpal tunnel compression frequently occur within days or weeks. A meta-analysis including 233 hypopituitary adults, with GH doses ranging from 0.08–0.3 IU/kg/wk documented fluid retention (37.4%), arthralgia (19.1%), and muscle pains (15.7%) in the first six months of treatment *(65)*. All symptoms resolve rapidly with dose reduction or frequently disappear without any action. Growth hormone's anti-natriuretic effect is not commonly associated with development of hypertension *(10,41)* although existing

hypertension may occasionally be aggravated in the early stages. Arthralgias involving small or large joints occur in some patients during GH treatment, but there is usually no evidence of effusion or inflammation and X-rays have shown no abnormality *(10)*. These changes also settle spontaneously or with dose reduction and may be related to swelling of articular cartilage or synovium.

There have been isolated reports of cerebral side effects resulting from GH replacement, in the form of encephalocele *(10)*, tinnitus *(11)*, and benign intra-cranial hypertension (BIH *[66]*). The majority of affected patients are children and the BIH has improved with cessation of therapy. Papilloedema was present in the majority of cases. Cessation of GH therapy has resulted in regression in all reported cases *(66)*. These effects are almost certainly a reflection of the changes in cellular hydration following GH replacement.

Of greatest concern are the potential effects of GH treatment on tumor development and recurrence. Little information is available on this topic in GH-deficient adults. Reassuringly data from long-term studies in children with both solid tumors and haematological malignancies, suggest that there is no increased risk of recurrence associated with GH therapy *(67–70)*.

CONCLUDING REMARKS

Our understanding of the role and importance of GH throughout life has increased dramatically in the last decade and the role of GH in adulthood is no longer disputed. GH-deficiency results in alterations in psychological well-being, cellular hydration, body composition, physical performance, and many aspects of metabolism. These alterations are associated with reduced physical and mental health, and emerging evidence suggests that affected subjects have a reduced life expectancy. Many of these alterations can be improved or completely corrected with GH replacement.

The major impediment to the use of GH replacement is its cost. It is anticipated that this will decrease significantly once the pharmaceutical industry recovers developmental expenditure. It is therefore likely that GH replacement will in the near future, become as routine as steroid, thyroid hormone and sex hormone replacement, in the management of the hypopituitary adult.

The prospect of GH replacement becoming a routine therapy does raise a number of important issues. Most importantly is the identification and selection of patients who may benefit from GH therapy. The studies to date have focused on those with profoundly reduced GH secretion, and little data is available on those with partial GH-deficiency. The gold-standard for the diagnosis of GH-deficiency remains the provocation test for GH secretion *(71)*. The best and most applied method is the insulin tolerance test *(72)*. The selection of patients is further complicated by the marked age associated decline in GH secretion *(71)*. Many elderly patients would meet diagnostic criteria for GH-deficiency and whether or not such patients are likely to benefit from GH therapy is unknown. Currently GH replacement is recommended for use in patients with severe GH-deficiency in whom replacement has been shown to be of benefit.

The "optimal" dose of GH replacement for the GH-deficient adult has been the subject of discussion. Most centers recommend initiation of GH replacement at a very low dose (eg., 0.01 IU/kg/d), with a gradual increase, tailored to the individual, with regular monitoring, particularly in the early phase, to avoid adverse effects. In addition, the serum IGF-1 concentration should be used as a marker of response to GH therapy. In practice,

most endocrinologists aim for an IGF-1 level, in the upper part of the aged-matched healthy control range, indicating 'physiological' replacement.

The majority of studies to date have addressed the effects of GH replacement over a period of 6–12 mo. The longer term effects have not been fully addressed, and it remains to be seen, whether GH treatment will reduce the incidence of cardiovascular and bone disease, over a lifetime. The prospect of life-long GH replacement for many GH-deficient adults means that the resources and facilities for long term monitoring must exist. Such monitoring will provide further information regarding the long-term issues of GH treatment, such as tumor development, but also determine whether GH replacement is associated with reduced health care expenditure and an increased life expectancy for the hypopituitary adult.

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14 Growth Hormone and Osteoporosis

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CONTENTS

INTRODUCTION

Since the first demonstration of the activity of human and monkey pituitary growth hormone (GH) extracts in humans *(1,2)*, research regarding the effect of GH on bone have focused primarily on the hormone's promotion of linear growth. In the last decade, however, the importance of the role of GH in bone metabolism has become increasingly apparent. Recent research has demonstrated that GH administration stimulates osteoblast proliferation and promotes bone formation in vitro and in vivo, and that GH deficient states are associated with osteoporosis. In addition, normal aging has been shown to be associated with both declining GH secretion and declining bone density, suggesting a possible link between GH and senile osteoporosis. A number of technical advances have aided this work. In vitro studies have been advanced by new cell-culture techniques and recombinant DNA technology. Studies of bone metabolism in humans have assessed bone turnover with a widening array of serum and urine markers. These include osteoblast markers, byproducts of bone formation, and urine markers of bone resorption. Furthermore, refinements in the measurement of bone mineral content and bone density have permitted long term studies of the effect of GH administration on bone mass. This chapter will address the relationship between GH and osteoporosis by reviewing in vitro studies of GH and bone cells, studies of patients with GH deficiency, and studies of GH administration using different model systems.

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MOLECULAR, CELLULAR, AND HISTOLOGIC EFFECTS OF GH

The effects of GH on longitudinal bone growth at the cellular level are well-established. GH acts at the growth plate, where it binds to chondrocytes and promotes local synthesis of insulin-like growth factor (IGF-1) *(3–5)*. GH stimulates the differentiation of growth-plate precursor cells and increases the responsiveness of these cells to IGF-1. Clonal expansion of differentiating chondrocytes, and thus longitudinal bone growth, is stimulated primarily by locally produced IGF-1, though GH and circulating IGF-1 may also have a role *(3)*.

An effect of GH on bone density, however, especially in patients whose epiphyses have fused, depends on the ability of GH to affect bone formation by osteoblasts, rather than simply to promote proliferation of growth-plate chondrocytes. GH has been shown to stimulate the proliferation of osteoblast-like cells cultured from fetal rat calvaria, and this effect is dependent on local synthesis of IGF-1 *(6)*. GH stimulates DNA synthesis as measured by labeled thymidine incorporation in fetal chicken osteoblasts, an effect which is enhanced by a serum factor, presumably IGF-1 *(7)*. In addition to promoting cell division, GH also stimulates collagen production and inhibits collagen breakdown in fetal rat osteoblasts *(8,9)*, suggesting a positive effect of GH on bone formation. Specific GH and IGF-1 receptors have been identified on rat osteoblasts *(10,11)*, supporting the hypothesis that effects of GH and IGF-1 on osteoblasts are receptor-mediated. IGF-1 enhancement of cell replication and collagen gene expression may depend on GH-induced local production of IGF binding protein 3 (IGFBP-3). IGF-1 effects on osteoblasts in cell culture are less pronounced when GH and the resultant synthesis of IGFBP-3 are absent and when IGF-1 binding to IGFBP-3 is blocked *(12)*.

GH may also influence bone density through effects on vitamin D metabolism. Hypophysectomized rats demonstrate a marked fall in plasma levels of 1,25 dihydroxyvitamin D, which is not explained by a change in circulating parathyroid hormone (PTH), calcium, or phosphorus levels *(13)*. Furthermore, hypophysectomy eliminates increases in plasma levels of 1,25 dihydroxyvitamin D as well as in vitro production of the vitamin by kidney slices ex vivo normally seen with phosphate deprivation. GH treatment of these animals restores normal 1α-hydroxylase activity *(14)*. Such GH effects on vitamin D metabolism could theoretically mediate increases in bone density through increased calcium absorption or local effects on bone mineralization.

Positive effects of GH on bone accretion have been characterized histologically in studies of GH administration in dogs *(15,16)*. Harris and Heaney published two studies in which dogs received pharmacologic doses of GH for 12 wk, and bone formation rates were determined using tetracycline labeling. Examination of cortical bone specimens from several skeletal sites showed that the formation of endosteal bone was markedly accelerated by GH administration, with significantly increased bone mineral accretion and a 30% decrease in fecal calcium loss owing to increased absorption *(15)*. However, these experiments have not been replicated, and their applicability to humans remains uncertain.

Extensive experimental evidence in vitro and in vivo therefore indicates that GH plays an important role in maintenance of the skeleton. These data have also provided a rationale to explore the possible therapeutic benefit of GH on bone density in states of GH deficiency and osteoporosis. In humans, however, these issues are complex, because GH deficiency (GHD) may vary in timing, etiology, and severity. Because GH is administered as an injection, whereas endogenous GH secretion occurs in pulses,

physiologic administration of GH may be difficult, and symptoms and signs of GH excess may result from GH administration, even with the most careful dosing schedules. Furthermore, GH has diverse metabolic actions apart from its effects on bone, which may limit its clinical utility, especially for therapy of osteoporosis in the GH sufficient patient.

GHD AND OSTEOPOROSIS

Childhood-Onset GHD and Osteoporosis

The positive effects of GH on osteoblast proliferation and bone formation clearly manifest in humans. This has been illustrated by several studies of patients with GHD in whom the absence of GH is associated with diminished bone-density. A relative bone density deficit has been reported in children with GHD before the start of GH replacement therapy *(17)*. However, these data are difficult to interpret because of the confounding variable of delayed skeletal maturation in such children.

Studies of adult patients with a history of childhood-onset GHD also demonstrate relative osteopenia compared to age-matched controls. Degerblad et al. *(18)* studied six young adults who had previously received GH replacement for GHD. Bone density of the proximal and distal forearm, primarily reflecting cortical and trabecular bone, respectively, was markedly diminished compared with healthy controls. A larger study of 30 GH-deficient men, 18–46 yr, reached a similar conclusion. Despite a history of GH replacement, bone density in the proximal forearm, distal forearm, and lumbar spine of these patients was significantly lower than normal *(19)*. To determine whether pituitary deficiencies other than GH were responsible for the lower bone density seen in the patients, the eight patients with isolated GH deficiency were analyzed separately. In this subgroup, bone density at all sites remained below that of the normal controls, although because of the small sample size, this difference remained significant only at the distal forearm. These data indicate that the osteopenia seen in patients with childhood-onset GH deficiency is at least partly attributable to GHD.

It is uncertain why such patients have low bone density despite GH replacement during childhood. It is possible that GH treatment was initiated too late or at too low a dose to achieve normal peak bone mass. Furthermore, GH replacement is typically discontinued when growth slows or ceases. It is likely that GH plays a role in the continued accretion of bone mass seen in normal adolescents and young adults after linear growth is complete. Finally, withdrawal of GH therapy may lead to an ongoing loss of bone after peak bone mass has been achieved.

Adult-Onset GHD and Osteoporosis

Studies of patients with adult-onset GHD have served to clarify the important role of GH in maintaining the skeleton once peak bone mass is reached. Such individuals have acquired hypothalamic or pituitary dysfunction in adult life, typically owing to neoplasms of the pituitary or adjacent structures. Compromise of secretory function may occur owing to mass effect of the lesion, or, as a result of surgical or radiation treatment. The best approach in diagnosing GHD in adults remains a major unresolved issue, and the presence of GH deficiency is typically determined by means of provocative tests. In contrast to children, in whom growth is an objective parameter used in the assessment of GHD, there is no defined physiological endpoint that can be used in the diagnosis of GH deficiency in adults. GH secretion is age-, sex-, and nutritional-dependent. The variabil-

ity of GH secretion in normals and the lack of objective endpoints have made the determination of absolute and relative GH deficiency problematic.

One study of 95 adults, ranging in age from 21–74 yr and identified as GH deficient on the basis of provocative tests, showed low bone density of the lumbar spine compared to normal controls. This significant deficit in bone density persisted when patients with untreated hypogonadism were excluded *(20)*. Two smaller studies showed reduced bone density of the total body *(21)*, femoral neck, Ward's triangle, and greater trochanter *(22)* in patients with adult-onset GHD compared to normals. In both of these studies, a significant correlation was found between bone density and serum levels of IGF-1, an integrated marker of GH secretion. Because patients with adult-onset GH deficiency were by definition endocrinologically intact through adolescence, they presumably had normal skeletal development and reached a normal peak bone mass. Therefore, osteopenia in such patients, can only be explained on the basis of accelerated loss of bone during adulthood.

Adult-onset GHD is often associated with other hormone deficiencies. Therefore, the studies cited previously could not entirely exclude gonadal steroid deficiency as a contributing cause of bone loss. Although patients in the majority of these studies received testosterone or estrogen replacement, it is possible that the period of gonadal steroid deficiency before the initiation of hormone treatment may have led to osteopenia. Holmes et al. *(23)* addressed this issue in a study of 26 GH-deficient adults who had been treated with surgery and/or radiation for tumors of the pituitary or brain. As a group, these patients displayed reduced bone density of the forearm and lumbar spine. A subgroup of patients with isolated GHD were also shown to have low bone density of the lumbar spine and forearm. These data indicate that GHD acquired in adulthood may lead to osteoporosis, presumably on the basis of bone loss occurring after the attainment of normal peak bone mass.

Growth Hormone and Age-Related Osteoporosis

Because both GHD and normal aging are associated with decreases in bone density, it has been hypothesized that reduced GH secretion may account in part for age-related loss of bone mass *(24)*. However, a causal relationship between GHD and osteoporosis has not been established. Nocturnal serum GH peaks average 20 ng/mL in 30-yr-old men, but this value declines steadily to 3 ng/mL by age 80 *(24)*. These values are reflected in the fall of IGF-1 levels, which also occurs with aging *(24)*. Because aging is associated with numerous physiological and hormonal changes, it has been difficult to determine definitively the degree to which the age-related decline in GH levels is responsible for bone loss. Bone density peaks at age 30, then declines progressively. In men, 25% of trabecular bone is lost by age 75 *(25)*. One study of women with osteoporosis and vertebral compression fractures showed no difference in the GH response to insulin-induced hypoglycemia in these patients compared to nonosteoporotic controls *(26)*. The GH response to hypoglycemia, however, may not reflect spontaneous GH secretion, and a relationship between declining GH production and osteoporosis could not be excluded. A later study showed that 141 men and women with osteoporosis and vertebral fractures had significantly reduced serum levels of IGF-1 compared to controls *(27)*. In addition, levels of the GH-dependent protein IGF-1 were positively correlated with bone density in osteoporotic women who were not receiving estrogen replacement. No such correlation was found in other subgroups of the experimental population, nor were correlations found between IGF-1 and bone density.

To date, the evidence for a relationship between age-related declines in GH secretion and bone density is circumstantial. More investigation is required to establish this link more definitively. Even in the absence of such investigation, studies of the effects of GH administration on bone in the elderly have been conducted, and are discussed later in the chapter.

EFFECTS OF GH ADMINISTRATION ON BONE TURNOVER AND BONE DENSITY

GH Administration in GH-Deficient Children

Although the effect of GH on linear growth in GH-deficient children has long been known, more recent technical advances in measurement of bone turnover markers and bone densitometry have allowed the investigation of osteoblast activity and bone density in this population. A number of studies have shown that in vitro observations, demonstrating positive GH effects on osteoblasts, can be applied to GH-deficient children receiving GH replacement. A study of GH-deficient patients, ages 11–19, who received increasing doses of GH in 2-wk blocks, showed increases in serum levels of osteocalcin *(28)* and the carboxyterminal propeptide of Type 1 procollagen *(29)*. These findings are indicative of increased osteoblast activity and increased synthesis of collagen in bone occurring in a dose-dependent manner in response to GH treatment.

Subsequent studies have evaluated both bone formation markers and bone density. Twenty-two GH-deficient children were studied while receiving GH either three or six times per week for six months *(30)*. Under both treatment protocols, significant increases in serum osteocalcin were seen. Only with more frequent dosing, however, did bone density increase at the ultradistal radius, a region consisting principally of trabecular bone. In a similar study, 26 GH-deficient patients were studied during 12 mo of GH administration. These patients showed significant increases in the carboxyterminal propeptide of Type 1 procollagen and in bone density at the distal third of the radius, a measure of cortical bone density *(31)*. The increase in bone density was significant whether normalized for chronologic age, statural age, or bone age.

Although the primary purpose of GH treatment of GH-deficient children is to stimulate linear growth, GH administration also increases bone density in this population. When taken together with the finding that adults with a history of childhood-onset GH deficiency are osteoporotic, these data suggest that GH plays an important physiologic role in the attainment of peak bone mass.

GH Administration in Adults with Childhood-Onset GHD

Before the introduction of recombinant human GH, provision of GH was limited in the clinical setting and—largely in clinical research—to children with GHD. Over the past 10 yr, the availability of virtually unlimited amounts of GH, has made possible the study of broader indications for its administration. One such area of research has been the administration of GH to adults with a history of childhood-onset GH deficiency, in whom GH therapy has generally been discontinued at the time of epiphyseal fusion or the attainment of predicted final height. In contrast to patients with adult-onset GHD, the initial diagnosis of GHD in adults with childhood-onset disease is made with the accepted pediatric standards during childhood. However, the investigation of bone metabolism in this population has been limited by a lack of long-term controlled studies and difficulties

in assessing physiologic vs pharmacologic GH replacement. A number of studies in such patients have shown consistent effects of GH administration on markers of bone turnover, but the long-term effects of GH on bone mass remain controversial. Because of this, the study of the effects of GH administration on bone in adults remains an active area of investigation.

Early studies of GH administration in adults with childhood GHD utilized changes in bone turnover markers as a primary clinical endpoint. These studies have demonstrated that GH reproducibly stimulates markers of bone formation and resorption. Johansen et al. *(32)* studied 21 adults, 11 of whom had isolated GHD and 10 of whom had panhypopituitarism diagnosed in childhood *(32)*. All had been treated with GH in childhood, but had discontinued GH for at least 6 mo before the investigation. GH administered daily for 4 mo in a placebo-controlled crossover trial produced a significant increase in the serum osteoblast marker osteocalcin (*see* Fig. 1). Urine levels of hydroxyproline, a marker of collagen breakdown, and a less specific marker of bone resorption also increased with GH administration. Further investigation in the same group of experimental subjects demonstrated that the more specific urine bone resorption markers, pyridinoline and deoxypyridinoline, also increased significantly and correlated highly with levels of osteocalcin *(32)*.

Later studies of GH administration in adults with childhood-onset GHD have evaluated GH effects on both bone-turnover markers and bone density. One such study of 14 GH deficient adults (eight of whom had a history of childhood-onset GHD) evaluated the effects of GH administration in a 6-mo, randomized, crossover design *(34)*. Although levels of alkaline phosphatase (a nonspecific marker of osteoblast activity) increased, no change in spinal bone density were seen at 6 mo. Degerblad et al. *(35)* studied six patients, five of whom had childhood-onset GHD, who received GH for 24 mo *(35)*. By 6 mo, a significant increase was seen in serum levels of the amino-terminal propeptide of type 3 procollagen, a serum marker of bone formation. At 18 mo, significant increases in both cortical (5%) and trabecular (18%) bone density were seen as measured by single photon absorptiometry of the proximal and distal forearm, respectively.

A subsequent study of men with a history of childhood-onset GHD receiving GH for a total of 18 mo reported that levels of the bone formation markers carboxyterminal propeptide of type I procollagen and osteocalcin increased by 3 mo, peaked between 6 and 12 mo, and then returned toward baseline *(36)*. Significant increases in bone density of the forearm and lumbar spine were demonstrated at the 18-mo point. Bone density was assessed in another open-label extension of a brief placebo-controlled trial of GH in patients with a history of childhood-onset GHD *(37)*. Single photon absorptiometry of the forearm was performed at 7 and 14 mo during GH administration and demonstrated a 4% increase in bone mineral content over this period. O'Halloran et al. *(38)* studied 12 patients ages 16–30 with childhood-onset GHD *(38)*. After 12 mo of GH administration, bone density increased in the proximal and distal forearm in an open label phase of the protocol. In contrast, Balducci et al. *(39)* administered GH for 12 mo to a group of 13 patients with adult-onset GHD, 10 of whom continued treatment for a total of 24 mo. Significant increases in the bone formation markers osteocalcin and alkaline phosphatase and the bone resorption marker urine hydroxyproline were seen at 12 mo. However, bone density of the lumbar spine as measured by dual photon absorptiometry showed no change at either time point.

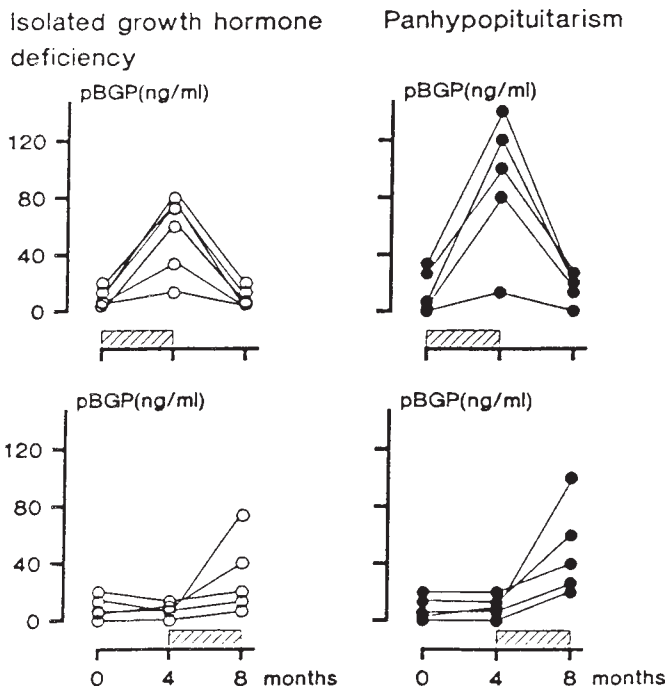

Fig. 1. Individual plasma concentrations of BGP (pBGP) in patients with isolated GH deficiency and panhypopituitarism during treatment with hGH or placebo. The hatched areas represent the active treatment periods.

Although increases in bone turnover have been consistently demonstrated in studies of GH administration to patients with childhood-onset GHD, increases in bone density have not. This may be owing in part to variability in doses of GH, duration of treatment, and methodology of bone densitometry. The majority of studies have shown improvements in bone density, but only during open label, noncontrolled trials. The role of such variables as drift in bone densitometer measurements and change in patient diet and exercise habits cannot be entirely excluded. Daily doses of GH also varied widely, from 0.04–0.1 IU/kg (approx 13–33 mcg/kg) with frequent reports of fluid retention and elevated levels of IGF-I. It is therefore unclear whether in some cases patients received a pharmacologic intervention that would be impractical or unsafe over the long term.

GH Administration in Adult-Onset GHD

Demonstration of a positive effect of GH administration on bone density in adults with childhood-onset GHD cannot be assumed to be applicable to patients who acquired GH deficiency as adults. It is unknown whether the abnormal course of skeletal maturation and the potential failure to reach peak bone mass despite GH therapy in patients with childhood-onset GHD may enhance the subsequent response of such patients to GH administered in adulthood.

Observations in patients with acromegaly, a condition in which the bones are exposed to high levels of GH in adulthood, provide some indication that GH may increase bone density in adults who develop normally. Two studies of patients with active acromegaly have shown increased bone density in the forearm *(40)*, femoral neck, and Ward's tri-

angle *(41)* compared to normals. This was not found in patients with a history of treated acromegaly who had normal levels of IGF-1 *(40)*. Interestingly, acromegalic patients who were simultaneously hypogonadal, displayed diminished spinal bone density despite the increase in cortical bone density *(40)*.

Studies of GH administration in patients with adult-onset GHD have shown results consistent with those in childhood-onset patients. Initial short-term studies have shown GH-induced increases in bone turnover, while subsequent longer-term, often noncontrolled studies have demonstrated improvements in bone density. Bengtsson and coworkers have published three studies of GH administration in patients with adult-onset GHD that have included evaluations of bone turnover markers and bone density. The first of these involved 10 patients and demonstrated GH-induced increases in osteocalcin and the aminoterminal propeptide of type 3 procollagen within 6 wk with further increases noted at 6 mo *(42)*. A subsequent study by this group involved 25 patients who participated in a 6 mo, placebo-controlled trial of GH administration, followed by an open-label trial for a total of 12 mo of GH treatment in 12 of the patients *(43)*. At 6 mo, significant increases in bone formation markers were seen, however, total body bone mineral density as measured by dual-energy X-ray absorptiometry decreased 2.1%. In the 12 patients who continued in the open-label phase of the study, total body bone mineral density remained slightly decreased compared to baseline, though a significant increase was seen in bone density of the femoral neck. The majority of the patients studied required reductions from the GH dose of 0.25 IU/kg/wk owing to symptoms of carpal tunnel syndrome and edema. The group's third study, an open-label trial, was larger and longer than the previous efforts (44 patients studied for 2 yr), and included downward dose adjustments in GH dose for patients with elevated levels of IGF-1 *(44)*. Despite the dose reduction protocol, 24 patients had elevated levels of IGF-1 at the conclusion of the study. Bone density measurements of the lumbar spine, femoral neck, greater trochanter, and Ward's triangle were increased at 18 mo compared to baseline and increased further at 2 yr for a total 3.8–5.6% increase over the entire study.

Two shorter randomized studies of GH administration in patients with adult-onset GHD have shown the expected increases in markers of bone without improvement in bone density after six *(45)* or 12 *(46)* mo of GH administration. The longer of the two studies showed a small but significant decrease in bone density of the whole body and the forearm in patients receiving GH *(45)*. Both studies employed doses of GH that frequently produced side effects. Another such study found no increase in bone density despite a total of 12 mo of GH administration to 13 patients in an open label extension of a 6-mo randomized study *(47)*.

We performed an 18-mo, placebo-controlled study of GH administration in 32 men with adult-onset GH deficiency and demonstrated a positive effect of GH on bone density of the lumbar spine (5.1% increase) and femoral neck (2.4% increase) over 18 mo with associated increases in markers of bone turnover (*see* Fig. 2) *(48)*. This study employed a starting dose of GH lower than that of the earlier studies (10 mcg/kg, approx 0.03 IU/kg), with dose adjustments that avoided supranormal IGF-1 levels in the majority of patients. These data indicate that physiologic replacement of GH can increase bone mass in this population. In addition, benefits were achieved with a much lower rate of side effects.

As with childhood-onset GHD, studies of GH administration in adult-onset GHD have consistently demonstrated increases in bone turnover, but not always increases in bone density. Only one controlled study has shown a benefit of GH on bone density, so that further studies are necessary to confirm this effect.

