

STRESS ADAPTATION, PROPHYLAXIS AND TREATMENT

Stress Adaptation, Prophylaxis and Treatment

Edited by

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Reprinted from *Molecular and Cellular Biochemistry*, Volume 196 (1999)



Springer-Science+Business Media, B.V.

Library of Congress Cataloging-in-Publication Data

Stress adaptation, prophylaxis, and treatment / edited by Dipak K. Das.

p. cm. -- (Developments in molecular and cellular biochemistry ; v. 26)

ISBN 978-1-4613-7319-3 ISBN 978-1-4615-5097-6 (eBook)

DOI 10.1007/978-1-4615-5097-6

1. Active oxygen in the body -- Congresses. 2. Stress (Physiology)--Congresses. 3. Adaptation (Biology)--Congresses. 4. Pathology, Molecular--Congresses. I. Das, Dipak Kumar, 1946- . II. Series. RB170.S77 1999

616.07--dc21

98-52740

CIP

ISBN 978-1-4613-7319-3

Printed on acid-free paper

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Originally published by Kluwer Academic Publishers in 1999

Softcover reprint of the hardcover 1st edition 1999

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Molecular and Cellular Biochemistry:

An International Journal for Chemical Biology in Health and Disease

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Preface

The very concept of 'Stress adaptation' was first developed by Professor Hans Selye in 1936 while searching for new hormones in placenta at the University of Montreal, Canada. His published article in 1946 described the term 'General adaptation syndrome'. He hypothesized that Stress reaction occurs in three phases: shock phase, countershock phase which then causes resistance to the stressor. After five decades, we have begun to realize the importance of the concept of 'Stress adaptation'.

Stress reaction is likely to play a crucial role in a variety of degenerative diseases including cancer and cardiovascular diseases. The last two decades witnessed a tremendous amount of growth in the development of our understanding in the stress adaptation, especially in the area of ischemic heart disease. In layman's language the term 'Stress adaptation' means that tissues can be adapted to withstand significant amount of stress by allowing them to condition against an identical or similar stressful condition. The conditioning phase results in stress reactions that alter the homeostasis of many intracellular mediators and hormones triggering a signal transduction that

leads to the upregulation of the body's defense system against the disease processes through the genetic manipulation.

The process of stress adaptation may appear to be simple, but in reality this is a very complex process and we are only beginning to understand the mechanism of adaptation. In January 1998, scientists from around the world got together to discuss potential applicability of the concept of 'Stress adaptation in Clinical arena'. The conference 'Stress adaptation, prophylaxis and treatment' was held in Calcutta, India. This special issue of *Molecular and Cellular Biochemistry* contains original research papers discussed on this subject during the conference, plus five additional papers that were not presented at the conference but which are topics that are related to the issue. The editor hopes that this special issue of the journal serves as an up-to-date source of information for scientists as well as clinicians interested to apply the concept of 'Stress adaptation' to cure diseases. The editor would like to thank the contributing authors for their cooperation and Dr. Naranjan S. Dhalla, Editor-in-Chief, for facilitating the publication of this focused issue.

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Myocardial preconditioning: Basic concepts and potential mechanisms

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Abstract

Preconditioning is a phenomenon, where brief periods of stress such as ischemia, heat shock or certain pharmacological agents make the heart tolerant to subsequent lethal ischemic injury. Preconditioning seems to involve a variety of stress signals which include activation of membrane receptors and signaling molecules such as protein kinase C, mitogen-activated protein kinases, opening of ATP-sensitive potassium channel and expression of a number of protective proteins. In this review, the potential role of these mechanisms is discussed. (*Mol Cell Biochem* **196**: 3–12, 1999)

Key words: ischemia, preconditioning, adenosine, protein kinase C, K_{ATP} channel, free radicals, heat shock proteins, antioxidants, nitric oxide

Introduction

Myocardial infarction (MI) continues to be among the most frequent causes of both debilitating illness and death. Tissue damage from MI occurs as a result of the initial ischemic event—primarily determined by its duration, and then subsequent injury resulting from reperfusion. Recent studies suggest that repeated **brief** episodes of ischemia and reperfusion render the myocardium more resistant against subsequent sustained ischemia and reperfusion. This phenomenon, known as ischemic preconditioning is an inherent ability of the myocardium to protect itself from ischemic damage. Murry *et al.* [1] were the first ones to describe the phenomenon of preconditioning. They found that the heart could be protected by a series of four 5-min coronary branch occlusions, each separated by 5 min of reperfusion. Protection was not the result of improved collateral flow, but rather represented a true improvement in the ability of the heart to tolerate ischemia. Reimer *et al.* [2] reported that four 10 min of coronary occlusion followed by reperfusion caused the same amount of ATP depletion as a single cycle of ischemia/reperfusion in dog. Ischemic preconditioning is not dependent on collateral blood

flow [1, 3, 4] and occurs in animals with little or no collateral circulation [5–7]. Preconditioning has been shown to occur not only in the territory of the artery which is being occluded as part of the preconditioning protocol, but also ‘at a distance’ in the territory of other coronary arteries [8]. It was suggested that preconditioning of the remote virgin myocardium may have been mediated by factor(s) activated, produced, or transported throughout the heart during brief ischemia/reperfusion.

The optimal duration of the ischemia used to induce preconditioning appears to be species dependent. Ischemic preconditioning has been demonstrated in rats after one to three cycles of ischemia/reperfusion (I/R) [9, 10], a single 5 min cycle of I/R in rabbits [11] and a 2.5 min of I/R in dogs [12–14]. In the isolated perfused mouse heart, two cycles of 2.5 min global ischemia and reperfusion significantly reduced infarct size following a 20 min of sustained ischemia [15].

Preconditioning and stunning

Ischemic preconditioning is not a consequence of myocardial stunning. Murry *et al.* [16] showed that 15 min of coronary occlusion in dogs resulted in post-ischemic myocardial

stunning during the first 2 h of reperfusion. However, the infarct size following sustained ischemia was significantly reduced suggesting that myocardial stunning and acute ischemic preconditioning were not related. Miura *et al.* [17] demonstrated that different degrees of stunning resulting from the variable preconditioning protocols were associated with identical reduction in the myocardial infarct size confirming that the protective effect of ischemic preconditioning and stunning were not related.

Preconditioning and second window of protection

Ischemic preconditioning has a short lifetime. It decays as the intervening reperfusion is extended. Ischemic preconditioning in pigs, was lost 1 h after a first cycle of brief coronary occlusion and reperfusion, and could not be renewed by a second cycle [18]. In dogs, ischemic preconditioning became less effective after 60 min [12] and lasted less than 3 h [1, 19, 20]. In rabbits, the decay of ischemic tolerance was more rapid, disappearing completely by 120 min [21]. A delayed resurgence in the cardioprotection afforded by ischemic preconditioning was demonstrated by Kuzuya *et al.* [19] and Marber *et al.* [11]. These investigators showed that significant effect of ischemic preconditioning reappeared when sustained ischemia was initiated 24 h after the repetitive short cycles of ischemia followed by reperfusion. This delayed effect of preconditioning, which has been referred to as the 'second window of protection' [11], has been demonstrated in rabbits [11] and dogs [19]. The duration of this second delayed period of protection is unknown, but it appears to last longer than the early cardioprotection resulting from preconditioning [22]. A similar delayed protective effect using whole body hyperthermia for preconditioning has been demonstrated 24 h post heat shock in rabbits [11, 23, 24], rats [25–27] and cultured myocytes [28]. In addition to ischemia and heat shock, the delayed protection can be reproduced by oxidative stress [29], pharmacological agents such as non-toxic derivative of endotoxin, monophosphoryl lipid A (MLA) [30] and A_1 -adenosine agonist 2-chloro-N⁶-cyclopentyl adenosine (CCPA) [31]. It has been proposed that calcium may also trigger potent endogenous protective mechanisms that may lead to protection against the so called 'calcium paradox' associated injury. The brief periods of ischemia evoke transient and mild elevation of calcium [32], which may lead to preconditioning in the isolated perfused heart [33]. However, it remains unknown whether calcium also leads to the delayed protection similar to MLA and CCPA.

Ischemic preconditioning in humans

Human myocardium has been preconditioned *in vitro* [34, 35]. Isolated human ventricular myocytes [36] and atrial

trabeculae [35] have also been successfully preconditioned *in vitro* using metabolic and functional end-points respectively. Not only that, ischemic preconditioning has been shown to occur in humans undergoing coronary angioplasty. Patients undergoing an elective PTCA of the LAD experienced significantly less ST segment deviation, lower mean pulmonary artery pressure, lower cardiac vein flow and less myocardial lactate production during the second balloon inflation [37].

In the TIMI-4 study, patients with acute MI and antecedent angina had a lower mortality, a smaller infarct size and a lower incidence of complications than patients whose acute MI was not preceded by angina [38]. A previously ischemic episode in patients with symptomatic significant coronary artery disease or variant angina appeared to lessen the severity of a subsequent ischemic episode [39–41] and reduced the frequency of ventricular arrhythmias during recurrent vasospasm [42]. Alkhulaifi *et al.* [43, 44] provided evidence that during cardiopulmonary by-pass, human myocardium can be preconditioned *in vivo* by two brief periods of ischemia and reperfusion.

Mechanisms of preconditioning

Since the first description of preconditioning by Murry *et al.* [1], a number of studies have been performed to discover the mechanism of this phenomenon, however its cellular basis is still not fully understood. As indicated in Fig. 1, preconditioning results in activation of a number of receptors such as adenosine [45], alpha-adrenergic [46] and delta-opioid [47]. One or more of these cascades may trigger protein kinase C, phosphorylation and/or gene transcription of proteins such as HSP 72, Bcl-2, MnSOD or inducible form of nitric oxide synthase (iNOS) and opening of the ATP-sensitive potassium (K_{ATP}) channel. A brief review of the various mechanisms is discussed as follows.

Adenosine

After the onset of myocardial ischemia, ATP depletion occurs rapidly [14] and adenosine is released in large amounts [48] in the interstitial space where it can interact with its receptors. The A_1 adenosine receptor, which is located on the cardiac myocytes [49], is involved in the cardioprotective effect of ischemic preconditioning. Administration of the broad acting adenosine receptor antagonist, 8-*p*-sulphophenyl theophylline (SPT), prior to the ischemic preconditioning protocol in rabbits, abolished the preconditioning [45]. Conversely, the intracoronary infusion of adenosine in isolated rabbit hearts [45] or the intravenous administration of the A_1 selective adenosine agonist, R-

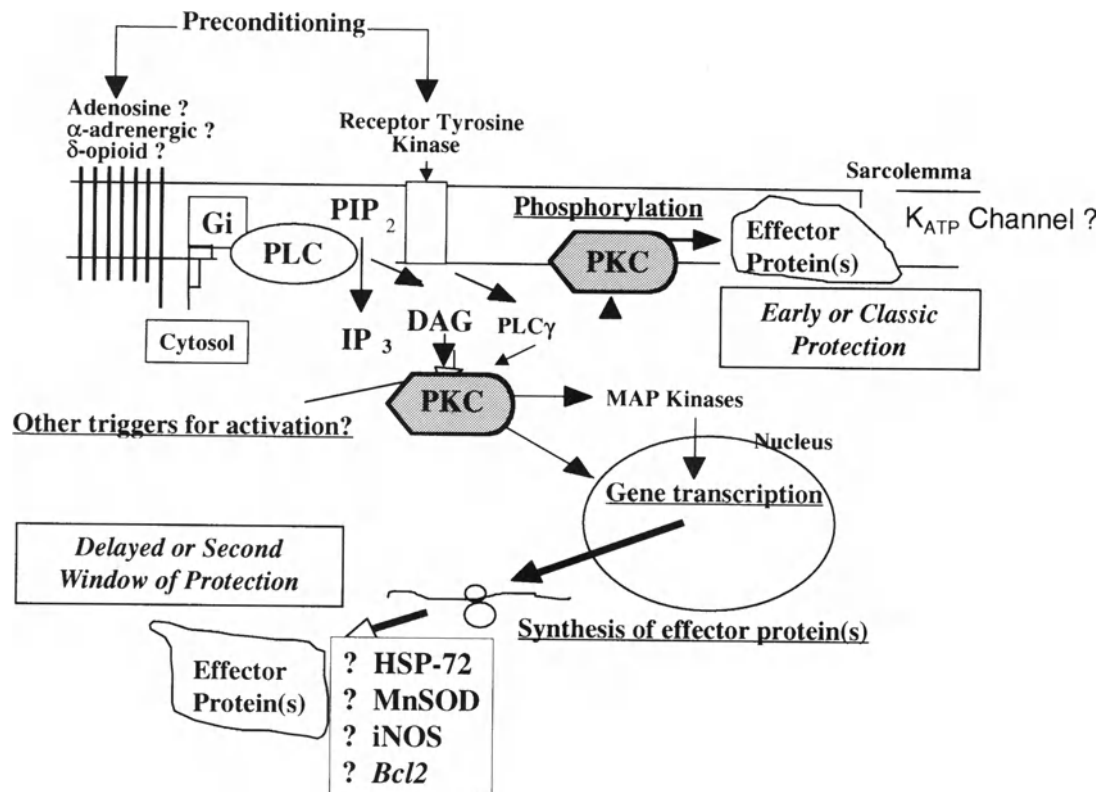


Fig. 1. Scheme outlining the mechanisms of early and delayed preconditioning. Activation of Gi protein coupled receptors (adenosine, bradykinin, alpha, adrenergic, delta opioid) by preconditioning triggers phospholipase C or D (PLC or PLD) which generates diacylglycerol (DAG) from phosphoinositol diphosphate (PIP₂). DAG causes translocation of PKC to cell membrane where it may participate in phosphorylation of unknown effector protein(s), which could be K_{ATP} channel. Preconditioning may also activate receptor tyrosine kinase, which may stimulate PKC via phospholipase C_g. Additionally, PKC may activate MAP kinases and cause gene expression via phosphorylation. It is also possible that one of the PKC subtypes can translocate to the nucleus where it may cause phosphorylation of the transcription factor leading to expression of new protein(s). Some of the possible candidates, that could lead to delayed cardiac protection may be HSP 72, antiapoptotic protein, Bcl-2, MnSOD or inducible form of nitric oxide synthase (iNOS). Although indirect evidence suggests the role of these proteins in cardiac protection, their direct cause and effect relationship in protection during delayed preconditioning remains to be determined.

phenylisopropyl adenosine (R-PIA) prior to the sustained period of ischemia and reperfusion resulted in a significant reduction in infarct size. Additionally, overexpression of myocardial A₁ receptor protects the heart from ischemic damage in transgenic mice [50]. The newly characterized A₃ adenosine receptor, which inhibits stimulated adenylate cyclase activity, has also been suggested to mediate in ischemic preconditioning in the rabbit [51, 52]. The activation of A₁ adenosine receptor also appears to be involved in the mechanism of the delayed cardioprotection induced by ischemic preconditioning. Baxter *et al.* [22] showed that the non-selective adenosine receptor antagonist, SPT abolished the delayed protection to sustained ischemia. Furthermore, three doses of the highly selective A₁ adenosine receptor agonist, CCPA significantly reduced infarct size in the rabbit heart [22, 31]. Continuous adenosine A₁ receptor activation with a high dose chronic infusion of CCPA leads

to down regulation of the signaling mechanism and loss of protection [53]. However, the administration of CCPA by intermittent dosing over a 10 day period preserved the myocardial protection 48 h after the last dosing [54].

Nitric oxide

Nitric oxide (NO) has been observed to protect tissue against ischemia/reperfusion injury by endothelium-dependent relaxation of the coronary circulation to counteract so called 'no-flow' phenomenon observed during reperfusion [55]. NO also inhibits the endothelium/neutrophil interaction which would decrease the infiltration of PMNs into cardiac tissue after reperfusion [56]. It has recently been reported that NO may be an important mediator of the brief ischemia induced first window of preconditioning [57]. Bradykinin is released

early in ischemia [58] and has been suggested to protect the heart via release of NO and prostacyclin. Additionally, delayed protection has also been proposed to be mediated by inducible enzymes including iNOS [59]. NO has been suggested to modulate K_{ATP} channels by increasing the second messenger cGMP. Using patch clamp techniques, Cameron *et al.* [60] provided direct evidence that NO enhances K_{ATP} channel activity in hypertrophied ventricular myocytes.

Protein kinase C

The transmission of stress signals into the intracellular targets is mediated by a network of interacting proteins that regulate cellular processes. Ischemic preconditioning appears to have a final common pathway involving protein kinase C (PKC). It modulates ion conductance by phosphorylating the serine and threonine residues of membrane proteins and it down-regulates cell surface receptors [61]. PKC has two separate functional domains: a hydrophobic regulatory domain which contains the binding sites for phospholipids, Ca, diacylglycerol (DAG) and phorbol esters; and a hydrophilic catalytic domain which contains an ATP and protein substrate binding site [61, 62]. In isolated rat hearts, blockade of α_1 -adrenergic receptors with prazosin, does not prevent neither the ischemia-induced translocation of PKC nor the sensitization of adenylyl cyclase [63]. However, prior perfusion with a PKC-inhibitor abolished the ischemia-induced sensitization of adenylyl cyclase [63]. Similarly, PKC inhibition abolished the phenylephrine-induced functional preconditioning in isolated rat hearts [64]. Administration of the endogenous PKC agonist resulted in myocardial preconditioning against subsequent ischemia/reperfusion in isolated rat hearts [64] and *in vivo* [65]. PKC inhibitors administered at the end of the ischemic preconditioning period *in vivo* [66, 67] blocked the protective effect of preconditioning. The critical period for PKC to exert its protective effect is its activation during the sustained ischemia but not the preconditioning stage [68].

A_1 adenosine receptors couple to the G-protein system since pertussis toxin pretreatment blocked the cardioprotective effect of preconditioning [69]. A number of other receptors, including the muscarinic M_2 [70–72], adrenergic α_1 [73, 74] and the bradykinin B_2 [75, 76], are also coupled with the G-protein system that can activate PKC [77, 78] and mimic ischemic preconditioning. Coupling of A_1 adenosine receptors to the G protein system activates phospholipase C which degrades phospholipids of the membrane to DAG. DAG activates PKC and causes translocation of PKC from the cytosol to the membrane via the incrotubules [79]. The fact that blockade of incrotubular activity with colchicine abolishes the protective effect of ischemic preconditioning,

supports the theory that translocation of PKC is necessary for ischemic preconditioning [67]. The translocation of PKC to the membrane is slow, requiring from 5 min [80] to 10 min [81]. Bogoyevitch *et al.* [82] reported that phorbol esters cause a rapid translocation of PKC-epsilon from the cytosol into the membranes of rat myocytes and that it remains in the membranes for about 1 h.

Persistent activation of PKC is not essential but the PKC induced phosphorylation of an unknown protein(s) before the period of sustained ischemia is required for the cardioprotective effect of preconditioning. In the translocation theory of ischemic preconditioning, A_1 adenosine receptor activation is necessary during both the preconditioning phase and the sustained ischemia period. Although activation of any receptor in the myocyte that couples to PKC can mimic ischemic preconditioning. Some studies suggested that adenosine is still required during the subsequent sustained ischemia [67, 83]. Since adenosine production in the ischemic myocardium is mainly attributable to ectosolic 5'-nucleotidase [84, 85], the activity of this enzyme was studied during preconditioning. Kitakaze *et al.* [86] showed that increases in ectosolic 5'-nucleotidase activity due to α_1 -adrenoreceptor activation may contribute to the infarct limiting effect of ischemic preconditioning. These authors demonstrated that the intracoronary infusion of a specific inhibitor of PKC, GF109203X, blocked increase in ectosolic 5'-nucleotidase activity [80].

Tyrosine-kinase dependent signaling

Receptor tyrosine kinases and mitogen-activated protein (MAP) kinases are important transducers of a variety of extracellular signals that regulate proliferation, differentiation and specific functions of differentiated cells [87]. Some key enzymes such as phospholipase C- γ 1 may be involved in the transduction pathway leading to PKC stimulation via tyrosine phosphorylation. The principal mechanism of signal transduction involves the binding of cytoplasmic SH2-containing proteins to the phosphorylated tyrosyl residues in a major substrate or on the tyrosine kinase itself. Three distinct MAP kinases, each with unique signaling pathways have been identified: the ERK group (p42/p44MAPK), the SAPK or JNK group and p38 MAP kinase which is a homologue of HOG1. The mitogenic signal is transmitted from the cytoplasm by the nuclear translocation of p42/p44MAPK isoforms [88]. Maulik *et al.* [89] demonstrated that repeated episodes of ischemia/reperfusion trigger a tyrosine-dependent signaling pathway leading to the activation of MAP kinase in the heart. Furthermore, blockade of tyrosine-dependent signaling pathway with genistein has been shown to block acute [89, 90] as well as delayed preconditioning in the heart [91].

K_{ATP} channel

ATP-sensitive K^+ channels are activated when intracellular ATP levels drop. Within 1–3 min of acute coronary occlusion, there is a pronounced decline in action potential duration (APD) secondary to the activation of ATP-regulated K channels [92]. De Weille *et al.* [93] reported that stimulation of PKC with phorbol ester resulted in the activation of the K_{ATP} channel in rat insulinoma cells. There is mounting evidence supporting the involvement of the K_{ATP} channels in the mechanism of preconditioning. Gross and Auchampach [94] demonstrated that blockade of the K_{ATP} channels with glibenclamide prevented the development of myocardial ischemic preconditioning in dogs. This was confirmed in rabbits by Toombs *et al.* [95] *in vivo* and by Tan *et al.* [96] *in vitro*. Yao and Gross [97] showed that activation of the K_{ATP} channels with the potassium channel opener bimakalim, lowered the threshold of ischemic duration necessary to precondition the myocardium in dogs. Opening of the myocardial K_{ATP} channel with aprikalim resulted in a marked cardioprotective effect in stunned myocardium, which was blocked by glibenclamide [98]. The K_{ATP} channel opener nicorandil also duplicated the effects of ischemic preconditioning, improving contractility and reducing contracture during reperfusion in the isolated perfused rat hearts [99].

Cole *et al.* [92] showed that guinea pig right ventricles subjected to no-flow ischemia failed to recover in the presence of glibenclamide while those subjected to ischemia after exposure to K_{ATP} channel opener, pinacidil, recovered completely. Using the same experimental model, Shigematsu *et al.* [100] found that the shortening of the APD during no-flow ischemia persisted in the early phase of reperfusion suggesting that shortening is mostly secondary to residual activation of the K_{ATP} channels. In addition, cromakalim, a K_{ATP} channel opener, significantly improved the contractile recovery in the early phase of reperfusion. However, recent reports suggest a lack of correlation between the APD shortening and cardioprotection with bimakalim and cromakalim, the openers of K_{ATP} channel, [101, 102]. Similarly, pyranil cyanoguanidine analogues, although retain glibenclamide-reversible cardioprotective effects, but they lack the APD shortening effect. Garlid *et al.* [103] proposed that mitochondrial K_{ATP} channels could be involved in the cardioprotective effect. Using diazoxide, a potent opener of the mitochondrial K_{ATP} channel, these authors showed significant cardioprotective effects in the isolated perfused heart. However this drug shortened ischemic APD significantly less than chromakalim at equicardioprotective concentrations.

A number of endogenous substances have been shown to mimic ischemic preconditioning, including adenosine [45] and acetylcholine [104] via K_{ATP} channel sensitive mechanism.

Yao and Gross [104] demonstrated a marked reduction in infarct size following a 10 min intracoronary infusion of adenosine, which was blocked by glibenclamide or 5-HD when infused in the coronary artery with adenosine. Grover *et al.* [106] reported significant reduction in infarct size induced by the A_1 adenosine agonist R-PIA was abolished by glyburide. Similarly the protective effect of acetylcholine was completely abolished by the simultaneous administration of the K_{ATP} channel blockers [72, 104]. These studies suggest that endogenous adenosine released and acetylcholine mediate cardioprotection via the K_{ATP} channel. The linkage between the A_1 adenosine receptor and the K_{ATP} channel has been shown to be mediated via the G-protein system [107–109].

Recent evidence from this laboratory suggests that K_{ATP} channel mediates the delayed phase of protection in the rabbit heart. Heat shock-induced protection was blocked by glibenclamide and 5-HD in the rabbit heart [110]. Additionally the second window of ischemic preconditioning as well as delayed protection by pharmacological agents such as CCPA and MLA was also abolished by K_{ATP} channel blockers [31, 111, 112]. Thus it appears that opening of K_{ATP} channel is the end effector of ischemic (early and delayed), heat shock and pharmacological preconditioning.

Heat shock proteins

A variety of cellular stresses result in an enhanced synthesis of a specific group of proteins: the heat shock or stress proteins (HSPs). HSPs exhibit the phenomenon of cross-tolerance in that their induction from one insult may confer protection from a different insult [113]. Currie *et al.* [26, 114] showed that exposure of rats to elevated temperature, with consequent cardiac HSP induction, resulting in an improved recovery of contractile function after subsequent ischemia and reperfusion with associated HSP synthesis. Both hyperthermia [25, 26] and ischemic preconditioning [11, 115] increased the synthesis of the major HSPs in the hearts of rats [26, 27], rabbits [24, 115]. Among the various members of stress proteins, HSP 72 is strongly induced in the myocardium by HS. In rabbits, a single 5 min coronary occlusion nearly doubled the steady state levels of HSP 70 mRNA while with multiple occlusions, HSP 70 mRNA levels were increased approximately 3 fold [115]. There is a graduated response to prior HS and ischemia. Prior HS to incrementally higher temperatures in rats, resulted in progressively increased amounts of HSP-72 induction and decreased infarct size after subsequent prolonged ischemia and reperfusion [27]. In addition, the improvement in contractile force of isolated rat papillary muscles which was observed at 24-, 48- and 96-h post HS, had a similar time course to the accumulation and subsequent decay of HSP 71(23). As HSP 71 was slowly degraded, the enhanced recovery of the hearts was decreasing

towards control values. Transgenic mice overexpressing HSP 70 have also been shown to be tolerant to ischemia/reperfusion injury [116, 117]. Recent studies from our laboratory examined the effect of heat shock on myocardial infarct size following ischemia/reperfusion *in vivo* and correlated with the level of HSP accumulation in the heart over time [118]. While the synthesis of HSP reached 80% of maximum within 4 h of initial insult and peaked between 12 and 30 h, heat shock failed to induce ischemic tolerance earlier than 24 h after the initial insult. These findings suggest that ischemic tolerance afforded by heat shock cannot be solely explained on the basis of HSP expression and may be dependent on other factors. One possibility was that heat-shock may cause phosphorylation of a protein(s) via PKC activation, that may have led to cardioprotection. A recent study from this laboratory proved that heat shock induced ischemic tolerance in the rat heart was abolished by chelerythrine, a specific inhibitor of PKC [119].

Anti-oxidant enzymes and preconditioning

Das *et al.* [120] showed that the activity of three major anti-oxidant enzymes: Mn-SOD, peroxisomal catalase and glutathione peroxidase, were increased in the hearts subjected to four preconditioning cycles as compared to the group which received one preconditioning cycle. Oxidative stress in hepatocytes, has been shown to induce translocation of the inactive PKC stored in the cytoplasm to the cell membrane where it can exert its activity [121]. In HeLa cells, Fujii *et al.* [122] showed that the phorbol ester which directly activates PKC, enhanced Mn-SOD mRNA expression which was abolished by actinomycin D treatment. PKC inhibitor, staurosporine, markedly inhibited the increases in Mn-SOD content and activity [123]. Hoshida *et al.* [124] showed that ischemic preconditioning, alters myocardial anti-oxidant activity immediately and 24 h later. Following four cycles of 5 min of ischemia and reperfusion, mitochondrial Mn-SOD activity in the ischemic myocardium was significantly increased as compared to the non-ischemic myocardium. In addition, glutathione peroxidase was significantly increased in the ischemic myocardium immediately after preconditioning. Oxidative stress induced by exogenously generated superoxide anion or with two cycles of hypoxia/reoxygenation increased early and late resistance of myocytes to reoxygenation injury. Interestingly, Mn-SOD activity in late preconditioning was considerably higher than in classic preconditioning in these studies [125]. Yamashita *et al.* [123] demonstrated a direct association between Mn-SOD induction in myocytes and the acquisition of tolerance to ischemia 24 h after hypoxic preconditioning. Antisense oligodeoxyribonucleotides corresponding to the initiation site of Mn-SOD translation, inhibited the induction of Mn-SOD and abolished the

acquisition of tolerance to hypoxia after hypoxic preconditioning in myocytes. These studies suggest that induction of Mn-SOD in myocytes by preconditioning plays a role in the acquisition of tolerance to ischemia 24 h after hypoxic preconditioning.

The A₃ adenosine receptor may be involved in the enhancement of cellular anti-oxidant enzyme activity [126]. Maggirwar *et al.* [126] reported that exposure of rat basophilic leukemia cells to the selective adenosine receptor agonist, R-PIA, resulted in a 2–3 fold increase in the activity of SOD, catalase and glutathione peroxidase, which was attenuated by theophylline and pertussis toxin, thus also implicating the G-protein system.

Preconditioning and apoptosis

Programmed cell death or apoptosis is the active form of cell suicide which plays a variety of important roles under physiological conditions and which when dysregulated can contribute to several diseases including cancer, autoimmunity and AIDS [127, 128]. Recent studies suggest that programmed cell death also plays an important role in the myocyte cell death following heart failure [129] as well as ischemia/reperfusion [130]. *In vivo* studies have demonstrated cardiomyocyte apoptosis during postnatal maturation [131], spontaneous hypertension in rats [132], rapid ventricular pacing [133] and microembolization-induced cardiac failure [134]. Apoptosis has been documented in ischemic rat myocardium beginning 2 h after ischemia [135]. Extensive cardiomyocyte apoptosis was also observed in reperfused rabbit [136] and rat [137] hearts as well as in myocardial autopsy tissue death from acute myocardial infarction [138]. Gottlieb *et al.* [136] and Fliss and Gattinger [130] showed that cell death during ischemia is mainly necrotic, whereas damage induced by reperfusion caused additional cell death principally through apoptosis. *Acute* ischemic preconditioning *in vivo* has been shown to inhibit apoptosis in the rat heart subjected to ischemia and reperfusion [139]. Furthermore, a recent study from our laboratory demonstrated showed that apoptosis is also inhibited by a second window of ischemic preconditioning [140]. This protective effect was associated with increased expression of antiapoptotic protein, Bcl-2 and down regulation of proapoptotic protein Bax.

Conclusions

Ischemic preconditioning is a physiologically relevant phenomenon which is the most powerful form of endogenous cardioprotection known to date. Its mechanism is complex and not entirely elucidated, but appears to involve activation of the membrane receptors, the translocation and activation

of PKC, tyrosine phosphorylation, phosphorylation of unknown target protein(s), the opening of the K_{ATP} channel and the induction of the synthesis of HSPs and anti-oxidant enzymes. It has immediate and delayed cardioprotective effects as evident from reduction of myocardial necrosis and inhibition of programmed cell death *in vivo*. Its induction by pharmacological means, such as adenosine agonist, calcium, acetylcholine, PKC agonists, bradykinin, MLA and other agents opens new avenues for the limitation of myocardial damage in patients at high risk for acute myocardial infarction. Further understanding of the mechanisms of preconditioning will enhance our ability to develop novel pharmaceutical applications for preconditioning human myocardium against ischemia/reperfusion insults.

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Regulation of cardiomyocyte apoptosis in ischemic reperfused mouse heart by glutathione peroxidase

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Abstract

Apoptosis, a genetically controlled programmed cell death, has been found to play a role in ischemic reperfusion injury in several animal species including rats and rabbits. To examine whether this is also true for other animals, an isolated perfused mouse heart was subjected to 30 min of ischemia followed by 2 h of reperfusion. Experiments were terminated before ischemia (baseline), after ischemia, and at 30, 60, 90 and 120 min of reperfusion. At the end of each experiment, hearts were processed for the evaluation of apoptosis and DNA laddering. The *in situ* end labeling (ISEL) technique was used to detect apoptotic cardiomyocyte nuclei while DNA laddering was evaluated by subjecting the DNA obtained from the cardiomyocytes to 1.8% agarose gel electrophoresis followed by photographing under UV illumination. The results of our study revealed that apoptotic cells appear only after 60 min of reperfusion as demonstrated by the intense fluorescence of the immunostained genomic DNA when observed under fluorescence microscopy. None of the ischemic hearts showed any evidence of apoptosis. These results were corroborated with the findings of DNA fragmentation showing increased ladders of DNA bands in the same reperfused hearts representing integer multiples of the internucleosomal DNA length (about 180 bp). Since our previous studies showed a role of glutathione peroxidase (GSHPx) in apoptotic cell death, we performed identical experiments using isolated hearts from GSHPx-1 knockout mice and transgenic mice overexpressing GSHPx-1. GSHPx-1 knockout mice showed evidence of apoptotic cell death even after 30 min of reperfusion. Significant number of apoptotic cells were found in the cardiomyocytes as compared to non-transgenic control animals. To the contrary, very few apoptotic cells were found in the hearts of the transgenic mice overexpressing GSHPx-1. Hearts of GSHPx-1 knockout mice were more susceptible to ischemia/reperfusion injury while transgenic mice overexpressing GSHPx-1 were less susceptible to ischemia reperfusion injury compared to non-transgenic control animals. The results of this study clearly demonstrate a role of GSHPx in ischemia/reperfusion-induced apoptosis in mouse heart. (Mol Cell Biochem **196**: 13–21, 1999)

Key words: apoptosis, DNA fragmentation, GSHPx-1 knockout mice, GSHPx-1 transgenic mice, ischemia/reperfusion

Introduction

Evidence is rapidly accumulating to implicate that cardiomyocytes undergo apoptotic cell death in a variety of coronary diseases including heart failure [1], myocardial infarction [2] and ischemia/reperfusion [3–11]. Characteristic signs of apoptosis appear in the ischemic myocardium only after several hours of ischemia. In the case of the rat heart, apoptosis first occurs after 2 h of ischemia and significant number of cells undergo apoptotic cell death after more

prolonged ischemia [3, 4]. In contrast, reperfusion even after a brief period of ischemia results in apoptosis [5–9]. Recent studies from our laboratory also demonstrated that in the rat heart apoptosis does not occur with up to 2 h of ischemia, but significant number of myocytes are subjected to apoptotic cell death and DNA fragmentation after even 90 min of reperfusion following 15 min of ischemia [10, 11]. These results suggest that although apoptosis occurs during prolonged ischemia or in frankly infarcted myocardium, reperfusion triggers a distinct signal for apoptosis which

would contribute to the pathophysiology of ischemic reperfusion injury.

Most of the studies on the contribution of apoptosis in myocardial ischemic reperfusion injury so far have been conducted using the rat heart [5–8,10–11]. To examine whether apoptosis contributes to myocardial reperfusion injury in other animals, we developed a perfused mouse heart model using the isolated mouse heart subjected to 30 min of global ischemia followed by 2 h of reperfusion. Oxidative stress and changes in the redox system are believed to be an important regulator of apoptosis [12–14]. A recent study from our laboratory demonstrated that upregulation of the antioxidant enzyme, glutathione peroxidase (GSHPx), by ebselen reduced the number of apoptotic cell death during reperfusion of the ischemic myocardium [6]. To further study the role of GSHPx in apoptosis, we performed identical studies using GSHPx-1 knockout mice and transgenic mice overexpressing GSHPx-1. Previous studies demonstrated that GSHPx-1 knockout mice were susceptible to and transgenic mice overexpressing GSHPx-1 were resistant to myocardial ischemia reperfusion injury as compared to non-transgenic control animals [15, 16]. The results of our study revealed reduced and increased number of apoptotic cells in the cardiomyocytes of transgenic mice overexpressing GSHPx-1 and GSHPx-1 knockout mice, respectively compared to those present in the non-transgenic animals.

Materials and methods

Generation of GSHPx-1 knockout and Transgenic mice

GSHPx-1 genomic clones were isolated from a bacteriophage FIX II genomic library, prepared with DNA of 1295VJ mouse (Stratagene, La Jolla, CA, USA), using a corresponding rat cDNA clone. The genomic insert in the clone was released from the bacteriophage vector by Sad digestion and further characterized by restriction mapping and DNA sequencing analyses. A 5.3 kb Sad genomic fragment was found to contain the entire mouse GSHPx-1 gene with sequence virtually identical to that published by Chambers *et al.* except for a few base substitutions in the region of intron [17]. This fragment of DNA was then used in construction of targeting vector (Fig. 1B). The coding sequence was disrupted by insertion of a neomycin resistance gene cassette (neo) derived from pMCIPolA into the EcoRI site located in exon 2. A herpes thymidine kinase gene expression cassette was placed 3' to the targeting sequence for performing negative selection with ganciclovir (a gift from Syntex Inc., Palo Alto, CA, USA). The targeting vector linearized with HindIII was transfected into R1 embryonic stem (ES) cells (Gift from Dr. Andras Nagy, Mount Sinai Hospital, Toronto, Canada) and selected with both G418 and ganciclovir. Resistant colonies

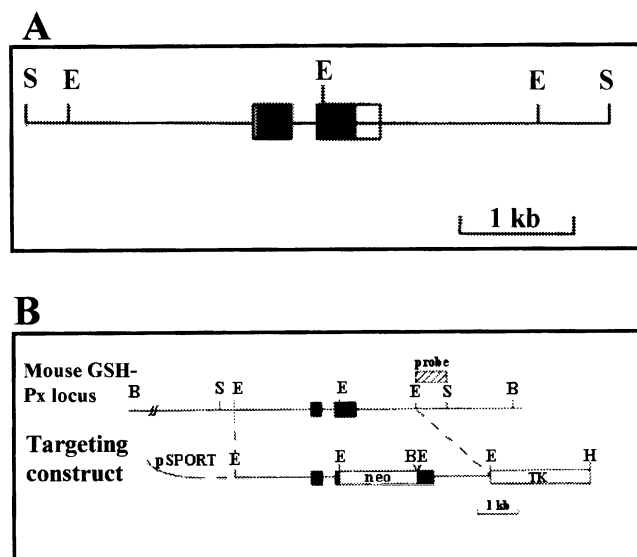


Fig. 1. (A) Restriction map of the mouse GSHPx-1 gene used to generate transgenic mice. Black and open boxes represent coding and non-coding region of the exons, respectively. (B) Targeting of the mouse GSHPx-1 gene in R1 ES cells. Genomic structure and partial restriction map of the mouse GSHPx-1 locus (top), the targeting vector (middle), and the targeted locus (bottom) are shown. Hatched box = probe used for DNA blot analysis. Various sites of restriction enzymes are shown by B = Bam HI, S = Sad, E = EcoRI, H = HindIII.

were screened by DNA blot analysis using a probe 3' external to the targeting sequence. Thirty percent of the colonies screened were found to contain the desired mutated structure in one of the two GSHPx-1 alleles. We have isolated approximately 100 homologous recombinant clones from about 300 colonies screened. Three clones were microinjected into C57B1 blastocysts according to the methods described by Bradley [18], and embryos were reimplanted into the uterine horns of foster mothers. A total of 23 chimeric mice were generated. Four male chimeric mice with more than 95% agouti coat color chimerism were bred with C57B1 female mice. One hundred percent of their F1 offspring carried the agouti coat color, suggesting that the reproductive organs of these chimeric mice are basically developed from the micro-injected ES cells. Germ line transmission of the targeted GSHPx-1 allele was evident by DNA blot analysis.