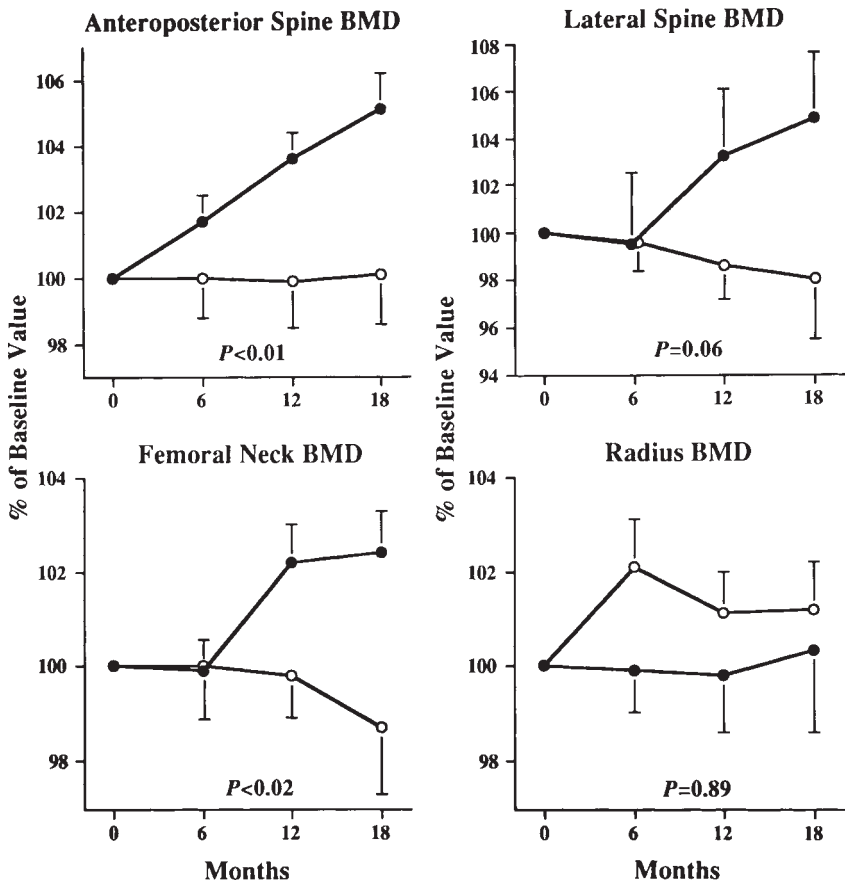

Fig. 2. Bone mineral density of the lumbar spine measured in the antero-posterior projection, lumbar spine measured in the lateral projection, femoral neck, and one-third distal radius in patients with GHD receiving GH (●) or placebo (○). Values are expressed as percentages of the baseline values. Error bars represent ± 1 SE. *P* values are for comparisons of rates of change between the two groups.

GH Administration for Age-Related Osteoporosis

Only a small number of studies have evaluated the effects of GH administration on bone density in women with postmenopausal osteoporosis and elderly men. The sample sizes of these studies have typically been small and overall fail to demonstrate a positive effect of GH on bone density. Aloia et al. *(49)* performed a series of three studies evaluating the effectiveness of GH in the treatment of post-menopausal osteoporosis. The first of these included eight patients who received pituitary-derived human GH for up to 12 mo *(49)*. Bone resorption increased as measured by urine hydroxyproline, and bone density of the radius decreased. A subsequent study compared 24 mo of combination treatment with GH and calcitonin to calcitonin alone in 25 post-menopausal women, and showed a deleterious effect of the addition of GH on radial bone density *(50)*. The third study compared a regimen of alternating GH and calcitonin to calcitonin alone in 14 women over 24 mo, and showed no significant difference in bone density between the two groups *(51)*.

Marcus and coworkers also investigated the effect of GH administration on bone turnover and bone mass in elderly subjects in two studies. In the first study, the effects of seven days of GH administration on bone turnover markers was examined in 12 women and six men over age 60, and both osteocalcin and hydroxyproline increased *(52)*. In a subsequent placebo-controlled study, bone density did not change in the group receiving GH, but decreased significantly in the placebo group *(53)*. Although these data suggest a possible protective effect of GH on bone density, the group was too small at the conclusion of the study, owing to a high dropout rate, to reach a meaningful conclusion. These data also highlight the potential high side effect profile of GH, particularly in high doses in an elderly population.

Rudman et al. *(54)* studied 21 elderly men with low levels of IGF-1, who, based on the investigator's previous work, were assumed to produce low levels of endogenous GH. This initial randomized trial demonstrated a small increase in lumbar bone density in the group treated with GH. However, when the study was expanded to include 45 subjects followed for up to 21 mo, a significant increase in bone density could no longer be demonstrated *(55)*. Because serum levels of IGF-1 have not been subsequently shown to separate GH sufficient and GH deficient patients, it remains unclear whether elderly patients diagnosed as GH-deficient on the basis of low IGF-1 levels may benefit from GH replacement.

Although GH administration to post-menopausal women and elderly adults does exert an effect on bone turnover, studies have failed to show a definite effect on bone density. It does not appear that a sufficient number of studies with large patient populations have been performed to rule out a possible benefit of GH in age-related or post-menopausal osteoporosis. Studies of GH in combination with a potent antiresorptive agent such as alendronate might be a productive future direction for this area of research. Long term studies of GH administration in GH-sufficient subjects, however, would have to be carefully designed to ensure that consequences of GH excess are not encountered.

CONCLUSIONS

A broad range of experimental data confirm that GH plays an important role in preserving the skeleton. GHD attributable to pituitary disease is associated with osteoporosis. Because of this it has been hypothesized that the decline in bone density seen in aging is attributable to declining GH secretion. In vitro, GH stimulates osteoblast proliferation and collagen production. Evidence from clinical studies indicates that GH replacement in GH-deficient adults improves bone density, though it is unclear whether this finding will be applicable to the relative GHD of aging.

Although the clinical use of GH in adults with GHD has been approved in the United States and Europe, a number of important questions remain unanswered. First, standards regarding the diagnosis of GHD must be established. Second, the best parameter to determine physiologic versus pharmacologic GH dosing is unknown. Third, the role, if any, of GH therapy in the treatment of osteoporosis in GH sufficient patients remains to be determined. Studies of GH administration to date have evaluated the hormone's effect on markers of bone turnover and bone density. The principal functional consequence of osteoporosis, however, is pathologic fracture, a potentially disabling or fatal condition. Larger studies of more prolonged duration will be required to determine whether GH administration affects this critical clinical endpoint.

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15 Growth Hormone and Syndrome X

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Contents

INTRODUCTION

The association and importance of several risk factors (obesity, dyslipoproteinemia, hepatic steatosis, insulin resistance, and hypertension) in the pathogenesis of noninsulin dependent diabetes mellitus (NIDDM) and myocardial infarction has been known in the literature for many years and was initially called "The Metabolic Syndrome" *(1,2)*. In 1988, Reaven introduced "Syndrome X" as the link between insulin resistance and hypertension *(3)*. Syndrome X is still used world-wide for the description of this association of risk factors and diseases. Other designations are "The Insulin Resistance Syndrome" *(4,5)* and "The deadly quartet" *(5)*.

As was pointed out by Vague in the forties, a specific distribution of adipose tissue is associated with both endocrine perturbations and human disease *(6)*. It has now been shown that the critical factor for the association between obesity, NIDDM, and cardiovascular morbidity is the mass of intra-abdominal fat *(7–10)*. This is probably explained by the unique metabolic characteristics and anatomical localization of visceral adipose tissue (omental and mesenteric fat) that comprises more than 80% of total intra-abdominal fat mass.

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PATHOGENIC MECHANISMS

The blood flow from visceral fat depot is drained via the portal vein to the liver, in contrast to other fat depots that are drained to the systemic circulation. Visceral adipose tissue has a higher turnover rate of fat, in both men and women, than other adipose tissue depots *(11)*. Both lipid accumulation, by the action of lipoprotein lipase (LPL), and the lipolytic response to catecholamines are elevated *(11–13)*. The increased lipolytic activity of visceral fat combined with its anatomical localization means that the liver is exposed to higher concentrations of free fatty acids (FFA) than any other organ. FFA have important influence on the liver metabolism. Increased levels of FFA attenuates the hepatic clearance of insulin from the pancreas and enhances the gluconeogenesis and the secretion of very low density lipoproteins (VLDL) from the liver *(14–17)*. Therefore, with enlarged visceral adipose tissue depots, as in visceral obesity, these effects of FFA on the liver would be expected to cause peripheral hyperinsulinemia, hyperglycemia, and elevated levels of VLDL, all of which are known to be important risk factors for NIDDM and arteriosclerosis.

The lipolytic process is mainly regulated by catecholamines in human adipose tissue *(18)*. Furthermore, visceral adipocytes have a higher density of lipolytic β-adrenergic receptors than other fat cells, mediating lipolysis by the action of norepinephrine *(19)*. The density of glucocorticoid as well as androgen receptors is also higher *(12,20)*. The effect of cortisol is mainly to increase visceral fat mass by increasing the expression of LPL *(21,22)*, while testosterone has the capacity to decrease fat accumulation by inhibiting LPL *(23,24)* and enhancing lipolysis by the increasing the expression of β-adrenergic receptors *(25,26)*. In addition to these intrinsic characteristics of the visceral adipocytes the surroundings of these cells are different from other adipocytes. Blood flow is higher than in other adipose tissues *(27)*, which is of fundamental importance for both lipid uptake and mobilization, and in addition visceral adipose tissue contains more catecholamines and catecholaminergic nerves than other adipose tissues *(28)*.

Taken together this means that visceral adipose tissue has a unique metabolic capability, hormone receptor density, blood flow and innervation to form a metabolic center of adipose tissue. This is of considerable and potential importance in view of the effects of FFA on the hepatic regulation of metabolism.

ENDOCRINE ABERRATIONS

During recent years of investigation abdominal/visceral distribution of adipose tissue has been found to be associated with endocrine disturbances, confirming the original observation by Vague *(6)*. These disturbances include an increased cortisol activity and a blunted secretion of growth hormone (GH) and sex steroids in both men and women *(29–34)*. These endocrine perturbations can theoretically be a consequence of the obese condition but it has also been suggested that the endocrine aberrations can have causal effects *(33,35)*.

Recent results from testosterone intervention studies in men with abdominal/visceral obesity *(33,36)* may support this hypothesis. A physiological amount of testosterone in middle-aged men with abdominal/visceral adiposity induced improved insulin sensitivity, plasma lipid levels, and diastolic blood pressure, as well as a specific decrease in visceral adipose tissue mass. This might theoretically be explained by direct effects of testosterone on adipose tissue. However, as testosterone treatment in men with

hypogonadotrophic hypogonadism increases GH secretion *(37)*, the observed effects could be explained by increased GH levels or by additional or synergistic effects by GH and testosterone on adipose tissue metabolism *(38)*.

Cortisol is of interest as it causes accumulation of abdominal/visceral adipose tissue *(39)* and an increased release of FFA. The latter will, in turn, cause reduced insulin binding in the liver and thereby higher circulating levels of insulin, glucose, and blood lipids by the mechanisms discussed above. The role of cortisol in obesity has been controversial during many years. Several authors have found decreased plasma cortisol levels in obese subjects while other have reported an increased cortisol secretion. We have recently found that these illusory contradictory findings can be explained by a disturbed diurnal secretion of cortisol, characterized by low morning plasma cortisol levels and increased 24-h free urinary cortisol values *(30)*. Furthermore, previous studies on abdominally obese subjects have shown increased cortisol secretion after corticotrophin releasing hormone (CRH) or ACTH challenge as well as after mental and physical laboratory stress tests *(30,31,40)*. In addition, dexamethasone inhibition of cortisol secretion is blunted in abdominally obese men, suggesting a down-regulation of glucocorticoid receptors in the brain controlling CRH secretion *(41)*.

There is considerable evidence that elevated secretion of CRH might attenuate other hypothalamic/pituitary hormonal axes including GH and gonadal steroids *(42)*. A plausible explanation of an increased activity of the hypothalamic-pituitary-adrenal (HPA) axis in Syndrome X could be chronic or intermittent stress challenges, or an impaired ability of the individual to cope with stress *(43)*. Furthermore, the neuro-endocrine findings discussed here show several similarities with conditions of oversecretion of cortisol such as Cushing's syndrome and depression, both of which have an increased activity of the HPA axis *(45)*.

GH SECRETION IN ABDOMINAL OBESITY

With increased adiposity, GH secretion is blunted with a decrease in the mass of GH secreted per burst but without any major impact on GH secretory burst frequency *(46)*. Moreover, the metabolic clearance rate of GH is accelerated *(32)*. The serum insulin-like growth factor (IGF)-1 concentration is primarily GH dependent and influences GH secretion though a negative feed-back system *(47)*. The serum levels of IGF-1 are inversely related to the percentage of body fat *(46)*. In addition, the low serum IGF-1 concentration in obesity is predominantly related to the amount of visceral adipose tissue and not to the amount of subcutaneous fat mass *(48)*. The relationship between regional fat distribution and GH secretion has only recently been considered. No significant correlation was found between the waist-to-hip ratio and 24-h GH secretion rates in a study of 21 healthy men *(49)*. However, measured by computed tomography, the amount of visceral adiposity was a major determinant of stimulated (arginine and clonidine) GH secretion in non-obese healthy adults *(50)*. Also the integrated 24-h GH concentration was found to be negatively associated with visceral fat mass in both young and old men and women *(51)*. These findings, together with other endocrine disturbances in central obesity, suggest that the low GH secretion and serum IGF-1 is secondary to a central disturbance of the neuroendocrine regulation.

Low levels of GH may be of importance for the metabolic consequences and the maintenance of the obese condition. One trial has, however, demonstrated near normalization of the 24-h GH secretion and serum IGF-1 in nine obese subjects after massive

weight loss *(52)* whereas others have not found a normalization of the GH response to provocative testing in response to weight loss *(53,54)*. Thus, whether the multiple endocrine aberrations including low GH secretion in abdominal obesity is primarily responsible or the consequence of the obese condition remains to be elucidated.

SYNDROME X AND GH DEFICIENCY IN ADULTS

Striking similarities exist between Syndrome X *(3,35)* and untreated GH deficiency in adults *(55)*. The most central findings in both these syndromes are abdominal/visceral obesity and insulin resistance *(35,56–58)*. Other features common to both conditions are high triglyceride and low high-density lipoprotein (HDL) cholesterol concentrations, an increased prevalence of hypertension, elevated levels of plasma fibrinogen and plasminogen activator inhibitor (PAI)-1 activity, premature atherosclerosis, and increased mortality from cardiovascular diseases *(35,56,59–62)*. Because of these similarities between GH deficiency in adults and Syndrome X, undetectable and low levels of GH, respectively, may be of importance for their metabolic consequences in these conditions.

Visceral Adiposity

Patients with acromegaly have reduced amount of adipose tissue mass *(63)* that normalizes with successful treatment *(64)*. Furthermore, successfully treated patients with acromegaly that normalize their GH secretion demonstrate an increase of predominantly the visceral adipose tissue mass *(65)*. The reverse scenario is seen in adults with hypopituitarism and untreated GH deficiency who have increased amount of body fat mass with abdominal preponderance *(57,58)*, which in response to GH administration results in a profound reduction of visceral adipose tissue and less marked effects on other adipose tissue depots *(66)*. Thus, GH has profound effects on adipose tissue distribution.

Insulin Resistance

Insulin resistance is a common condition and can be seen, for example, in NIDDM, obesity, and hypertension. The inter-relationship between insulin resistance and these conditions, as well as the exact mechanisms for insulin resistance, have not yet been fully clarified. It has recently been clear that GH-deficient adults are also insulin resistant in peripheral tissues (as measured using the hyperinsulinemic euglycemic clamp technique *[67,68]*). In our study, glucose disposal rate (GDR) in the GH-deficient group was less than half that of controls, when calculated according to body weight and when corrected for body fat *(67)*. The decreased lean body mass and the increased abdominal obesity in GH deficiency may be of importance for this finding as the association between increased body fat mass and insulin resistance is stronger in the presence of abdominal obesity *(69)*. Low levels of serum IGF-1 may also contribute to insulin resistance *(70)* as IGF-1 stimulates the glucose transport in skeletal muscle *(71)*. Other factors such as different composition in skeletal muscle fibers *(72)* and decreased physical activity in adults with GH deficiency may be of importance.

Dyslipoproteinemia

GH has important effects on the lipoprotein metabolism. For example, hypophysectomy changes the lipoprotein pattern from a predominantly HDL to one with a distinct low density lipoprotein (LDL) peak in the rat *(73)*, suggesting that the presence of GH

is essential for maintaining a normal lipoprotein pattern. Moreover, in response to GH the serum LDL-cholesterol and apolipoprotein B concentrations decrease *(73)*, probably a result of the increased clearance of these lipoproteins through increased hepatic LDL receptor activity *(74)*.

A common finding in both GH deficiency and Syndrome X is high levels of serum triglycerides and low HDL-cholesterol concentrations. This may be associated with their increased abdominal adiposity *(75)* and insulin resistance *(67,68,76)*. However, although a dramatic reduction in visceral adipose tissue occurs in response to GH treatment, serum triglyceride concentration is not reduced *(66,75,77)* and the concentration of HDL-cholesterol is increased *(77,78)*. The lipolytic action of GH treatment probably increases the flux of FFA to the liver *(79)* and increases the synthesis and secretion of VLDL from the liver. The LPL activity in adipose tissue is attenuated *(80)* and the post-heparin plasma LPL is not affected by GH treatment *(81)*. As serum triglyceride concentrations do not increase under conditions of increased VLDL secretion the peripheral catabolism must be enhanced. Increased LPL activity in other tissues such as muscle is therefore likely *(81)*. Furthermore, the strong association between glucose/insulin homeostasis and VLDL metabolism *(56)* might be reflected in the response to GH. The unaffected triglyceride levels might thus be explained by essentially unchanged insulin sensitivity *(82)* and glucose tolerance during more prolonged GH treatment in GH-deficient adults.

Fibrinolysis

Plasminogen activator inhibitor (PAI)-1, the fast-acting tissue plasminogen activator (t-PA) inhibitor, is the major regulator of fibrinolytic activity in plasma. Increased PAI-1 activity acts in a thrombogenic direction. Elevated PAI-1 activity has been associated with coronary artery disease *(84,85)*, increased risk of myocardial infarction in young patients *(86)*, recurrent myocardial infarction *(87)*, and deep vein thrombosis *(88)*. High PAI-1 activity has previously been found in patients with hypertension, insulin resistance, and abdominal obesity *(89–91)*. In addition, we have recently shown that elevated PAI-1 activity in GH-deficient adults as compared with healthy controls matched for age, sex, and body mass index *(58)*.

Previous population-based studies have shown that fibrinogen is an independent risk factor for stroke as well as myocardial infarction, and is at least as important as blood lipids and blood pressure *(92,93)*. Obesity has been associated with both increased fibrinogen levels and increased PAI-1 activity *(90,91,94–96)*. Also the fibrinogen levels were higher in the GH-deficient group *(58)*: Although patients and controls were matched for BMI, we observed both higher fibrinogen levels and PAI-1 activity in the GH-deficient patients, suggesting that other factors, in addition to obesity per se, are of importance. Both the elevated fibrinogen levels and PAI-1 activity may be linked to the abdominal and visceral obesity, indicated by a high waist to hip ration in these patients *(58)*.

Blood Pressure

Both GH deficiency in adults and Syndrome X are associated with increased prevalence of hypertension. The insulin resistance in Syndrome X has been linked with hypertension through increased activity of the sympathetic nervous system *(3)*. In adults with hypopituitarism and untreated GH deficiency enhanced activity of the sympathetic nervous system has been measured by intraneural recordings in muscle

(97) linking this condition to increased prevalence of hypertension. In addition, GH deficiency has been found to be associated with low levels of nitric oxide (NO), a paracrine vasodilator produced in endothelial cells, which normalize in response to GH treatment *(98)*.

GH TREATMENT OF PATIENTS WITH ABDOMINAL OBESITY

As GH promotes lipolysis low levels of GH has therefore been suggested to be of importance for the maintenance of the obese condition. The calorigenic effects of GH in obese subjects has also been known for many years *(99)*. Some trials have therefore addressed the question of whether GH administration through its calorigenic and lipolytic action might enhance weight loss during dietary restriction in obese subjects. Both short-term *(100)* and several weeks of GH treatment *(101,102)* in combination with dietary restriction were unable to enhance the loss of body fat or body weight as compared with saline treatment. The GH administration may, however, decrease the loss of lean body mass during dietary restriction *(100,102)*. These results, therefore, suggest that GH is not useful in the induction or enhancement of weight loss in obese subjects.

We have learned that GH can improve several of the aberrations that occur both in GH deficiency and Syndrome X. Thus, in GH-deficient adults the lipolytic effects of GH results in a preferential reduction in visceral adipose tissue *(66)*. Furthermore, GH reduces the diastolic blood pressure *(103)*, reduces total cholesterol, LDL-cholesterol *(57,104–106)*, and increases HDL-cholesterol concentrations *(78,105,107)*. Furthermore, long-term GH treatment does not impair insulin sensitivity *(82)*. With this background we have studied the effects of GH on the metabolic, circulatory, and anthropodometric aberrations associated with abdominal/visceral obesity and Syndrome X *(108)*.

The men who were studied were moderately obese with a preponderance of abdominal and/or visceral localization of body fat. As a group, they had slight to moderate metabolic changes known to be associated with abdominal/visceral obesity with serum IGF-1 concentrations in the low normal range and moderate insulin resistance as judged from the GDR values obtained during the euglycemic glucose clamp. None had overt diabetes. Nine months of GH treatment in a randomized, double-blind placebo controlled trial, in these middle-aged men with abdominal/visceral obesity reduced their total body fat and resulted in a specific and marked decrease in both abdominal subcutaneous and visceral adipose tissue (Fig. 1). Moreover, insulin sensitivity improvement (Fig. 2) and serum concentrations of total cholesterol and triglyceride decreased. Diastolic blood pressure decreased while plasma fibrinogen increased slightly.

GH exerts direct insulin-antagonistic effects even after the administration of physiologic doses of GH. GH has been considered to be the principal factor in the decrease in insulin sensitivity observed in the early morning, the so-called "dawn phenomenon" *(109)* and the insulin resistance following hypoglycemia *(110)*. Thus, our observation of increased insulin sensitivity during prolonged GH treatment is unexpected although not inexplicable. This improvement could be explained by the decrease in visceral adipose tissue mass induced by GH, followed by a decrease in FFA exposure to the liver counteracting the insulin-antagonistic effects of GH. Alternatively, as the major site of glucose disposal is in the skeletal muscle *(111)*, the possibility has to be considered that the improvement in GDR in response to the more prolonged GH treatment might also be an effect of increased glucose transport in the skeletal muscle, possibly mediated through the IGF-1 receptor *(71)* and an increased proportion of insulin-sensitive type 1 muscle fibers *(72)*.

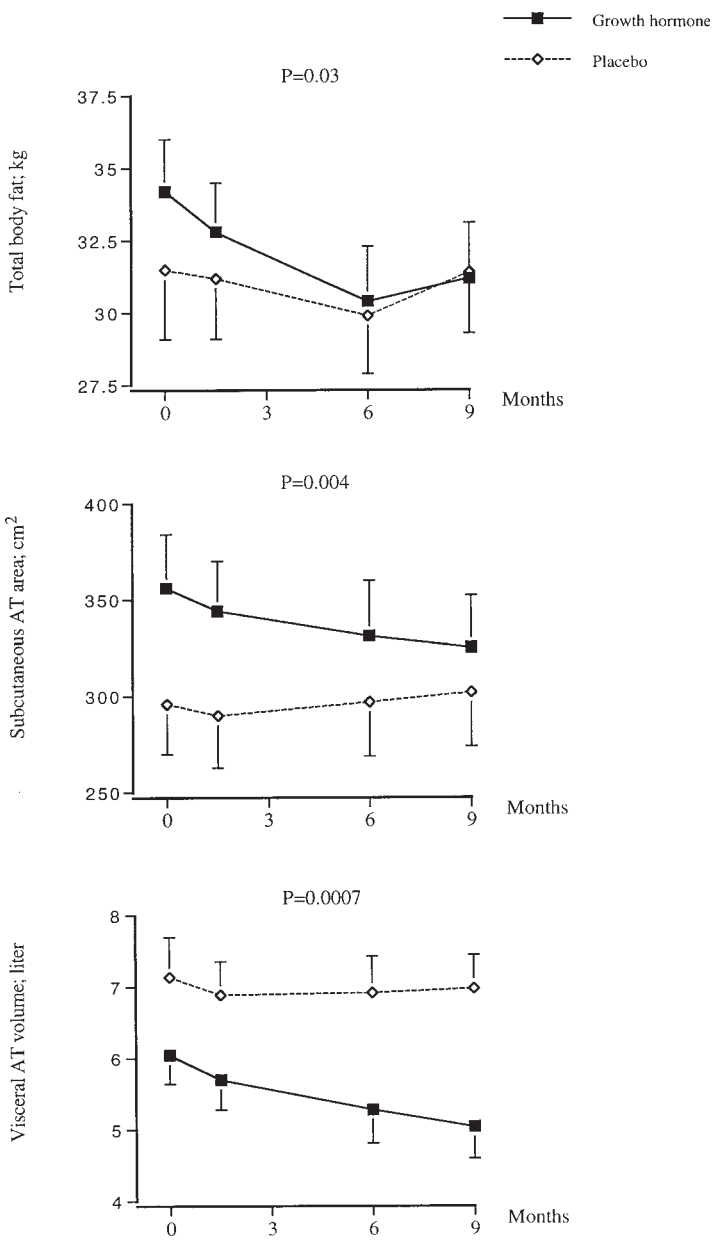

Fig. 1. Mean total body fat calculated from total body potassium, abdominal subcutaneous adipose tissue (AT) area at the level of L4-L5 and total volume of visceral AT assessed with computed tomography during 9 mo of treatment with rhGH or placebo in 30 men with abdominal/visceral obesity. The horizontal bars indicate the SE for the mean values shown and *p*-values denote the differences between the two groups by two-way ANOVA for repeated measurements.

The reduction in total cholesterol is conceivably an effect of enhanced hepatic LDL-receptor activity in response to GH *(74)*. In healthy adults, short-term GH administration has been reported to increase serum triglyceride concentrations *(112)*. In this study, the serum triglyceride concentration also displayed an initial increase in response

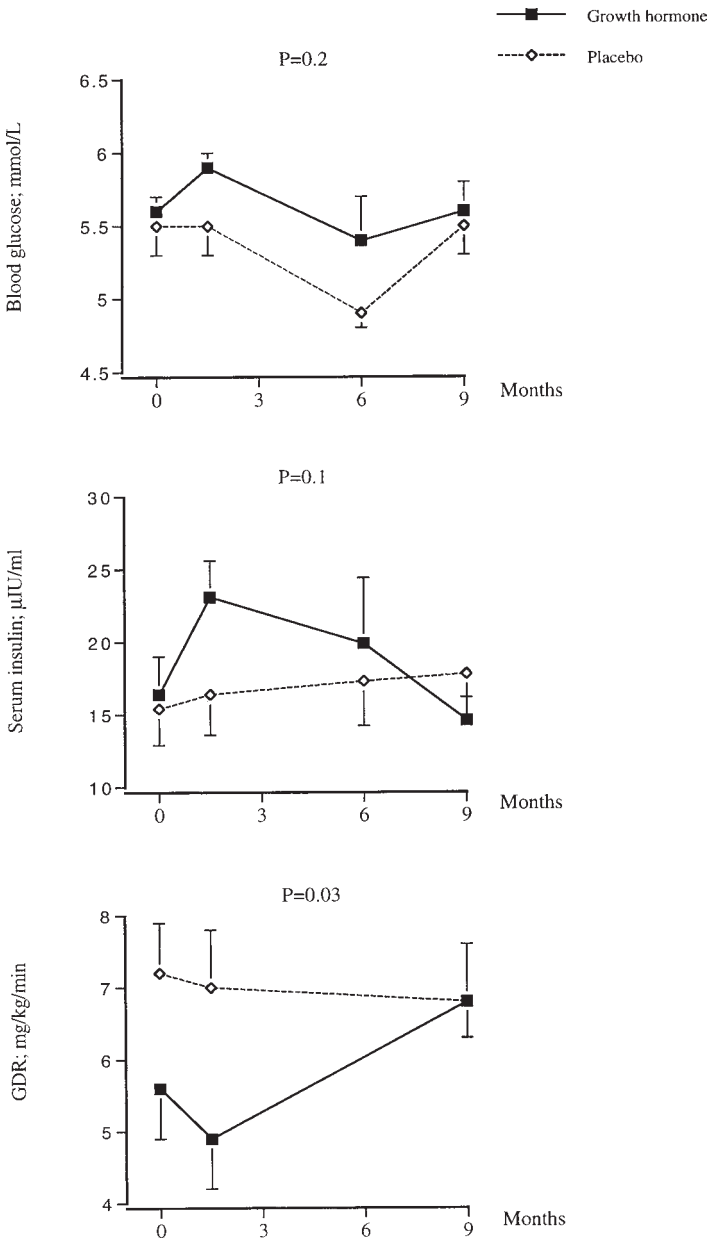

Fig. 2. Mean fasting blood glucose, serum insulin, and glucose disappearance rate (GDR) assessed with a euglycemic hyperinsulinemic glucose clamp during 9 mo of treatment with rhGH or placebo in 30 men with abdominal/visceral obesity. The horizontal bars indicate the SE for the mean values shown and *p*-values denote the differences between the two groups by two-way ANOVA for repeated measurements.

to GH treatment. This could be an effect of both an increased flux of FFA to the liver and a direct stimulatory effect on the esterification of oleic acid into triglyceride and phospholipids in hepatocytes *(113)* in response to GH, which in turn enhances the very low density lipoprotein production from the liver. However, after nine months of GH treat-

ment, the serum triglyceride concentration had decreased again, probably as an effect of the increased insulin-stimulated glucose uptake. The GH treatment reduced diastolic blood pressure without affecting systolic blood pressure. This is in line with results from GH-deficient adults where GH administration reduced diastolic blood pressure, possibly as an effect of reduced peripheral vascular resistance *(103)*. The mechanisms behind the reduction in peripheral vascular resistance might be indirect through the reduced abdominal obesity and increased insulin sensitivity *(114)* or more direct through the action of IGF-1 on the vascular wall *(115)* with increased levels of NO *(98)*.