To prepare transgenic mice overexpressing GSHPx-1, the 5.3 kb sad genomic fragment of DNA including approximately 2.0 kb of 5' flanking sequence was used in micro-injecting into fertilized mouse eggs derived from B6C3F1 x B6C3F1 mating [15]. Two lines of transgenic mice carrying extra copies of the mouse GSHPx-1 gene were generated (Fig. 1A). The transgenic line Tg[MGP]-41 was used in this study. GSHPx activity in heart muscles were determined by standard enzymatic assay method [19]

Isolated mouse heart preparation and measurement of contractile functions

Twenty four mice were divided into three groups : GSHPx-1 knockout (n = 8), Transgenic overexpressing GSHPx-1 (n = 8) and nontransgenic control (n = 8) . They were anesthetized with an intraperitoneal injection of sodium pentobarbital (200 mg/kg) and heparin (sodium) (500 U/kg) administered at the same time to prevent intravascular coagulation of blood. The heart was excised immediately after thoracotomy, and placed in cold perfusion buffer [15, 16]. The aorta was cannulated, and the heart was perfused with Krebs-Henseleit bicarbonate (KHB) buffer by the retrograde Langendorff method (Fig. 2). A small incision was made at the main trunk of the pulmonary artery to drain coronary effluent. The effluent was collected for one minute before ischemia, after ischemia and during reperfusion and stored at -20°C for subsequent measurement of creatine kinase (CK). A 4-0 silk suture on a round bodied needle was passed through the apex of the heart and attached to the apex which in turn was attached to a force transducer. The heart rate (HR), force developed by the heart (DF) and first derivative of developed force (dF/dt) were recorded. Data of myocardial contractile function were recorded and analyzed in real time using the Cordat II data acquisition, analysis, and presentation system [9] (Data Integrated Scientific Systems,

Pinckney, MI, USA; Triton Technologies, Inc. San Diego, CA, USA). After stabilization, preischemic baseline contractile function was measured. The hearts of all mice were subjected to 30 min of ischemia under normothermia by clamping the aortic cannula, followed by 2 h of reperfusion. Coronary flow was calculated before ischemia and during reperfusion by quantifying the release of coronary effluent over 1 min.

Estimation of creatine kinase (CK) release

CK release from the heart was estimated in the perfusate collected from the heart before ischemia, and during reperfusion using a CK assay kit obtained from Sigma Chemical Company (St. Louis, MO, USA). The enzyme activity was expressed as units/ml perfusate. The results are expressed as total CK release from the heart after the indicated time periods.

Measurement of infarct size

At the end of reperfusion, a 10% (w/v) solution of triphenyl tetrazolium in phosphate buffer (Na_2HPO_4 88mM, NaH_2PO_4 1.8 mM) was infused into a side arm of the aortic cannula until the myocardium stained deep red [15, 16]. The heart was excised, weighed and stored at -70°C . Frozen hearts were sliced perpendicularly to the long axis from apex to base in 0.8 mm thick sections. Sections were then fixed in 2% paraformaldehyde. Thin mouse heart cross sections were placed between two cover slips and digitally imaged using an IBM-compatible PC and a *Microtek ScanMaker 600z*, a 600 dot per inch, flat-bed, full color scanner. The cross section was imaged at the maximum scaling and dot resolution that the scanner would allow. The digitized image was stored in *Adobe TIFF* file format by the software package *PhotoStyler*, v.1.0.3, by U-Lead Systems, Inc. For analysis of infarct areas, some enhancement of the image was necessary at times to more clearly visualize the areas of staining by *Corel Photo-Paint 4.0* (Corel Inc.). Corel was also used to mark the stained areas. To quantitate the areas of interest in pixels, a *NIH Image 5.1* (a public-domain software package) was used. The entire area of risk (transmural) was quantified in pixels using the computer software, and the measured infarct areas were compared to the entire area at risk in a blinded fashion.

Evaluation for apoptosis

Apoptotic cell death was evaluated by *in situ* end labeling (ISEL) of cardiomyocyte nuclei [6, 7]. In short, cryostat sections of heart biopsies were fixed with glutaraldehyde (1%) followed by permeabilization with methanol:acetone (1:1 v/v). After incubating with proteinase K (20 $\mu\text{g}/\text{ml}$) for

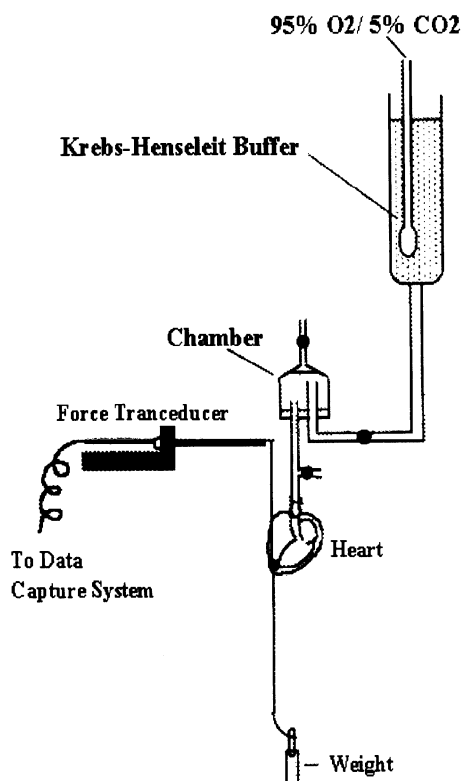


Fig. 2. Langendorff set up for mouse heart. Heart was perfused with oxygenated KHB-buffer retrogradely as described in Materials and methods.

20 mm at ambient temperature, the sections were stained with Hoechst 33258 for 30 min in the dark. After repeated washings with PBS buffer, the sections were incubated in a solution containing potassium cacodylate (200 mmol/l), CoCl_2 (2 mmol/l), bovine serum albumin (0.25 mg/ml), Tris-HCl, pH 6.6 (25 mmol/l), biotin-16-dUTP (10 $\mu\text{mol/l}$) and terminal transferase (25 units) for 1 h at 37°C. For control, sections were treated with DNAase I (10 U/ml) before adding terminal transferase. The reaction was terminated by repeated washing with PBS, and then the sections were incubated with a staining solution containing avidin-FITC (2.5 $\mu\text{g/ml}$), saline-sodium citrate buffer (4x), triton X-100 (0.1%), powdered milk (5%) for 30 min in the dark. The sections were washed again with PBS, and the intensely fluorescent nuclei were observed by fluorescence microscopy, and ISEL-positive myocytes were counted.

DNA fragmentation

Apoptosis is best characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180 bp or multiples thereof that are readily detected as a DNA ladder by gel electrophoresis. DNA was isolated by standard techniques [6, 7]. To perform DNA laddering, 10 μg of DNA obtained from control, ischemic, and reperfused myocardium was subjected to electrophoresis on 1.8% agarose gel containing 0.5 $\mu\text{g/ml}$ ethidium bromide, and photographed under UV illumination.

Statistical analysis

For statistical analysis, a two-way analysis of variance (ANOVA) followed by Scheffe's test was first carried out using Primer Computer Program (McGraw-Hill, 1988) to test for any differences between groups. If differences were established, the values were compared using Student's *t*-test for paired data. The values were expressed as mean $\pm$ S.E.M. The results were considered significant if *p* was less than 0.05.

Results

Characterization of GSHPx-1 gene knockout and transgenic mice

The 3' external probe was hybridized with a 5.3-kb Sad and an approximately 11-kb BamHI genomic fragment of normal mouse DNA. Insertion of the neo selective marker resulted in hybridizing Sad and BamHI fragments with sizes of 7.1 kb and 4.3 kb, respectively. Germ line transmission of the targeted GSHPx-1 allele was similar.

The specific activity of GSHPx-1 in the hearts of transgenic mice was found to increase by approximately 500% relative

to controls. Compared with normal heart homogenate which had a GSHPx-1 activity of $0.037 \pm 0.003 \mu\text{M NADPH/min/mg protein}$, the transgenic hearts showed an activity of $0.188 \pm 0.014 \mu\text{M NADPH/min/mg protein}$ (Fig. 3). Increased GSHPx-1 activity was also noticed in brain, lung and muscle of the transgenic mice. Over-expression of cardiac GSHPx-1 activity had no effects on the levels of other antioxidant enzymes, including manganese superoxide dismutase, copper/zinc superoxide dismutase, glutathione reductase, catalase, and glucose-6-phosphate dehydrogenase, in the hearts of the transgenic mice (data not shown).

Myocardial performance

All hearts recovered their beats spontaneously after 30 min of global ischemia followed by reperfusion. After reperfusion, all groups had the heart rates similar to those of baseline. No significant difference was found in heart rate between groups throughout the experiment. Recovery of Developed force (DF) defined as (peak systolic force-diastolic force) during reperfusion was lower for the knockout hearts and higher for the transgenic hearts compared to the control nontransgenic hearts throughout the reperfusion (Table 1). In control hearts, DF recovered to the level of baseline after 30 min of reperfusion, and this level was maintained through the end of the experiment. On the other hand, DF did not recover beyond 85% level of baseline for the knockout mice. Above all DF showed significantly lower recovery for knockout hearts after 30 min of reperfusion ($p < 0.05$). A Similar trend was seen in $\text{dF/dt}_{\text{max}}$. A Significantly lower recovery of $\text{dF/dt}_{\text{max}}$ occurred in knockout hearts after 30 minutes of reperfusion ($p < 0.05$).

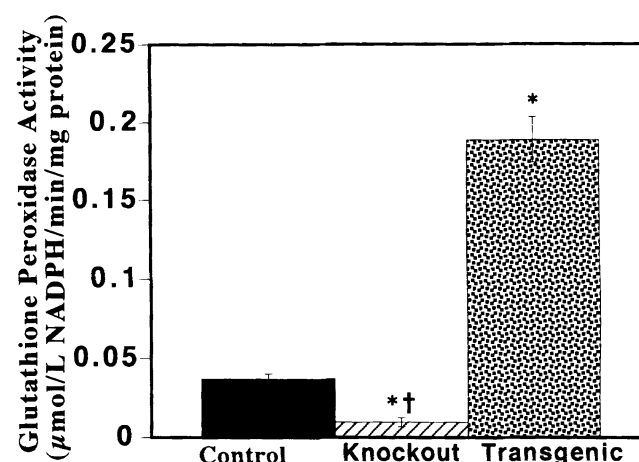


Fig. 3. Estimation of the level of Glutathione peroxidase activity in the heart of control transgenic and GSHPx knockout mice. Hearts were excised, and Glutathione peroxidase was estimated in heart by using enzymatic assay methods as described in Materials and methods. Results are mean $\pm$ S.D. for $n = 6$ per group. * $p < 0.01$ compared to control, † $p < 0.01$ compare to transgenic.

Table 1. Heart rate, DF and dF/dt in the hearts of normal mice, transgenic mice overexpressing GSHPx-1 and GSHPx-1 knockout mice

		Heart rate (bpm)	Recovery of DF (%)	Recovery of dF/dt (%)
Control	Baseline	260 ± 4.1	100 ± 6.2	101 ± 7.6
	R30	245 ± 5.2	52 ± 4.4	58 ± 4.2
	R60	234 ± 6.3	58 ± 4.3	65 ± 4.0
	R120	235 ± 3.2	65 ± 4.5	78 ± 3.6
Knockout	Baseline	260 ± 5.1	110 ± 4.8	100 ± 5.2
	R30	243 ± 6.3	40 ± 3.6*	45 ± 2.5*
	R60	260 ± 5.5	37 ± 4.2*	39 ± 3.1*
	R120	245 ± 3.8	30 ± 4.0*	42 ± 3.2*
Transgenic	Baseline	273 ± 5.2	108 ± 5.2	100 ± 3.4
	R30	244 ± 7.4	65 ± 5.9	78 ± 4.4*
	R60	240 ± 6.6	80 ± 5.1*	84 ± 3.0*
	R120	248 ± 5.3	95 ± 4.2*	92 ± 2.9*

Results are expressed as Means ± S.E.M. of at least six animals per group. *p < 0.05 compared to corresponding control.

These results demonstrate that knockout mice hearts showed significantly lower myocardial contractile recovery after 30 min of ischemia. To the contrary, post-ischemic ventricular recovery for the transgenic mice overexpressing GSHPx-1 was significantly higher compared to nontransgenic control animals. No significant difference was noticed in coronary flow between the three groups of hearts (data not shown).

Creatine kinase release from heart

Total creatine kinase release from the heart (Fig. 4) which reflects cellular injury or tissue necrosis and membrane permeability, was only about 5 units/ml for all the groups prior to ischemia. After ischemia, CK release was increased in all groups, but the amount of release was much higher for knockout hearts and much lower for the transgenic hearts. For example, at 30 min of reperfusion CK release was 108 ± 5.3 units/ml for knockout mice and 38 ± 2.1 units/ml for transgenic mice as compared to 52 ± 6.2 units/ml (p < 0.05) for non-transgenic controls. The amount of CK release progressively increased as the duration of reperfusion increased. At 60 min of reperfusion, CK release was 161 ± 6.7 units/ml for knockout and 51 ± 1.8 units/ml for transgenic hearts compared to 83 ± 8.1 units/ml (p < 0.05) for non-transgenic controls. Similarly, after 120 min of reperfusion, CK release from the knockout and transgenic mouse hearts amounted to 193 ± 9.1 and 71.2 ± 3.8 units/ml, respectively, compared to those from non-transgenic controls which was 109 ± 7.5 units/ml.

Myocardial infarction

In this study global ischemia for 30 min was adopted, therefore the whole ventricle was regarded as the area of risk. The area that was not stained by triphenyl tetrazolium was measured and calculated as the infarct area. Infarct size for

each heart was defined as: (Σ infarct area of each slice / Σ Total risk area) X 100. Mean value of infarct size in knockout group was significantly higher than that in control group ($27.6 \pm 4.4\%$ versus $17.0 \pm 1.0\%$, p < 0.05) (Fig. 5). For transgenic hearts, the infarct size was 9.0 ± 0.7 .

Induction of apoptosis by ischemia/reperfusion

We were unable to detect apoptotic cells in the control and in the ischemic hearts which were not subjected to reperfusion. Even 30 min of ischemia could not induce apoptosis, there was no sign of fragmented nuclear DNA in these biopsies. Apoptotic cells were identified only in the reperfused hearts. The extent of apoptosis increased with the progression of reperfusion time. Apoptotic cells were first evidenced after

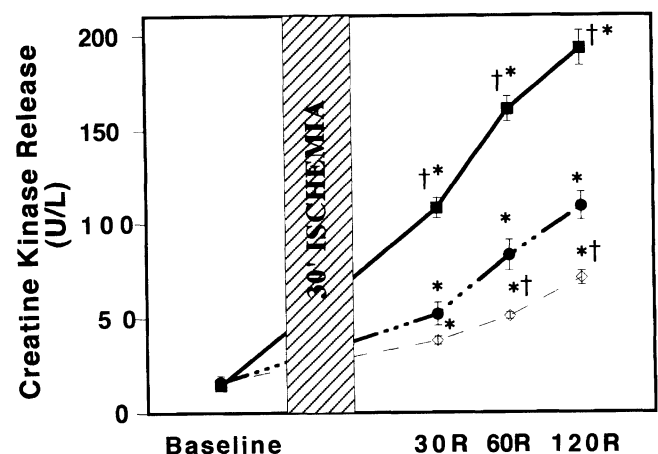


Fig. 4. Creatine Kinase release was estimated from the heart during post-ischemic reperfusion. CK release was mentioned as described in Materials and methods. Results are expressed as Mean ± S.E.M. for n = 10 per group. Each CK assay was determined in duplicate. *p < 0.05 compared to control, *compared to the baseline, †p < 0.05 compared to the control value. ●—● Control, ■—■ knockout, ◇—◇ transgenic mice.

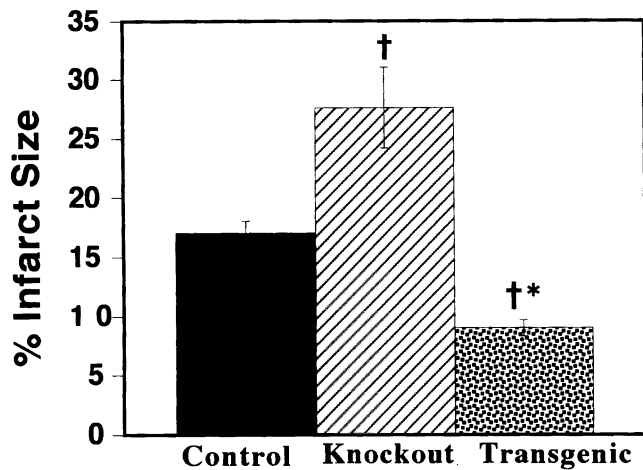


Fig. 5. Estimation of the infarction caused by ischemia and reperfusion in the transgenic and GSHPx knockout mice compared to the normal control. Normalized infarction was evaluated as described in the Materials and methods Section at 2 h of reperfusion followed by 30 min of ischemia. Results are expressed as mean $\pm$ S.E.M. for 10 animals in each group. $^{\dagger}p < 0.05$ compared to the control, $*p < 0.05$ compared to knockout, $^{\dagger}p < 0.05$ compared to control.

60 min of reperfusion. The number of apoptotic cells increased by 20% after 2 h of reperfusion in the control nontransgenic mouse heart as evidenced from the immunohistochemical staining of the extended DNA in these hearts. The number of apoptotic cells were significantly higher (48%) in the knockout hearts. Only 1.1% apoptotic cells were found in transgenic hearts as compared to perfused control hearts. The results are shown only after 120 min of reperfusion (Fig. 6).

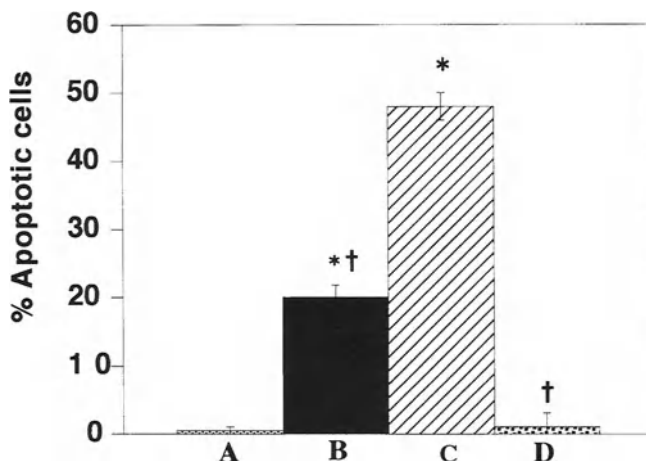


Fig. 6. Estimation of the apoptotic cells. Apoptotic cells expressed as % of total number of cardiomyocytes in the control, knockout and transgenic mice. Results are expressed as mean $\pm$ S.E.M. for 10 animals in each group. $^{\dagger}p < 0.05$ compared to the knockout, $*p < 0.05$ compared to the perfused control mice. A = perfused baseline control, B = Control (30 min ischemia followed by 120 min reperfusion), C = Knockout (30 min ischemic followed by 120 min of reperfusion), D = Transgenic (30 min ischemia followed by 120 min reperfusion).

DNA fragmentation induced by ischemia/reperfusion

Like apoptotic cell death, 30 min of ischemia did not cause DNA laddering. DNA fragmentation became obvious after 60 min of reperfusion following 30 min of ischemia. Hearts perfused for 90 min and 2 h after 30 min of ischemia demonstrated more clear DNA fragmentation as compared to 90 min reperfusion hearts. The amount of DNA fragmentation was significantly lower in the hearts of the transgenic mice while opposite results were noticed for the hearts of knockout animals as shown in Fig.7.

Discussion

The results of our study clearly demonstrated that similar to rat and rabbit hearts, up to 30 min of normothermic ischemia do not induce apoptotic cell death in mouse hearts. To the contrary, cardiomyocytes underwent apoptosis and DNA fragmentation after 60 min of reperfusion following 30 min of ischemia. Number of apoptotic cells increased pro-

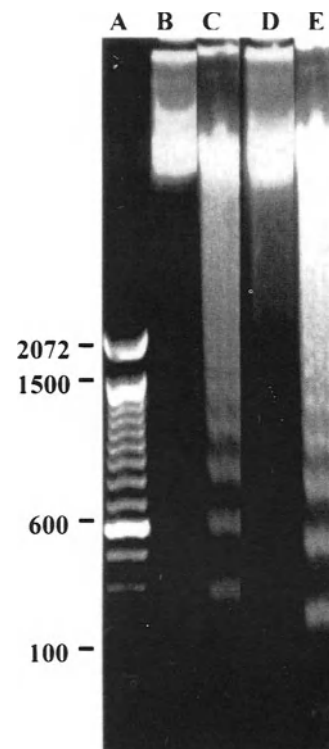


Fig. 7. Estimation of DNA fragmentation. DNA extracted from 15 mins of perfused baseline control (lane B), 30 min of ischemia followed by 120 min of reperfusion control (lane C), transgenic (lane D), and knockout mice (lane E) were subjected to 1.8% agarose gel electrophoresis as described in Materials and Method. A 100 bp DNA ladder (lane A) was used as Molecular weight marker.

gressively as a function of the reperfusion time. Significantly reduced number of apoptotic cells were found in the cardiomyocytes obtained from transgenic mice overexpressing GSHPx-1. To the contrary, increased number of apoptotic cells were present in the hearts of GSHPx-1 knockout mice. Hearts of the GSHPx-1 knockout mice were more and transgenic mice overexpressing GSHPx-1 were less susceptible to ischemic reperfusion injury as compared to non-transgenic control animals.

A growing body of evidence exists in the literature to support the notion that both necrosis and apoptosis contribute to the pathophysiology of ischemic and reperfusion injury. Most of the studies so far have been reported for rat hearts where no evidence of apoptosis were found in hearts which were subjected to up to 2 h of ischemia. For example, recent studies from our laboratory found no evidence for apoptosis in the rat hearts subjected to 2 h of ischemia, but apoptosis became evident when these hearts were subjected to 15 min of ischemia followed by 90 min of reperfusion [6, 7, 10, 11]. Another related study showed that apoptotic and necrotic myocyte cell deaths have been shown to independently contribute to infarct size in rats, but apoptosis did not become evident for up to 2 h of ischemia [3]. In a more recent study, characteristic signs of apoptosis were shown to appear only after 2.25 h of ischemia [4]. To the contrary, hearts subjected to relatively short periods of ischemia followed by reperfusion results in apoptosis, and this was independently shown by our group [5, 10, 11] and two other groups [4, 9]. In the present study, like rat and rabbits hearts, cardiomyocytes from mouse heart also did not show any signs of apoptosis and DNA fragmentation for up to 30 min of ischemia, but these cells underwent apoptosis only after 60 min of reperfusion following 30 min of ischemia. Taken together, this raises the interesting possibility that reperfusion of ischemic myocardium triggers some distinct signal for apoptosis which is not mediated by up to 30 min of ischemia.

Substantial evidence exists in the literature to support that reperfusion of the ischemic heart generates oxygen free radicals which contribute to the pathogenesis of reperfusion injury [20–22]. Interestingly, free radicals and/or oxidative stress are also common mediators of apoptosis, perhaps via the formation of lipid peroxidation and lipid hydroperoxides [23–25]. A direct role of oxygen free radicals has also been implicated in the pathogenesis of apoptosis. For example, SOD or an expression vector containing SOD cDNA was found to delay apoptotic cell death [26] and free radical mediated apoptosis was found to be modulated by proto-oncogene expression [27]. In fact, reactive oxygen species and prooxidants may act through mobilization of Ca^{2+} [28]. In cultured sympathetic neurons, when injected with SOD or with an expression vector containing SOD cDNA, apoptosis was delayed by a considerable amount [26]. Injection of antisense SOD expression vector into the neurons decreased

the amount of SOD simultaneously delaying apoptosis. This study also demonstrated that if SOD was injected after the development of oxidative stress, it had no effect on apoptosis. A recent study from our laboratory demonstrated that a seleno peroxide mimic, ebselen, could reduce apoptotic cell death and DNA fragmentation in concert with reduction of myocardial ischemic reperfusion injury. The precise mechanism by which ebselen reduced programmed cell death was not clearly understood. Ebselen, a synthetic selenium-containing heterocycle, is known to function as an antioxidant by its ability to reduce lipid hydroperoxides [29]. These authors demonstrated that ebselen suppresses free radical formation from hydroperoxides and hydrogen peroxide which have been implicated in the pathogenesis of ischemic reperfusion injury. Indeed, this compound was also shown to reduce ischemia reperfusion injury [30]. Recently, 2-(methylseleno) benzanilide, the main metabolite of ebselen *in vivo*, was shown to possess antioxidant activity against peroxynitrite which may be formed *in vivo* during pathologic conditions [31]. More recently, ebselen was found to inhibit apoptosis and DNA fragmentation in mouse thymocytes with simultaneous inhibition of lipid peroxidation. Thus, it appears that GSHPx play a crucial role in the regulation of reperfusion-mediated cardiomyocyte apoptosis.

The role of GSHPx is further confirmed from the results of the present study. Significantly increased number of apoptotic cells were apparent in the cardiomyocytes obtained from GSHPx knockout mice. Corroborating with this finding, hearts of the transgenic mice overexpressing GSHPx-1 showed reduced number of apoptotic cells. Cardiomyocytes contain a GSH redox cycle, in which GSHPx reaction accepts peroxides and peroxide-derived alkoxyl and peroxy radicals as substrates [32]. GSH-reductase replenishes the loss of GSH using NADPH as donor for reducing equivalents. In heart, GSHPx functions in three ways: first, it removes excessive H_2O_2 that are formed during reperfusion by the action of superoxide anions; second, it converts peroxidized fatty acids formed during lipid peroxidation into hydroxy fatty acids; and third, it reverses the oxidation of protein sulphhydryl groups. Catalase is also capable of removing H_2O_2 , but not only is it present in relatively low amounts in the heart (only 1–2% as compared to liver), especially in the mitochondrial and cytosolic compartments (>90% catalase is present in peroxisomes) where free radicals are generated, but also the K_m value of catalase for H_2O_2 is higher than that of GSHPx [33], further implying the primary importance of this enzyme in the heart. GSHPx also plays a crucial role in the maintenance of the structural and functional integrity of the cytosolic and mitochondrial compartments. The presence of a higher GSHPx activity may be vital for the heart to survive the attack of reactive oxygen species that are produced in the ischemic reperfused myocardium. The GSHPx reaction removes H_2O_2 at the expense of GSH. Oxidative stress can result in the

depletion of myocardial GSH and NADPH and an efflux of GSSG [34]. The fact that GSHPx-1 is present in much higher concentration in heart compared to catalase and that it possesses a high affinity for H_2O_2 (K_m for H_2O_2 degradation is about 40 μM) [35] suggests that GSHPx-1 plays a major role in removing the cytotoxic H_2O_2 formed during the reperfusion of ischemic myocardium. Indeed a number of reports exist in the literature to support the notion that enhancement of GSHPx-1 makes the cells more resistant to oxidative stress [36] and that GSHPx-1 is more important than catalase in this respect [37]. Because a cyclic detoxification pathway (glutathione-glutathione peroxidase cycle) exists in the heart which appears to play an essential role in the defense against stress such as ischemia/reperfusion, the maintenance of GSH levels seems to be crucial, and thiol (SH) groups are essential for the tissues to protect themselves against free radical attack.

In summary, our results confirmed that the cardiomyocytes of mouse hearts undergo apoptosis during reperfusion following ischemia. We also showed for the first time that the antioxidant enzyme, GSHPx, play a significant role in ischemia/reperfusion-mediated apoptotic cell death. Since oxidative stress developed in the reperfused heart is one of the causative factors for the development of apoptosis, the results also suggested that free radical scavengers/antioxidants previously known to reduce reperfusion injury, may at least in part, function by abrogating apoptotic cell death.

Acknowledgement

This study was supported by NIH HL 34360, HL 22559, HL 33889, HL 56803 as well as by a Grant-in-Aid from the American Heart Association. The authors thank Dr. Ye Shih Ho of Wayne State University for providing the transgenic and knockout mice used in this study.

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Thrombin releases calcium from internal stores of ultraviolet C-treated V79 fibroblasts independent of phosphatidylinositol biphosphate hydrolysis: Role of oxidative stress

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Abstract

V79 fibroblasts were treated with ultraviolet (UV) C radiation alone as well as in conjunction with chronic oxidative stress. The effects of these treatments on calcium signaling were observed at 30 min post-irradiation. In the absence of extracellular calcium, thrombin released calcium from internal stores of UVC-irradiated V79 fibroblasts even after exposure to neomycin. In neomycin-treated control and chronic oxidative stress cells, no calcium release by thrombin was observed after chelation of external calcium. Calcium release by thrombin from internal stores of UV-irradiated and neomycin-treated cells was completely abolished by pretreatment with N-acetyl cysteine and dexamethasone. Cellular total soluble thiol content which is a good indicator of cellular reduced glutathione (GSH) level was significantly elevated 30 min after ultraviolet radiation, indicating an adaptive response after oxidative stress. Chronic oxidative stress alone resulted in a much smaller increase in GSH but chronic oxidative stress in conjunction with UVC produced a very prominent elevation in GSH levels. Our data suggest that thrombin can cause calcium release from internal stores of ultraviolet-irradiated fibroblasts which is independent of phosphatidylinositol biphosphate hydrolysis and is directly related to the level of oxidative stress. Involvement of phospholipase A_2 and a role for its products as possible mediators of calcium release from intracellular stores, is strongly indicated. (Mol Cell Biochem **196**: 23–30, 1999)

Key words: ultraviolet radiation, oxidative stress, calcium, phospholipase A_2 , thrombin, V79 fibroblast

Abbreviations: UVR – ultraviolet radiation; UVC – ultraviolet C; PLA_2 – phospholipase A_2 ; NDGA – nordihydroguaiaretic acid; GSH – reduced glutathione; MAPK – mitogen-activated protein kinase

Introduction

Thrombin acts through a G-protein coupled receptor which is coupled to phospholipase C activation through G_q and G_o and inhibition of adenylate cyclase through G_i [1, 2]. Activation of phospholipase D and cytoplasmic phospholipase A_2 (PLA_2) are also observed during thrombin activation in a number of cell types [3, 4]. Thrombin causes elevation of internal calcium and activates protein kinase C (PKC) and mitogen activated protein kinase (MAPK) [2, 4–6]. Thrombin-

induced increase in internal calcium is caused by both inositol trisphosphate-mediated release from internal stores and calcium influx from the extracellular compartment [7].

Ultraviolet radiation (UVR) affects mammalian cells through DNA damage and also through changes in key signal transduction pathways involving growth factor receptor activation and tyrosine phosphorylation [8–10]. Oxidative stress has been identified as the mediator of some of these effects [10]. Changes in the cellular level of soluble thiols like cellular reduced glutathione (GSH) and anti-

oxidant enzymes are good indicators of oxidative stress conditions [11–16]. Initial cellular response to oxidative stress is usually reflected in a reduction in GSH and antioxidant enzymes [12, 13]. These parameters may increase after the initial decline as a compensatory response [14–16]. Elevation in internal calcium levels is frequently observed in connection with cellular damage [17–19]. In the eye lens, tissue damage has been associated with UV-induced increases in intracellular calcium [19].

We observed an unusual increase in internal calcium by thrombin solely from internal stores in the absence of phosphatidylinositol biphosphate hydrolysis in UV-irradiated V79 fibroblasts. Our study was designed to characterize the nature of this novel effect induced by environmental stress.

Materials and methods

Materials

Fura-2 AM, thrombin from bovine plasma, Genistein, Dexamethasone, Nordihydroguaiaretic acid (NDGA) and myelin basic protein were obtained from Sigma Chemical Co, USA. Media and reagents for cell culture were obtained from Gibco-BRL, USA.

Cell culture

Chinese hamster lung fibroblast line V79 was cultured as previously described [20]. Cells were grown in minimal essential medium supplemented with 10% dialyzed goat serum and antibiotics (penicillin and streptomycin) in plastic petri dishes. In order to subject the cells to chronic oxidative stress, the growing cells were treated with hydrogen peroxide (30 μ M), for 1 h at 37°C 5 days a week for a period of 15 weeks. UVC irradiation was carried out by a G.E. germicidal UV lamp (maximum output at 254 nm) for 10 sec. The incident dose rate was 2.0 J/m²/sec.

Determination of intracellular free calcium concentration

Cells were loaded with 1 μ M Fura-2 AM in Hank's buffer (135 mM NaCl, 5 mM KCl, 5 mM glucose, 3 mM NaH₂PO₄, 4 mM K₂HPO₄, 4 mM NaHCO₃, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, pH 7.2) for 20 min at 37°C. For deesterification of the dye, the cells were incubated for another 20 min in dye-free buffer. After one wash in dye-free buffer, the cells were given UV radiation. Cells were finally suspended in Hank's buffer with or without calcium (2 x 10⁵/ml) for measurement of fluorescence at 35°C in a Hitachi F-4010

Spectrofluorimeter with excitation at 340nm and emission at 500 nm. Calcium concentration was calculated from the equation $[Ca^{2+}] = K_d \times (F - F_{min}) / (F_{max} - F)$, the equilibrium dissociation constant (K_d) of Fura 2 being 224 nM [21]. Cells were disrupted with 0.1% Triton X 100 and F_{min} and F_{max} was determined in the presence of 3 mM EGTA and of 3mM CaCl₂, respectively. F_{min} and F_{max} were checked before and after UV irradiation and no change in these parameters due to UVR was detected.

Treatment with inhibitors

All the inhibitors were tested, before use, for fluorescence properties which may interfere with Fura-2 measurements. Presence of genistein, dexamethasone, N-acetylcysteine and NDGA did not interfere with Fura-2 measurements. However, when neomycin and N-acetylsalicylic acid were present, fluorescence measurements were affected. Therefore, after treatment with these inhibitors, the cells were washed and resuspended in inhibitor free medium for experiments. Cells were exposed to dexamethasone (5 μ M), genistein (25 μ M), and N-acetyl cysteine (5 mM) individually for 20 min at 37°C before UVR and the inhibitors remained in the suspension during the experiment.

NDGA (40 μ M), and N-acetylsalicylic acid (500 μ M) exposure was given by incubation at 37°C for 10 min after UV-irradiation. In some experiments, when the direct effect of NDGA was being measured, NDGA (40 μ M) was added directly to the cell suspension in the fluorimeter cuvette. Neomycin (50 μ g/ml) was added to the culture medium and cells were exposed to it for 48 h in culture.

Estimation of total soluble thiol

Total soluble thiol was measured to provide an assessment of GSH. Clear supernatants were collected after precipitation of cell lysates with 10% sulfosalicylic acid. For the estimation of total soluble thiol, the clear supernatants were mixed with 0.4 mM DTNB (5,5' Dithiobis 2 nitrobenzoic acid) in 0.2 M sodium phosphate buffer, pH 8.0. The absorbance was read at 412 nm [11, 22].

Myelin basic protein (MBP) kinase assay

Cell lysates were incubated at 30°C for 30 min in a medium containing 25 mM Tris, 50 mM NaF, 20 mM MgCl₂, 0.5 mM EGTA, 0.1 mM sodium ortho vanadate, 10 μ g MBP, 10 μ M PKA inhibitor (Sigma), 0.05 mM ATP and ADP, 10 μ M γ -³²P-ATP. Phosphorylated proteins were separated through centrifugation in phosphocellulose units (Pierce) and counted for

radioactivity by scintillation counting.

Results

Release of calcium from internal stores of V79 fibroblasts by thrombin

Thrombin releases small amounts of calcium from internal stores of control V79 fibroblasts in the absence of extracellular calcium (33 ± 13 nM increase). In UVC-irradiated cells, the peak calcium increase by thrombin from internal stores was significantly ($p < 0.001$; 130% increase) greater (79 ± 12 nM). When control V79 cells were exposed to neomycin, an inhibitor of phosphatidylinositol bisphosphate hydrolysis [23], subsequent calcium response by thrombin was completely blocked in the absence of external calcium (Fig. 1A). However, in cells which received both neomycin and UVR, thrombin was able to release calcium under similar circumstances (Fig. 1B). This calcium increase (53 ± 12 nM) was significantly lesser ($p < 0.01$; 34% lower) than that observed in UV-irradiated cells without neomycin treatment. No calcium release from internal stores was observed, in the absence of external calcium, in cells subjected to chronic oxidative stress and neomycin (data not shown). Under similar circumstance, cells treated with both chronic oxidative

stress and UVR demonstrated a marked elevation in internal calcium (67 ± 11 nM).

Effect of inhibitors on calcium release by thrombin from internal stores of UV-irradiated and neomycin-treated V79 fibroblasts

Pretreatment with genistein, a tyrosine kinase inhibitor [24], caused no significant change in calcium release from internal stores of UVC-irradiated V79 fibroblasts (Table 1). Pretreatment with N-acetylcysteine (NAC), a potent antioxidant, did not affect calcium release from internal pools of control fibroblasts (data not shown) but the same parameter was affected drastically (83% inhibition) in UV-irradiated and neomycin treated cells. Pretreatment with dexamethasone, an inhibitor of PLA_2 [25], did not cause any impairment of thrombin-induced Ca^{2+} release from internal stores of control fibroblasts (data not shown). Dexamethasone caused a moderate but not significant ($p > 0.05$) inhibition of thrombin-induced calcium release from internal pools of UVC-irradiated fibroblasts (Table 1). When UVC-irradiated fibroblasts were pretreated with neomycin, this inhibitory effect became even more pronounced and resulted in an almost complete abolition (96% inhibition) of the calcium release by thrombin (Table 1).

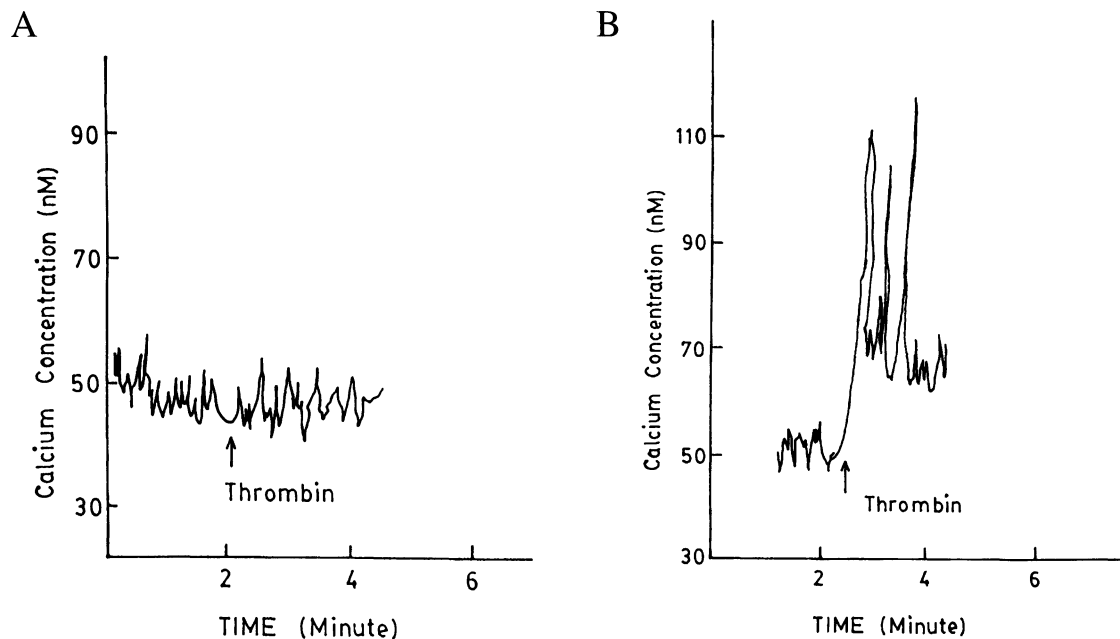


Fig. 1. Thrombin-induced calcium release from internal stores of V79 fibroblasts. A = Control V79 cells + neomycin; B = UV-irradiated V79 cells + neomycin; External medium contained 2 mM EGTA. UV-irradiated cells were examined 30 min after irradiation. Details of the UV irradiation procedure, Fura-2 loading, fluorescence measurements, neomycin treatment are given in the Materials and methods section. Traces are representative of 5–10 experiments.