The multiple endocrine alterations associated with abdominal/visceral obesity can either be primarily responsible or be the consequence of the obese condition. This is the first trial to clearly demonstrate favorable effects by GH on the multiple perturbations associated with abdominal/visceral obesity. We therefore suggest that a blunted GH secretion could be an important factor in the development of the metabolic and circulatory consequences of abdominal/visceral obesity.

GENERAL CONCLUSION

The high and abnormal activity of the HPA axis, low levels of sex steroids, and attenuating GH secretion in abdominal obesity suggests a central neuroendocrine dysregulation in abdominal obesity. Whether this is of primary importance for the evolution of abdominal obesity or merely a secondary phenomenon to the obese condition remains to be elucidated.

The finding that replacement with testosterone and GH to men with abdominal obesity are able to diminish the negative metabolic consequences of the visceral obesity suggest that the low levels of these hormones are of primary importance for the metabolic consequences associated with visceral/abdominal obesity.

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16 Interactions Between Growth Hormone Secretion and Sleep

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Contents

THE ROLE OF THE SLEEP-WAKE CYCLE IN THE TEMPORAL ORGANIZATION OF HUMAN GH SECRETION

Association Between GH Release and Sleep in Normal Adults

The fact that the secretion of growth hormone (GH) is markedly stimulated during sleep has been recognized for more than three decades. Early studies using the first available radioimmunoassays for GH demonstrated that the peripheral levels of this hormone increased rapidly following sleep onset *(1–5)*. In normal adult subjects, the 24-h profile of plasma GH levels consists of stable low levels abruptly interrupted by bursts of secretion. The most reproducible pulse occurs shortly after sleep onset *(3,4)*. This relationship between sleep onset and GH secretion appears to be most consistent in the human species, because it is more difficult to evidence in other mammals, although elevated blood GH levels have been observed during sleep in baboons, rhesus monkeys, dogs, lambs, and both immature and adult rats *(6–12)*. Species differences could be

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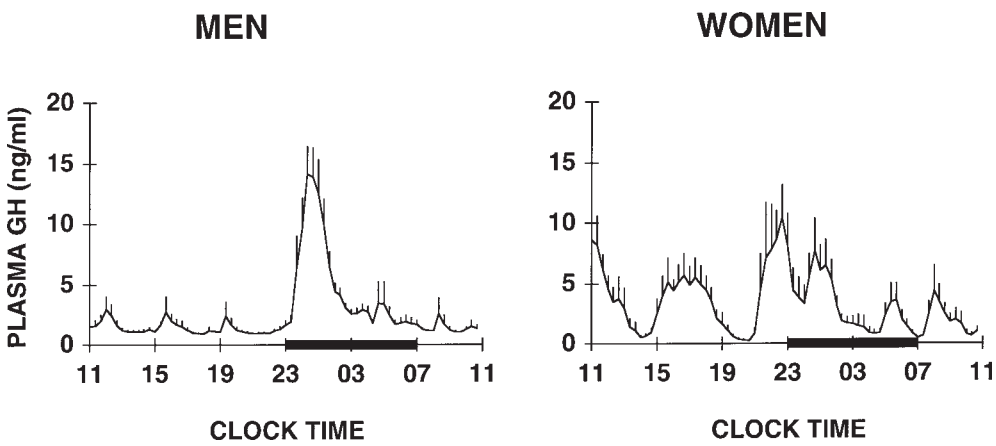

Fig. 1. Mean (+SEM) 24-h GH profiles in 6 men, ages 21–30 yr, (left) and in 8 women, ages 21–33 yr (right). The black bars represent the sleep periods. Unpublished data.

related to the fact that human sleep is consolidated in a single 7 to 9-h period, whereas multiple sleep bouts are the rule in other mammals. Thus, the sleep-wake transition in the human is generally associated with more pronounced variations in endocrine, metabolic, and other parameters than in other species.

In men, the sleep-onset GH pulse is generally the largest, and after the third decade of age, it is often the only pulse observed over the 24-h span. This is well illustrated by the mean 24-h GH profile from normal young men, shown in the left panel of Fig. 1. In women, daytime GH pulses are more frequent and the sleep-associated pulse, although still present in most cases, does not generally account for the majority of the 24-h GH release. The right panel of Fig. 1 shows mean profiles of plasma GH in healthy young women studied during the midluteal phase of the menstrual cycle. The increased daytime activity of the somatotropic axis in women has been shown to be correlated with circulating free estradiol levels *(13)*.

Effects of Manipulations of the Sleep-Wake Cycle

Sleep onset will elicit a pulse in GH secretion whether sleep is advanced, delayed, or interrupted and re-initiated. Figure 2 illustrates the maintenance of this relationship in subjects who underwent a 12-h shift of the sleep-wake cycle. A pulse of GH secretion following sleep onset has been observed in subjects submitted to a variety of manipulations of the sleep-wake cycle, including a 3-h delay *(5)*, a 5-h delay *(14)*, an 8-h delay *(15)*, a 12-h delay *(4,16)*, a 16-h delay *(16)*, daytime recovery sleep following 28 h of continuous wakefulness *(17)*, nocturnal recovery sleep following 40 h of continuous wakefulness *(18)*, a 7-h advance associated with transmeridian travel *(19)*, and a 7-h advance following 33 h of sleep deprivation in the laboratory *(19)*. Daytime naps are more consistently associated with GH release when they occur in the afternoon and the propensity for SW sleep is relatively high, as opposed to the morning when REM sleep predominates *(20,21)*. The relationship between sleep onset and GH release was largely maintained in subjects exposed to a 3-h sleep-wake cycle for 10 d *(22)* as well as in subjects living in temporal isolation without any environmental time cues (i.e., "free-running conditions") *(23,24)*. An early study where sleep was interrupted by one hour of enforced wakefulness

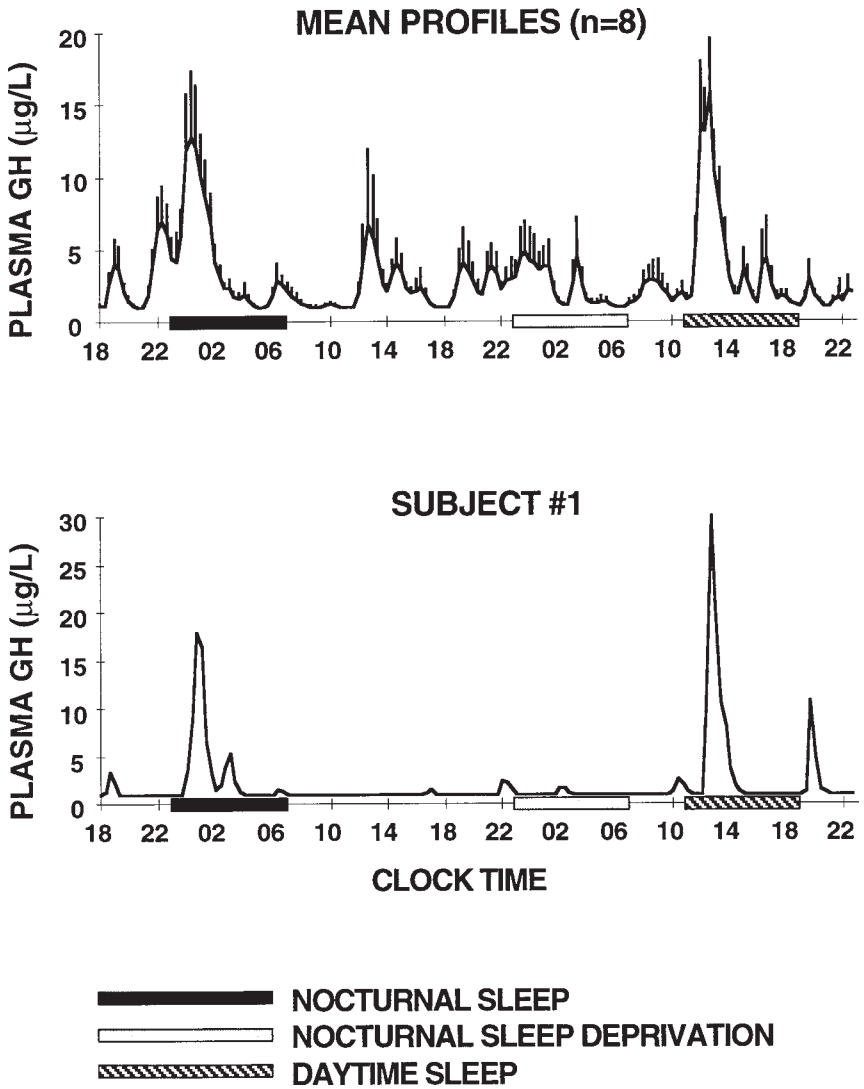

Fig. 2. Upper panel: Mean (+SEM) profiles of plasma GH in 8 normal young men studied during a 53-h period including 8 h of nocturnal sleep, 28 h of sleep deprivation and 8 h of daytime sleep. The black bar represents the nocturnal sleep period. The open bar represents the period of nocturnal sleep deprivation. The dashed bar represents the period of daytime sleep. Lower panel, Individual plasma GH profile from one representative subject. Symbols are as in upper panel.

demonstrated an increase of GH levels following the re-initiation of sleep *(25)*. Finally, a study of night workers indicated that the main GH secretory episode still occurred during the first half of the daytime sleep period *(15)*.

Circadian Modulation of GH Secretion

Although sleep is clearly a major determinant of the 24-h profile of GH secretion in man, there is also evidence for the existence of a circadian modulation, i.e., an intrinsic effect of the time of day. A careful re-analysis of an early study of normal subjects

submitted to a 3-h sleep-wake cycle for a prolonged period of time indicated that the elevation of GH concentration following sleep onset was largest when the sleep episode occurred in the late evening, i.e., around the usual bedtime *(26)*. The late evening and early part of the night appear to represent a period of increased propensity for GH secretion, as studies involving abrupt delays of the sleep period in very young adults have shown modest increases in GH pulsatility at this time of day (despite enforced wakefulness), suggesting the existence of a weak circadian modulation of GH secretion *(14,15,27,28)*. A recent study using repeated injections of GHRH at 3-h intervals demonstrated the existence of a diurnal variation in peak GH response that was interpreted as reflecting a diurnal rhythm in somatostatinergic tone *(29)*. Interestingly, elevated GH responses were already apparent in the early evening, prior to sleep onset.

ASSOCIATIONS BETWEEN SLEEP STAGES AND NOCTURNAL GH RELEASE

Increased GH Release During Slow-Wave Sleep

Sleep does not involve a constant state of reduced brain activity but instead an approximate 90-min oscillation between non-REM (rapid eye movement) stages and REM stages, which is normally repeated 4–6 times per night. Polygraphic sleep recordings include electroencephalographic (EEG), electromyographic (EMG), and electrooculographic (EOG) recordings. The so-called polysomnogram is visually scored in 20- or 30-s epochs in stages I, II, III, IV, REM, and Wake using standardized criteria *(30)*. In the normal sequence, waking is followed by the lighter stages of non-REM sleep (i.e., stages I and II) and then within 10–20 min by so-called slow wave sleep (SWS: stages III and IV), which is maintained for nearly one hour in normal young subjects. Lighter stages of non-REM sleep then reappear and the first REM period is initiated. As the night progresses, non-REM sleep becomes more shallow, the duration of REM episodes becomes longer and the number and the duration of awakenings increase. During a normal night in normal young subjects, approx 20% of the night are spent in SW, 25% in REM, 50% in stages I and II, and 5% in wake. The upper panel of Fig. 3 shows a typical polysomnogram of a young normal subject.

An alternative, and perhaps more informative, way to analyze sleep is to submit the EEG recordings to power spectral analysis. This procedure provides a more detailed quantification of changes in EEG frequency and amplitude than sleep stage scoring but is not as standardized and may be more readily affected by artifacts. The EEG signal is digitalized and after appropriate filtering, submitted to a Fast Fourier Transform with calculation of the spectral density in standard frequency bands. The low frequency delta waves that are apparent during SWS are reflected in an increase in spectral power in the so-called delta range (0.5–3 Hz). The second panel of Fig. 3 illustrates the profile of delta power corresponding to the polysomnogram shown in the upper panel. It is noteworthy that delta activity may be present in stage II sleep and therefore precede the appearance of stages III and IV.

Already in the late sixties, well documented studies involving analyses of polygraphically recorded sleep and concomitant GH levels concorded in indicating that there is a consistent relationship between the appearance of delta waves in the EEG and GH secretion during early sleep as well as during the later part of the night *(3–5,31)*. Moreover, selective slow-wave sleep deprivation was shown to result in diminished (but not totally

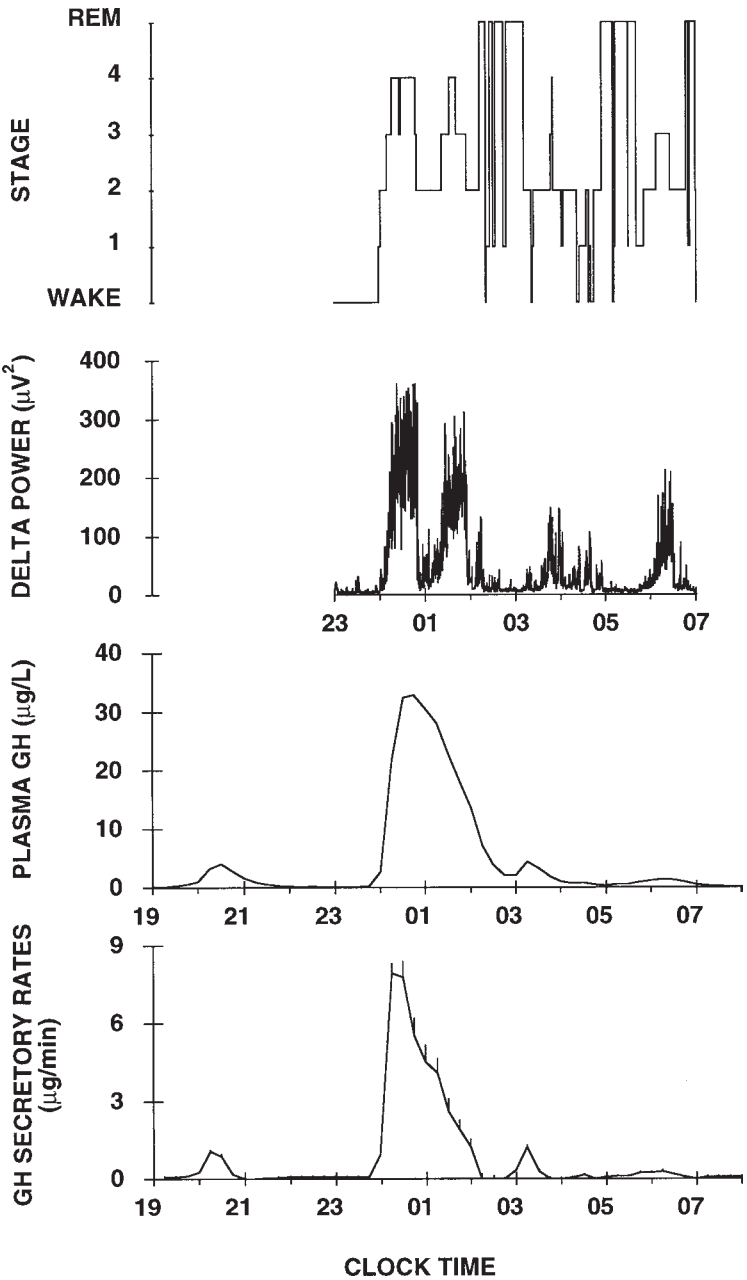

Fig. 3. Profiles of visually scored sleep stages (at 30-s intervals), delta power, plasma GH levels (sampled at 20-min intervals) and GH secretory rates from a typical normal young subject. Note that the profile of GH secretory rates clearly shows a rapid decrease in GH secretion coinciding with the end of the first period of increased delta wave activity and a re-initiation of secretion (reflected in a shoulder on the descending limb of the secretory pulse) at the beginning of the second period of increased delta wave activity. A complete interruption of secretion was not observed during the trough of delta activity, most likely because neither REM nor Wake interrupted the first non-REM period. Note also that the cessation of active secretion occurred shortly after the end of the second burst of delta wave activity, whereas plasma GH levels did not return immediately to baseline.

suppressed) GH secretion during sleep *(32,33)*. These initial findings, which supported the concept that the daily GH secretory output is critically dependent on the occurrence of SW sleep, were confirmed in a number of later reports *(19,24,34)* but not in others, which suggested that the relationship with SW sleep is fortuitous *(35–37)*. Indeed, nocturnal GH surges occurring independently of the presence of SW sleep were reported in one study *(37)* and in another, selective partial SW stage deprivation failed to suppress or delay the sleep-onset GH pulse *(36)*. Several investigators reported marked rises in GH secretion prior to the onset of sleep *(35–38)*. An analysis of the association of GH secretion during sleep and delta wave activity failed to demonstrate a significant linear "dose-response" relationship between the two processes *(37)* and it was suggested that sleep onset per se, rather than the occurrence of SWS, is the primary determinant of sleep-related GH secretion *(36,37)*.

Later studies of the relationship between sleep stages and GH release used deconvolution (a procedure that allows secretory rates to be derived from plasma concentrations by eliminating the effects of hormonal distribution and clearance using a mathematical model) to examine GH secretory rates, rather than plasma GH levels *(14,39)*. The analysis of variations in GH secretory rates during the various stages of sleep is more accurate than the analysis of plasma concentrations because the temporal limits of each pulse are more accurately defined and additional pulses which were masked by hormonal clearance are revealed. This is illustrated in the lower panels of Fig. 3. Using deconvolution calculation, a detailed study with 30-s sampling of plasma GH during sleep has indicated that maximal GH release occurs within minutes of the onset of SW sleep *(39)*. Furthermore, in studies examining GH secretion in normal young men of similar height and weight, it was found that approx 70% of GH pulses during sleep occurred during SW sleep and that there was a quantitative correlation between the amount of GH secreted during these pulses and the duration of the SW episode *(14)*. Furthermore, the longer the SW episode, the more likely it was to be associated with a GH pulse. These relationships remained significant even when sleep-onset pulses were not included in the calculations. This quantitative correlation between various markers of slow wave activity and amount of concomitant GH release has been confirmed in a more recent study *(40)*. The temporal and quantitative associations between GH secretory rates, SW stages, and delta power are illustrated in Fig. 4.

Nevertheless, the relationship between slow wave activity and GH secretion is not obligatory, because nocturnal GH secretion can occur in the absence of SW sleep and approx one third of the SW periods are not associated with significant GH secretion *(14)*. Because GH secretion is also under inhibitory control by somatostatin, variability of somatostatinergic tone may underly dissociations between SW sleep and nocturnal GH release. The short-term negative feedback inhibition exerted by GH on its own secretion may also explain observations of absent GH pulse during the first SW period when a secretory pulse occurred prior to sleep onset. Such pre-sleep GH pulses are likely to reflect the fact that, even during waking, the late evening period appears to correspond to an increased propensity to secrete GH, probably the result of a reduction in somatostatinergic activity *(29)*. In addition to variable somatostatin activity as a likely explanation to dissociations between nocturnal GH release and SW sleep, a number of studies (reviewed in Effects of GHRH on Sleep Quality) have demonstrated that hypothalamic GHRH stimulates non-REM sleep and have suggested that stimulation of GH release and promotion of sleep may represent two independent outputs of distinct popu-

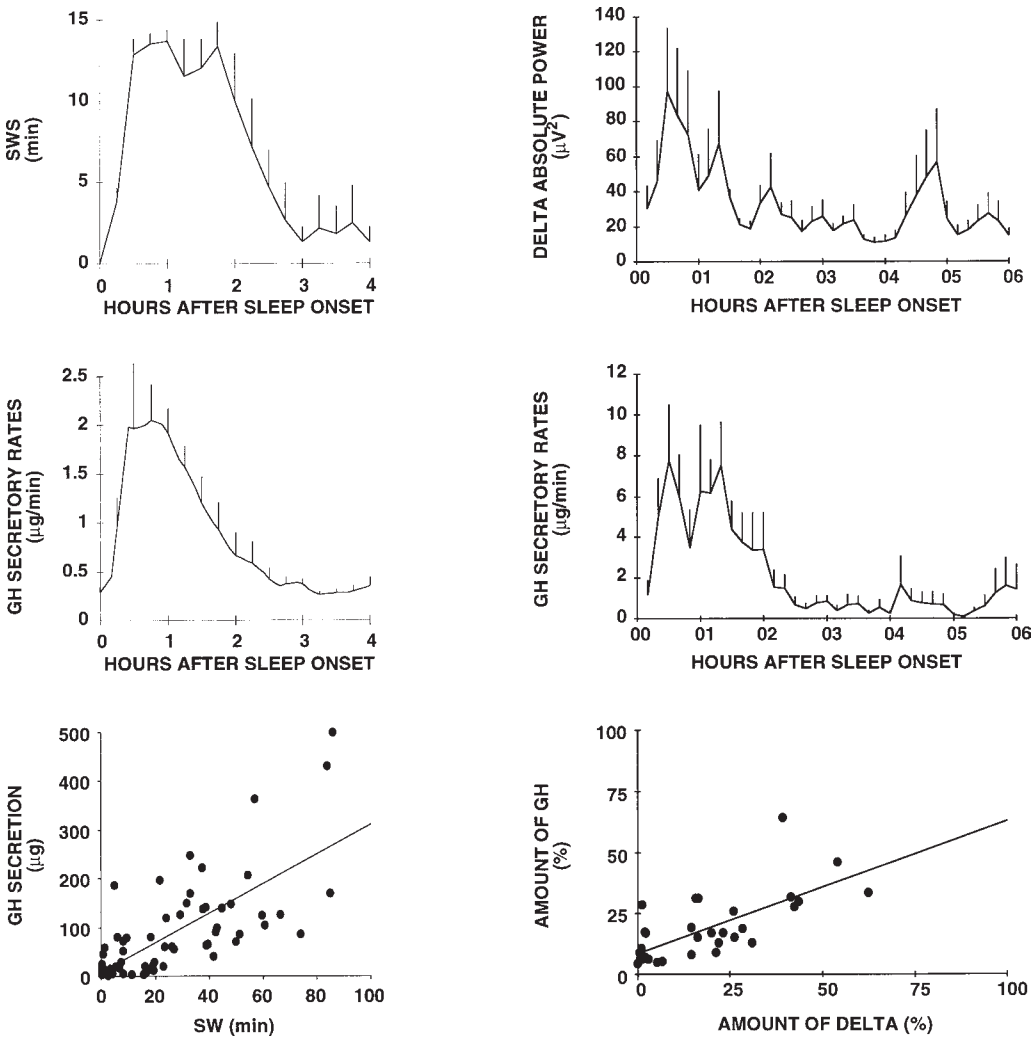

Fig. 4. Left: Mean (+SEM) profiles of visually scored SW sleep (expressed as min of SW sleep in each 15-min interval separating successive blood samplings; top panel), simultaneous GH secretory rates (middle panel), and correlation between amount of GH secreted and duration of SW periods (lower panels). The data were obtained in 8 normal subjects *(14)*. Right: Mean (+SEM) profiles of delta power (top panel), profiles of GH secretory rates (middle panel) and correlation between amount of GH secreted and delta power (lower panels). The data were obtained in 10 normal subjects *(40)*.

lations of hypothalamic GHRH-containing neurons. The available evidence concerning the mechanisms underlying sleep-related GH release is further reviewed in the section "Putative Mechanisms Underlying Interactions Between Somatotropic Axis and Sleep."

In normal young men, sleep stages markedly modulate the amount and dynamic characteristics of GH secretion in response to a bolus injection of GHRH at a dose eliciting a GH response in the physiological range. In a study examining GH responses to GHRH during wake and during the various stages of sleep, it was found that the GH response to GHRH given at the beginning of the first SW period was approx 30% higher than that

observed during wake and lasted almost one hour longer *(41)*. However, there appeared to be no synergy between the stimulatory effect of exogenous GHRH and that of SW sleep and in fact, their cumulative effects were slightly less than additive. When given during REM sleep, the response to GHRH was less than that observed during SW but was similar to that observed during wake if the subject remained asleep following the injection *(41)*.

Inhibitory Effects Associated with Awakenings

In a study where GH secretion was stimulated by the injection of growth hormone releasing hormone (GHRH) at the beginning of the sleep period, it was found that whenever sleep was interrupted by a spontaneous awakening, the ongoing GH secretion was abruptly suppressed *(41)*. This inhibitory effect of awakenings on the GH response to GHRH was further demonstrated in a detailed study where sleeping subjects who had received a GHRH injection were awakened 30 min after the injection and then allowed to re-initiate sleep 30 min later *(42)*. The subjects who were able to resume sleep rapidly showed a secondary smaller GH pulse. A near complete inhibition of the GH response to GHRH was also observed when the injection was given 20 min after a forced awakening around the end of the first third of nocturnal sleep *(42)*. It has been suggested that this inhibitory effect of nocturnal awakenings on the GH secretory response to GHRH could be mediated by an increase in somatostatin release *(42)*. This increase in somatostatinergic activity could be effected by an increase in corticotropic activity. Indeed, awakenings during sleep are consistently associated with a pulse of cortisol secretion *(43)*, and corticotropin-releasing hormone (CRH) administration may inhibit the GH response to GHRH stimulation *(44)*.

These findings suggest that sleep fragmentation (a hallmark of aging *[45]*) will generally decrease nocturnal GH secretion and are particularly interesting in view of the well documented age-related decreases in GH secretion that occur in both men and women *(13,46)*. Furthermore, the important effects of sleep and awakening on the secretory response to GHRH injection indicate that the state of wakefulness of the subject should be carefully monitored during testing with GHRH to prevent naps which could markedly influence the response.

Pharmacological Stimulation of Slow-Wave Sleep

The existence of a robust relationship between SW activity and increased GH release raised the possibility that compounds that increase slow-wave sleep may also be GH secretagogs. Commercially available hypnotics tend to inhibit, rather than increase, SW sleep and do not stimulate GH release *(47–49)*. However, reliable stimulation of SW sleep in normal subjects has been obtained with oral administration of low doses of gamma-hydroxybutyrate (GHB), a simple four carbon fatty acid that is used as an investigational drug for the treatment of narcolepsy *(50–55)* as well as with ritanserin, a selective $5HT_2$ receptor antagonist.

GHB is a metabolite of gamma-aminobutyric acid (GABA) that is normally present in the mammalian brain and is thought to be acting as a neurotransmitter *(56–58)*. GHB readily crosses the blood-brain barrier but has a short duration of action, which limits its use for the treatment of insomnia. A recent study showed that bedtime administration of GHB, even at a very low dose, results in a twofold increase in the amount of GH secreted during sleep *(59)*. This effect of GHB on nocturnal GH secretion resulted from an increase in the amplitude and the duration of the normal secretory pulse associated with sleep

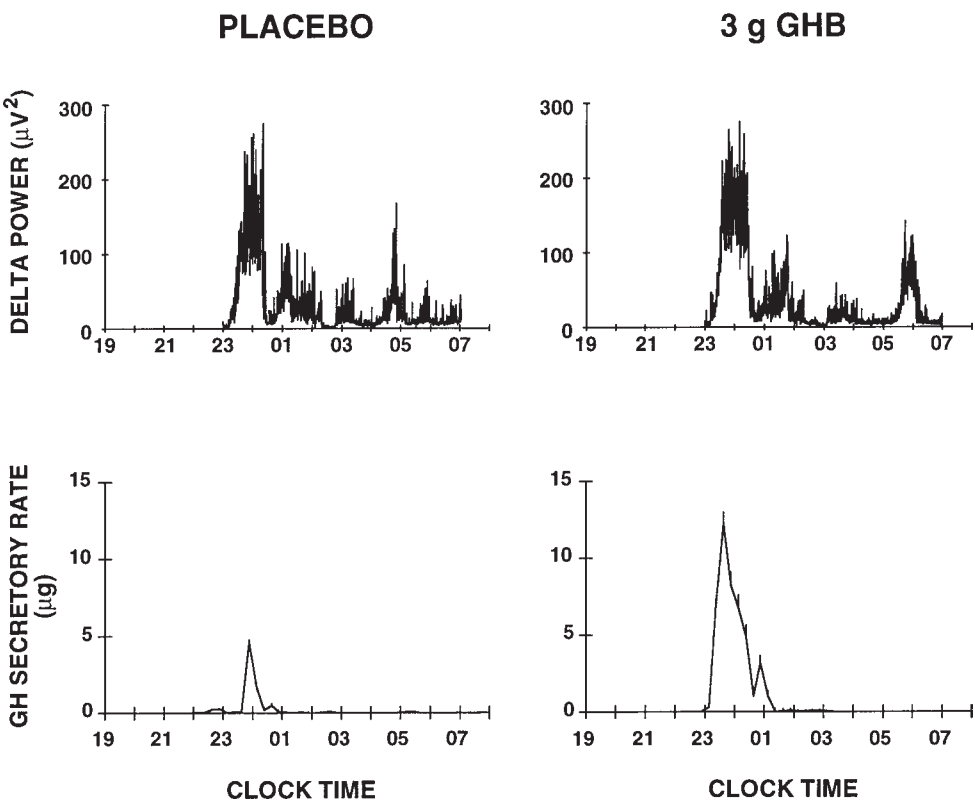

Fig. 5. Profiles of GH secretory rate and delta power in a representative subject after oral administration of placebo (left) or 3 g GHB (right). Data source: ref. *59*.

onset, rather than from the induction of additional pulses. The increase in GH secretion was only initiated after the first epoch of stage II sleep had been recorded and was quantitatively correlated with an increase in amount of stage IV during early sleep *(59)*. Representative examples of the profiles of delta power and GH release following bedtime administration of either placebo or 3 g GHB are shown in Fig. 5.

Administration of the selective $5HT_2$ receptor antagonist, ritanserin, was also found to result in parallel and highly correlated increases between delta wave activity and nocturnal GH release *(40)*. The stimulation of delta power and GH secretion obtained by ritanserin is of a lesser magnitude than that observed following treatment with low clinical dosages of GHB.

Conversely, the reduction of slow wave sleep and delta power observed following flumazenil, a benzodiazepine antagonist, was found to be associated with a decrease in GH concentration *(28)*.

SLEEP-RELATED GH SECRETION DURING DEVELOPMENT AND AGING

Development

The total amount and the temporal distribution of GH secretion is strongly dependent on age. Spontaneous GH secretion is detectable in term infants who appear to have

a high level of tonic, i.e., non pulsatile, secretion *(60)*. As the infant matures, GH pulse frequency and amplitude decrease and tonic secretion diminishes *(60)*. A pulsatile pattern of GH release, with increased pulse amplitude during sleep, is present in prepubertal boys and girls *(61)*. During puberty, the amplitude of the pulses but not the frequency is increased, particularly at night *(62,63)*. Maximal overall GH concentrations are reached in early puberty in girls and in late puberty in boys *(63)*. Because of the robust nature of the relationship between sleep and GH release in both prepubertal and pubertal children, it has been proposed that a "sleep test", i.e., repeated measurements of plasma GH during overnight sleep, may provide a reliable index of GH secretion and a useful test of GH deficiency *(64,65)*. A number of reports examining the relationship between nocturnal GH secretion and SW sleep in children have indicated that the temporal association observed in adult is already present both in prepubertal and pubertal children and remains detectable in growth retarded children without growth hormone deficiency and in hyperactive children of small stature *(66–69)*.

Aging

Aging is associated with dramatic decreases in GH and insulin-like growth factor (IGF)-1 secretions and with pronounced alterations of sleep quality *(13,45,46,70–72)*. When similar bedtime schedules are enforced in young and old men, the sleep onset time and the total sleep period are not significantly modified by age but sleep stage distribution is markedly altered, with more frequent and prolonged awakenings, a pronounced reduction in the duration of SW stages, a decrease in the total amount of REM stages, and an earlier timing of the appearance of REM stages. In healthy elderly men over the age of 65, the total amount of GH secreted over the 24-h span is generally less than one third of the daily output of men under 30 yr *(13,46,73–76)*. Similarly, the amount of SW sleep in older adults is reduced in the same proportion *(46)*. This decline in overall GH secretion appears to be achieved primarily by a decrease in amplitude, rather than frequency, of GH pulses. In a retrospective analysis involving nearly 100 simultaneous recordings of sleep and 24-h GH secretion in adult men ages 18–82 yr, we have recently shown that these dramatic effects of aging on SWS and GH secretion occur early in adulthood in an exponential fashion and are essentially complete by the beginning of the fifth decade *(77)* (Fig. 6). Similar observations in a smaller subject population studied overnight have been recently reported, although the non-linear decrease in the amount of SW sleep failed to be detected *(78)*. Although early studies had generally concluded that sleep-related GH pulses were absent in the elderly, the findings of more recent studies are concordant in showing persistent, but reduced, GH secretion during sleep *(13,46,74,76)*. In our retrospective analysis, it was apparent that the proportion of daily GH output that occurs during the first few hours of sleep, does not decrease with age, but remains stable or even slightly increases. A significant correlation between levels of IGF-1 and delta power has been reported in older adults *(79)*. The parallelism between decreased amount and quality of deep sleep and diminished somatotropic activity raises the interesting possibility that some of the peripheral effects of the hyposomatotropism of the elderly, such as the reduction in lean body mass, may partially reflect a central alteration in sleep control.

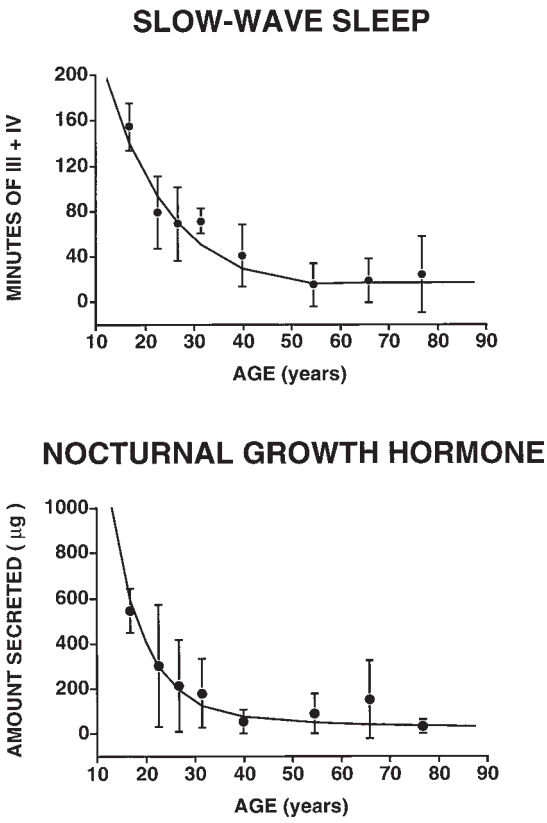

Fig. 6. Chronology of aging of SW sleep (scored visually) and nocturnal GH secretion (mean ± SEM for each age bracket). The subjects were 102 healthy non-obese men, ages 18–83 yr, who were grouped according to age bracket. Unpublished data.

RELATIONSHIP BETWEEN GH SECRETION AND SLEEP IN PATHOLOGICAL STATES

In untreated acromegaly, studies that have examined the GH profile during polygraphically recorded sleep have reported the absence of sleep-related GH pulses despite the presence of SW sleep *(80,81)*.

A few studies have examined nocturnal GH secretion in patients with obstructive apnea before and after treatment *(82–84)*. As expected, nocturnal GH release is decreased in untreated apneic subjects. Because adult patients with this pathology are frequently obese, the low overnight GH levels could reflect the hyposomatotropism of obesity, rather than result from the shallow and fragmented nature of their sleep. However, two studies that have examined the nocturnal GH profile before and after treatment with continuous positive airway pressure (CPAP) have demonstrated that treatment of the sleep disorder resulted in a clear increase in the amount of GH secreted during the first few hours of sleep *(83,84)*. An example is illustrated in Fig. 7. In children, surgical correction of obstructive sleep apnea may restore GH secretion and normal growth rate *(82)*.

In obese subjects who do not have sleep apnea, a normal relationship between the first SW episode and GH release may be observed both during nocturnal sleep and during

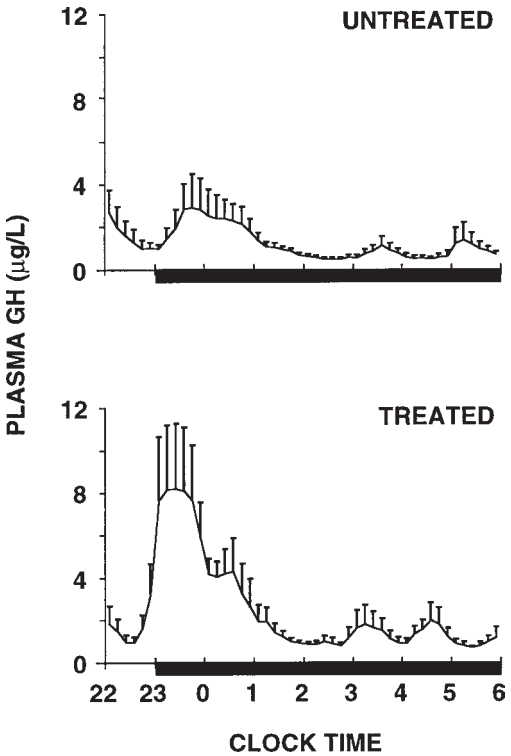

Fig. 7. Mean (+SEM) nocturnal plasma GH profiles in patients with sleep apnea before (top) and after (bottom) CPAP treatment. Black bars represent the scheduled sleep periods. Data source: ref. *83*.

daytime recovery sleep after sleep deprivation but, as expected, the amount of GH secreted in the sleep onset pulse is markedly decreased as compared to nonobese control subjects *(85)*.

In a recent study in which polysomnographic recordings and hourly GH levels were obtained for 24 h in African patients infected with trypanosomiasis (i.e., African sleeping sickness), significant correlations between plasma GH levels and SW sleep were identified despite the fact that the temporal distributions of both sleep and GH secretion across the 24-h cycle were markedly disrupted *(86)*.

A number of studies have examined sleep quality and GH secretion in depression and have generally observed a decrease in nocturnal GH release as well as a reduced amount of SW sleep, although quantitative relationships between the magnitude of these two alterations were not demonstrated and cause-effect associations remain elusive *(38,87,88)*.

MODULATORY EFFECTS OF COMPONENTS OF THE SOMATOTROPIC AXIS ON SLEEP

Although the association between sleep and GH release has been well documented, there is also good evidence to indicate that components of the somatotropic axis are involved in regulating sleep quality. Although the roles of each hormone could not be identified, the findings of a recent study in transgenic mice with a deficiency in the somatotropic axis have been particularly convincing as a robust loss of non-REM sleep

was demonstrated in these animals as compared to their wild-type littermates *(89)*. The studies reviewed in the present section are most consistent in indicating a role for GHRH in promoting non-REM and/or SW sleep via central, rather than peripheral, mechanisms. The current findings implicating GH in sleep regulation are more controversial.

Sleep Abnormalities in Conditions of Deficient or Excessive GH Secretion

A limited number of human studies, all originating from the same group of investigators, have examined sleep quality in subjects with congenital isolated GH-deficiency and in acromegalic patients before and after treatment *(90)*. In GH-deficient adults, a decrease in duration of SW sleep and a significant suppression in delta power were observed but there were no significant differences in REM sleep *(90,91)*. After six months of daily treatment with 2 IU/m^2 GH, the relative amount of REM sleep increased and there was a trend for an increased duration of SW sleep *(92)*.

In untreated acromegalic patients without sleep apnea, standard polysomnography revealed a reduction in REM sleep as well as a reduction in amount of SW sleep as compared to control subjects *(92)*. However, a more complex picture emerged when power spectral analysis of the EEG was performed *(92)*. Indeed, though the minutes of REM and SW were decreased, the spectral energy per min spent in both REM and SW was increased, indicating that the amplitude of the EEG waves during both SW and REM sleep is higher in acromegalics than in control subjects *(92)*. The amplitude of the EEG is the sum of the post-synaptic potentials of the cerebral cortex, and thus a higher energy presumably reflects an increased neuronal activity in the cerebral cortex. One year after adenomectomy, an important increase in REM time was observed, resulting in normal values for this age group *(92)*. A moderate increase in SW sleep was also observed. Treatment normalized the EEG energy per minute in both SW and REM stages *(92)*.

As will become apparent from the review given below in Effects of Exogenous GH Administration on Sleep Quality, some of the alterations of sleep quality found in patients with GH deficiency or acromegaly and the effects of treatments of these conditions are not consistent with the findings from studies on the effects of acute administration of GH or GHRH on sleep in normal subjects. However, it must be recognized that chronic pathological conditions and their correction by treatment may involve indirect effects on sleep quality in addition to those putatively mediated by the hormones of the somatotropic axis.

Rodent studies of sleep in hypothysectomized animals or in animals with lesions of the arcuate nucleus have provided conflicting observations and are difficult to interpret because neither model is specific for the somatotropic axis (reviewed in ref. *89*). An association between chronic GH excess and increased sleep duration was demonstrated in giant "supermice" genetically engineered with extra GH genes *(93)*.

Effects of Exogenous GH Administration on Sleep Quality

Early studies in rats and in cats indicated that injections of exogenous GH may stimulate REM sleep *(94,95)*. In humans, the stimulation of REM sleep was confirmed in a study involving an intramuscular GH injection administered 15 min before bedtime *(96)*. In addition, this treatment resulted in a decrease in SW sleep. A more recent study reported no effects on sleep quality when GH levels were elevated either by intravenous infusion or by intramuscular injection given approx 3 h before sleep onset *(97)*. As indicated above, in GH-deficient subjects, prolonged treatment with daily injections of exogenous GH resulted in a marked increase in REM sleep *(92)*.

Effects of GHRH on Sleep Quality

A number of studies have demonstrated effects of GHRH on sleep quality and it has been suggested that GH secretion and sleep may share common regulatory mechanisms *(98)*. In rodents, intracerebral as well as systemic injections of GHRH stimulate non-REM sleep, even in hypophysectomized animals *(99–102)*. Systemic injections of GHRH also stimulate REM sleep in intact, but not in hypophysectomized rodents *(101,102)*. Conversely, inhibition of endogenous GHRH using GHRH antagonists or antibodies to GHRH decreases both non-REM sleep and GH secretion *(98,103)*. In humans, discrepant data have been reported. No effects of GHRH on visually scored sleep stages were found when the peptide was injected during daytime or before sleep onset *(104,105)*, or when it was given as an infusion *(97,106)*. However, delta power during the first 100 min of sleep was significantly enhanced following bedtime injection of GHRH *(105)*. When the intravenous injections were performed during sleep, stimulatory effects on the duration of stages III and IV *(106–108)* and modest increases in REM sleep *(106,108)* were observed in normal young subjects. Similar—though weaker—effects were reported in healthy elderly controls *(109)*. Sleep-promoting effects of the peptide may depend on the timing of administration *(108,110)*. The persistence of stimulatory effects of GHRH on non-REM sleep in hypophysectomized rodents indicates that these effects are not mediated by GH *(101,102)*. In contrast, the finding that increases in REM sleep following GHRH administration are seen only in intact animals suggests that REM-enhancing effects of GHRH may be mediated by GH *(101,102)*. This hypothesis is consistent with the effects of exogenous GH administration on human sleep, which, as indicated above, have been mostly in the direction of a stimulation of REM, rather than non-REM, sleep.

Effects of Somatostatin on Sleep

Inconsistent data have been reported concerning the action of somatostatin on sleep. In the rodent, REM sleep was inhibited by immunoneutralization of endogenous somatostatin *(111)* and enhanced by intracerebroventricular administration of exogenous somatostatin *(112)*, while non-REM sleep was inhibited by subcutaneous injections of a long-acting somatostatin analog *(113)*. In humans, repeated intravenous injections or infusion of somatostatin did not influence sleep quality in normal young subjects *(107,114)*, but REM sleep was decreased by somatostatin in the elderly *(115)*.

Growth Hormone-Releasing Peptide (GHRP) and Related Molecules

Recent studies have indicated that the release of GH is also under the control of an as yet unidentified stimulatory pathway that may be activated by synthetic compounds such as the GH-releasing peptides (GHRPs) and their functional agonists *(116,117)*. These compounds are thought to act as functional somatostatin antagonists *(118)*. It is not known whether this second axis for GH stimulation is also involved in sleep regulation. Indeed, the findings of the only study that has examined the effects of injections of GHRP-6 around bedtime were an enhancement of the amount of stage II sleep without any other significant effect on either SW sleep or REM sleep *(119)*. These data do not exclude the possibility that, as was previously shown for GHRH, GHRP may have a stimulatory effect on SW sleep when given during the later part of the night, at a time when SW sleep is not naturally abundant. However, a recent study indicates that, in contrast with GHRH, single injections of GHRP, at a dosage resulting in similar GH

elevations, have no stimulatory effects on SW sleep, even when given at a time when SW sleep is not predominant *(120)*.

Recently, we have shown that 7-d oral treatment with MK-677, a functional agonist of GHRP acting via the GHRP receptor *(121)*, is associated with an increase in both stage IV and REM in normal young men *(122)*. This intriguing finding is difficult to interpret because plasma GH levels were not elevated at the time of the sleep study (although acutely MK-677 is a powerful GH secretagogue) but plasma IGF-1 levels were markedly increased. Multiple complex mechanisms could be involved in the chronic effects of MK-677 on sleep with dubious relevance to the effects of direct, acute, stimulation of the GHRP axis.

PUTATIVE MECHANISMS UNDERLYING INTERACTIONS BETWEEN SOMATOTROPIC AXIS AND SLEEP

Based on the review of studies using various pharmacological agents, it may be concluded that sleep-onset GH secretion is regulated by GHRH stimulation occurring during a period of relative somatostatin withdrawal. Indeed, in humans, GH secretion during early sleep may be nearly totally suppressed by the administration of a specific GHRH antagonist, thus demonstrating an important role for GHRH in the control of sleep-related GH release *(123)*. On the other hand, the late evening and nocturnal hours appear to coincide with the trough of a diurnal variation in hypothalamic somatostatin tone *(29)*. Cholinergic muscarinic blockade by a variety of drugs, including methscopolamine, scopolamine, atropine, and pirenzepine suppresses sleep-related GH secretion *(124–127)*. Conversely, piperidine, a nicotinic cholinergic receptor stimulator, enhances GH secretion during early sleep *(128)*. Thus, cholinergic mechanisms must be partially involved in the control of sleep-related GH secretion. A large body of evidence has suggested this cholinergic control is effected via the regulation of hypothalamic somatostatin.

There is evidence that, under physiological conditions, sleep-related GH secretion may be less sensitive to somatostatin inhibition than spontaneous daytime GH secretion or daytime GH secretion in response to a variety of stimuli. Indeed, during sleep, GH secretion is not suppressible by acute hyperglycemia *(129)*, a potent mechanism of inhibition of daytime GH release mediated in part by increased hypothalamic somatostatin activity. Similarly, aging, which is thought to be associated with a progressive increase in somatostatinergic tone *(130)*, seems to affect GH secretion less during sleep than during wake, as the secretory output associated with the sleep-onset GH pulse remains relatively preserved *(77)*. Reductions in somatostatinergic tone achieved by pharmacological treatment will generally enhance sleep-related GH secretion in adults *(128,131)* but in children, such treatments affect daytime, but not nocturnal, GH release because hypothalamic somatostatin activity is thought to be already minimal at night in this population *(132–134)*. Differences in sensitivity of sleep-related vs daytime GH release to variations in hypothalamic somatostatin activity suggest that distinct mechanisms underly GH secretion during wake and during sleep.

Indeed, based on rodent data, it has been proposed that the stimulation of GH release and the promotion of non-REM sleep by GHRH are two separate processes that involve GHRH neurons in two distinct areas of the hypothalamus *(135–137)*. The control of pituitary GH release would primarily involve GHRH neurons in the arcuate nucleus *(138)*. The promotion of non-REM sleep by GHRH would implicate another area of the

mediobasal hypothalamus where GHRH neurons are concentrated, within and around the ventromedial nucleus *(138)*. The majority of GHRH neurons in this latter region project to various parts of the basal forebrain which are involved in sleep regulation and may therefore be part of the mechanism linking somatotropic activity and sleep *(135–137)*. The association between GH release and SW sleep could represent synchronous activity in these two regions. However, the findings in several studies of a quantitative relationship between amount of GH secreted and various measures of SW activity suggest that the GHRH neurons which are implicated in the promotion of non-REM sleep also participate to some extend in the control of pituitary GH release *(14,40,59)*. The data suggestive of a lesser somatostatinergic control of GH release during sleep than during wake would be interpreted as evidence for weaker somatostatinergic control in the areas involved in sleep regulation and sleep-related GH secretion than in the area controlling daytime GH release. Although the concept of a dual control of daytime and sleep-related GH secretion remains to be directly demonstrated, it allows for the reconciliation of a number of experimental observations, including occasional dissociations between nocturnal GH secretion and SW sleep.

CONCLUSIONS

A large body of evidence indicates the existence of a robust relationship between SW sleep and GH release in the human and is consistent with the hypothesis that activation of hypothalamic GHRH activity is involved in the control of both SW sleep and nocturnal GH release *(136)*. The remarkable correlations between increased SW sleep and augmented GH release demonstrated in studies using SW-enhancing drugs indicate that pharmacological agents that reliably stimulate SW sleep may represent a new class of powerful GH secretagogues.

Aging is associated with marked decreases in both the duration of SW stages and the amount of GH secretion *(13,46,74,139)*. Although the clinical implications of decreased SW sleep are unclear, multiple studies have indicated that the relative GH deficiency of the elderly is associated with increased fat tissue and abdominal obesity, reduced muscle mass and strength, and reduced exercise capacity *(140–142)*. Compounds that stimulate SW sleep may represent a novel approach to increase endogenous GH secretion in older adults via the pharmacological enhancement of a physiological stimulus acting at a normal time of day. Such approaches will require the development of new lines of research since commercially available hypnotics, including the benzodiazepines, improve sleep efficiency but do not increase SW sleep *(143)*.

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17 Growth Hormone in AIDS

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Contents

INTRODUCTION

Body wasting is a frequent and potentially devastating complication of human immunodeficiency virus (HIV) infection. As widespread use of prophylaxis against opportunistic infections and other treatment strategies has effectively delayed the onset of classic AIDS-indicating illnesses, the prominence of the wasting syndrome as an AIDS-defining condition has increased *(1,2)*. In a large cohort study in the United States, the wasting syndrome was the AIDS-defining condition in 18% of those who had received prophylaxis against *P. carinii* pneumonia (PCP), when compared with 6% of those who did not *(2)*. Among patients followed at a naval medical facility, wasting accounted for 31% of AIDS diagnoses in 1992 as compared to only 6% in 1988 *(1)*. Epidemiological data suggest that women are at similar risk for wasting as men *(3,4)*.

In malnourished patients with HIV infection, timing of death was found to be related to the magnitude of depletion of body weight and body cell mass (BCM) *(5)*. Both extrapolated and observed values for body weight and BCM at the time of death (66 and 54% of normal, respectively) were similar to historical reports of death from starvation *(6,7)*. Multiple prospective *(8–11)* and retrospective *(5,12,13)* studies have demonstrated significant relationships between loss of weight *(5,8,11–13)* or lean body mass (LBM),

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particularly BCM *(5,9,10)*, and mortality in HIV-infected individuals. Notably, the impact of weight and body composition on survival in these studies was independent of other factors thought to influence mortality, including the $CD4^+$ lymphocyte count. Recently, a weight loss of as little as 5% in patients with HIV infection has been shown to increase risk not only of death but also of opportunistic complications *(11)*. In addition to affecting survival and disease progression, wasting can impact one's quality of life. In a study of clinically stable outpatients with HIV infection, with and without an AIDS-defining illness, BCM adjusted for height was significantly and independently associated with an important aspect of quality of life, namely physical functioning, even after controlling for age and disease severity *(14)*.

Weight loss in HIV infection tends to be episodic *(15–18)* and features depletion of both fat and LBM *(9,19–25)*. Individuals with HIV infection may also experience periods of weight stability and weight gain *(15,16,18)*. Although patterns of weight loss in individuals with HIV infection vary considerably, a typical scenario is rapid weight loss during infection *(17,18)* and a failure to fully regain the weight during the subsequent recovery phase *(26)*. The accumulated loss of weight and LBM resulting from such episodes leads eventually to clinically significant wasting.

MECHANISMS OF WEIGHT LOSS

Several metabolic disturbances that could theoretically contribute to weight loss have been found in patients with HIV infection. For example, increased rates of resting energy expenditure (REE), averaging from 8 to more than 25% greater than controls, have been reported in stable patients across the spectrum of HIV infection *(17,27–32)*. Even greater increases in REE may occur in patients with secondary infection *(17,29)*. However, the presence of metabolic disturbances such as hypermetabolism cannot account fully for the magnitude of weight loss seen in many HIV-infected individuals. Instead, a growing body of evidence points to decreased energy intake as the most important contributor to HIV-associated weight loss. Grunfeld et al. *(17)* demonstrated that energy intake was decreased in HIV-infected patients with active secondary infections. Weight decreased by an average of 5% in four weeks during secondary infection. Weight trend correlated with energy intake but not with REE. Macallan et al. *(31)* measured total energy expenditure (TEE), REE, and energy intake in 27 men with HIV infection at different stages of disease. Rates of TEE were decreased in patients studied during periods of rapid weight loss, but energy intake was decreased to an even greater extent, thus accounting for the weight loss. Notably, energy intake in these patients was reduced to such an extent that it was not even sufficient to cover the cost of REE, much less that of energy required for activity. Certainly, the inability to decrease REE in the face of decreased energy intake can serve as a co-factor in accelerating weight loss, but reduced energy intake, rather than metabolic disturbances, was the primary contributor to weight loss *(17,31)*.