Table 1. Effect of inhibitors on calcium release from UV-irradiated V79 fibroblasts

Inhibitor	(%) Inhibition of thrombin induced Ca^{2+} release from intracellular stores	
	(+) Neomycin	(-) Neomycin
Genistein	11.18 $\pm$ 8.48	14.72 $\pm$ 14.51
N-acetyl cysteine	82.79 $\pm$ 13.99 ^a	–
Dexamethasone	96.33 $\pm$ 4.22 ^a	39.12 $\pm$ 25.91
N-acetylsalicylic acid	11.05 $\pm$ 9.83	–
Nordihydroguaiaretic acid (NDGA)	88.33 $\pm$ 3.53 ^a	68.16 $\pm$ 7.30 ^{a,b}

Data are presented as percentage inhibition of thrombin-induced calcium release from UV-irradiated V79 fibroblast. Details of the experiments are described in the Materials and methods section. Mean calcium release from 2×10^5 neomycin pretreated fibroblasts was 53 nM and that from 2×10^5 cells without neomycin was 79 nM. Values are represented as means $\pm$ S.D. of 2–5 experiments. ^a $p < 0.01$ significantly different from corresponding control value. ^b $p < 0.05$ significantly different from corresponding value in neomycin pretreated cells.

Effect of cyclooxygenase and lipoxygenase inhibitors on thrombin-induced calcium release from internal stores of V79 fibroblasts

When UV-irradiated V79 fibroblasts were pretreated for 15 min with 500 μM acetylsalicylic acid, a cyclooxygenase inhibitor [26], no significant changes were observed in subsequent release of calcium from intracellular stores by thrombin (Table 1). However, pretreatment of UV-irradiated fibroblasts by nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase [27], produced a very pronounced inhibition of subsequent thrombin-induced calcium release from intracellular stores (Table 1). The degree of inhibition was significantly ($p < 0.01$) greater in UVC-irradiated cells which also received neomycin, when compared with UVC-irradiated cells without neomycin treatment. Also, NDGA itself caused a prominent calcium release from UVC-irradiated cells with or without neomycin treatment but not from control cells (Table 2). NDGA-induced calcium release from internal stores of UVC-irradiated fibroblasts was almost completely blocked ($> 90\%$ inhibition) by pretreatment with dexamethasone (Table 2).

Table 2. Nordihydroguaiaretic acid (NDGA)-induced calcium release from internal stores of V79 fibroblasts

	(+) Neomycin	Increase in free intracellular Ca^{2+} (nM)		
		Control (-) Neomycin	UV-irradiated (+) Neomycin	(-) Neomycin
NDGA	3 $\pm$ 1	17 $\pm$ 13	139 $\pm$ 28 ^a	176 $\pm$ 50 ^a
Dexamethasone + NDGA	–	–	2 $\pm$ 1	10 $\pm$ 10

Data are presented as mean $\pm$ S.D. of 2–4 experiments. Details of the experiments are presented in the Materials and methods section. Dexamethasone (5 μM) was added just before UV-irradiation and was present in the cell suspension till the experiments were performed. ^a $p < 0.001$ significantly different from the corresponding value in the dexamethasone treated group.

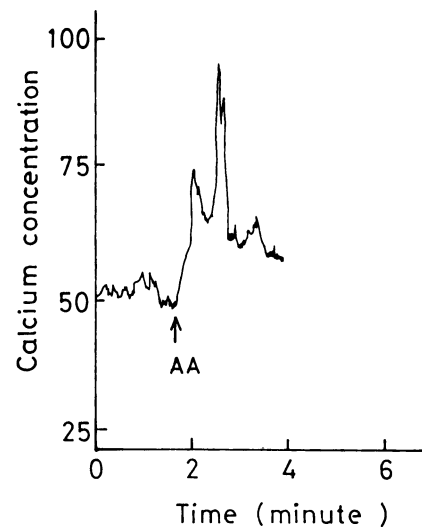


Fig. 2. Calcium release by arachidonic acid from internal stores of V79 fibroblasts. Control V79 cells were treated with arachidonic acid (20 μM) in the presence of 2 mM EGTA. Arrow shows addition of arachidonic acid. Trace is representative of 3 experiments.

Unsaturated fatty acid sensitive calcium pool in V79 fibroblasts

Arachidonic acid could release calcium from V79 fibroblasts both in the presence (data not shown) or absence of extracellular calcium (Fig. 2).

Cellular total soluble thiol content

Cellular total soluble thiol levels [11, 22] were measured as a direct indicator of cellular reduced glutathione (GSH) levels. GSH levels increased slightly but did not undergo significant alterations ($p > 0.05$) by treatment with chronic oxidative stress (Fig. 3). However, UVR produced a more than 2-fold elevation ($p < 0.05$) in GSH levels when measured at 30 min post-irradiation. Also, UVR treatment of cells which had received chronic oxidative stress, brought about a significant increase ($p < 0.01$) in GSH.

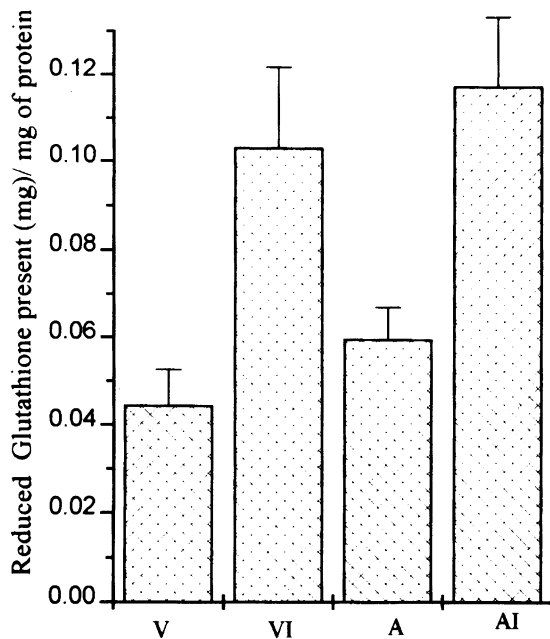


Fig. 3. Effects of UVC and chronic oxidative stress on non-protein bound thiol levels of V79 fibroblasts. V = Control V79 cells; A = V79 cells under chronic oxidative stress; VI = V79 cells treated with UVC radiation; AI = V79 cells under chronic oxidative stress treated with UVC radiation. All cells were exposed to neomycin as described in the Materials and methods section. Details of the UVC irradiation procedure, chronic oxidative stress procedure and NP-SH estimation are given in the Materials and methods section. Data are presented as mean $\pm$ S.D. of 2–4 experiments.

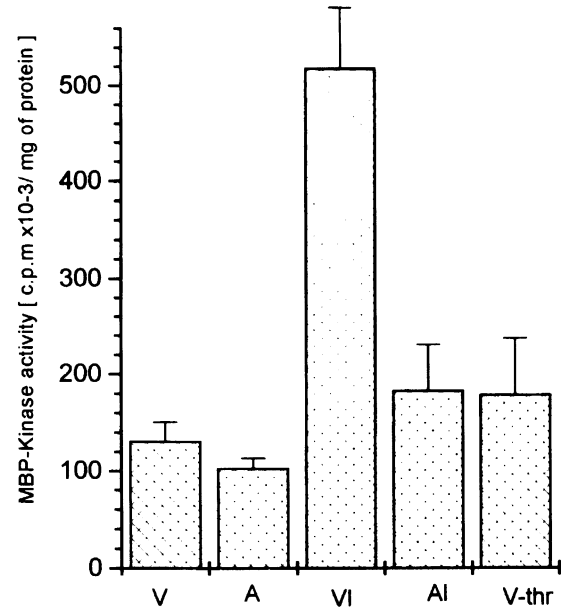


Fig. 4. Myelin basic protein kinase activity of V79 fibroblasts. V = Control V79 cells; A = V79 cells under chronic oxidative stress; VI = V79 cells treated with UVC radiation; AI = V79 cells under chronic oxidative stress treated with UVC radiation; V + Thr = V79 cells treated with 1 unit/ml thrombin at 37°C for 3 min in the presence of 2 mM EGTA. All cells received neomycin treatment as described in the Materials and methods section. Details of the UVC irradiation procedure, chronic oxidative stress procedure and Myelin basic protein kinase assay method are given in the Materials and methods section. Data are presented as mean $\pm$ S.D. of 3–5 experiments.

Myelin basic protein (MBP) kinase activity

As PLA_2 is known to be activated through phosphorylation by mitogen-activated protein (MAP) kinase [28], myelin basic protein kinase activity was measured under conditions inhibitory for protein kinase C and A. Chronic oxidative stress alone or chronic oxidative stress together with UVR failed to significantly elevate ($p > 0.05$) MBP kinase activity over control levels (Fig. 4). In contrast, cells which received UVR alone showed a 4 fold increase ($p < 0.01$) in MBP kinase activity. Thrombin alone produced a slight increase in MBP kinase activity in neomycin-treated V79 fibroblasts in the absence of external calcium.

Discussion

In recent years, UVR has been shown to affect signaling pathways in addition to its well-known damaging effect on DNA [9, 10, 29–31]. UVR causes tyrosine phosphorylation and activation of growth factor receptor and src in many cell types [8, 10, 32]. A number of studies has demonstrated UVR induced activation of ras, raf and mitogen activated protein

kinase (MAPK) [8, 30, 31]. Activation and synthesis of PLA_2 by UVR have also been documented [32]. Damage of eye lens tissue by UVR has been linked to a disturbance in cellular calcium homeostasis [19].

In our study, we have observed an unusual release of calcium by thrombin from internal stores of UV-irradiated fibroblasts which was independent of phosphatidylinositol biphosphate hydrolysis. This release was not dependent on UVR-induced tyrosine phosphorylation as inhibition of this process did not have any effect on this unusual calcium release. UVR is known to produce oxidative stress in cells and many of the UVR induced alterations including the activation of PLA_2 are mediated through oxidative stress [10, 32, 33]. N-acetyl cysteine, a potent antioxidant, has completely blocked this calcium release by thrombin in UV-irradiated V79 fibroblasts. Furthermore, this unusual release from UVR-treated cells also has correlated well with the level of oxidative stress, as has been demonstrated by the induction of the antioxidant defence mechanism – such as an increase in cellular GSH. Adaptive increases in cellular antioxidant defence system after oxidative stress have been

noted also by other investigators [14–16]. Elevation in cellular GSH after oxidative stress in cultured cells may occur through increased uptake of precursor amino acids and increased synthesis of glutathione [34]. Chronic oxidative stress cells have shown no neomycin-insensitive calcium release from internal pools by thrombin and a mild alteration in cellular GSH, whereas UVC-treated cells have demonstrated both neomycin-insensitive release of internal calcium and a very prominent change in GSH. Therefore, it is most likely that this effect of UVC is mediated through production of oxidative stress. Recently, the role of oxidative stress in UVC-induced cellular alterations has been emphasized by the finding that p53 activation by UVC could be prevented by prior exposure to N-acetyl cysteine [33].

The involvement of PLA₂ has become clearer as dexamethasone, an inhibitor of PLA₂ [25], has completely blocked calcium release by thrombin from internal stores of UV-irradiated and neomycin-treated cells. As thrombin could not produce this calcium mobilizing effect in control cells, a synergism between UVR and thrombin may be visualized. Thrombin is a potent activator of PLA₂ [2], and the effects of both thrombin and UVR-induced oxidative stress seem to converge at the point of activation of PLA₂. As both thrombin and UVR are able to activate P42/44 and P38 MAP kinase [4, 6, 30, 31, 35, 36], which could lead to the activation of PLA₂ by phosphorylation, it is important to establish whether MAP kinase activation has been involved. Our data have clearly indicated that members of the MAP kinase family which readily phosphorylate MBP such as P42/44 and P38 MAP kinases [37], are not involved in the activation of PLA₂, as cells subjected to both chronic oxidative stress and UVR have shown this novel Ca²⁺ increase but have failed to demonstrate significant elevation ($p > 0.05$) of MBP kinase activity above control levels (Fig. 4). However, our data have not excluded the possibility that stress activated protein (SAP) kinases which do not phosphorylate MBP [37], are involved in the activation of PLA₂ under these circumstances. As the activation of PLA₂ would lead to accumulation of free fatty acids (FFA), possibly arachidonic acid, we have explored the possibility whether a PLA₂ product, i.e., an unsaturated fatty acid or its metabolite, is the substance responsible for calcium mobilization. To this end, we have used N-acetyl salicylic acid, a cyclooxygenase inhibitor to see whether prostaglandins or thromboxanes act as the calcium mobilizer in UV-irradiated fibroblasts. Inhibition of the cyclooxygenase pathway has not adversely affected subsequent calcium mobilization by thrombin, demonstrating that prostaglandins or thromboxanes are clearly not involved in the unusual release of calcium from UV-irradiated fibroblasts. However, pretreatment with the lipoxygenase inhibitor, NDGA, has almost completely abolished (88% inhibition) subsequent release of calcium by thrombin from UVR and neomycin treated cells. In UVR treated cells which have not received neomycin, this inhibition

was significantly ($p < 0.001$) lesser, indicating the existence of a calcium release pathway insensitive to NDGA treatment, namely the phosphatidylinositol bisphosphate hydrolysis. This effect of NDGA could possibly have two explanations. On the one hand, products of the lipoxygenase pathway such as the various leukotrienes may be responsible for this unusual calcium release and on the other hand, blockage of lipoxygenase could result in the accumulation of its substrate arachidonic acid which may itself act as a mediator of calcium release from internal stores inhibiting subsequent calcium release from the same pool. Indeed, it has been noted that NDGA itself could release calcium from internal stores of UV-irradiated fibroblasts and this release has not been observed in control fibroblasts. It is conceivable that blockage of further metabolism of the calcium mobilizing agent, possibly a polyunsaturated fatty acid such as arachidonic acid, has culminated in the accumulation of that compound. This effect of NDGA is completely inhibited by pretreatment with dexamethasone (Table 2), further underscoring the fact that the unusual release of calcium is mediated by a PLA₂ product, possibly arachidonic acid. Calcium mobilization by polyunsaturated fatty acids from internal stores of many cell types, has been described [38, 39]. We have also established that a polyunsaturated fatty acid-sensitive calcium pool exists in V79 fibroblasts as arachidonic acid could release calcium from internal pools of V79 fibroblasts. Figure 5 provides a schematic diagram of the possible events initiated by UVR-induced oxidative stress. The effect of thrombin and that of UVR-induced stress converge at the point of activation of PLA₂, resulting in an increased released of arachidonic acid which, in all likelihood triggers a calcium release from internal pools of V79 fibroblasts.

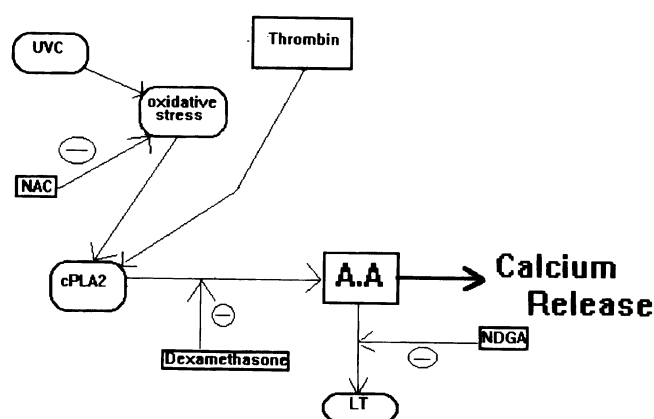


Fig. 5. Schematic diagram of the events evoked by UVC and mediated by oxidative stress in V79 fibroblasts. UVC produces oxidative stress in V79 fibroblasts. Both thrombin and oxidative stress synergistically activate PLA₂. When PLA₂ is blocked by Dexamethasone, formation of PLA₂ products such as arachidonic acid is inhibited. In contrast, when breakdown of arachidonic acid is blocked by NDGA, accumulation of arachidonic acid could occur.

In conclusion, our data provide evidence for a calcium release from internal stores of UV-irradiated V79 fibroblasts by thrombin which is independent of phosphatidylinositol biphosphate hydrolysis and is mediated by the activation of PLA₂ by UVR through production of oxidative stress.

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Glutathione homeostasis in response to exercise training and nutritional supplements

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Abstract

Glutathione plays a central role in the maintenance of tissue antioxidant defenses and in the regulation of redox sensitive signal transduction. In muscle cells, the level and redox status of GSH regulates activity of the redox sensitive transcription factor NF- κ B. Physical exercise may cause oxidation of GSH in tissues such as the blood, skeletal muscle and liver. Endurance training strengthened GSH dependent tissue antioxidant defenses in most studies. Although studies investigating the effect of sprint training are few, current results show that sprint training may also have a beneficial effect on tissue GSH homeostasis. Skeletal muscle GSH level appears to be tightly regulated by the state of physical activity. Regular exercise enhances and chronic inactivity decreases the level of GSH in this tissue. N-acetyl-L-cysteine (NAC) and α -lipoic acid (LA) are two antioxidant dietary supplements that are able to enhance cellular GSH levels. Because LA can be recycled to its potent dithiol form, dihydrolipoate, by enzymes present in the human cell it has a clear advantage over NAC. Recently an improved form of LA, a positively charged analogue (LA-Plus), has been discovered. LA-Plus has more potent immuno-modulatory activity compared to LA. Both LA and NAC have been shown to have beneficial effects in protecting tissue GSH homeostasis against exercise induced oxidative stress. (Mol Cell Biochem **196**: 31–42, 1999)

Key words: thiols, antioxidant, redox, adaptation, oxidative stress, skeletal muscle, dietary supplement

Introduction

Physical exercise increases energy demand, particularly that of the active tissues such as the skeletal muscle. Depending on the type of activity we get this extra energy from aerobic or anaerobic sources. For example in a 100 m sprint that lasts for about 10 sec or weight lifting events, the required energy supply rate is so high that there is no time for aerobic processes to contribute. All of the energy is spent from energy-rich pyrophosphate bond reserves in the tissue. In longer activities such as the 1500 m run that lasts for around 4 min, however, energy is supplied by both anaerobic (35%) and aerobic (65%) metabolic processes. For endurance events such as the 26 mile marathon run, almost all of the energy is supplied by aerobic metabolism. The metabolic cost of and oxygen consumption by skeletal muscles during exercise also depends on the nature of muscle contraction

that is predominantly involved. For example eccentric contraction (muscle lengthens) as during downhill running is associated with much less metabolic cost and oxygen consumption than other types of muscle contractions where the muscle group shortens (concentric) or remains unchanged in length (isometric). Standard exercise forms such as running or bicycling usually involve some specific muscle groups contracting concentrically, and some other muscles eccentrically.