INEFFICACY OF CALORIC SUPPLEMENTATION

Simply increasing energy intake by oral, enteral, or parenteral routes does not consistently restore LBM in individuals with HIV infection. Failure to increase LBM was particularly evident in a group of patients with systemic infections, predominantly cytomegalovirus or *M. avium* complex, who were given total parenteral nutrition (TPN) *(33)*. These individuals gained weight while receiving TPN, but they experienced no net

increase in BCM, estimated by total body potassium counting. In contrast, patients with malabsorptive disorders but no systemic infection gained weight and BCM. These results were recently confirmed in a study in which administration of TPN to patients with diarrhea or other obstacles to enteral supplementation produced increases in weight and LBM during two months of therapy *(34)*.

Similarly, although treatment with the appetite stimulant, megestrol acetate, has produced increases in energy intake and body weight in patients with HIV-associated wasting *(35,36)*, the weight gain in patients treated with this agent consists predominantly or exclusively of fat. For example, in one recent trial, treatment with megestrol acetate (800 mg/d for 12 wk) resulted in a 4.5 kg increase in fat with no change in LBM *(36)*. In another trial, weight gain averaged 3.5 kg, but only 1.1 kg was LBM and the remainder, fat *(35)*. Although increases in body fat in this setting may not be intrinsically harmful, there is no correlation between body fat content and survival *(5,9)*.

CHANGES IN GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-1 IN HIV INFECTION

Disturbances in the growth hormone (GH) and insulin-like growth factor-1 (IGF-1) axis have also been described in HIV infection. Decreased levels of IGF-1 have been noted in some malnourished individuals with HIV infection *(37,38)*, but normal levels of IGF-1 were reported in two other groups of patients with prior weight loss *(30,39)*. One potential explanation for these discrepant findings is that the patients in these latter two groups were studied during periods of relative clinical and weight stability, whereas the two former groups included patients who were losing weight at the time of study.

Frost et al. *(38)* noted a pattern of increased serum GH levels, coupled with decreased IGF-1 levels in 3 of 11 patients with HIV-associated wasting. On the other hand, in a recent study of GH secretory profiles in patients with HIV infection, growth hormone deficiency was evidenced by decreased GH peak amplitude and area under the curve *(40)*.

Evidence of growth hormone resistance has been noted in several studies, as well. When weight-stable HIV-infected patients with normal levels of IGF-1 were given pharmacologic doses of rhGH, circulating IGF-1 levels increased to a lesser extent than was seen in healthy control subjects *(30,39)*. Pharmacologic doses of GH failed to increase muscle protein synthesis in HIV-infected men, whereas protein synthesis increased significantly in a similarly treated control group *(40)*. However, it should be noted that another group of men with HIV-associated wasting who were treated with rhGH for one week experienced significant retention of nitrogen and potassium, suggesting accrual of lean tissue *(30)*, despite an apparently blunted increase in IGF-1 . The results of this latter study will be discussed in greater detail in the following section.

The failure of nutritional or appetite-stimulating therapies to consistently restore LBM and suggestions of disturbances in the GH/IGF-1 axis and hypogonadism have prompted considerable interest in both GH and IGF-1 as potential therapies for HIV-associated wasting.

GROWTH HORMONE THERAPY FOR HIV-ASSOCIATED WASTING

Because pharmacologic doses of GH have induced nitrogen retention in catabolic patients after surgery *(41)*, burns *(42)*, and hypocaloric feeding *(43)*, a study was under-

taken to determine whether short-term treatment with GH could produce a comparable anabolic response in persons with HIV-associated weight loss *(30)*. Six HIV-positive men with an average weight loss of 19% and six healthy weight-stable HIV-negative controls were hospitalized on a metabolic ward for 19 days. Throughout the study, all subjects consumed a constant diet at a level of energy intake that maintained body weight during the pre-treatment period. After successive five-day equilibration and seven-day baseline periods, subjects received a pharmacologic dose of recombinant human GH (rhGH; 0.1 mg/kg/d) by subcutaneous injection for seven days.

Body weight, which remained constant during the baseline period, increased progressively throughout rhGH treatment. Weight gain averaged 2.0 ± 0.3 and 1.6 ± 0.2 kg (3.4 ± 0.5 and 2.4 ± 0.3%) in HIV-positive and HIV-negative subjects, respectively, during one week of treatment. Plasma levels of IGF-1 increased significantly in both groups. An approximately threefold increase in IGF-1 was noted on d 7 in the HIV-negative controls, whereas values in the HIV-positive subjects increased approximately twofold. Urine nitrogen excretion declined within the first 24 h of rhGH treatment and remained below baseline levels throughout the treatment phase. Despite the apparently blunted IGF-1 response in the HIV-positive subjects, average daily nitrogen retention was similar in the two treatment groups (4.0 ± 0.2 vs 4.0 ± 0.6 g/d in HIV-positive and HIV-negative, respectively). The ratio of retained potassium to nitrogen was consistent with retention of these elements in lean tissue *(44)*. If all of the nitrogen and potassium that were retained were incorporated into lean tissue *(44)*, net gains of 0.8–0.9 kg of lean tissue were realized during one week of rhGH treatment.

Additional preliminary results were obtained in an open-label outpatient study of rhGH in patients with HIV-associated wasting. Krentz et al. *(45)* treated four patients with 5 mg rhGH every other day (approximately half the dose given in the metabolic ward study described above) for three months. This group experienced significant increases in weight and LBM, measured by bioelectrical impedance analysis, and a trend toward increased skeletal muscle power and endurance. Plasma levels of IGF-1 increased significantly, but to a lesser extent than in the aforementioned patients treated with 0.1 mg/kg of rhGH for seven days *(30)*. Three patients who received physiologic doses of rhGH (2.5 mg every other day) experienced no significant increases in IGF-1, weight, or LBM *(45)*.

On the basis of the promising results obtained in open-label studies, a three-month randomized, double-blind, placebo-controlled multicenter trial was performed to determine whether the protein-anabolic effects of pharmacologic doses of rhGH could be sustained in a large group of patients with HIV-associated wasting studied in an outpatient setting *(46)*. Assessments included body weight, body composition by dual-energy X-ray absorptiometry (DEXA), functional performance by graded treadmill testing, viral load by the branched DNA technique, and safety. A total of 178 patients with >10% weight loss or whose weight was <90% of ideal for body size were randomized to receive the same pharmacologic dose of rhGH used in the aforementioned nitrogen balance study *(30)* (0.1 mg/kg/d; $N = 90$) or placebo ($N = 88$) for 3 mo. Treatment with rhGH resulted in a sustained and significant increase in weight (+1.6 ± 0.2 kg) and an even greater increase in LBM (+3.0 ± 0.4 kg), accompanied by a decrease in fat (–1.7 ± 0.2 kg). In contrast, changes in weight, LBM, and fat in the placebo group were not significantly different from baseline. Differences between treatment groups at week 12 were highly significant *(46)*.

Treadmill work output at volitional exhaustion increased significantly in the rhGH-treated group in comparison to those given placebo (13.2 vs 2.5% in rhGH and placebo, respectively). For the group as a whole, changes in both work output and time to volitional exhaustion correlated positively and significantly with change in LBM ($r = 0.320$, $p < 0.001$ and $r = 0.225$, $p = 0.012$, respectively) but not fat. However, quality of life, as assessed by an HIV-specific instrument *(47)*, was unaffected by rhGH treatment. Days of disability and use of ambulatory, hospital, and home care services did not differ between the rhGH and placebo groups *(46)*.

Overall, treatment with rhGH was well tolerated. Side effects possibly related to rhGH (swelling/puffiness, arthralgia/myalgia, diarrhea) were generally mild to moderate in severity and usually resolved with symptomatic treatment or dose reduction. There were no significant differences between the groups in clinical events, AIDS progression, or death over the 12-wk study period. Likewise, viral load did not change in either the rhGH or placebo groups. It should be noted that all patients were required to be maintained on antiretroviral therapy throughout the study period.

In another placebo-controlled study, a lower dose of rhGH (1.4 mg/d; $N = 15$) was evaluated over a 12-wk period *(48)*. Lean body mass, measured by DEXA, increased modestly after 6 wk of treatment (approx 1 kg; $p = 0.002$ vs placebo), but this increase was not significant after 12 wk. Indices of muscle function and quality of life increased significantly ($p = 0.008$ and 0.02, respectively, by paired *t*-rest), although these differences were not significant when compared with the placebo group.

The effects of rhGH on intermediary energy metabolism have also been studied. In the aforementioned metabolic ward study in which patients were fed a constant diet *(30)*, rates of REE, which were 10% higher in the HIV-infected subjects when compared with HIV- controls during the baseline period, increased further (7.5%) during rhGH treatment. Protein oxidation rates decreased by 40% ($p < 0.001$) and lipid oxidation increased by 29% ($p < 0.05$), while rates of carbohydrate oxidation were unaffected. The increases in lipid oxidation were consistent with a trend to increased whole-body lipolysis, as measured by the rate of appearance of [d_5] glycerol *(30)*.

Inpatient assessments of body composition and energy metabolism were also performed in six men with HIV-associated wasting before and at the end of three months of rhGH treatment *(49)*. All six patients had increased levels of REE after three months of rhGH treatment, and, in five of six patients, the increase in REE exceeded a level that could be accounted for by increases in LBM. Lipid oxidation increased, protein oxidation decreased, and carbohydrate oxidation was unchanged relative to baseline levels. The magnitude of these changes was comparable to that seen in patients studied after one week of rhGH treatment *(30)*.

The effects of rhGH on self-selected dietary intake were evaluated in a separate subset of 11 patients from San Francisco General Hospital who enrolled in the aforementioned multicenter study *(49)*. These patients kept written 7-d food records before and at the end of 3 mo of treatment with rhGH. Energy intake did not change significantly during rhGH treatment (+203 ± 262 kcal/d; 1.3 ± 4.0 kcal/kg/d) and was of a magnitude that would have been obviated by the aforementioned increase in REE noted during a comparable period. These results suggest that rhGH does not chronically stimulate appetite and that the increases in lean tissue are achieved through increased reliance on fat as fuel.

INSULIN-LIKE GROWTH FACTOR-1 (IGF-1)

It is generally assumed that most, of not all, of the protein-anabolic effects of rhGH are mediated by IGF-1 *(50)*. Indeed, as described earlier, significant increases in plasma levels of IGF-1 have been observed following rhGH administration to patients with HIV-associated wasting *(30,39)*. However, the magnitude of these increases is less than that seen in HIV-negative controls. These observations have prompted speculation that there may be a degree of rhGH resistance in patients with HIV-associated wasting and that IGF-1 may be a more effective treatment than GH. Previously, in healthy humans consuming hypocaloric diets, administration of recombinant human IGF-1 (rhIGF-1) resulted in significant improvement in nitrogen balance *(51,52)*.

In a metabolic ward study in patients with HIV associated wasting fed a weight-maintaining diet, daily infusions of rhIGF-1 (4 μg/kg/h for 12 h) produced significant short-term retention of nitrogen, averaging approx 1.7 g/d *(39)*. However, a waning effect was noted after 9 d of therapy. Notably, infusion of a higher dose of rhIGF-1 (12 μg/kg/h for 12 h) produced no significant nitrogen retention. This reverse dose-response relationship may have resulted from the suppression of IGF binding protein-3 (IGFBP-3) in patients given the higher dose, reducing thereby the bioavailability of the exogenous rhIGF-1. Leucine and glycine flux, measured by stable isotope techniques, were unaffected by treatment at either dosing level. The predominant side effect of this treatment was headache, which occurred in 10 of 13 subjects treated.

Preliminary studies have also been performed to identify the optimal subcutaneous dosing regimen for rhIGF-1 *(53)*. First, an HIV-negative subject was treated with increasing doses of rhIGF-1 for a total of 23 d. Because a tendency to hypoglycemia occurred at doses greater than 90 μg/kg/d, that dose was chosen for subsequent studies. In one HIV-negative and one HIV-positive subject who received a constant dose of 90 μg/kg/d, given as a single subcutaneous injection for 14 d, decreases in urine urea nitrogen excretion averaging +1.6 and +3.4 g/d in the HIV-negative and HIV-positive patient, respectively, were observed during the entire treatment period. Thus, there was no evidence of the tachyphylaxis reported to occur during IV infusion of rhIGF-1 in a study in HIV-positive subjects *(39)*. Interestingly, increases in REE also occurred in a dose-dependent manner, and increases in lipid oxidation rates were observed in all three patients given rhIGF-1 *(53)*.

Overall, the nitrogen-retaining effects of rhIGF-1 in metabolic ward studies did not consistently attain levels seen with rhGH *(30)*, whereas the increases in REE were comparable to those seen with rhGH. Moreover, the insulin-like effect of IGF-1 poses a potential obstacle to its use in patients with HIV-associated wasting, many of whom may be at increased risk of hypoglycemia because of limited energy stores, anorexia, malabsorption, or increased insulin sensitivity *(54,55)*.

COMBINED THERAPY WITH GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-1

Because GH and IGF-1 have opposite effects on circulating glucose levels, some investigators have hypothesized that these two agents used in combination would be more effective than either agent alone *(56)*. Indeed, when such a combination was given to healthy patients consuming hypocaloric diets, the degree of nitrogen retention was significantly greater than that achieved with IGF-1 alone *(52)*. Similarly, in

a study in which a total of 60 patients with HIV-associated wasting were randomized to receive either rhGH (1.4 mg/d), rhIGF-1 (10 mg/d), a combination of these agents, or placebo for 12 wk, patients in the group that received the combination treatment experienced an increase in LBM (+3.2 ± 0.6 kg; $p < 0.001$ by DEXA) that was greater than those achieved with either agent alone *(48)*. However, this increase in LBM was not accompanied by improvements in quality of life, muscular strength, or immune function.

In a separate multicenter trial designed to evaluate the efficacy and safety of a combination of rhIGF-1 and rhGH, patients with weight loss >10% were randomized to receive either rhIGF-1 (5.0 mg twice daily) plus rhGH (0.34 mg twice daily; $N = 93$) or placebo ($N = 49$) for 12 wk *(56)*. Weight in patients receiving active therapy increased transiently to a peak of approx 1.5 kg at wk 3, but returned to near baseline levels by wk 12. There were no significant differences in weight between treatment groups at any timepoint. Plasma levels of IGF-1 increased significantly in the treatment group, while remaining constant in those who received placebo. Despite the significant increases in IGF-1 levels in the treatment group, fat-free mass, estimated by anthropometry, increased only transiently at wk 6 before returning to baseline levels by wk 12. No significant differences between groups were noted in isokinetic muscle strength or exercise endurance measured by cycle ergometry. Peripheral edema occurred more frequently in the treatment group *(56)*.

A subset of men enrolled in this trial underwent more extensive evaluation of changes in body composition *(57)*. This substudy included measurements of total body potassium (TBK) by 40K counting, total body nitrogen (TBN) by prompt ± in vivo neutron activation, and fat and LBM by DEXA. A total of 44 patients who received active treatment and 22 on placebo were so studied. No significant changes in weight, TBK, or TBN were noted in either treatment group at wk 12. However, in the group who received active therapy, fat declined by approx 1.2 kg ($p < 0.001$), while LBM increased by approx 1.1 kg ($p < 0.05$).

SUMMARY

As of now, three placebo-controlled studies of rhGH, alone *(46)* or in combination with rhIGF-1 *(48,57)*, have been performed. Each featured a similar study design and duration and included measurement of body composition by a state-of-the art technique, DEXA, in all or in sizable subgroups of patients. The increases in LBM achieved with combinations of rhIGF-1 and rhGH at the doses employed were either no greater *(48)* or actually less than *(57)* those achieved with treatment with a pharmacologic dose of rhGH alone during a comparable period *(46)*. Taken together, these results provide little justification for using a combination of two recombinant drugs, requiring three or four subcutaneous injections daily, in preference to a single dose of rhGH alone. Interestingly, these results provide a suggestion of a dose-response relationship between rhGH and LBM. For example, the increase in LBM in patients who received rhGH at an average dose of 6 mg/d *(46)* was approximately three times greater than that seen in a smaller group of patients who received 1.4 mg/d *(48)*. Similarly, in patients who received 10 mg/d of IGF-1, those who were treated concurrently with rhGH in a dose of 1.4 mg/d *(48)* experienced an approximately threefold greater increase in LBM those who received a total of 0.7 mg/d *(57)*.

CONCLUSIONS

Studies using a variety of statistical approaches have demonstrated that losses of weight and LBM are associated with impaired quality of life, accelerated disease progression, and reduced survival in patients with HIV infection. Collectively, these results suggest that reversal or mitigation of wasting could improve survival and the overall clinical course in such patients and, thus provide a strong rationale for investigating the effectiveness of anabolic therapies such as GH. Studies evaluating the safety and efficacy of this pharmacologic intervention against wasting have produced promising results, but several important issues surrounding the use of anabolic agents such as GH in this setting remain.

First, the appropriate dosing and maintenance regimens must be identified. These questions are important not only for the purpose of limiting side effects, but also because the costs of GH are considerable and may ultimately limit its accessibility. In evaluating the pharmacoeconomic implications of the use of GH in this population, consideration must be given to potential savings from any potential increase in the patient's ability to live independently or reduction in HIV-associated complications or reliance on TPN that might result from this therapy. Other important issues such as quality of life cannot be assigned a monetary value but must be considered. Certainly, the ultimate question is whether amelioration of wasting can improve survival, but placebo-controlled studies probably cannot be ethically conducted for periods sufficient to detect such an effect, should it exist. Overall, future studies should be designed not just to evaluate the best regimens and the best ways to use anabolic therapies in patients with HIV-associated wasting, but also to determine whether clinically relevant functional benefits accompany increases in weight and/or LBM.

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18 Clinical and Physiological Studies with Growth Hormone-Releasing Hormone

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Contents

INTRODUCTION

The circumstances and the timing of the discovery of growth hormone-releasing hormone (GHRH) encouraged early clinical studies of its effects in humans and influenced the direction of the first work. Because the structure of GHRH was derived from human tissue—tumors overproducing GHRH *(1,2)*—it was known with confidence that an authentic replica would be biologically active in man, and the first studies in human subjects could be planned with some knowledge of its physiological activities and potency. Because of extensive earlier studies with the hypothalamic releasing factors whose discovery had preceded that of GHRH—thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), corticotropin-releasing hormone (CRH), and the GH-inhibiting peptide somatostatin (somatotropin-release inhibiting factor, or SRIF)—there were ample precedents for clinical protocols to evaluate potential diagnostic and therapeutic uses. Studies of both of these types of applications began immediately upon the availability of sufficient quantities of synthetic GHRH.

PHYSIOLOGIC RESPONSES

The first clinical studies with GHRH were conducted in normal adult men and women and in patients with acromegaly and idiopathic GH deficiency. GHRH reliably and

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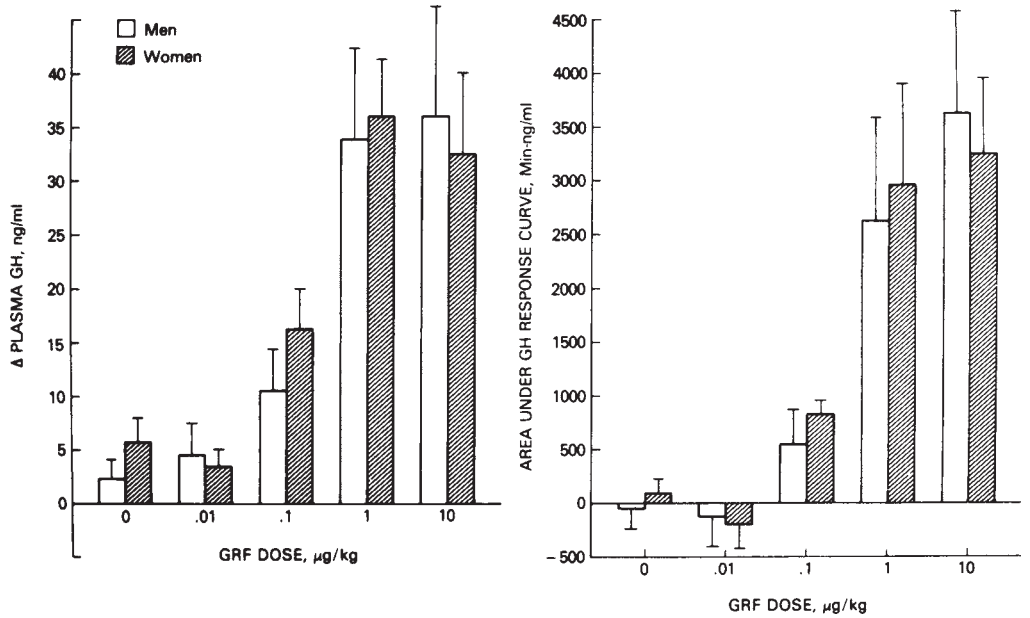

Fig. 1. Dose-response relationships for the acute stimulation of GH by intravenous bolus injections of GHRH(1–44)NH_2 in normal men and women. The left panel shows the increment in peak GH. The right panel shows the integrated area under the GH response curve. From ref. *5*.

specifically stimulated GH secretion, with similar responses in men and cycling women *(3,4)*. Dose-response studies showed that the ED_{50} for intravenous bolus administration was approx 0.3 μg/kg body weight; a dose of 1 μg/kg evoked a maximal peak GH (Fig. 1) *(5)*. The range of individual responses, however, was extremely wide. This was initially noted on testing of different subjects, but then also on repeated testing of the same individual (Fig. 2) *(6)*. Studies of the effects of continuous infusions of GHRH, reproducing some of the pathophysiology of the patients with GHRH-secreting tumors, also showed a wide variation in GH responses, with a pattern of pulses of increased magnitude compared to baseline or placebo infusions, but occurring at approximately the same times of day and night, with an increase during sleep, as seen in spontaneous GH secretion (Fig. 3) *(7–9)*.

Both of these results were interpreted as showing the variable effects of endogenous somatostatin secretion. When hypothalamic SRIF was high, GH responses to GHRH would be low; when SRIF was low, responses to GHRH would be brisk. Since the bolus injections were generally timed randomly relative to endogenous secretion, GH responses would vary widely from one testing session to the next. If endogenous pulses of GH secretion normally coordinated a reduction of SRIF with a surge of GHRH, it stood to reason that SRIF could control the timing of GH pulses even during continuous GHRH infusions.

Awareness of the endogenous GH-releasing peptide (GHRP) ligand system makes it less certain that the full explanation for these phenomena involves only SRIF, but there is now ample evidence to believe that these effects do occur and are at least a significant part of the explanation. GH responses to GHRH are markedly increased by pretreatment with drugs that are believed to reduce hypothalamic SRIF secretion, such as β-adrenergic antagonists, dopaminergic agonists, or cholinergic agonists *(10,11)*. SRIF and its analogs block the GH response to GHRH, and in circumstances where endogenous SRIF secre-

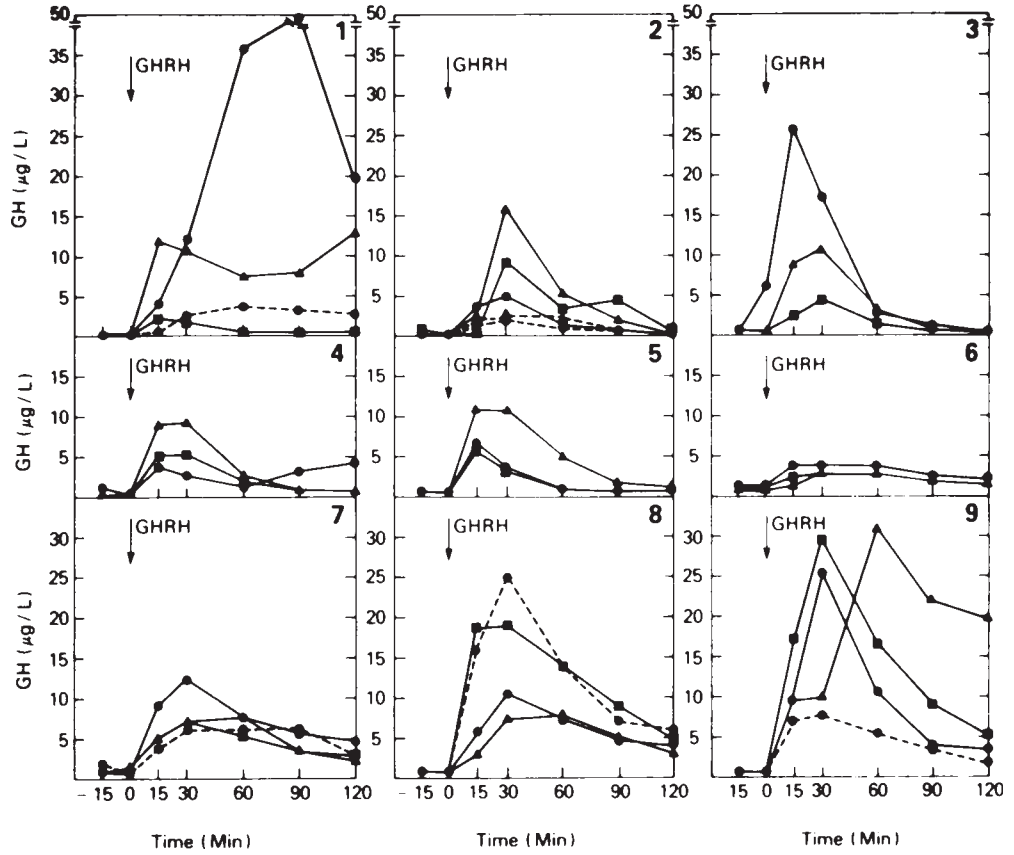

Fig. 2. GH responses to the acute injection of GHRH(1–44)NH_2, 1 μg/Kg iv, on separate occasions in healthy young men. Each panel shows the responses in a different subject, and each curve shows the responses for a different testing session. From ref. *6*.

tion is believed to be increased, such as obesity, GHRH responses are blunted but can be restored by weight loss *(12)*.

There is now an extensive literature on the effects of various agents or conditions to modulate the responses to GHRH. Although some of these have been taken as indicating the clinical neuropharmacology of somatostatin regulation, these interpretations are subject to the usual caveats for clinical studies, where direct measurement of hypothalamic peptides is not possible, and some of these effects may reflect other mechanisms or indirect effects. For example, the marked suppression of GH responses, including the GH response to GHRH, which is seen in Cushing's syndrome can be largely reversed using inhibitors of free fatty acid secretion, an effect which may be partly somatostatin-independent *(13)*.