Under resting conditions, oxygen content in arterial and venous blood of the skeletal muscle tissue is 20 and 15 ml per 100 ml blood, respectively. Physical exercise may increase skeletal muscle arterio-venous oxygen difference by 3-fold and blood flow through the tissue by 30-fold. As a result we may have up to 100-fold increase in oxygen flux through the active skeletal muscles during exercise. In 1978 Dillard *et al.* [1] tested whether physical exercise associated

increased oxygen consumption may cause oxidative tissue damage. They reported that in humans moderate intensity physical exercise increased the content of pentane, a lipid peroxidation by-product, in expired air. Electron paramagnetic resonance spectroscopy allows a direct detection of the short-living free radical species. Using this technique Davies *et al.* [2] showed for the first time in rats that exhaustive treadmill exercise may increase skeletal muscle and liver free radical concentration by 2–3 fold. Recent studies show that repeated exercise augments oxidative stress and that exercise-induced oxidative stress may cause damage to the genetic material [3]. The different mechanisms that may contribute to exercise induced oxidative stress have been recently discussed in a international Olympic committee publication [4].

Exercise training results in a large number of beneficial adaptive changes in various tissues, especially in the skeletal muscle and heart [5–13]. Several studies show that exercise training also enhances the ability of tissues to counteract oxidative damage [14]. In organs such as skeletal muscle, heart and liver, antioxidant defenses appear to be upregulated by physical training. In 1973, Caldarera *et al.* [15] were the first to show that acute exercise increases catalase activity in rat liver, heart and skeletal muscle. Since then a relatively large number of studies have tested the effect of a variety of endurance exercise training regimes on antioxidant defenses [16]. Jenkins *et al.* [17] studied the antioxidant enzymes of the muscle. Needle biopsy samples were collected from the vastus lateralis muscle of healthy men. The subjects were split into high-fit ($\text{VO}_{2\text{max}} > 60 \text{ ml/Kg min}$) and low-fit ($\text{VO}_{2\text{max}} < 60 \text{ ml/Kg min}$) groups. The high aerobic capacity group had significantly greater activities of catalase and superoxide dismutase in their muscle. A strong positive correlation ($r = 0.72$, $p < 0.01$) between the subject's maximum oxygen uptake and muscle catalase was noted. A similar correlation was also observed between the subject's maximum oxygen uptake and muscle superoxide dismutase ($r = 0.60$, $p < 0.05$). The study also found that there was a rank order relationship in both the tissue oxygen consumption and antioxidant enzyme activity [17].

Glutathione has emerged to be one of the most fascinating molecules virtually present in all animal cells often in quite high (millimolar) concentrations. It is known to have multifaceted physiological functions including antioxidant defense, detoxification of electrophilic xenobiotics, modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotide synthesis, regulation of immune response, and regulation of leukotriene and prostaglandin metabolism. The effect of exercise training and the role of nutritional supplements in regulating tissue glutathione homeostasis has been briefly reviewed in this work.

Physical exercise and tissue glutathione response

Human blood plasma contains low amounts of reduced glutathione (GSH). Almost all of blood glutathione represents blood cell content, mainly that of the erythrocyte. Oxidation of GSH to glutathione disulfide (GSSG) is a sensitive marker of oxidative stress. In 1988, Gohil *et al.* [18] were the first to report that even submaximal exercise induces blood GSH oxidation. A 100% increase in blood GSSG level was reported within the first 15 min of exercising at 65% $\text{VO}_{2\text{peak}}$ [18]. Ji *et al.* [19] exercised 8 healthy male cyclists at 70% $\text{VO}_{2\text{max}}$. In contrast to the finding of Gohil *et al.* [18], a bout of exercise that lasted for more than 2 h did not elevate blood GSSG. Exercise induced blood glutathione oxidation has been shown in a number of later studies [20–22] and is consistent with previous human experimental studies showing that exhaustive exercising of rats remarkably increases GSSG level in the plasma [23].

The association between exercise intensity and related oxidative stress in healthy young men who exercised for 30 min at their aerobic and anaerobic thresholds one week apart has been studied in our laboratory [21]. Blood samples were drawn before, immediately after and 24 h after tests. In line with the observation of Gohil *et al.*, all four exercise bouts (at aerobic and anaerobic thresholds for 30 min each, and two maximal tests [mean duration ~ 14 min]) remarkably increased the level of blood GSSG. Exercise induced perturbations in the blood glutathione redox status and plasma lipid peroxide level were no more observed in the 24 h post-exercise recovery samples. Viguie *et al.* [24] also observed that there was no evidence of persistent or cumulative effects of repeated leg cycling exercise (at 65% $\text{VO}_{2\text{peak}}$, 90 min, for 3 consecutive days) on blood glutathione redox status. In moderately trained men, a 50% decline in the blood GSH level was observed during the first 15 min of exercise. This effect was accompanied by an increase in the level of blood GSSG. Total glutathione level in the blood did not change significantly during the exercise. Blood GSH level returned to baseline after 15 min of post-exercise recovery. Although high speed running for brief time intervals (~ 20 sec) to exhaustion did not influence blood GSH oxidation, Sastre *et al.* [25] observed that in trained men blood GSSG levels were 72% higher immediately after exercise than at rest. In young men, intermittent exercise bouts to exhaustion increased blood GSSG by 35% [26]. More recently, Laaksonen *et al.* [22] also observed exercise-induced blood GSH oxidation in young insulin-dependent diabetic men and their corresponding healthy controls. Results obtained so far show that physical exercise may enhance the utilization of blood GSH resulting in decreased GSH/GSSG ratio.

Physical exercise influences GSH metabolism in skeletal muscles and liver of rats [16]. Lew *et al.* [23] reported that exhaustive exercise decreases both liver and muscle glutathione consistently. We investigated the influence of exhaustive treadmill run on the tissue GSH status of rats. Exhaustive exercise decreased total glutathione reserves of the liver and active skeletal muscles red gastrocnemius and mixed vastus lateralis, and that this effect was not observed in the less active longissimus dorsi muscle [27]. Exercise-induced decrease of total glutathione pool in the liver, red gastrocnemius muscle, mixed vastus lateralis muscle and heart of rats was also seen in another independent study carried out in our laboratory. This effect was, however, not seen in the lung [28]. Duarte *et al.* [29] confirmed that a single bout of exercise results in glutathione loss from skeletal muscle. Exercising resulted in a 50% decrease in left soleus muscle total glutathione content, an effect that was interpreted as an index of oxidative stress. Recovery of muscle glutathione level was slow in the post-exercise recovery period. This recovery was remarkably faster in mice supplemented with allopurinol, an inhibitor of the superoxide producing enzyme xanthine oxidase. It was suggested that exercise-induced increase in xanthine oxidase dependent superoxides causes oxidative stress to muscle tissues located in close proximity, and that this stress is manifested as a loss of tissue glutathione.

Role of tissue glutathione in exercise

Although the importance of glutathione in protecting against oxidative stress is well recognized, the role of physiological levels of glutathione and other endogenous antioxidants in protecting against exercise induced oxidative stress is less clear. We evaluated the role of glutathione and selected antioxidant enzymes as determinants of lipid peroxidation at rest and in response to exercise in men ($n = 13$ – 14) aged 20–30 years, who cycled for 40 min at 60% of their maximal oxygen consumption (VO_{2max}) [30]. Blood GSSG increased by about 50% in response to exercise. Mean blood reduced GSH decreased by 13% with exercise. Of the measured red blood cell antioxidant enzyme activities, only selenium dependent GSH peroxidase activity increased following exercise. In univariate regression analysis, plasma lipid peroxidation by-product levels at rest predicted exercise induced change in blood total glutathione. Blood GSSG levels at rest were a strong determinant of post-exercise levels. Subjects with a favorable blood glutathione redox status at rest maintained a more favorable redox status in response to exercise induced oxidative stress. Changes in blood GSH, GSSG and total glutathione in response to exercise were closely associated with both resting and exercise induced plasma lipid peroxidation. Results of this study underscores

the critical role of glutathione homeostasis in modulating exercise induced oxidative stress, and conversely, the effect of oxidative stress at rest on exercise induced changes in glutathione redox status [30].

A critical role of tissue glutathione in protecting against exercise-induced oxidative stress has been evident particularly from studies involving GSH deficiency. Tissue GSH synthesis is dependent on dietary amino acid supply [31]. Thus, food-deprivation decreases tissue GSH content and refeeding corrects such effect. Exhaustive treadmill exercise tends to further lower hepatic GSH level of food-deprived rats. This effect was more prominent in rats that were refed and had higher levels of baseline hepatic GSH level [32]. Glutathione deficient rats have been prepared by the intraperitoneal injection of L-buthionine-[S,R]-sulfoximine [28]. The L-buthionine-[S,R]-sulfoximine treatment approach selectively inhibits the first enzyme of GSH synthesis, γ -glutamylcysteine synthetase, and turns off intracellular GSH synthesis. L-buthionine-[S,R]-sulfoximine treatment resulted in (i) ~ 50% decrease in the total glutathione pools of the liver, lung, blood and plasma, and (ii) 80–90% decrease in the total glutathione pools of the skeletal muscle and heart. Compared to the placebo treated controls, endurance to exhaustion of glutathione deficient exercising rats was reduced to half. Results of this experiment indicated a crucial role of endogenous GSH in the circumvention of exercise induced oxidative stress and as a determinant of exercise performance [28]. In another recent study, diethyl maleate induced depletion of glutathione also significantly decreased swim-performance of rats [33].

The effect of a more long term GSH deficiency has been studied in mice that were swim-exercised [34]. Global GSH-deficiency in mice was induced by the intraperitoneal injection of L-buthionine-[S,R]-sulfoximine combined with supplementation of 20 mM L-buthionine-[S,R]-sulfoximine in drinking water for 12 days. Using such a protocol, GSH contents in the plasma, liver, kidney heart and skeletal muscles were decreased by 65, 77, 85, 90 and 93% of the control values, respectively. A more enduring exercise bout that lasted for 4–6 h was tested, and no effect of GSH-deficiency on swim-endurance was observed in these mice. Marked decrease in hepatic GSH level was observed during exercise. Consistent with our observation [28], exercise-induced oxidative lipid damage was more in GSH-deficient mice, particularly in skeletal muscle. Increased tissue lipid peroxidation in GSH-deficient state is consistent with the hypothesis that GSH plays a central role in the antioxidant-network, and that impaired GSH defense weakens the efficacy of lipid phase antioxidants as well. Leeuwenburgh and Ji [34] showed that GSH deficiency also influences the activity of antioxidant enzymes. For example, GSH-deficiency was associated with decreased GSH peroxidase activity in the liver, whereas activities of GSSG reductase

and GSH S-transferase were elevated. GSH deficiency may also influence oxidative metabolism in tissues [34]. Results from the heart of GSH deficient mice subjected to prolonged exercise show that GSH is actively used in the myocardium during prolonged exercise at moderate intensity and that GSH deficiency is relatively well tolerated by the heart, possibly compensated for by an increased GSH uptake from the plasma [35].

Response to exercise training

A properly selected exercise training regime improves cardiovascular health and the gross functional capacity of the human body. Does exercise training improve the ability of tissues to defend against oxidative stress? Yes, indeed this has been observed to be the case in several independent studies [14, 36, 37] with a very few exceptions [38]. A recent human study measured resting muscle and blood antioxidant status in untrained and jump-trained humans. Activities of GSH redox cycle enzymes, GSH peroxidase and GSSG reductase, were significantly higher in jump-trained compared with untrained subjects [39]. Endurance training dependent increase in tissue antioxidant defense has been also linked with improved physical performance [40]. We observed that in diabetic patients, a bout of exercise induces oxidative stress and that physical fitness may have a protective effect against such oxidative stress [22, 41]. The protective effect of exercise training against ethanol-induced oxidative injury in specific regions of the brain has been also observed [42]. Adaptation of tissue antioxidant defense systems in response to exercise training appears to be age-sensitive. In an interesting study by Leeuwenburgh *et al.* it was observed that although exercise training selectively increased the activity of antioxidant enzymes in tissues of young rats, there was no such protection against oxidative stress in the senescent muscle.

Kihlström [43] showed that endurance swim training provides enhanced protection to the heart against oxidative stress. This added capacity to detoxify reactive oxygen species was mainly because of elevated glutathione level and a more efficient NADPH supplying system in the trained heart. The training program decreased the activity of GSSG reductase in the myocardium, and increased the activity of thioredoxin reductase. These results concerning the swim training induced decrease of antioxidant enzyme activities in the heart were also observed by this author in a previous investigation [44]. GSSG reductase requires NADPH to maintain a favourable redox status of glutathione. Training increased the activity of the NADPH supplying enzyme glucose 6-phosphate dehydrogenase in the right ventricle. Also, the level of GSH was higher in the trained heart, especially in the left subepimyocardium.

In response to similar sub-maximal exercise, endurance trained rats are able to maintain tissue glutathione redox status better as reflected by the GSSG/total glutathione ratio compared to their untrained counterparts. In this case, the endurance training program significantly increased the activities of GSH peroxidase, GSSG reductase and glucose-6-phosphate dehydrogenase in the skeletal muscle and heart tissues [45].

The effects of aging and exercise training on rat skeletal muscle antioxidant enzyme activities have been tested [46]. Superficial glycolytic and deep oxidative vastus lateralis muscles were collected from rats aging from 2.5 months (young) to 27.5 months (senescent). Old rats had significantly lower GSH peroxidase activity in the deep vastus lateralis muscle. After progressive treadmill training, activity of the hydroperoxide-metabolising enzyme in deep vastus lateralis muscle significantly increased to a level higher than that observed in sedentary young rats. Thus, although aging adversely affects the antioxidant enzyme capacity in skeletal muscle, regular exercise can preserve such protective function. In a different model where Kanter *et al.* [47] tested the effect of swim-training, consistent results were obtained showing that training enhanced GSH peroxidase activity in the blood and liver. In contrast to some of the other studies described above, Tiidus and Houston [48] observed that in female rats, six week treadmill training does not influence the GSH peroxidase activity in skeletal muscle, heart and liver. In a human study, however, endurance training did increase erythrocyte GSH peroxidase activity [26].

We observed that treadmill training of rats increased skeletal muscle citrate synthase activity indicating enhanced oxidative capacity [27]. Hepatic total glutathione content was elevated in the trained rats. However, such an effect was not observed in any of the skeletal muscles studied (i.e. red gastrocnemius, mixed vastus lateralis, and longissimus dorsi). Leg muscle GSH peroxidase activity was higher in trained rats. Treadmill training decreased GSSG reductase activity in red gastrocnemius muscle. This effect may be related to the high intensity of training that may have increased flavoprotein turnover and breakdown in the muscle. Endurance training also increased the activity of γ -glutamyl transpeptidase in both leg muscles, the effect being more pronounced in red gastrocnemius. In the trained leg muscles, activated γ -glutamyltranspeptidase may facilitate the import of substrates required for GSH generation. Decreased γ -glutamyl transpeptidase activity was observed in the control leg muscles after exercise [27]. This effect, however, was not observed in the trained leg muscles indicating that during exercise the trained muscles have a more active substrate import system for GSH generation compared to the untrained controls. γ -Glutamyl transpeptidase activity of the trained liver decreased (~ 50%) after the exercise bout. This response might ensure that

fewer γ -glutamyl compounds are re-trapped in the liver when the needs of the active peripheral tissues are acute. The contention that exercise training strengthens GSH dependent tissue antioxidant defense was further supported by another study where swim-training of rats was associated with a marked increase in the activities of GSH peroxidase and GSSG reductase in the skeletal muscle, heart and liver [49].

GSH dependent antioxidant protection in the skeletal muscle appears to be tightly regulated by the state of physical activity; endurance training enhances and chronic activity-restriction diminishes such protection [27]. Dogs are more naturally endowed to be aerobic runners as compared to the rats that have been the experimental animal in other studies concerning the response of glutathione redox cycle to endurance training and exercise. Beagle dogs, commonly used as a laboratory animal, possess a well developed musculoskeletal system apparently suited for running. Thus, we studied the influence of treadmill training on beagle dogs. Treadmill training (5.5–6.8 km/h, 40 km/day, 5 days/week, 15% uphill grade, for 40 weeks) increased the oxidative capacity of red gastrocnemius, extensor carpi radialis, and triceps muscles of the leg. Training induced changes in the components of GSH metabolism was most pronounced in the red gastrocnemius muscle that is predominantly oxidative by composition. Hepatic and red gastrocnemius total glutathione levels were elevated in response to training. In all three leg muscles mentioned above, training elevated GSH peroxidase activity. This effect was also most pronounced in the red gastrocnemius muscle. GSSG reductase activities in extensor carpi radialis and triceps muscles were higher in the trained dogs. Trained animals with higher hepatic total glutathione reserves also had higher GSH S-transferase activity indicating that the liver of the trained animals had a higher detoxicant status. Training effects were not observed in the splenius muscle of the neck and trunk region which were least active during the training process. In a separate dog experiment [27], the effect of chronic activity-restriction on red gastrocnemius muscle of beagles was studied. The knee and ankle joint of right pelvic limb of each dog was immobilized for 11 weeks in a light fibre-glass cast. The left leg was used as the paired control. Chronic physical inactivity did not influence the activity of GSH dependent enzymes, however, the total glutathione level of the red gastrocnemius muscle was remarkably decreased in the immobilized leg [27]. Decreased total glutathione level and increased GSSG have been also observed to be associated with skeletal muscle atrophy [50].

A 55 week endurance training study with beagle dogs showed that physical training may enhance hepatic GSH S-transferase activity [27]. GSH S-transferases are a family of GSH dependent enzyme that play a central role in drug detoxification and xenobiotic metabolism. In addition, GSH S-transferases may also contribute to hydroperoxide

metabolism because they have non-selenium GSH peroxidase activity. Later it was confirmed in rats that swim-training also increases hepatic GSH S-transferase activity when compared to non-trained controls. Electrophoretic and Western blot analyses revealed that a Ya-sized subunit of the transferase is specifically induced by exercise training. Analyses of affinity-purified GSH S-transferases further revealed that a Ya₁ subunit of Ya was most sensitive to exercise training. Non-trained control rats had Ya-subunits predominantly made up of Ya₂, whereas the trained animals had 4.3-fold increased in Ya₁ [51]. GSH S-transferases of exercise trained animals had increased peroxidase activity, an effect that was consistent with the changes in subunit composition. Studies on the regulation of Ya gene expression have revealed that the gene contains a regulatory sequence known as the antioxidant response element or ARE in the 5'-flanking region. Transcription of Ya is activated by oxidants such as hydrogen peroxide by a mechanism acting through the ARE [52]. Ya₁ is known to be induced in hydroperoxide overload situations such as selenium deficiency [53]. Thus, exercise-induced regulation of Ya₁ is expected to be oxidant mediated.