ACROMEGALY

Since GHRH was initially isolated from patients with tumors chronically overproducing it, it was clear from the beginning that continuous exposure to GHRH could support continuous somatotroph overstimulation. Although in vivo and in vitro studies of perifused pituicytes showed a measurable desensitization to the effects of GHRH *(8,9,14)*,

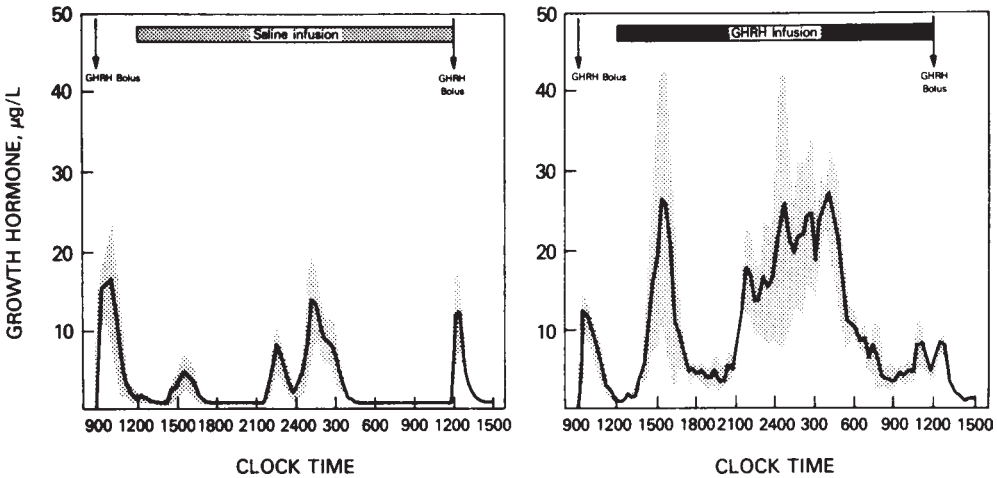

Fig. 3. Mean (± SD) plasma levels of GH during the continuous intravenous infusion of saline (left) or of GHRH, 1 μg/Kg/h (right), for 24 h in 6 healthy young men. Infusions were preceded and followed by bolus injections of GHRH, 1 μg/Kg iv. There is an increase in GH secretion during GHRH infusions, but in an episodic pattern, with periods of higher GH occurring at approximately the same time as during saline infusions. The response to GHRH injections is unaltered following saline infusions but decreased after GHRH infusions, showing attenuation of the response or depletion of releasable GH stores. From ref. *9*.

the magnitude of this was much less than that seen with GnRH and did not lead to a similar paradoxical inhibition of pituitary secretion. Also, even with sustained high levels of GHRH in the tumor patients, GH secretion remained pulsatile, as discussed above in Physiologic Responses. This provided early evidence that chronic treatment with GHRH could increase GH production as long as the pituitary was intact; that long-acting agonists, when they were developed, would behave primarily as agonists; and that it was not necessary to provide a pulsatile pattern of GHRH stimulation in order to elicit a pulsatile response.

Assays for GHRH were developed rapidly and showed that plasma GHRH levels in the tumor-bearing index patients were in the range of 1 ng/mL or higher. A survey of nearly 200 samples collected from patients with acromegaly at several centers showed low levels in all but the index patients, suggesting that ectopic overproduction of GHRH is an uncommon cause of acromegaly *(15)*. Several additional cases of ectopic GHRH syndrome have since been identified, many of them identified by the other hormone overproduction syndromes seen in patients with pancreatic adenomas, carcinoid tumors, or small-cell carcinomas of the lung; but the general finding of this early study has held up. The incidence is sufficiently low that it is still debated whether a measurement of the plasma GHRH level is a cost-effective part of the initial evaluation of patients with acromegaly.

Although the general view is that the great majority of patients with low circulating levels of GHRH have autonomous GH-producing pituitary adenomas, it is possible that some of these patients have hypothalamic ("eutopic") rather than ectopic GHRH overproduction. These patients would presumably have somatotroph hyperplasia rather than adenomas, and would respond to GHRH antagonists with a reduction in GH secretion, as do normals and patients with ectopic GHRH secretion *(16,17)*. Although most observers believe that this situation is also a rare one, the frequency of eutopic overproduction of GHRH has not yet been characterized.

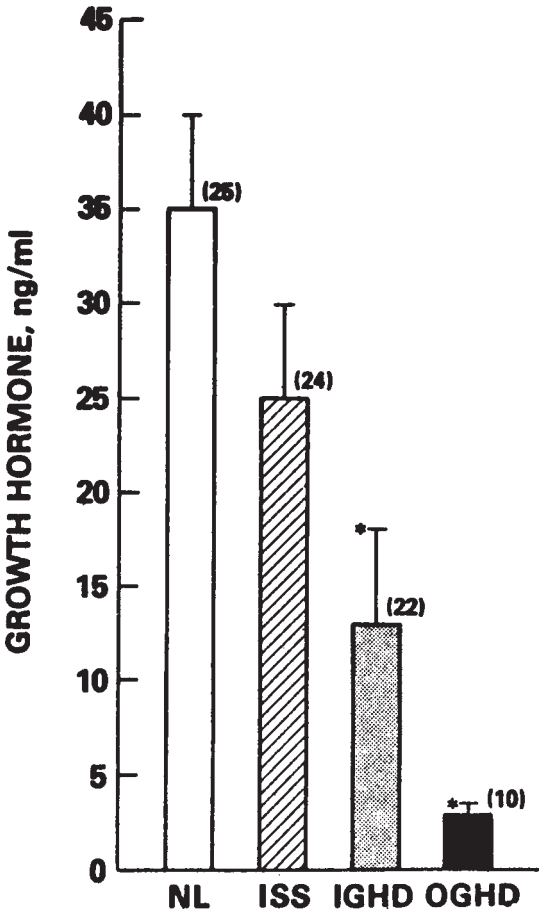

Fig. 4. Growth hormone (GH) responses (± SEM) to the intravenous bolus injection of 1 μg/Kg GHRH in groups of normal (NL) prepubertal boys and girls, in children with idiopathic short stature (ISS), and in children with either idiopathic (IGHD) or organic (OGHD) GH deficiency. The number of subjects in each group is shown in parentheses. *, $p < 0.05$ vs normal, and IGHD vs OGHD. From ref. *21*.

GHRH injections or infusions stimulate a further rise in GH secretion even in acromegalic patients with presumed pituitary adenomas *(18)*. Since responses of the normal somatotrophs are blunted by the high levels of IGF-1, this has been taken as evidence that most somatotroph adenomas are sufficiently well differentiated to retain GHRH receptors linked to GH responses. It has been suggested that cytotoxic agents such as ricin A chain or boron-containing compounds could be linked to GHRH analogs to ablate these tumors selectively *(19)*, but this potential therapeutic approach has not reached clinical trials.

GROWTH HORMONE DEFICIENCY

Early studies showed that a significant proportion of growth hormone deficient (GHD) children respond to the acute administration of GHRH with a rise in GH levels *(20–22)*. These responses are lower on average than those of normal children or non-GH deficient children with short stature, but can overlap into the normal range (Fig. 4) *(21)*. Depending

upon the series, the fraction of patients who respond to GHRH ranges between 40 and 80% *(23–25)*. Failure to respond to a first single bolus of GHRH does not necessarily exclude pituitary responsiveness. We have demonstrated that some GH deficient children who fail to respond initially may convert to a positive response after repeated GHRH stimulation, presumably reflecting an increase in GH synthesis and storage in a releasable pool *(26)*. These observations suggest that most patients with idiopathic GHD do not have an intrinsic pituitary defect in the production of GH, but rather appear to have a deficiency in the hypothalamic secretion of GHRH (and possibly of the endogenous GHRP-like substance as well).

The situation is different in adults with acquired GHD, most of whom have pituitary disease or iatrogenic pituitary damage. In this setting, GHRH cannot be used for treatment, but the absence of a GH response to GHRH can be used as a diagnostic test for GHD, particularly in combination with the simultaneous administration of arginine to reduce the blunting of GH responses by age or obesity *(42)*. The combined GHRH-arginine test is one of the diagnostic tests recommended by the Growth Hormone Research Society consensus workshop *(54)*.

Treatment of GH Deficiency

More than 10 years ago, Thorner et al. demonstrated that repeated administration of GHRH via pulsatile infusion pump (1–3 μg/kg/pulse sc for 6 mo) increased the growth velocity of two growth hormone deficient children (Fig. 5) *(27)*. During GHRH treatment, the growth velocity of these patients increased to rates similar to those observed during therapy with conventional doses of growth hormone. Shortly thereafter, we showed a similar effect in a group of GH-deficient children treated over two weeks with pulsatile GHRH (1 μg/kg/pulse q 3 h iv) or placebo *(28)*. These studies also demonstrated that prolonged treatment with pulses of GHRH continued to stimulate pulses of GH secretion (Fig. 6) and increased the circulating concentration of IGF-1. Others have subsequently confirmed these results *(29,30)*.

The finding that continuous infusions of GHRH still yielded pulsatile GH secretion indicated that it might not be necessary to provide GHRH treatment in a paraphysiologic pattern, as is necessary for a continued stimulatory response to GnRH. During the last few years numerous publications have documented that much simpler regimens of GHRH administration can also increase growth velocity in GH-deficient children. Rochiccioli et al. *(31)* and Ross et al. *(32)* demonstrated that GHRH given in doses of approx 10–20 μg/kg/d sc once or twice daily for 6–18 mo could stimulate growth velocity to approximately twice the basal values. Similar results were observed by Duck et al. *(33)* and by Thorner et al. *(34)*, who documented that growth velocity increased from approx 4 cm/yr to 8 cm/yr during once or twice daily GHRH administration in relatively large groups of GH deficient children (Fig. 7). There appears to be a correlation between the total daily dose of GHRH administered and the growth velocity effect *(35)*; however, the optimal dose and frequency of administration of GHRH has not yet been defined *(36)*.

Although the growth velocities achieved with these GHRH trials often reach the normal range, they are generally lower than the mean first-year growth velocities seen with conventional doses of GH. This is not necessarily an inappropriate response, and may in fact reflect the preservation of feedback regulation at the pituitary level. Because no well designed dose-response studies for long-term GHRH treatment have been performed in GH deficient patients, however, it is not possible to say whether the differences

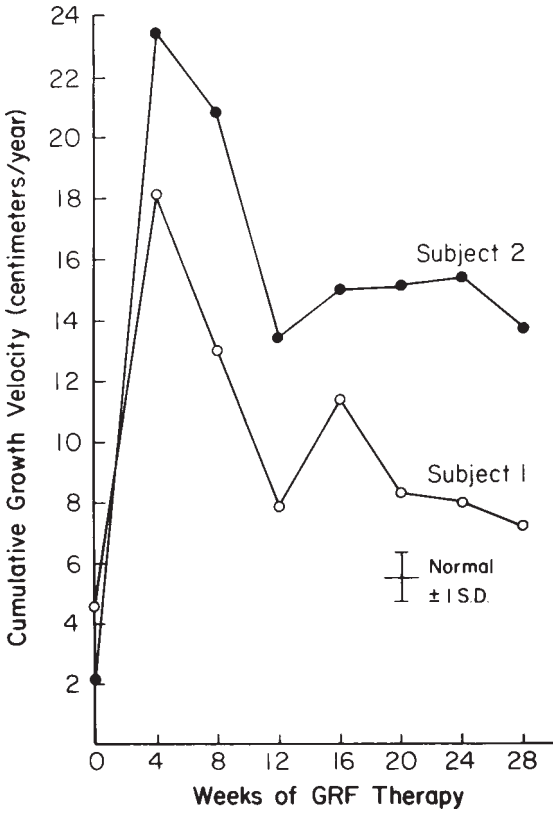

Fig. 5. Cumulative linear growth rate before and during 6 mo of therapy with pulsatile subcutaneous GHRH in two children with GH deficiency. The reference normal growth rate is for 8-yr-old boys. From ref. *27*.

in responses reflect qualitative or merely quantitative differences in GH exposure between the two therapies. Since the supraphysiologic growth rates ("catch-up growth") seen early in GH treatment may help reduce the height lag in these children, it has been suggested that patients might be treated initially with GH and then switched to GHRH for growth maintenance. This approach has not yet been tested.

In general, the chronic administration of GHRH has not produced undesirable side effects. Although a small number of patients have demonstrated apparent allergic responses to GHRH, the induction of anti-GHRH antibodies during chronic GHRH therapy has not been a clinically relevant problem and antibody titers have not correlated with these clinical reactions. The progression of bone age during chronic GHRH therapy has closely paralleled chronological age in these patients, suggesting that if GHRH therapy were to be maintained for sufficient time it might increase final height. However, no published information is available regarding final height in GH deficient children treated chronically with GHRH.

Adjuvant Enhancement of GHRH Therapy

Since endogenous somatostatin secretion can blunt the GH response to GHRH, and agents which inhibit somatostatin secretion can enhance acute GH responses to GHRH,

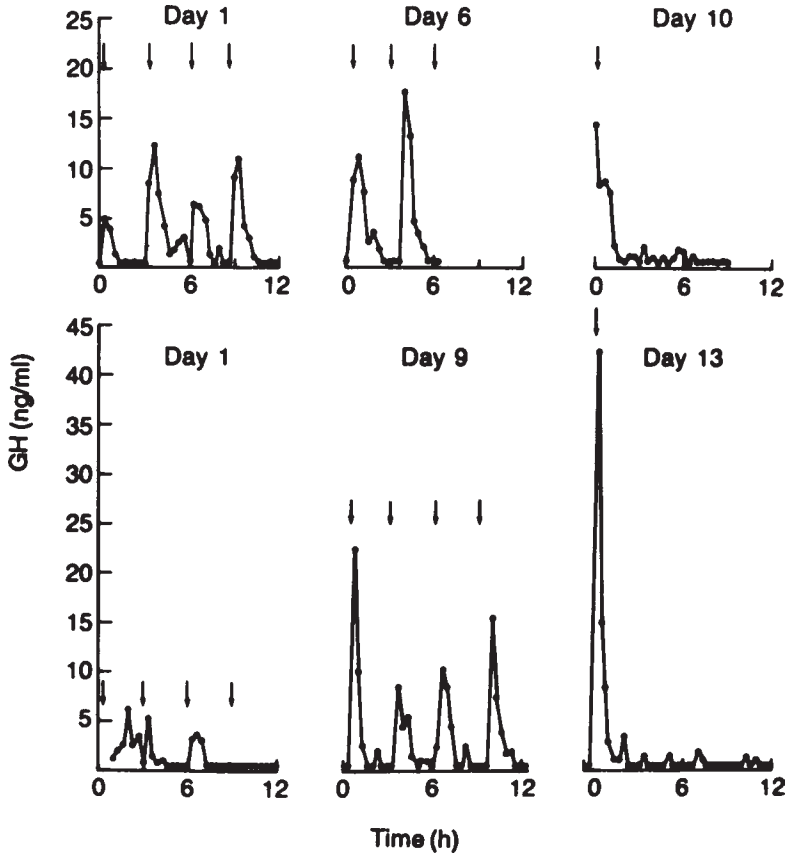

Fig. 6. Growth hormone (GH) responses to the pulsatile administration of GHRH(1–44)NH_2, 1 μg/Kg iv at 3-h intervals, to two children with idiopathic GH deficiency. The times of the injections are shown by the arrows. The magnitude of the GH responses to the injections varies over time, and in the subject shown in the lower panel the average response increases as treatment continues. GH levels return to low values immediately after the injections are discontinued. Modified from ref. *28*.

it seemed reasonable to speculate that chronic co-treatment with an inhibitor of somatostatin secretion might induce a greater increase in growth velocity than treatment with GHRH alone. Confirming this speculation, we found that co-treatment with the β_1-adrenergic antagonist atenolol increased the growth velocity response to GHRH during the first year of therapy, without side effects or a disproportionate increase in bone maturation (Fig. 8) *(37)*. This approach could potentially also work using other inhibitors of somatostatin secretion, such as dopaminergic agonists, or using other agents which enhance the GH response to GHRH, notably the GHRPs. We showed that the acute synergistic effect of GHRH and GHRPs on GH seen in normal subjects is also observed in GH deficient children; the GH response to the two peptides given together is much greater than to either given alone *(38)*.

Unlike adrenergic antagonists, GHRP's also stimulate growth of GH deficient children in their own right. Thus the combination of the two categories of GH secretagogue might stimulate growth to a greater degree than either agent given alone. Since both may be deficient in GHD children with hypothalamic lesions, this may also be a more com-

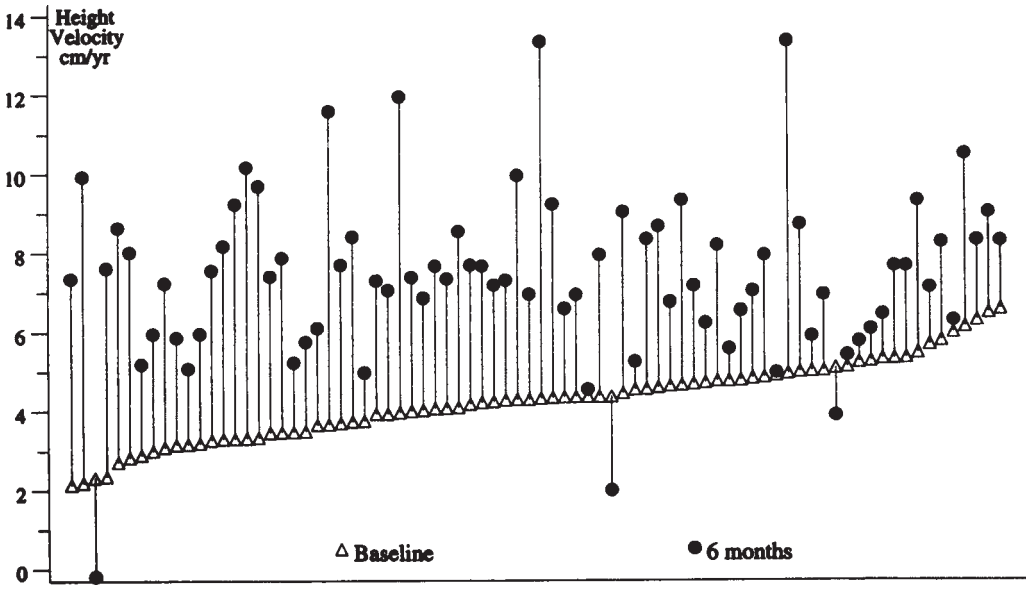

Fig. 7. Individual height velocities for 80 patients who completed 6 mo of treatment with GHRH, at baseline (Δ) and after 6 mo of treatment (○). The subjects are shown in order of ascending baseline height velocities. From ref. *34*.

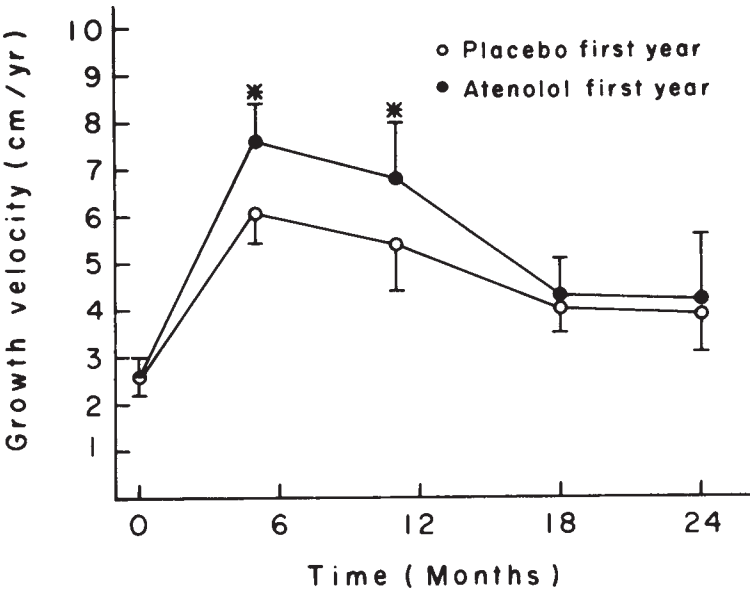

Fig. 8. Growth velocities in GH deficient children treated with once nightly injections of GHRH, 20 mg/Kg sc, plus either placebo or the β_1-adrenergic antagonist atenolol, 1 mg/kg orally once daily. There is a significantly greater acceleration of growth during the first 12 mo of treatment in patients treated with GHRH plus atenolol than with GHRH plus placebo. *, $p < 0.05$ vs GHRH plus placebo. From ref. *37*.

plete physiologic therapy. We have conducted a pilot study in which a GHRP, GHRP-2, was given daily to six children with GH deficiency, followed by the combination of GHRH plus GHRP for 2 mo *(39)*. Growth accelerated into the normal range during both treatment programs, and GH responses to the two agents together continued to be greater than to GHRP alone. No longer-term studies comparing the growth velocity responses to single vs combined treatments have been reported.

Because of rapid proteolysis, the duration of the effect of GHRH administered sc is relatively brief, and efforts are being directed at developing superagonist analogs *(40)* or long-acting formulations of this peptide. This strategy might help further simplify and enhance the efficacy of this form of therapy.

AGING

The age-related decrease in GH secretion is associated with changes in functional capacity, body composition, and hormonal status which mimic those observed in adults with GH deficiency. These changes have raised questions similar to those focused around the decline in sex steroids with aging or menopause, including whether there is a net benefit in reversing this decline; if so, how; and who should be treated. Since the aging pituitary remains responsive to GHRH *(41)*, GHRH has come under study both as a potential probe for assessing the status of the GH axis and as a potential therapeutic agent as an alternative to GH administration.

It is difficult to place these roles in context when the broader questions which frame them are still open. As with GH itself (*see* Chapter 12), we do not yet know whether reversing the age-related decline in GH secretion with GHRH would provide long-term benefits in excess of its side effects; which specific populations might benefit, whether with short-term or chronic treatment; or whether evaluating the functional status of the GH axis is an important part of identifying the people (if any) who would benefit the most. Answers to these questions are not now available.

Use of GHRH in Diagnosis

Part of the challenge of considering treatment in a heterogeneous aging population is identifying those individuals with the most severe reductions in endogenous GH secretion before testing whether that process helps define who might most benefit from treatment. Measuring 24-h pulsatile or even mean GH levels is impractical for population screening, and thus there has been a search for simpler tests or markers, including GH responses to GHRH, which might correlate with reductions in endogenous secretion. As with GH deficiency, a simple index has been an elusive goal. In early studies we found that the GH response to GHRH was well maintained in very healthy older subjects (Fig. 9) *(41)*, but other authors have reported a decrease in responsiveness. This discrepancy may reflect differences in the populations studied, since the increase in adipose tissue which accompanies aging can blunt GH responses. This reduction is presumably related at least in part to an increase in somatostatin tone, given that arginine pretreatment, which reduces somatostatin, can almost completely restore the age-related decline *(42)*.

In contrast to its use in adult-onset GHD, the variability in GH responses to GHRH alone, and the alteration of baseline responses by the use of somatostatin inhibitors both make GHRH a relatively poor tool for gaging the endogenous activity of the GH axis. Other tests, such as insulin-induced hypoglycemia, have so far proven to be better

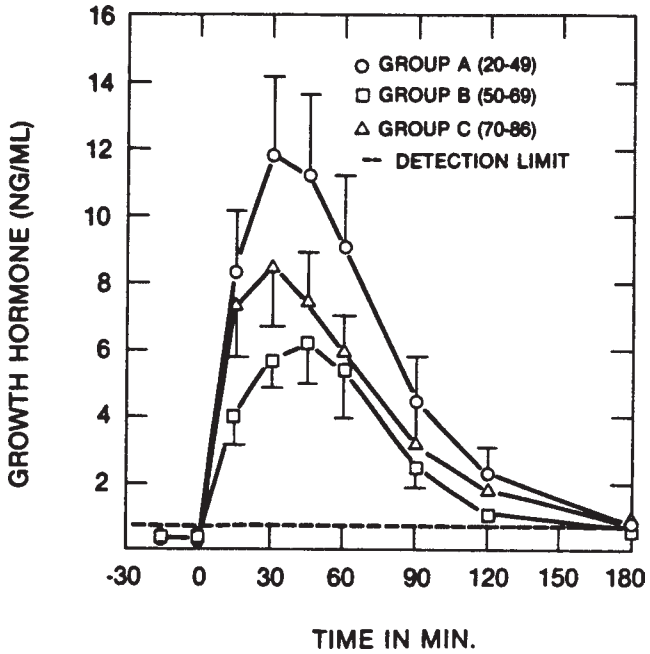

Fig. 9. GH responses (± SEM) to the acute intravenous injection of GHRH(1–44)NH_2, 1 μg/Kg iv, in groups of healthy adults aged 20–49 (○), 50–69 (□), or 70–86 (Δ). From ref. *41*.

discriminators between normal aging and adults with frank GH deficiency *(43,44)*; but there are still no data to indicate whether this test or others might extend to the more general question of identifying those older individuals with the greatest decrement in GH secretion.

Use of GHRH in Therapy

Even when the acute GH response to GHRH is reduced, repeated doses of GHRH can produce increases in GH and IGF-1 levels in normal older subjects, and thus GHRH could potentially be used to stimulate GH secretion chronically as an alternative to GH treatment. The overall utility of this type of treatment is subject to many of the same unresolved questions as concern GH administration, but there are several physiologic and practical considerations that may eventually favor the use of secretagogues over GH in those settings (if any) in which GH enhancement ultimately proves useful. The GH response to GHRH is modulated by negative feedback inhibition by IGF-1 and somatostatin, and these physiologic modulators may partially buffer against overtreatment. In some settings, the biologic response to GH is modulated by its pattern of administration (pulsatile vs continuous) as well as by the total quantity administered *(45)*. In this context the pulsatile pattern of GH evoked even by continuous infusions of GHRH may prove advantageous. Although the GH response to GHRH treatment in aging may be blunted by increased somatostatin tone, a variety of enhancing adjuvants, including β-adrenergic antagonists, arginine, and the combination of GHRH with GHRP's, can boost those responses. As noted, in the context of pediatric GH deficiency chronic suppression of somatostatin can also augment the therapeutic response *(37)*, but it is not known whether this is also true in aging.

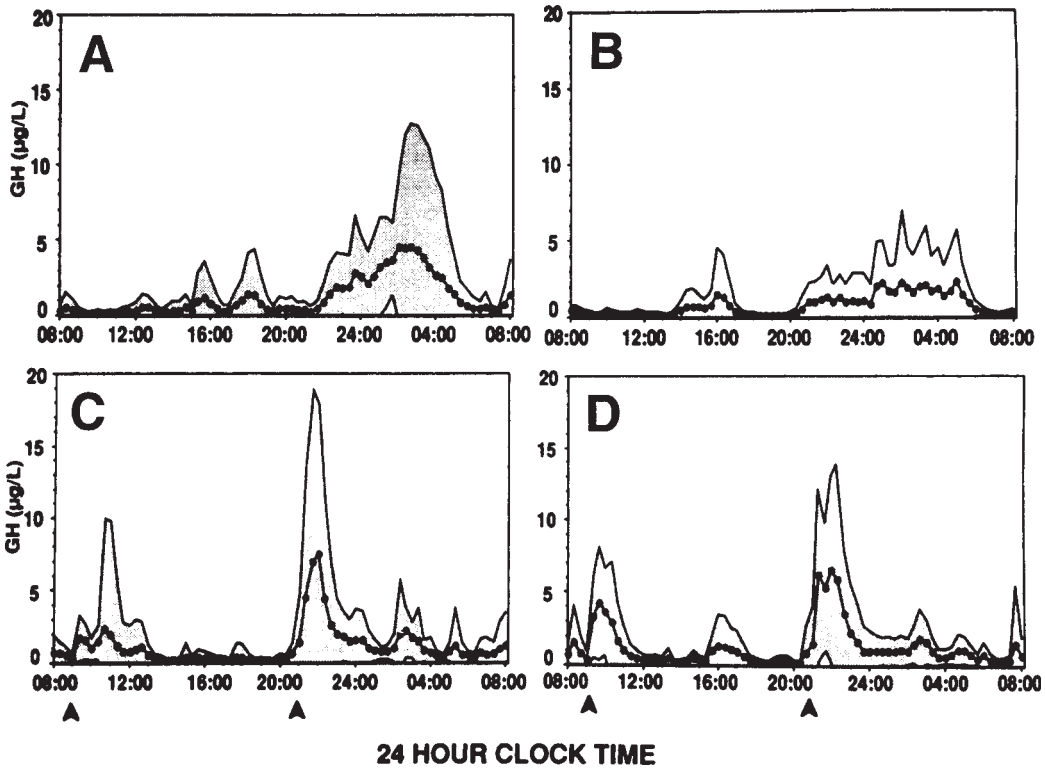

Fig. 10. GH plasma levels (± SD) measured over 24 h in young **(A)** and older men **(B)** at baseline, and in older men during treatment with GHRH(1–29)NH_2, 0.5 mg **(C)** or 1 mg **(D)** sc twice daily. Arrowheads show the times of the GHRH injections. From ref. *47*.