Endurance training can upregulate certain antioxidant enzyme activities in rat diaphragm muscle, indicating the potential for improvement of the resistance to intracellular reactive oxygen species [6, 36]. Acute exercise may cause oxidative damage in rat diaphragm through activation of inflammatory pathways and endurance training minimizes oxidative stress caused by acute exercise [54]. The effect of intensity and duration of exercise on training induced tissue antioxidant enzyme responses has been studied [55]. Rats were exercised at low, moderate or high intensity at one of three exercise durations (30, 60 or 90 min/day). The coastal and crural diaphragm, plantaris muscle and parasternal intercostal muscles were investigated. Training effects were highly tissue specific. All training programs markedly increased GSH peroxidase activity in the costal diaphragm, but not in crural diaphragm. Exercise-intensity or duration did not have any major influence on training-induced elevation of GSH peroxidase activity in the coastal diaphragm. In crural diaphragm, however, moderate and high intensity exercise training decreased tissue GSH peroxidase activity when the daily exercise duration was as long as 90 min. None of the training programs influenced GSH peroxidase activity of the parasternal intercostal muscle, although remarkable effects were observed in the plantaris muscle. In the plantaris, daily exercise duration had a marked effect on GSH peroxidase activity response. Longer daily exercise duration triggered a more marked response. Results of another similar study further support that training effects are indeed highly tissue specific [56]. Although exercise training increased GSH peroxidase activity in the red gastrocnemius muscle of rats, such effects were not consistently seen in soleus or even white gastrocnemius muscles. Similar to the

previous results obtained from plantaris muscle, daily exercise duration had a marked effect on GSH peroxidase activity response. Recently it has been shown that porcine skeletal muscle adapts to endurance exercise training in a manner similar to muscle of humans and other experimental animals [57]. Criswell *et al.* [58] observed that high intensity interval exercise is superior to moderate intensity continuous exercise in the elevation of GSH peroxidase activity in rat soleus muscle. Training dependent enhancement of human tissue antioxidant defenses, in certain cases, is thought to be so remarkable that it may completely offset oxidative stress expected to be caused by a severe bout of triathlon race [59].

Both strenuous long duration exercise and exhaustive sprint training may cause oxidative stress [60]. Yet, studies investigating the influence of physical training on tissue antioxidant status have mostly tested the effect of endurance training, which enhances tissue oxidative capacity. Information on the effect of sprint training, which relies primarily on non-oxidative metabolism, on tissue antioxidant defenses is scanty. We examined the effect of a sprint training regimen on rat skeletal muscle and heart GSH system [61]. Soleus muscle, predominantly made up of slow-oxidative fibers, was studied as representative of slow-twitch muscle. Plantaris and extensor digitorum longus muscles, consisting mainly of glycolytic fibers and the superficial white portion of the quadriceps femoris muscle, mainly consisting of fast-oxidative-glycolytic fibers were studied as representative of fast-twitch muscles. Mixed gastrocnemius muscle was examined as an antagonist of extensor digitorum longus muscle. Lactate dehydrogenase and citrate synthase enzyme activities were measured in muscle to test the effects of training on glycolytic and oxidative metabolism, respectively. The efficacy and specificity of the 6 week sprint training protocol was attested by markedly increased anaerobic but not aerobic metabolic capacity in mixed and fast twitch fiber muscles. Endurance training consistently upregulates GSH dependent defenses and other antioxidant enzymes, with effects most marked in highly oxidative muscle. In contrast, sprint training enhanced antioxidant defenses primarily in fast glycolytic muscle. Compared with the control group, GSH peroxidase activities in gastrocnemius, extensor digitorum longus muscles and heart increased following sprint training. The training program also increased GSSG reductase activity in the extensor digitorum longus muscle and heart. Sprint training did not influence glutathione levels or GSH related enzymes in the oxidative soleus muscle. The effect of intermittent sprint cycle training on the level of muscle antioxidant enzyme protection has been also investigated in humans [62]. Resting muscle biopsies, obtained before and after 6 weeks of training and 3, 24, and 72 h after the final session of an additional 1 week of more frequent training, were analyzed for activities of the antioxidant enzymes GSH peroxidase,

GSSG reductase, and superoxide dismutase. Intermittent sprint cycle training that induces an enhanced capacity for anaerobic energy generation also improved the level of antioxidant protection in the muscle.

Regulation of NF- κ B in skeletal muscle derived cells

Oxidation-reduction (redox) based regulation of signal transduction and gene expression is emerging as a fundamental regulatory mechanism in cell biology [63, 64]. Electron flow through side chain functional $\text{CH}_2\text{-SH}$ groups of conserved cysteinyl residues in proteins account for their redox-sensing properties. At least two redox sensitive transcription factors, nuclear factor κ B (NF- κ B) and activator protein-1 (AP-1) have been well defined [63, 64]. Intracellular thiol redox status appears to be a critical determinant of NF- κ B activation. At low levels of cytosolic GSSG, T-cells fail to activate NF- κ B in response to appropriate stimuli, whereas high GSSG concentration inhibits the binding of activated NF- κ B to its cognate DNA site. Thus, it appears that an intermediate optimal level of intracellular GSSG is required for effective NF- κ B activation. Droge *et al.* have found that GSH deficiency of T-cells is associated with a suppression of NF- κ B function. This effect was suggested to be related to very low levels of GSSG, thought to be necessary for NF- κ B activation in some models, in GSH deficient cells [65].

Tumor necrosis factor α (TNF α), a cytokine product of monocytes and macrophages [66] is a rapid and potent activator of NF- κ B (Figs 1 and 2). TNF α is suggested to be implicated in muscle wasting of cachexia [67–70]. In support of this it has been observed that sustained increase in serum levels of TNF α contributed by tumor cells [71–74], or in TNF α transgenic mice [75] can induce muscle wasting. Muscle wasting in cachexia is a common phenomenon observed in a large population of individuals suffering from chronic diseases such as AIDS, cancer, some inflammatory disorders, sepsis and trauma [76]. Muscle wasting has been recognized as the single most common cause of death among cancer patients [77]. Other muscle pathologies such as eosinophilia myalgia syndrome [78] have been also observed to be associated with increased levels of TNF α . Recently it has been shown that exhaustive exercise of athletes results in increased TNF α levels in the serum [79] suggesting that TNF α may be also implicated in exhaustive exercise induced muscle damage. We investigated the role of endogenous glutathione status in TNF α induced NF- κ B activation in skeletal muscle-derived cells [80]. TNF α proved to be a potent inducer of transient NF- κ B activation in L6 myoblasts. In buthioninesulfoximine treated GSH

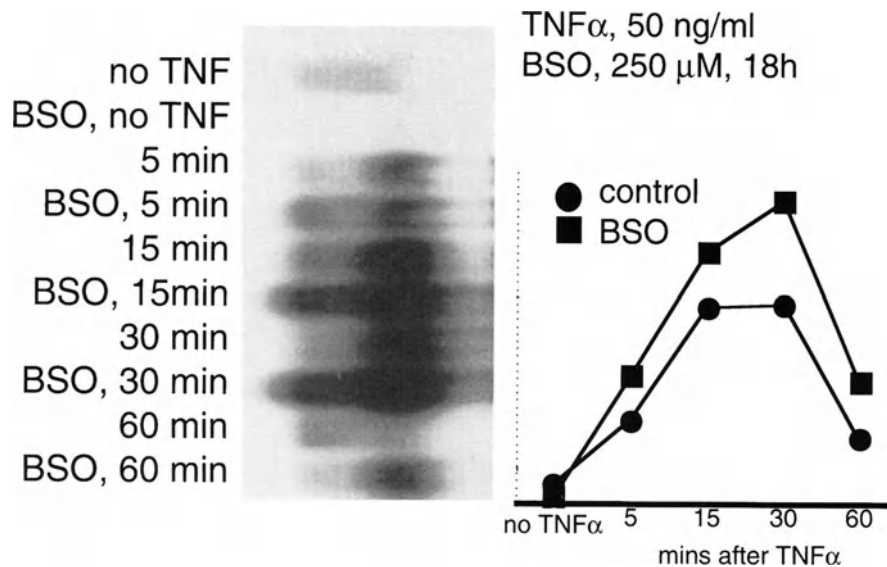


Fig. 1. GSH depletion potentiates TNF α induced NF- κ B activation in skeletal muscle derived L6 myoblasts. GSH depletion was caused by buthionine sulfoximine (BSO) treatment (250 μ M, 18 h). Lines represent densitometry values of the NF- κ B autoradiograph as a function of TNF α treatment time. Circles, BSO non-treated controls; squares, BSO treated GSH depleted cells. Cells were either pre-treated or not with BSO as indicated in the respective lanes, and treated or not with TNF α for the time intervals indicated. The specificity of NF- κ B band was verified by cold κ B consensus probe competition (not shown) [80].

deficient cells, TNF α induced NF- κ B activation was potentiated (Fig. 1) suggesting that such activation is sensitive to cellular GSH, but may have been independent of high levels of intracellular GSSG as previously proposed [65]. Enhancement of cell GSH reserves by treatment of myoblasts with pyrrolidinedithiocarbamate inhibited TNF α induced NF- κ B activation (Fig. 2). Bischloroethylnitrosourea treatment is known to inhibit GSSG reductase activity and thus impair NADPH dependent recycling of GSSG to GSH leading to elevated cell GSSG/GSH. In 1,3-bis(chloroethyl)-1-nitrosourea treated cells, TNF α induced NF- κ B activation was markedly potentiated suggesting that GSSG may participate in TNF α induced NF- κ B activation. The inhibitory effect of pyrrolidinedithiocarbamate on induced NF- κ B

activation correlated with its effect on intercellular adhesion molecule - 1, the expression of which is known to be NF- κ B regulated, expression suggesting changes in cell GSH status not only influences NF- κ B activation but also regulates κ B dependent transcription [80].

Nutritional supplements

Administered GSH *per se* is not effectively transported into cells [81] except in the small intestine [82–85], it is mostly degraded in the extracellular compartment. The degradation products, i.e. the constituent amino acids, may be used as substrates for GSH neosynthesis inside the cell. Two brief



Fig. 2. Inhibition of TNF α induced NF- κ B activation in L6 derived skeletal myoblasts with elevated GSH level. Cells were treated (200 μ M, 4 h) or not with pyrrolidinedithiocarbamate (PDTC) before TNF α treatment. PDTC treatment increased cell GSH level [80]. Cells were treated or not with TNF α for the time intervals indicated. Bars represent densitometry values of the NF- κ B autoradiograph. The specificity of NF- κ B band was verified by cold κ B consensus probe competition (not shown) [80].

rodent studies claimed that exogenous GSH may remarkably increase endurance to physically exercise [86, 87]. Compared to placebo treated controls 0.5, 0.75 and 1 g/kg intraperitoneal doses of GSH increased endurance to swimming by a marked 102.4, 120 and 140.7%, respectively [87]. At a dose 0.25 g/kg, GSH did not affect endurance when injected once but such a dose could significantly increase endurance when injected once a day for seven consecutive days. In another study, oral GSH at dosages 0.25–1 g/kg caused a dose-dependent significant improvement in swim endurance [86]. Both above-mentioned studies employed brief bursts of swimming as the exercise challenge and did not report any biochemical data related to either glutathione metabolism or other indices of oxidative stress. We attempted to clarify the possible mechanism of such beneficial effect of GSH supplementation. An extensive biochemical investigation was necessary before any hypothesis regarding the role of exogenous GSH in endurance enhancement could be formulated. Almost all the evidence supporting the contention that a single bout of exercise may induce oxidative stress have been obtained from studies using exercise types that were long in duration, and mostly running or cycling in nature. Because we aimed to test the efficacy of exogenous GSH in controlling exercise induced oxidative stress, an enduring (~ 2 h) treadmill run protocol was used. Intraperitoneal injection of GSH solution (1 g/kg body weight) resulted in a rapid appearance of GSH in the plasma and was followed by a rapid clearance of the thiol. Following the injection, excess plasma GSH was rapidly oxidized. GSH injection did not influence GSH status of other tissues studied. Following repeated administration of GSH, blood and kidney total glutathione levels were increased. Plasma total glutathione of GSH supplemented animals was rapidly cleared during exhaustive exercise. The GSH administration protocol, as used in this study, did not influence the endurance to exhaustive physical exercise of rats. In another report, we showed that treadmill run to exhaustion is associated with a remarkable increase in immunoreactive Mn-SOD (manganese superoxide dismutase, a mitochondrial protein) in the plasma. Glutathione supplementation (500 mg/kg body weight) marginally suppressed such release of the mitochondrial protein to the plasma [88]. The inability of exogenous GSH to provide added antioxidant protection to tissues may be largely attributed to the poor availability of exogenous GSH to the tissues. In another part of this GSH supplementation study conducted in our laboratory we tested the effect of GSH supplementation on exercise-induced leukocyte margination and neutrophil oxidative burst activity [89]. Exercise-associated leukocyte margination was prevented by GSH supplementation. Peripheral blood neutrophil counts were significantly higher in GSH-supplemented groups compared to the placebo control groups. Also, exercise-induced increase in peripheral blood

neutrophil oxidative burst activity as measured by luminol-enhanced chemiluminescence per volume of blood tended to be higher in the GSH-supplemented group, and lower in the GSH-deficient rats suggesting that high plasma GSH may have augmented exercise dependent neutrophil priming. In these experiments, for the first time it was shown that GSH supplementation can induce neutrophil mobilization and decrease exercise-induced leukocyte margination, and that exogenous and endogenous GSH can regulate exercise-induced priming of neutrophil for oxidative burst response [89].

N-acetyl-L-cysteine (NAC) and α -lipoic acid (LA) are two nutritional supplements that have remarkable ability to increase cell GSH (Fig. 3) [31]. Both of these agents have been found to be safe for human use. After free NAC enters a cell, it is rapidly hydrolyzed to release cysteine, the rate limiting substrate for intracellular GSH synthesis [31]. α -Lipoic acid is also known as thioctic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid or 6,8-thioctic acid [31, 64, 90–93]. At physiological pH, α -lipoic acid is anionic and referred to as lipoate. When treated to cells, lipoate is rapidly reduced to dihydrolipoate (DHLA) and released outside the cell. Members of the pyridine nucleotide-disulfide oxidoreductase family of dimeric flavoenzymes e.g. lipoamide dehydrogenase, thioredoxin reductase, and glutathione reductase reduce intracellular lipoate to dihydrolipoate in the presence of cellular reducing equivalents NADH or NADPH (Fig. 3). Thus, a unique advantage of lipoate is that it is able to utilize cellular reducing equivalents, and thus harnesses the metabolic power of the cell, to continuously regenerate its reductive vicinal dithiol form. Because of such recycling mechanism, the lipoate-dihydrolipoate couple can be continuously maintained in a favorable redox state at the expense of the cell's metabolic power [31, 93]. Low concentrations of lipoate has been shown to increase cellular GSH levels by improving the availability of cysteine inside the cell [91, 94]. Because NADH is rapidly consumed to reduce lipoate to dihydrolipoate, treatment of cells with lipoate decreases the NADH/NAD⁺ ratio. This effect on intracellular reducing equivalent homeostasis is thought to be beneficial in situations such as diabetes and ischemic injury [95]. Because LA can be recycled from its oxidized form to the potent reduced DHLA form by enzymes of the human cell and NAC or its metabolites cannot be regenerated by such mechanism, LA has often been found to be more potent than NAC on a concentration basis [90, 96].

Dihydrolipoate, the reduced form of lipoate, is a potent biologically safe reducing agent and antioxidant [93]. After being enzymatically generated inside the cell, dihydrolipoate rapidly escapes from the cell to the extracellular culture medium [97]. To improve retention in cells, recently we modified the α -lipoic acid (LA) molecule to confer a

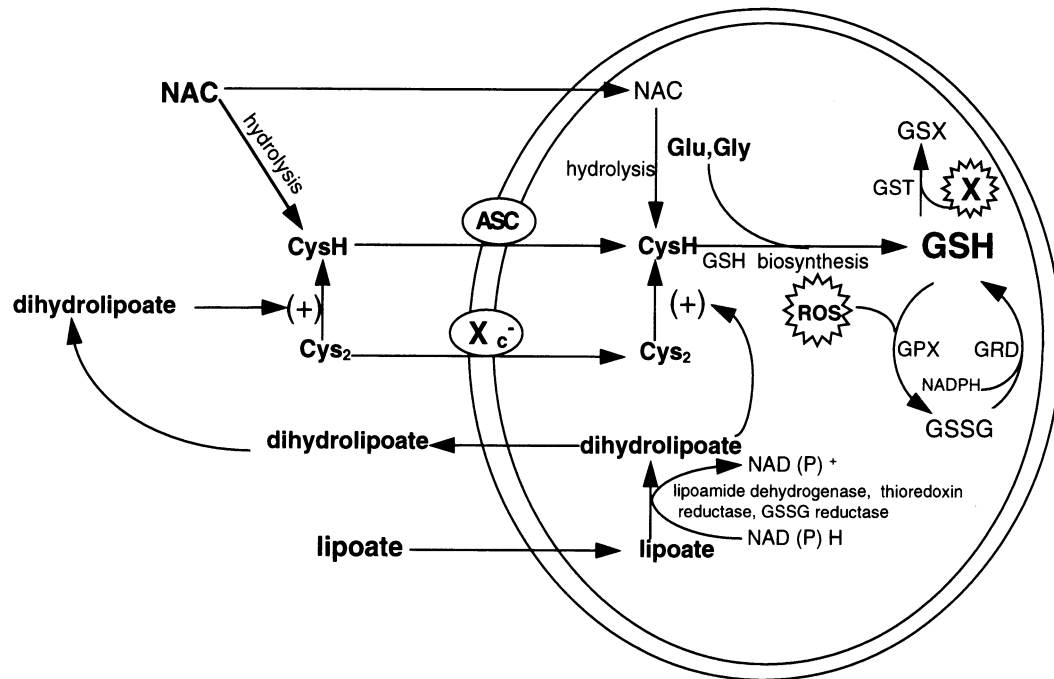


Fig. 3. Improved cysteine supply inside the cell represents an important mechanism by which intracellular GSH may be increased. Hydrolysis of N-acetyl-L-cysteine (NAC) generates cysteine (CysH). Following lipoate supplementation, extracellular dihydrolipoate reduces cystine (Cys₂) outside the cell to cysteine. The cellular uptake mechanism for cysteine by the ASC system is approximately 10 times faster than that for cystine by the X⁻ system. Thus, dihydrolipoate markedly improves cysteine availability within the cell resulting in accelerated GSH synthesis [31]. Glu – glutamine; Gly – glycine; X – electrophilic xenobiotic; GST – GSH S-transferase; GPX – GSH peroxidase; GRD – GSSG reductase; ROS – reactive oxygen species. For more information [31].

positive charge at physiological pH [98]. The protonated form of the new molecule is referred to as LA-Plus (Fig. 4). The uptake of LA-Plus by human T cells was higher compared to that of LA. Several-fold higher amounts of DHLA-Plus, the corresponding reduced form of LA-Plus, was detected in LA-Plus treated cells compared to the amount of DHLA found in cells treated with LA. On a concentration basis, LA-Plus was found to be more biologically potent than LA [98].

The effect of oral N-acetylcysteine supplementation on exercise-associated rapid blood GSH oxidation in the subjects who performed two identical maximal bicycle ergometer exercises three weeks apart has been tested in our laboratory [21]. Before the second maximal exercise test, the men took N-acetylcysteine tablets (200 mg × 4 /day) for two days, and an additional 800 mg in the test morning. In all experiments, blood samples were drawn before, immediately after and 24 h after tests. N-acetylcysteine supplementation increased free radical scavenging capacity of human plasma. Maximal bicycle ergometer test associated rapid blood GSH oxidation was markedly attenuated by N-acetylcysteine supplementation indicating that the treatment spared exercise-associated blood thiol redox status perturbation. In a separate study, trained athletes were orally supplemented with a combination of 1 g GSH and 2 g vitamin C daily for 7

days to test the possible effect of this treatment on exercise-induced blood GSH oxidation [25]. In all five men studied, linearly progressive-intensity treadmill exercise induced blood GSH oxidation. The magnitude of this effect ranged from 34–320% increase in blood GSSG compared to pre-exercise levels. The antioxidant-supplementation protocol was effective in completely protecting against blood GSH oxidation induced by exercise [25].