To date, most studies of GHRH treatment in aging have been of short duration (six weeks or less), long enough to assess endocrine-metabolic responses but not changes in body composition or function. Corpas et al. showed that either continuous infusions *(46)* or twice-daily subcutaneous injections *(47)* of 0.5 or 1 mg GHRH(1–29)NH_2 could stimulate GH secretion (Fig. 10) and elevate plasma levels of IGF-1 in healthy older men, reaching normal young adult IGF-1 values with the higher dose. Vittone et al. recently described the effects of six weeks of open-label treatment in 11 healthy older men with the same total daily dose of GHRH (2 mg sc) as in the earlier high-dose study, but given as a single bedtime injection *(48)*. Two measures of muscle strength improved; but in this study plasma IGF-1 and IGFBP-3 levels did not rise. The authors concluded that divided doses of GHRH may be more effective than a single higher dose.

The only published study of longer duration to date is the report of Khorram et al., in which single nightly injections of a GHRH analog were given for 16 wk *(49)*. Lean body mass increased in men but not in women in this study group. Two longer-term studies using single nightly injections of 1 mg GHRH(1–29)NH_2 are in progress at the University of Washington. The first examines the combined effects of six months' treatment with GHRH or placebo, together with strength or endurance conditioning exercise, upon metabolism, body composition, and physical functional performance *(50)*. The second focuses upon effects upon pulsatile GH secretion, sleep, and cognition.

Preliminary results from this latter study are encouraging, but also highlight the practical limitations of the current formulation of GHRH. Once-nightly GHRH stimulates an increase in 24-h GH secretion in both men and estrogen-replaced women. In men there is an approx 40% increase in circulating IGF-1, with a lesser effect in the women; and there is an approx 5% decrease in body fat in both sexes *(55)*. These preliminary findings are consistent with the gender difference in responses reported by Khorram and colleagues *(49)*, and with reports of a lesser response in women to treatment with GH *(56)*. The stimulation of GH secretion, however, is restricted to the acute response in the 2–3 h immediately following the GHRH injections. There is no enhancement of spontaneous GH secretion later in the night, and the rise in IGF-1 may even inhibit late-night GH pulses. Thus, a longer-acting GHRH preparation will be needed to stimulate all-night episodic GH secretion.

Effects on Sleep and Psychological Function

The frequency of sleep disorders increases markedly with aging. It has long been known that nocturnal secretion of GH is correlated with episodes of slow-wave (delta) sleep (SWS); but it is not clear whether this means only that SWS stimulates GH secretion, or whether the stimuli to GH secretion, such as GHRH, can also stimulate SWS. Kerkhofs and colleagues reported that GHRH could acutely promote sleep in normal men, with the specific effects depending upon the timing of drug administration *(51)*. This topic is reviewed by Van Cauter and colleagues in this volume (Chapter 15).

Treatment Population

The studies reported so far have been conducted in healthy older subjects with no major functional impairments. This study population provides relevant information for a chronic treatment aimed at the general older population, but it is not yet clear whether this is the appropriate target population. It may be that a more appropriate or cost-effective use of GHRH in the elderly will be in much more focused or short-term settings—for example, in the frail elderly, in patients with serious debilitating illness, or in the treatment of patients with fractures, wounds, or burns, to assist in bringing them to a point when they can enter an active rehabilitation or exercise program. These are also settings in which the utility of GH and of other secretagogues is being investigated, but there are no published data on the effects of GHRH in these contexts.

Potential Adverse Experiences

The definition of appropriate treatment populations depends in part upon the balance of specific benefits and risks. A preliminary report indicates that a low plasma level of IGF-1 may correlate with increased mortality in patients over 70 yr old *(52)*, suggesting a potential benefit in treatments which elevate IGF-1. However, just as thyroid hormone replacement can worsen the prognosis of patients with "low-T_3 syndrome," a recent study of high-dose GH treatment in critical illness, a state of GH resistance, showed a worse outcome in the GH treatment group *(53)*. This is a somewhat different situation from the reduced GH secretion seen in aging, but it raises caution about the potential risks in reversing changes that may be in part adaptive.

At this early stage, the reported side effects of treatment studies using GHRH have been few compared to studies of the use of GH in aging. So far, published reports of GHRH treatment have reported no adverse effects upon fasting glucose, and a relatively

low incidence of clinical side effects such as edema or carpal tunnel syndrome. The number of subjects and the duration of treatment are still small, however, and it is also still not clear whether this generally favorable experience reflects the qualitative differences between GHRH and GH, or simply the differences in the effective potency of the doses used. As in the pediatric setting, there has been no direct comparison between GH and GHRH treatment in doses that produce similar increments in circulating GH.

SUMMARY: FUTURE DIRECTIONS

GHRH is a physiologic replacement for patients with reduced GH secretion owing to hypothalamic causes. Its range of potential clinical applications is thus almost as broad as the potential applications of GH treatment, except for patients with pituitary lesions—both the traditional indication of idiopathic GH deficiency, and the nontraditional indications such as wasting illness, wound and fracture healing, and aging, which largely remain to be clarified. Compared to GH, it has several physiologic advantages. It stimulates an episodic pattern of GH secretion that may produce different responses from the broad elevation in GH levels seen with GH treatment, and it preserves feedback at the pituitary level to help buffer against overtreatment. This latter effect is not an absolute protection, since the patients with GHRH-producing tumors manifest GH excess; but it may help in settings with the lower levels of GHRH used in treatment. Older patients in particular appear to be sensitive to the side effects of GH in doses which are well tolerated in children.

GHRH antagonists are in early clinical trials; they will provide a helpful physiologic probe, but whether they will be useful in any but the small fraction of cases of acromegaly owing to GHRH overproduction remains doubtful. Despite intensive study, a long-acting superagonist analog of GHRH in man has not yet become available. Native GHRH is rapidly inactivated by proteolysis at the N terminal, and thus the response to acute GHRH administration is very brief, falling short of the goal of recreating a normal pattern of pulsatile overnight secretion (Fig. 10). Since GHRH is less well suited to oral or nasal administration than secretagogues in the GHRP family, development of a long-acting analog or formulation of GHRH is critical if it is to have a major role in clinical treatment. The recognition that an endogenous substance binding to the GHRP receptor(s) probably also plays a major role in the physiologic regulation of GH means that a full physiologic replacement program for hypothalamic GH deficiency may entail substitution with both classes of secretagogues.

Thus the challenges for future studies with GHRH are threefold. On a technical level, a long-acting preparation is acutely needed. The number of patients even with classical GH deficiency who have been treated with GHRH is still relatively small, and the definition of other appropriate indications for GH augmentation remains largely for the future. Careful clinical investigation using both GHRH, its antagonists, and GHRP's will assist in defining the interaction among these converging systems in the physiologic regulation of GH in humans.

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19 Clinical Use of Growth Hormone Secretagogues

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and Glenn J. Gormley, MD, PhD

Contents

INTRODUCTION

In 1977, Bowers et al. observed that met-enkephalin analogs could stimulate secretion of growth hormone from pituitary cells *(1)*. In 1981, Momany and Bowers *(2)* described additional work on the conformational analysis of small peptides that stimulate GH secretion (growth hormone releasing peptides, GHRPs), followed by their demonstration in 1984 that the hexapeptide His-DTrp-Ala-Trp-DPhe-Lys (GHRP-6) caused secretion of GH from the pituitary cells of several species, including the chick, monkey, lambs, calves, and rats *(3)*. It was subsequently demonstrated that as a class, GHRPs elicit GH release in humans when delivered intravenously *(4)*, intranasally *(5)*, or orally *(6)*.

In parallel with the development and refinement of the GHRPs, several groups have developed low molecular weight compounds that mimic the action of the GHRPs *(7–9)*. These compounds were found to have improved oral bioavailability compared to peptides, which will expand the clinical utility of GH secretagogues. The first of these, described in 1993 by Smith et al. *(10)*, at Merck Research Laboratories, is a nonpeptidyl GH secretagogue in the benzolactam family. In 1995, the same group described L-163,191 (MK-0677), an orally active GH secretagogue with a spiropiperidine structure *(11)*. These compounds, as well as GHRP-6, GHRP-2, and hexarelin have been studied in man in several patient populations and act through the same recently described receptor *(12)*.

Interest in growth hormone has grown dramatically since 1985, largely fueled by the unlimited availability of recombinant human growth hormone for clinical and experi-

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mental use. The approved clinical uses of recombinant human GH around the world now include GH deficiency in adults and children, short stature in association with renal insufficiency, AIDS related wasting, and treatment of Turner's Syndrome. The clinical use of GH is being further explored for a number of other conditions including non-GH deficient short stature, age-related muscle loss (sarcopenia), and a variety of catabolic states. For several of these conditions, growth hormone secretagogues could provide an attractive alternative to recombinant GH. Potential advantages of these compounds include oral bioavailability as well as the ability to provide more physiologic GH replacement than is possible with exogenous GH.

In addition to the therapeutic potential of the growth hormone secretagogues, the discovery of the GHRPs represents a breakthrough that will ultimately lead to a deeper understanding of the regulation of the somatotropic axis. It is likely that the growth hormone secretagogue receptor binds an endogenous ligand (GH secretion factor) that regulates GH secretion within the central nervous system by a mechanism distinct from that of growth hormone releasing hormone (GHRH) and somatostatin. Characterization of this new pathway will expand our understanding of the somatotropic axis and may provide insight into the etiology of the age-related decrements in GH secretion. This chapter will summarize the current literature on the pharmacologic and physiologic responses to growth hormone secretagogues in man and will identify potential clinical applications of these compounds.

PHARMACOLOGY

Mechanism of Action

Physiologic secretion of GH is normally pulsatile, with the majority of secretion during the first few hours of sleep *(13)*. Maintenance of this pattern is dependent upon the balance between stimulation by GHRH and inhibition by somatostatin, both secreted by the hypothalamus. However, the factor or factors responsible for regulating secretion of these hormones are unknown. The mechanism of GH release by the growth hormone secretagogues is complex and not completely understood. Both animal and human data demonstrate that the secretagogues bind to pituitary somatotrophs and cause direct stimulation of GH secretion *(3,12,14–23)*. The secretagogues also bind to cells within the hypothalamus *(24)* where the growth hormone secretagogue receptor has been identified *(12)*. Most studies suggest that the physiologic action of the secretagogues occurs both at the pituitary and at the level of the hypothalamus, and therefore an intact hypothalamic-pituitary axis is required for a vigorous GH response (Fig. 1). Consistent with this, animal studies have shown stimulation of hypothalamic GHRH secretion in response to hexarelin, but no change in hypophysial portal somatostatin levels *(25)*. Clinical data are also supportive of hypothalamic and pituitary sites of action. Pombo et al. *(26)* studied patients with neonatal pituitary stalk transection and found the GH response to GHRH (1 μg/kg iv), GHRP-6 (1 μg/kg iv) and the combination of GHRH and GHRP-6 was dramatically reduced compared to controls with normal hypothalamo-pituitary anatomy (Fig. 2). In the control subjects studied by this group, the mean peak GH response to the combination treatment was nearly 70 ng/mL, compared to a GH mean peak in the subjects with stalk transection of <5 ng/mL. However, the response to GHRH was also suppressed in these patients. Thus it is likely that the limited GH secretion was owing to an unresponsive pituitary rather than to lack of hypothalamic-pituitary communication. Additional con-

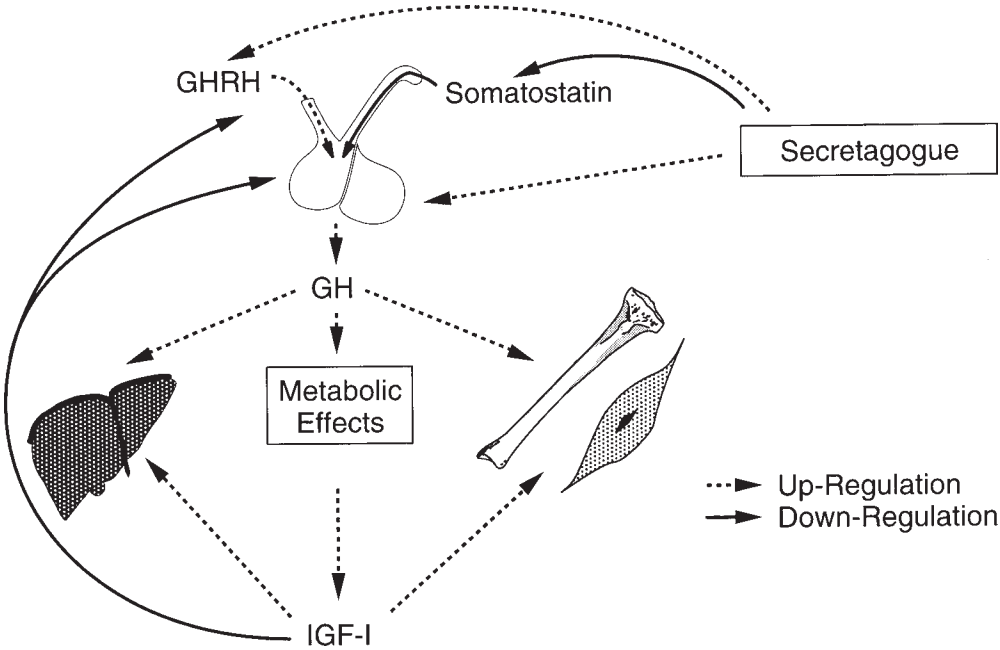

Fig. 1. Schematic representation of sites of action of growth hormone secretagogues. The primary sites of action in vivo are thought to be at the level of the hypothalamus as well as at the pituitary.

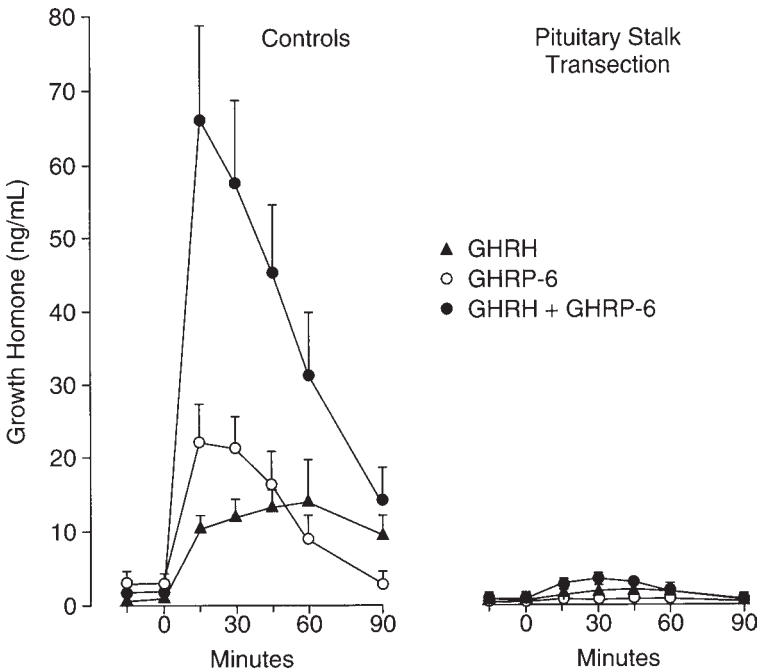

Fig. 2. Controls (n = 7) or subjects with perinatal pituitary stalk transection (n = 7) were treated on separate occasions with GHRH (1 μg/kg, iv), GHRP-6 (1 μg/kg, iv), and GHRH + GHRP-6. Samples were collected for serum GH analysis for 90 min post-dosing. Adapted from Pombo et al. *(26)*.

vincing data have been reported by Loche et al. *(27)*. This group found that among growth hormone deficient patients with anatomical pituitary abnormalities on magnetic resonance imaging, 10 of 11 subjects demonstrated a blunted GH response to a single dose of hexarelin. In contrast, patients with idiopathic GH deficiency had a significantly higher peak GH response to hexarelin equal to that of short normal children. Similar findings were reported by Popovic et al. *(28)* in a group of 12 patients with hypothalamo-pituitary disconnection who received a single dose of GHRH, GHRP-6, or the combination. Compared to age and sex-matched normal controls, these patients had a similar GH response to GHRH, implying normal pituitary function. However, the GH response to GHRP-6 and the combination of GHRP-6 and GHRH was much lower in the patient group, consistent with GHRH deficiency at the pituitary level (hypothalamo-pituitary disconnection) and suggestive of a primarily hypothalamic site of action for GHRP-6.

Several lines of evidence indirectly support an interaction of growth hormone secretagogues and somatostatin. Stimulation of GH by the secretagogues is synergistic with GHRH *(29–32)*. Coadministration of atropine with GHRP-6 completely inhibits the stimulation of GH secretion, whereas coadministration with pyridostigmine increases GH secretion, as does insulin-induced hypoglycemia *(33)*. To explain these data, these authors propose that somatostatin tone was increased by atropine, a cholinergic receptor antagonist, and decreased by pyridostigmine and hypoglycemia. They conclude that GHRP-6 induced GH secretion is dependent upon somatostatin tone, but does not act through mediating somatostatin release. In explaining the results of GHRP-6 infusion in healthy male volunteers, Huhn et al. *(34)* have suggested that growth hormone secretagogues act as functional somatostatin antagonists. Several other investigators have provided indirect data to support this hypothesis. Maccario et al. *(35)* studied the interaction of hexarelin with glucose and free fatty acids (FFA) in six healthy men. Glucose is thought to inhibit GH secretion by stimulation of somatostatin secretion and FFA may act directly at the pituitary. Both oral glucose and FFA dramatically decreased the GH secretory response to GHRH, but only blunted the response to hexarelin. These data support a mechanism of action for the GHRPs of antagonizing the action of somatostatin at the pituitary. Jaffe et al. *(36)* reached a similar conclusion in a study in which a 34-h iv infusion of GHRP-6 or saline was administered to nine healthy young men. During the GHRP infusion there was a significant increase in GH secretion, especially during non-sleep hours, when somatostatin tone is highest. Similarly, once daily dosing with MK-0677, a long-acting growth hormone secretagogue, resulted in a greater increase in GH and IGF-1 when dosed in the morning than in the evening *(37)*. Taken together, data in humans are consistent with animal data, suggesting that the primary mechanism of action of the growth hormone secretagogues in vivo is within the central nervous system at the level of the hypothalamus or higher, with effects including both an increase in GHRH secretion and functional somatostatin antagonism.

Long vs Short-Acting Compounds: GH Secretory Pattern and Hormonal Specificity

In clinical studies, single doses of growth hormone secretagogues, given intravenously, intranasally, or orally have resulted in a dramatic elevation in serum GH levels (to approx 40–70 ng/mL) *(38–45)* accompanied by modest post-dose increases in serum cortisol (mediated by ACTH) and prolactin *(46,47)*. Since growth hormone and prolactin secreting cells are derived from the same embryonic lineage, stimulation of prolactin can

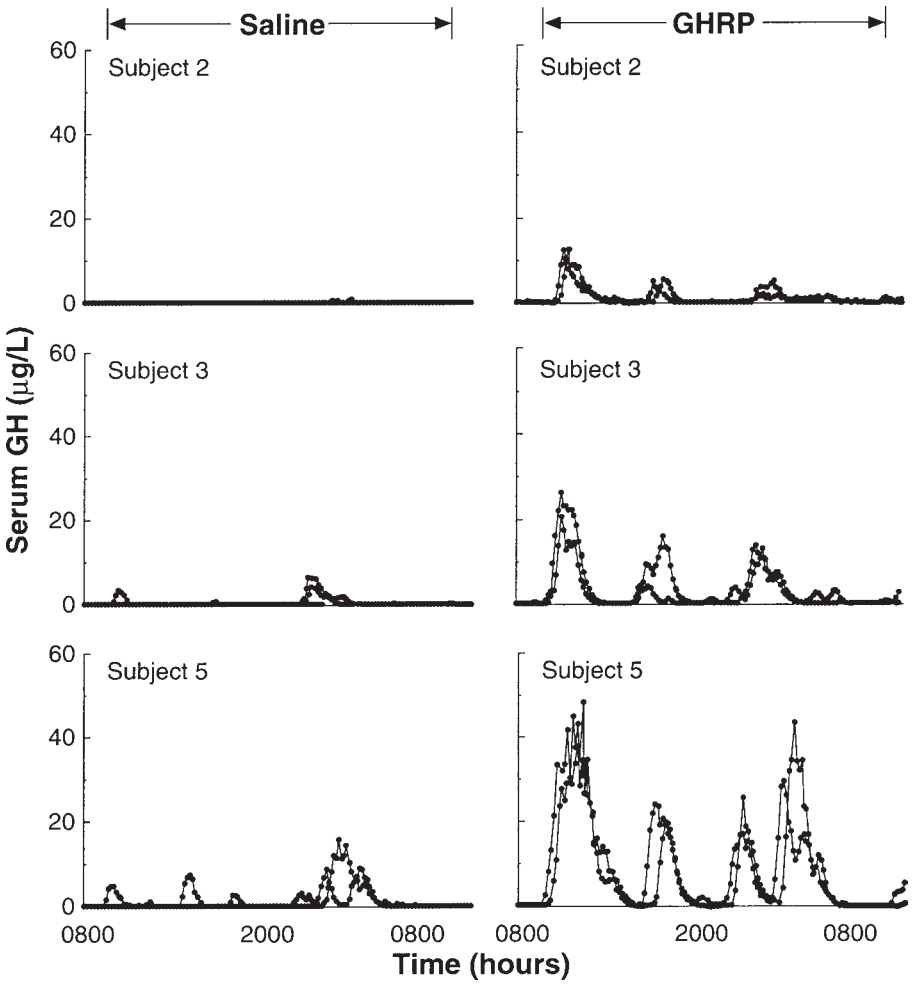

Fig. 3. Serum samples were collected every 10 min from each of three normal subjects during 24-h saline or GHRP-6 (1 μg/kg · h, iv) infusions. Samples were analyzed for GH by immunoradiometric assay. Adapted from Huhn et al. *(34)*.

occur if lactotrophs and/or somatomammotrophs in the pituitary expressed the secretagogue receptor. The mechanism of stimulation of ACTH is less clear. Although a direct stimulation of ACTH by the secretagogues is possible, there is no evidence that corticotrophs express the secretagogue receptor.

Continuous infusion of short acting peptide secretagogues results in a different GH profile than that observed after single bolus doses. Huhn et al. *(34)* administered a 24-h iv infusion of GHRP-6 to healthy young men and observed increased pulsatile GH secretion during the infusion. These investigators noted an increase in the number of GH pulses, as well as in the pulse height and interpeak nadir GH concentration (Fig. 3). Since MK-0677 is a long-acting GHRP-mimetic that is orally active *(11)*, such a compound might be expected to have an effect similar to that of an infusion. In a double-blind placebo controlled crossover design, Copinschi et al. *(48)* dosed nine healthy young males with MK-0677 or placebo orally once daily for 7 d. An increase in GH pulse

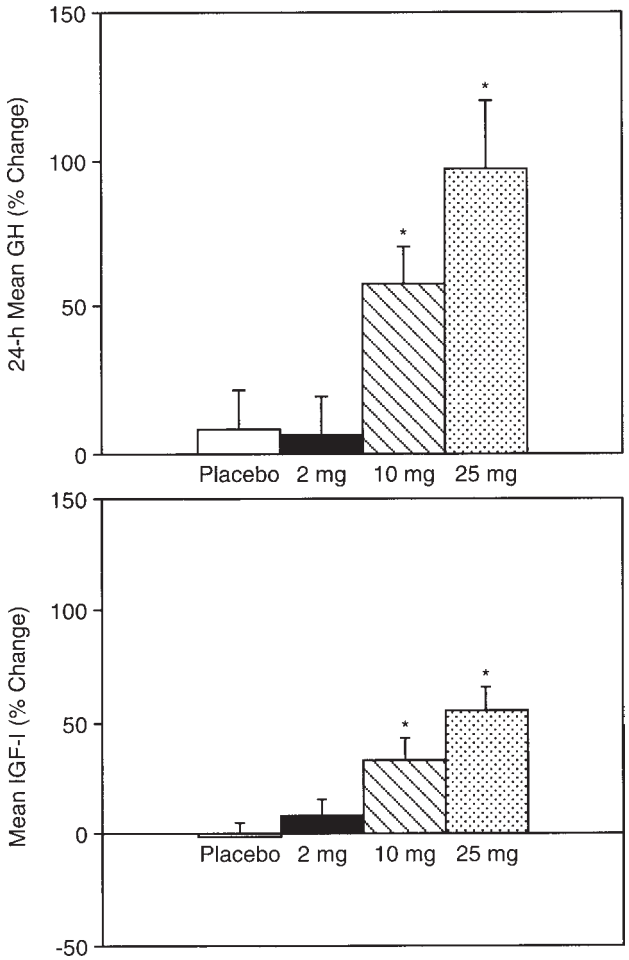

Fig. 4. Healthy elderly subjects were treated with MK-0677 2 mg (n = 10), 10 mg (n = 12) or 25 mg (n = 10), or placebo (n = 10) daily at 10–11 PM. Subjects underwent serial sampling every 20 min for 24 h for GH at baseline and after 14 d of treatment (geometric mean ± geometric SE). Serum IGF-I levels were obtained at baseline and post-treatment (geometric mean ± geometric SE). *, $p < 0.05$ from baseline. Adapted from Chapman et al. *(37)*.

frequency, but not in amount of GH secreted, was observed. Chapman et al. *(37)* performed a double-blind placebo controlled study with the same compound in healthy elderly men and women with somewhat different results. Subjects were dosed daily with MK-0677 (or placebo). GH was measured every 20 min for 24 h prior to treatment and after two weeks. In the MK-0677 treated subjects, increases in GH peak amplitude, peak area, and interpeak nadir were observed, but no difference in peak number was detected (Figs. 4 and 5). Serial samples were also collected for determination of cortisol and prolactin levels. There was no change in serum cortisol levels or diurnal cortisol secretory pattern compared to baseline or to placebo-treated controls *(37)*. However, a modest (approx 20%) increase in mean serum prolactin levels was seen in elderly subjects after 2 wk of treatment with MK-0677. This increase was well within the physiologic range, and was not associated with clinical signs or symptoms of hyperprolactinemia. The

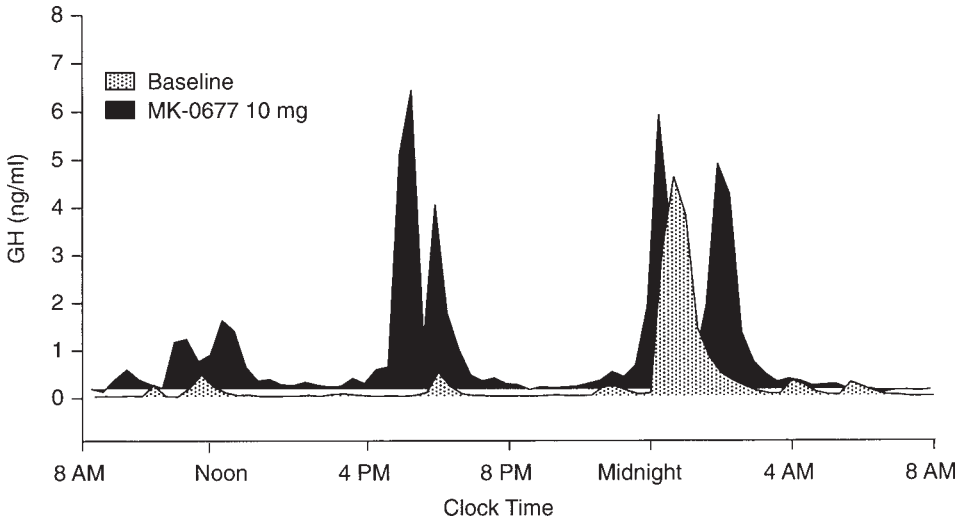

Fig. 5. Twenty-four hour GH concentrations from a 69-yr-old man before and after 14 daily doses of MK-0677 10 mg. Blood was collected every 20 min from 8 AM until 8 AM.

apparent inconsistency between the former two studies *(34,48)* and the latter one *(37)* may be related to the different GH assays employed. Chapman et al. employed a highly sensitive chemiluminescent GH assay with a lower limit of detection of 0.002 ng/mL. The apparent increase in GH peak number reported in the former two studies may be owing to the inability to detect very small peaks at baseline that subsequently have detectable magnitude after treatment. In addition, an increased interpeak nadir, which contributes to an increase in mean 24-h GH secretion, may not be appreciated in less sensitive assays. Based on infusion studies as well as on data from Chapman et al. using MK-0677, it appears that continuous exposure to a secretagogue results in upregulation of the endogenous GH pulsatile profile without perturbing the underlying pulse frequency generator.

POTENTIAL INDICATIONS

A clear role for the growth hormone secretagogues has yet to be demonstrated. The secretagogues have both potential advantages and disadvantages compared to recombinant human GH. GH replacement therapy requires parenteral administration and results in a nonphysiologic serum GH profile with often supraphysiologic levels. Particularly in older individuals, these characteristics may contribute to the poor clinical tolerability that has been reported in several clinical trials *(49–52)*. In contrast, the longer acting secretagogues, or frequent dosing with the shorter acting compounds, may result in increased GH secretion in a physiologic pattern, resulting in improved tolerability. In addition, oral (or intranasal) dosing is possible. In other indications where a modest physiologic increase in GH and IGF-1 levels is desirable, secretagogues may have advantages compared to the use of GH. However, it is likely that with chronic dosing with a secretagogue, supraphysiologic levels of GH similar to those achieved with recombinant GH therapy will not be sustained. In conditions of relative GH resistance where GH therapy is currently being explored (e.g., wound healing, post-surgical rehabilitation) *(53–56)* or used (e.g., AIDS wasting) *(57)*, the secretagogues may have limited or no efficacy. With any

new chemical entity, use of these compounds may be accompanied by unknown risks that will require careful evaluation.

Responses in Specific Populations

Short Stature: Diagnosis and Therapy

GH deficient short stature may be owing to defects at the pituitary, the hypothalamus, or at the level of the CNS pulse generator. Idiopathic GH deficiency, probably as a result of hypothalamic or neurosecretory defects, accounts for the majority of GH deficiency in children *(58)*. One of the potential clinical applications of the secretagogues could be to aid in identifying the etiology of GH deficiency. Just as with the more classical GH testing agents such as insulin, L-DOPA and clonidine, values for normal responsiveness could be established. Abnormal responders could be tested in combination with GHRH to differentiate between pituitary and higher defects. Determination of normal values for responsiveness to the secretagogues will need to take pubertal development into account since in short normal children, the GH secretory response to hexarelin also appears to vary with pubertal status *(59)*.

GH Deficient Children. Several groups have tested growth hormone secretagogues in GH deficient children. Loche et al. *(27)* administered hexarelin (2 μg/kg iv) to 15 children and 4 adults who met classical criteria for GH deficiency and in whom GH therapy was discontinued 2–4 wk prior to testing. Forty-five short normal children were tested as controls. In patients with organic defects based on MRI, hexarelin stimulated a mean peak GH response of 5.5 ± 2.3 ng/mL. In contrast, short normal children and children with idiopathic GH deficiency exhibited mean peak GH responses of 51.7 ± 3.7 ng/mL and 63.0 ± 6.5 ng/mL, respectively. Several other groups have found that a subset of GH deficient children respond to growth hormone secretagogues with GH secretion. In a group of nine Russian children with GH deficiency, Tiulpakov et al. *(60)* tested a single intravenous dose of GHRP-2 (1 μg/kg). One child had a peak GH response >30 ng/mL; three children exhibited a peak GH response of 1–1.5 ng/mL, and the remainder had responses <1 ng/mL. In five children with idiopathic short stature, the peak GH response ranged from 8.7 to >100 ng/mL (Fig. 6). Mericq et al. *(61)* administered GHRP-1 (1 μg/kg iv) to 22 prepubertal GH deficient children. Approximately 60% of tested subjects had a significant response, defined as fourfold times the standard deviation of the GH assay. However, the mean response was 7.5 ± 8.0 ng/mL, substantially less than the response reported in normal subjects. Subsequently, patients who responded to a single dose were treated daily with GHRP-2 (0.3 μg/kg sc). Doses were increased to 1 μg/kg and 3 μg/kg at 2-month intervals. After six months of treatment, mean growth velocity approximately doubled (2.5 ± 0.5 cm/yr to 5.6 ± 1.5 cm/yr), but there was no significant change in serum IGF-1 level *(62)*.

Several factors must be considered in determining the therapeutic potential of the growth hormone secretagogues for treatment of GH deficiency in childhood. First, it is unknown whether a single dose GH response is an adequate predictor of long-term growth response. Although the response to a single dose demonstrates that the hypothalamic/pituitary axis is intact, some nonresponders may have the potential to respond after a period of 'priming' with repeated exposure to secretagogues. Additionally, in other populations, down-regulation of the GH response to growth hormone secretagogues has been described after chronic dosing *(48)*. Since the endogenous feed-back mechanisms

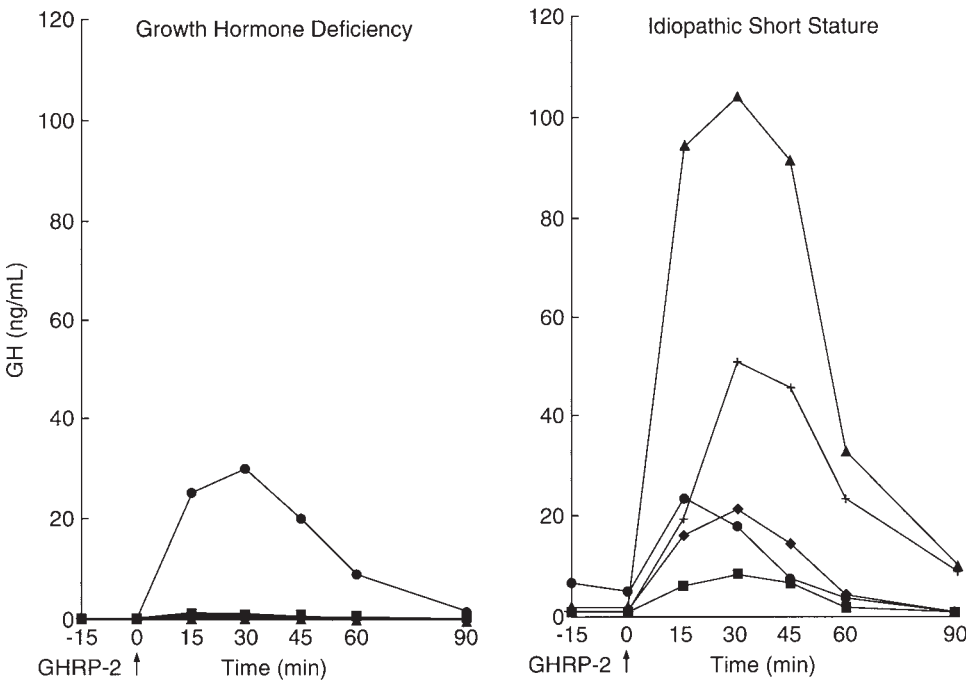

Fig. 6. GH secretory response to a single bolus dose of GHRP-2 (1 μg/kg, iv) in children with GH deficiency (Group A, $n = 9$) and children with idiopathic short stature (Group B, $n = 5$). Adapted from Tiulpakov et al. *(60)*.

are intact, achieving clinically similar growth velocities to those seen with GH treatment may not be possible with secretagogues. Alternatively, the supraphysiologic GH levels (and growth velocities) achieved with recombinant GH may result in a state of GH receptor downregulation *(63)* leading to the declining growth velocities seen with increasing duration of GH treatment. The more physiologic levels and patterns of GH attained with secretagogues may result in growth velocities initially slower than those seen with GH, but more constant over a period of years, possibly resulting in similar gains in adult height.

Additionally, the studies described above *(27,60,62)* were performed with short-acting peptide secretagogues. Clinical use of a longer acting secretagogue may provide different pharmacodynamic responses, leading to a different degree of clinical efficacy. Finally, the cohorts of GH deficient children tested in the latter two studies may not be representative of the majority of children currently diagnosed as GH deficient and on replacement therapy. Based on the mechanism of action of the growth hormone secretagogues, one might expect that the most profoundly deficient children, i.e., those most likely to have pituitary defects, would be least likely to respond to secretagogues. In contrast, as GH has become widely available since 1985, the criteria for diagnosis of deficiency have become less stringent. Thus, a larger fraction of the GH deficient population in the United States (and possibly Europe) may be 'responders' to growth hormone secretagogues. In spite of the modest clinical efficacy described *(62)*, additional clinical trials of growth hormone secretagogues, with efficacy assessed by growth velocity, seem warranted.

Non-GHD Short Stature. Several groups have investigated the use of growth hormone secretagogues in non-GH deficient short stature. These studies include the group reported by Tiulpakov *(64)*, Laron *(65)*, and Bellone *(66)*. In each case, the GH response to growth hormone secretagogues was higher than that of GH deficient children and similar to historical reports of the response in children of normal stature. As these children would be expected to have normal hypothalamic/pituitary axes, a 'normal' response to the growth hormone secretagogues is not surprising. Whether this would translate into increased adult height after chronic therapy is unclear since the benefits of GH in this population are also not well defined. Use of growth hormone secretagogues in non-GH deficient short stature should be limited to controlled clinical studies in children with extreme short stature where clinical use may be more justified and efficacy can be defined.

Renal Failure. Recombinant human GH is approved for treatment of growth failure in children with renal failure. In adults with chronic renal failure, hexarelin has been demonstrated to stimulate secretion of GH *(67)*. Thus, it is possible that the growth hormone secretagogues may be useful in improving growth in this population. However, it is unknown whether sufficiently high levels of GH can be stimulated chronically, in order to demonstrate clinical efficacy. Prior to pursuing efficacy trials in this population, pilot studies to demonstrate hormonal responsiveness to growth hormone secretagogues would be needed.

Turner's Syndrome. Recombinant human GH is approved for treatment of short stature in Turner's Syndrome. As these patients would be expected to have normal hypothalamic-pituitary axes, a pilot study to demonstrate hormonal responsiveness to growth hormone secretagogues would be warranted prior to further exploration of clinical efficacy.

Potential Indications in Adults

Several clinical conditions are associated with decreased growth hormone (GH) bioactivity. GH deficiency by classical criteria as well as normal aging and are associated with decreased GH secretion. In addition, a wide variety of catabolic conditions, including Type 1 diabetes, post-operative recovery and malnutrition from a variety of causes are characterized by relative GH resistance, resulting in elevated levels of GH and decreased circulating IGF-1 *(68)*. The rationale for treatment and the therapeutic potential of growth hormone secretagogues in these conditions are discussed below in GH Deficient Adults.

GH Deficient Adults. Adults who meet the classical criteria for GH deficiency, either as a continuing condition from childhood or through secondary (acquired) deficiency, comprise the most obvious category of adults who might benefit from secretagogue treatment. Adults with growth hormone deficiency have been shown to have decreased muscle mass, increased fat mass, and are at increased risk for cardiovascular disease and early mortality *(69)*. The clinical use of GH in these individuals has been approved in several countries in Europe, and was recently approved in the US. However, the majority of these patients, with secondary GH deficiency after irradiation or surgery, are unlikely to be responders. Nevertheless, the subset who are responders may benefit from the use of growth hormone secretagogues to the same extent that GH replacement is beneficial in this population. Additionally, since GH is less well tolerated with increasing age, if secretagogues could result in a more physiologic GH profile, they may exhibit a better tolerability profile.

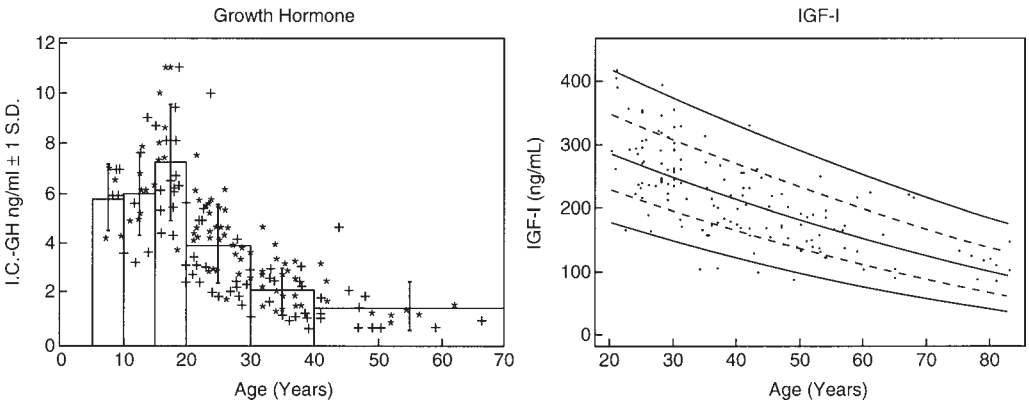

Fig. 7. Decrease in circulating IGF-I and GH secretion with advancing age. Integrated concentration of GH was determined every 20 min for 24 h in 173 nonobese subjects from 7–65 yr of age. Adapted from Juul et al. *(107)* and Zadik et al. *(70)*.

Aging. GH secretion wanes with age, such that by the seventh decade, secretion may be only 60% of that of a young adult *(70)*. Many elderly subjects have endogenous GH and IGF-1 levels that fall in the range of classically GH deficient patients *(71,72)* (Fig. 7). In parallel with the decrease in GH, there is an age-related loss of muscle mass and strength that contributes to the musculoskeletal impairment associated with the frailty of old age. Acute illness, surgery, or traumatic injury resulting in immobilization contribute to additional loss of muscle mass. GH receptors are present on muscle tissue, and GH has an important anabolic effect on muscle *(73)*. Therefore, replacement of GH to young adult levels may have a beneficial effect on strength and function in the frail or immobilized elderly.

Rudman et al. *(74)* tested this hypothesis in a landmark study in which 12 healthy elderly men with low serum IGF-1 treated with recombinant human GH after a 6-mo baseline period. Results were compared to nine untreated controls. The treated group demonstrated an increase in IGF-1 and lean mass and a decrease in fat mass (Fig. 8). Since that time, several other groups have been able to replicate the effects of GH on body composition and muscle mass. Despite these accomplishments, the goal of demonstrating an increase in strength and/or function with growth hormone treatment of elderly subjects has proven elusive in most studies to date *(50,52,75)*. However, in a double blind, placebo controlled trial, one group *(76)* recently demonstrated a 10–12% increase in muscle strength in healthy elderly after three months of treatment with relatively low doses (0.03 mg/kg tiw) of recombinant human GH. Limited demonstration of efficacy in the majority of studies may have been owing to several factors. In most cases, the study sample size was small. In addition, all groups to date have studied healthy elderly subjects, where demonstration of functional improvement may be most difficult. In addition, poor tolerability of exogenous GH resulted in the need to decrease doses in most studies.

Tolerability in this population may be related to the non-physiologic manner of delivery and often, supraphysiologic levels of GH attained. Growth hormone secretagogues may therefore have a distinct advantage over rhGH in this population. Secretagogues with oral bioavailability are under evaluation *(6,11,37,40,48,66,77–80)*. Hexarelin 2 μg/kg iv has been demonstrated to stimulate GH secretion in elderly subjects *(81,82)*.

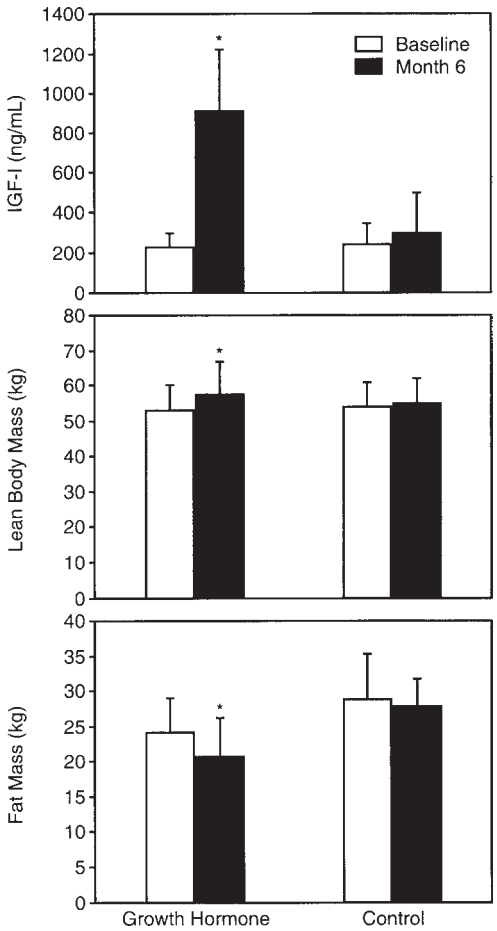

Fig. 8. Healthy elderly men with low serum IGF-1 levels ($n = 12$) were treated with recombinant human GH (0.03 mg/kg three times weekly) for six months. Control subjects ($n = 9$) received no treatment. Serum IGF-1 levels, and body composition were measured pre- and post-treatment (mean ± SD; *, $p < 0.05$). Adapted from Rudman et al. *(73)*.

However, lack of hormonal specificity remains a concern, as serum ACTH, cortisol and prolactin show post-dose increases after dosing with this compound *(83)*. A GHRP-mimetic, MK-0677 has been tested in elderly subjects and has been demonstrated to have good general tolerability and to stimulate sustained increases in pulsatile GH secretion and serum IGF-1 levels in elderly subjects (Figs. 4 and 5) *(83)*. If beneficial effects on strength, similar to those demonstrated using with GH *(76)* can be shown, the growth hormone secretagogues present the potential for a well-tolerated clinical approach to treating or preventing musculoskeletal impairment associated with aging.

Nevertheless, this clinical target presents formidable obstacles to drug development. Age-related musculoskeletal impairment as a result of muscle wasting (sarcopenia) is not well recognized as a clinical syndrome. Thus, selection of an appropriate patient population remains difficult. In addition, given the inherent day to day variability in function in the 'frail' target population as well as the presence of a host of concomitant conditions, demonstration of clinically meaningful efficacy would be difficult.

Catabolic States. Exploratory studies have been performed using rhGH in a variety of catabolic states including as an adjuvant to post-operative nutrition, in wound healing after skin grafting for burns, and in AIDS associated wasting syndromes. The latter condition has recently received US FDA approval as a treatment indication. In addition, exploratory studies have been performed using GH in hip fracture and hip replacement patients *(84,85)*. As these conditions are characterized by GH resistance, it is unclear whether growth hormone secretagogues, with intact endogenous feedback mechanisms, will be able to stimulate sufficiently high levels of GH to achieve clinical benefit. To address this, a study was performed in which calorically deprived healthy young adult males were used as a model for a catabolic state *(86)*. Subjects were treated with MK-0677 or placebo and nitrogen balance was measured using techniques similar to studies performed with GH and IGF-1 *(87–90)*. MK-0677 improved nitrogen retention, resulting in a positive shift in nitrogen balance, suggesting potential clinical utility of the growth hormone secretagogues in clinical catabolic states.

Obesity. GH has a lipolytic effect and has been proposed as a potential therapy for obesity *(91)*. As treatment with GH is parenteral and expensive, growth hormone secretagogues have been considered as a possible alternative therapy for obesity. However, in obese subjects, the GH response to classical stimuli of GH secretion is generally suppressed *(92–94)*. Several investigators have explored the responsiveness of obese subjects to growth hormone secretagogues. Loche et al. *(59)* administered hexarelin (2 μg/kg iv) to a group of 10 obese prepubertal children (body weight approx 45–85% above ideal body weight). The GH peak and AUC in response to hexarelin was approx 50–60% lower than the response in 24 short normal prepubertal children. These results corroborate those previously described by Cordido et al. *(38)* who demonstrated a significant GH response to GHRP6 in obese adults (>30% above ideal body weight). As growth hormone secretagogues stimulate GH secretion in obese subjects, the potential for GH-mediated lipolytic effects exists. However, Svensson et al. *(95)* recently presented results of a study in which obese middle aged men were treated with MK-0677 for eight weeks. Serum IGF-1 levels and 'lean mass' were increased, but fat mass was unchanged. It appears, however, that some of the growth hormone secretagogues may exhibit a direct appetite stimulatory effect *(96)*. This may provide an explanation for the lack of fat loss in the former study, and could compromise the utility of using growth hormone secretagogues with this activity as a therapy for obesity.

Diabetes. Poorly controlled Type 1 diabetes (IDDM) is characterized by relative malnutrition as a result of insulin deficiency. As with other states of malnutrition, diabetics tend to demonstrate GH resistance to a degree inversely proportional to the adequacy of their diabetic control *(97)*. Thus, poorly controlled diabetes is characterized by elevated GH and low circulating levels of IGF-1 *(98,99)*. In an effort to understand the mechanism of this GH resistance, Guistina et al. recently administered hexarelin (100 μg iv bolus) to 10 nonobese adult men with Type 1 diabetes, and compared their GH responses to those of seven healthy adult men matched for age and body mass index (BMI) *(100)*. Hexarelin stimulated a greater GH peak and AUC in men with diabetes than in normals ($p < 0.05$). As the growth hormone secretagogues are thought to have activity as functional somatostatin antagonists, the relatively greater GH response in diabetics may be owing to underlying increased somatostatin tone. The use of recombinant human IGF-1 is being explored as a potential therapeutic intervention in diabetes. However, it is unlikely

that secretagogues would have clinical utility in this condition since increasing GH levels would tend to worsen glycemic control.

Chronic Corticosteroid Exposure. Chronic exposure to corticosteroids is characterized by nitrogen loss and muscle wasting. It is possible that the anabolic action of GH may ameliorate some of the muscle wasting associated with Cushing's Syndrome or chronic corticosteroid therapy. Similar to the case of obesity, the GH response to standard GH secretory stimuli is decreased in patients chronically exposed to corticosteroids. Gertz et al. used L-692,429, a nonpeptide GHRP analog, to test whether growth hormone secretagogues can overcome this suppression *(101)*. In a double blind, placebo controlled crossover design, nine healthy young men received L-692,429 0.2 mg/kg iv preceded by prednisolone 20 mg orally three times per day or placebo for 4 d. GH peak and AUC were decreased approx 55–60% after prednisolone compared to placebo treatment. Using a higher dose of L-692,429 (0.75 mg/kg) partially overcame the steroid-induced suppression of GH secretion. The findings in this model of corticosteroid exposure are similar to those in a study of patients with untreated Cushings Syndrome. In that study, 10 patients with Cushing's Syndrome and five normal adults received GHRP-6 100 μg iv *(102)*. The post-dose GH AUC was suppressed approx 77% in Cushing's Syndrome patients compared to normals. In contrast, Dieguez et al. *(103)* report lack of a GH response to GHRP-6 (1 μg/kg) in four patients with Cushing's Syndrome. This apparent disparity could result from the smaller sample size in the latter report and to individual variation in responsiveness to GHRP-6. It is also possible that the difference in reported responsiveness is a result of different doses of GHRP-6, or to different etiologies of Cushing's Syndrome in the two reports.

Although the response to growth hormone secretagogues appears to be suppressed in the presence of corticosteroid excess, the presence of a significant GH response in at least a subset of patients suggests the possibility of anabolic benefit. However, as most growth hormone secretagogues identified to date exhibit imperfect hormonal specificity, stimulation of small quantities of ACTH (and therefore cortisol) may complicate assessment of treatment status and could affect eventual clinical benefit. Thus, any long-term study in this population would require close monitoring of the pituitary-adrenal axis.

Miscellaneous. One or more of the growth hormone secretagogues have been tested for GH secretory capacity in a number of other populations, including Down Syndrome *(104)*, hyperthyroidism *(105)*, and patients with polycystic ovary disease *(106)*. In each of these populations in which the hypothalamic-pituitary axis is intact, an apparently normal GH secretory response to growth hormone secretagogues was documented.

SUMMARY

GHRPs and GHRP-mimetic secretagogues present a tool for furthering our understanding of the control of GH secretion, as well as a unique therapeutic opportunity. The GH secretagogues likely mimic an endogenous ligand that is critical to normal GH secretion. The pharmacologic effects of these secretagogues have been well characterized in a variety of patient populations and across many species. Degree of responsiveness to a single dose of GH secretagogue may assist in the diagnosis of specific defects in the hypothalamic-pituitary axis.

The GH secretagogues may hold specific advantages compared to recombinant human GH. Oral dosing is possible, as has been demonstrated with both peptide and nonpeptide

secretagogues. With MK-0677, sustained (4-wk) elevations of IGF-1 have been reported *(37)*. In addition, a physiologic pulsatile pattern of GH secretion can be achieved with secretagogues *(37)*. GH treatment, particularly in older subjects, has been associated with dose-related problems with tolerability. As physiologic feedback mechanisms remain intact with secretagogue use, it is possible that a better tolerability profile can be achieved as well.

To date, little long-term safety and efficacy data are available for GH secretagogues. As the GH secretagogues demonstrate some degree of lack of specificity for stimulation of GH secretion, chronic use of these compounds will require careful evaluation of their effects on cortisol and prolactin secretion. In addition, as with any new drug, there is the possibility of non-GH-mediated side effects that have not yet been identified.

As the GH secretagogues demonstrate some degree of lack of specificity for stimulation of GH secretion, chronic use of these compounds will require careful evaluation of their effects on cortisol and prolactin secretion. In addition, as with any new drug, there is the possibility of non-GH-mediated side effects that have not yet been identified.

Potential clinical targets for GH secretagogues include classical GH deficiency in adults and children, Turner's syndrome, a variety of catabolic conditions and frailty associated with age-related hyposomatomedinemia. Clinical studies are currently under way to evaluate the utility of secretagogues in several of these conditions. It is likely that secretagogues will only have efficacy in a subset of GH deficient patients as an intact hypothalamic-pituitary axis is necessary. In addition, it is unknown whether secretagogues can overcome the GH resistance associated with catabolic states. In the treatment of frail elderly, if clinical efficacy can be demonstrated GH secretagogues have the potential to change the acceptance of the inevitability of functional decline and dependence with aging. In an aging population, the social impact of such changes could be significant. Although significant scientific and clinical challenges to the clinical development of these exciting compounds remain, the therapeutic potential remains tantalizing.

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INDEX

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CONTEMPORARY ENDOCRINOLOGY™

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Human Growth Hormone

Research and Clinical Practice

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In *Human Growth Hormone: Research and Clinical Practice,* Roy Smith, Michael Thorner, and a distinguished panel of researchers and clinicians combine a review of GH regulation and its action at the molecular level with a state-of-the-art description of the basis for GH deficiency and the use of GH therapy in a variety of clinical situations. The clinical uses of GH discussed here move beyond the treatment of GH-deficient children to include the genetics of GH deficiency, GH deficiency in adults, osteoporosis, Syndrome X, sleep quality, GH in AIDS patients, and GHRH in clinical studies. Also described are a family of GH secretagogues that bind to a new orphan receptor controlling the physiology of GH release. The discussion of the new GH therapeutics includes their design, their action as regulators in the pituitary gland and in the central nervous system, and their use as new agents for treating growth hormone deficiency states.

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- **Reviews the latest developments in growth hormone physiology**
- **Discusses the clinical applications of growth hormone**
- **Details the molecular pharmacology of GH receptors**
- **Describes a new orphan receptor controlling GH release**
- **Elucidates both the regulation and the effects of aging on GH release**

CONTENTS

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