Lipoic acid is widely used as a food supplement. Almost all of the evidence showing the beneficial effect of lipoic acid on cell GSH has been obtained from *in vitro* studies, however. There is only scanty information regarding whether orally supplemented lipoic acid influences the level of intact lipoate in tissues such as the skeletal muscle and liver. In a recent study we sought to assess the effect of oral supplementation (150 mg/kg, 8 weeks) of lipoic acid on (1) tissue lipoate concentration, GSH levels, and the activities of GSH related enzymes; and (2) exercise induced changes in tissue GSH homeostasis and lipid peroxidation [99]. Lipoic acid supplementation increased the level of lipoate in the red gastrocnemius muscle, and increased total glutathione levels in the liver and blood. Exercise-induced decrease in heart glutathione S-transferase activity was prevented by lipoic acid supplementation. Exhaustive exercise

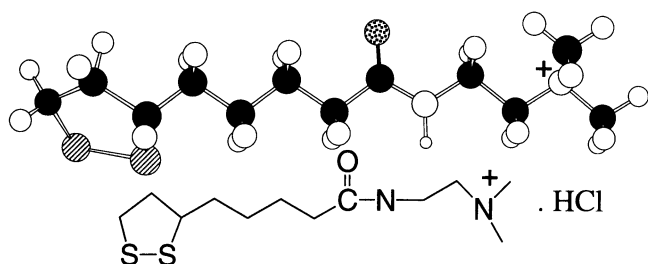


Fig. 4. LA-Plus: A novel analogue of α -lipoic acid with increased biological activity. Chemical name: N,N-dimethyl,N'-2-amidoethyl-lipoate. LA-Plus is positively charged, as shown, at physiological pH. For more information see ref [98].

significantly increased lipid peroxidation metabolite levels in the liver and red gastrocnemius muscle. Lipoic acid supplementation protected against oxidative lipid damage in the heart, liver as well as red gastrocnemius muscle. This study showed that orally supplemented lipoic acid is indeed able to favorably influence tissue antioxidant defenses and counteract oxidative damage at rest and in response to exercise [99].

Acknowledgments

Work supported by research grants from the Finnish Ministry of Education, Juho Vainio Foundation, Helsinki, and in part by NIH grants GM 27345 and DK 50430.

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Mechanisms of beneficial effects of probucol in adriamycin cardiomyopathy

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Abstract

Probucol, a lipid-lowering drug, has been shown to offer protection against adriamycin-induced cardiomyopathy. In order to define the mechanism of this protection, we examined changes in antioxidants and lipid peroxidation in hearts as well as lipids in hearts and plasma from rats treated with either adriamycin or adriamycin and probucol with appropriate controls. Any potential free radical quenching as well as growth inhibitory effects of probucol were also examined using Chinese hamster ovary (CHO) cells in culture. In animal model, adriamycin caused a significant depression in glutathione peroxidase and increased plasma and cardiac lipids as well as lipid peroxidation. Probucol treatment modulated adriamycin-induced cardiomyopathic changes and increased glutathione peroxidase and superoxide dismutase activities. In the presence of adriamycin under hypoxic conditions, formation of adriamycin semiquinone radical was detected by ESR. The cell growth in these cultures was also inhibited by adriamycin in a dose-dependent manner. Probucol had no effect on adriamycin-induced growth inhibition as well as formation of semiquinone radicals. It is proposed that probucol protection against adriamycin cardiomyopathy is mediated by increased antioxidants and lipid-lowering without any effect on free radical production. (*Mol Cell Biochem* **196**: 43–49, 1999)

Key words: semiquinone radical, antioxidants, lipid peroxidation, oxidative stress

Introduction

Adriamycin (also known as doxorubicin), an extremely potent antitumor antibiotic, was discovered in the early 1960's [1]. However, early excitement about its great antitumor potency waned when a serious cardiotoxicity of the drug became obvious [2]. Most of the research has focussed on understanding the mechanisms of antitumor as well as cardiomyopathic effects of adriamycin.

Different mechanisms of antitumor action of adriamycin including free radical induced DNA strand break have been proposed and extensively reviewed [3]. Evidences for [4, 5] and against this proposition [6, 7] have been reported. Other suggested mechanisms of antitumor action are binding of adriamycin-iron complex to DNA [8], intercalation of adriamycin between base pairs in the DNA strands [9], and inhibition of the topoisomerase II [10, 11].

For adriamycin-induced cardiomyopathy, several different mechanisms that have been suggested are: inhibition of nucleic acid and protein synthesis [12]; release of vasoactive amines [13]; changes in adrenergic function [14]; lysosomal alterations [15]; free radical formation and oxidative stress [13, 16–18], reduction in myocardial antioxidants [20, 21] and lipid peroxidation [18, 22]. It appears that oxygen radical injury is involved in most of the above proposed mechanisms.

Using a rat model of adriamycin-induced cardiomyopathy, we have shown that probucol, an antioxidant and lipid-lowering drug, offered complete protection against adriamycin-induced cardiomyopathy without interfering with its antitumor effect [23]. Probucol may provide protection by acting as: (1) a direct antioxidant; (2) a promoter of endogenous antioxidants; (3) a lipid-lowering drug; and/or (4) a radical quencher. The present study on adriamycin cyto-

toxicity was undertaken to examine some of these mechanisms of probucol action.

Materials and methods

In vivo studies

Animal model

Male Sprague-Dawley rats (body weight, 250 ± 10 g) were maintained on normal rat chow. Rats were divided into four groups: CONT (control), ADR (adriamycin treated), PROB (probucol treated) and PROB+ADR (probucol + adriamycin treated). Adriamycin (doxorubicin hydrochloride) was administered intraperitoneally in six equal injections (2.5 mg/kg ADR, each) to animals in ADR and PROB+ADR groups over a period of 2 weeks for a cumulative dose of 15 mg/kg of body weight as described previously [21, 23]. Probucol was given to animals in PROB and PROB+ADR groups in a cumulative dose of 120 mg/kg body weight, in 12 equal, injections (i.p.). PROB treatment in these groups was started two weeks prior to the ADR treatment and was then continued for another two weeks concurrently with ADR, as described before [23]. CONT animals were injected with the vehicle alone (lactose, 75 mg/kg in saline) following the same regimen as ADR.

After the last injections, all animals were monitored for 3 weeks for general appearance, behaviour and mortality. At the end of the 3-week post-treatment period, the animals were sacrificed by decapitation and blood was collected in heparinized tubes. The latter was immediately centrifuged at $1500 \times g$ for 10 min, separating plasma for the assessment of plasma lipids. Atria and other connective tissue from hearts were dissected away and ventricles were used to assess myocardial lipids, antioxidant enzyme activities and lipid peroxidation.

Catalase assay

Ventricles were homogenized in 9 vol of 0.05 M potassium phosphate buffer (pH 7.4) and centrifuged at $40,000 \times g$ for 30 min [24]. Supernatant (50 μ l) was added to the cuvette containing 2.95 ml of 19 mM H_2O_2 in potassium phosphate buffer. The colour was read at 240 nm on a Zeiss spectrophotometer every min for 5 min. Commercially available catalase was used as a standard. Specific activity of the enzyme was expressed as units per milligram tissue protein.

Superoxide dismutase assay

Supernatant ($20,000 \times g$ for 20 min) was assayed for superoxide dismutase (SOD) activity by following the inhibition of pyrogallol autooxidation [25]. Pyrogallol (24 mM) was prepared in 10 mM HCl and kept at $4^\circ C$ before use. Catalase

(30 μ M stock solution) was prepared in an alkaline buffer (pH 9.0). Aliquots of supernatant (150 μ g protein) were added to Tris HCl buffer containing 25 μ l pyrogallol and 10 μ l catalase stock solution. The final volume of 3 ml was made up of the same buffer. Changes in absorbance at 420 nm were recorded at 1-min intervals for 5 min. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol autooxidation with a known SOD activity. This assay was highly reproducible, and the standard curve was linear up to 250 μ g protein with a correlation coefficient of 0.998. Data were expressed as SOD units per milligram protein as compared with the standard.

Glutathione peroxidase assay

Glutathione peroxidase activity was expressed as nanomoles of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized to nicotinamide adenine dinucleotide phosphate (NADP) per min per milligram protein, with a molar extinction coefficient of 6.22×10^6 at 340 nm [26]. The ventricles were homogenized (10% wt/vol) in 0.05 potassium phosphate buffer (pH 7.4). Cytosolic glutathione peroxidase was assayed in a 3 ml cuvette containing 2.0 ml of 75 mM phosphate buffer, pH 7.0. The following solutions were then added: 50 μ l of 60 mM reduced glutathione, 100 μ l glutathione reductase (30 U/ml), 50 μ l of 0.12 M Na_3N , 100 μ l of 15 mM Na_2EDTA , 100 μ l of 3.0 mM NADPH and 100 μ l of cytosolic fraction obtained after centrifugation at $20,000 \times g$ for 25 min. Water was added to make a total volume of 2.9 ml. The reaction was started by the addition of 100 μ l of 7.5 mM H_2O_2 , and the conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm at 1-min intervals for 5 min.

Thiobarbituric acid reactive substances (TBARS)

Myocardial lipid peroxidation was assessed by determining thiobarbituric acid reactive substances using a modified thiobarbituric acid method [27]. The ventricles were homogenized (10% wt/vol) in buffered 0.9% KCl (pH 7.4). The homogenate was incubated for 1 h at $37^\circ C$ in a water bath. A 2-ml aliquot was withdrawn from the incubation mixture and pipetted into an 8 ml Pyrex tube. One millilitre of 40% trichloroacetic acid and 1 ml of 0.2% thiobarbituric acid were promptly added. To minimize peroxidation during the subsequent assay procedure, 0.02% butylated hydroxytoluene was added (final concentration 0.0125) to the thiobarbituric acid reagent mixture [28]. Tube contents were vortexed briefly, boiled for 15 min and cooled in a bucket of ice for 5 min. Two millilitres of 70% trichloroacetic acid was then added to all tubes and the contents were again vortexed briefly. The tubes were allowed to stand for 20 min. This was followed by a centrifugation of the tubes for 20 min at 3500 rpm. The colour was read at 532 nm on a Zeiss spectrophotometer and compared with a known malondialdehyde standard.

Lipid analysis

Plasma triglycerides, total cholesterol, high density lipoproteins and low density lipoproteins were determined enzymatically using kits obtained from Sigma Diagnostics (#352, #352-3, #336) and expressed as mg/dl plasma. For cardiac lipids, ventricles (1 g) were homogenized in 10 ml 0.05 M potassium phosphate buffer (pH 7.4), centrifuged at 40,000 x g for 30 min and supernatants were assayed using the same Sigma kits, and expressed as mg/g tissue.

In vitro studies

Cell culture and growth inhibition assays

Chinese hamster ovary (CHO) cells (type AA8; ATCC CRL-1859) were grown in α -MEM (alpha minimum essential medium) containing 20 mM HEPES, 100 units/ml penicillin G, 100 μ g/ml streptomycin, 10% calf serum (Gibco, iron supplemented and enriched) in an atmosphere of 5% CO₂ and 95% air at 37°C (pH 7.4). Cells in exponential growth were harvested and seeded (typically 2000 cells/well) in 96 well microtiter plates (100 μ l/well) and allowed to attach for 24 h. The drugs were dissolved either in α -MEM (adriamycin), or DMSO (probucol), and were added to give a final volume of 200 μ l/well. When DMSO was used, the final concentration of DMSO did not exceed 0.5% (v/v). This amount of DMSO was shown through the use of appropriate controls to have no significant effect on cell growth. The cell growth was determined by 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay [29]. Briefly, 20 μ l of MTT (2.5 mg/ml in PBS, Dulbecco's phosphate buffered saline) was added to each well and the plate was incubated for a further 4 h. After careful aspiration of the medium, 100 μ l of DMSO was added and the absorbance was read at 490 nm in a Molecular Devices plate reader with reference to the absorbance at 650 nm and appropriate blanks. Typically 6 replicates were measured at each drug concentration. The IC₅₀ values for growth inhibition were obtained from a non-linear least squares fit of the absorbance-drug concentration data to a 3-parameter logistic equation with the absorbance at high adriamycin concentration set equal to 0.

Electron spin resonance (ESR) experiments

To ensure that viable cells were used in the ESR experiments, cell viability was measured on a haemocytometer by the ability of the cells to exclude 0.3% trypan blue. Over the maximum time that the cells were suspended in α -MEM/HEPES (5 h) or PBS (1 h), the cells were greater than 90% viable. Cells used for the ESR studies were concentrated by centrifuging a one ml aliquot of this suspension at 20 x g for 5 min, resuspended in PBS (pH 7.4), and centrifuged again. After the addition of drugs or inhibitors in PBS, the sample for ESR analysis was made up to a final cell density of 10⁸

cells/ml. A 50 μ l aliquot of the cell suspension was injected into an 8 cm length of gas permeable Teflon tubing (1 mm i.d.), folded at both ends and inserted into a quartz ESR tube, and placed in the ESR cavity. Gas permeable ultra thin wall capillary Teflon tubing (1 mm i.d., 0.04 mm wall thickness) was used in the ESR experiments. Pre-purified grade thermostatted (37°C) nitrogen was flowed continuously (200 l/h) over the sample while the spectra were recorded. Recording of the first-derivative ESR spectra was started approximately 25 min after the sample tube was placed in the cavity under nitrogen. A total of 16 spectra (50 sec/scan) were recorded over a period of 20 min and their signals were averaged. The ESR spectra were recorded with a Varian E-12 spectrometer with Nicolet 1180 data system and a Bruker variable temperature controller. The instrument settings were: microwave power 20 mW, modulation frequency 100 kHz, microwave frequency 9.0 GHz, modulation amplitude 1.0 G, time constant 0.1 sec, gain 8000, 1024 data points/scan, magnetic field centred at 3215 G, and a 40 G scan range.

Proteins and statistical analysis

Proteins were determined by the Lowry method [30]. Data were expressed as the mean $\pm$ S.E.M. For a statistical analysis of the data, group means were compared by one-way ANOVA, and Bonferroni's test was used to identify differences between groups. Statistical significance was acceptable to a level of $p < 0.05$.

Results

In vivo studies

General observations

Seven to ten days following completion of ADR treatment, difference in appearance and behaviour of ADR group rats compared to all other groups became obvious. These rats were slower, lethargic, and much lighter. Their fur became scruffy and yellowish. By the end of 3 week post-treatment period, enlarged abdomen due to accumulation of ascitic fluid in the abdominal cavity became a very dominant feature. About 30% of the ADR group rats had to be removed before the end of the study due to deterioration of their condition (in compliance with animal care regulations). PROB + ADR group rats did not have any of the above characteristics of the ADR group, except significant body weight loss.

Antioxidant studies

Ventricles from CONT, ADR, PROB and PROB + ADR groups were examined for endogenous antioxidant activities as well as TBARS (as a measure of lipid peroxidation) (Table 1). ADR treatment caused significant decrease in GSHPx activity as well as increase in the TBARS quantity (Table 1).

Table 1. Effects of probucol on adriamycin-induced changes in antioxidant enzyme activities and lipid peroxidation

Animal Group	GSHPx (nmol/mg protein)	SOD (U/mg protein)	Catalase (U/mg protein)	TBARS (nmol/g heart)
CONT	49.53 ± 2.1	32.85 ± 3.0	27.77 ± 3.0	50.30 ± 4.3
ADR	35.84 ± 2.9*	30.27 ± 4.6	30.99 ± 2.3	93.12 ± 3.8*
PROB	52.48 ± 1.9	43.60 ± 4.0 [†]	32.12 ± 4.5	47.85 ± 4.4
PROB + ADR	60.88 ± 2.6 [†]	52.10 ± 3.8 [†]	29.70 ± 3.0	53.18 ± 2.1

CONT – control; ADR – adriamycin; PROB – probucol; and PROB + ADR – probucol + adriamycin. Data are mean ± S.E.M. of 5–7 animals. * $p < 0.05$ from all other groups; [†] $p < 0.05$ different from CONT and ADR groups.

Probucol treatment in PROB+ADR groups normalized TBARS to control levels and significantly increased GSHPx activity. Both PROB and PROB + ADR groups had significantly higher levels of SOD activity. Catalase did not show any change in any group.

Plasma and cardiac lipids

Levels of plasma and heart triglycerides and total cholesterol, as well as plasma low density lipoproteins (LDL) and high density lipoproteins (HDL) were examined in all four groups (Table 2). ADR treatment resulted in a significant increase in all measured lipid parameters, i.e. plasma triglycerides, total cholesterol, LDL, HDL, and heart triglycerides and total cholesterol (Table 2). In the PROB group, the only significant change was a decrease in plasma HDL and cardiac total cholesterol, compared to control levels (Table 2). PROB treatment normalized plasma triglycerides and total cholesterol as well as cardiac total cholesterol and triglycerides to control values (Table 2) in PROB + ADR group compared to the CONT and ADR groups. Plasma LDL was significantly decreased compared to ADR group, while still higher than in CONT and PROB groups (Table 2).

In vitro studies

Effect of probucol on adriamycin-mediated cell growth

In order to determine if probucol had any effect on adriamycin-mediated growth inhibition, experiments were undertaken to

see if continuous exposure of the cells to probucol alone had any effect on CHO cell growth. The addition of probucol (0.1–100 μ M) to the cells resulted in a small (approximately 15%) enhancement in growth that was significant ($p < 0.02$) for the 0.1–5 μ M probucol concentration range (Fig. 1).

Probucol (0, 50 and 100 μ M) was preincubated with the attached cells for 20 min before adriamycin was added. The cells were allowed to grow in the presence of both drugs for a further 48 h before cell survival was determined using an MTT assay. It was observed to be a highly reproducible feature of the adriamycin cell survival curve that partial stimulation of growth was observed in the low micromolar range. This effect has been noted previously [31, 32]. The IC_{50} values for adriamycin growth inhibition in the presence of 0, 50 and 100 μ M probucol were obtained from a 3-parameter fit of the data to a logistic equation and yielded IC_{50} values of 0.16 ± 0.05 , 0.16 ± 0.05 and 0.14 ± 0.04 μ M, respectively (Fig. 2). Clearly, probucol does not affect the growth inhibitory effects of adriamycin towards CHO cells.

ESR studies

In order to investigate whether the cardioprotective effects of probucol might be occurring through a reduction in the amount of semiquinone free radical known to be formed by adriamycin, ESR experiments were undertaken to see if probucol had any effect on the amount of semiquinone observed. In a control experiment in which 100 μ M probucol was incubated with CHO cells in the absence of adriamycin no significant ESR signal was seen (Fig. 3a). Increasing the

Table 2. Effects of probucol on adriamycin-induced changes in plasma (mg/dL) and heart (mg/g tissue) lipids

Animal Group	PLASMA				HEART	
	Triglycerides	Total Cholesterol	HDL	LDL	Triglycerides	Total Cholesterol
CONT	193.22 ± 14.70	84.03 ± 13.19	30.12 ± 4.20	38.48 ± 4.10	26.26 ± 1.89	14.20 ± 1.01
ADR	703.82 ± 63.22*	348.17 ± 30.60*	51.05 ± 3.43*	145.12 ± 4.48*	47.40 ± 2.95*	19.98 ± 1.48*
PROB	151.44 ± 10.28	69.12 ± 5.50 [†]	14.05 ± 1.12 ^{††}	29.92 ± 3.40	23.16 ± 0.72	9.72 ± 0.84**
PROB + ADR	240.14 ± 18.77	120.12 ± 8.40 [†]	19.26 ± 1.90 ^{††}	74.25 ± 5.12 [†]	28.42 ± 2.80 [†]	12.18 ± 0.78

Data are mean ± S.E.M. of 5–6 animals. *Significantly different from all other groups ($p < 0.05$). **Significantly different from CONT ($p < 0.05$). [†]Significantly different from ADR ($p < 0.05$). ^{††}Significantly different from CONT and ADR ($p < 0.05$).

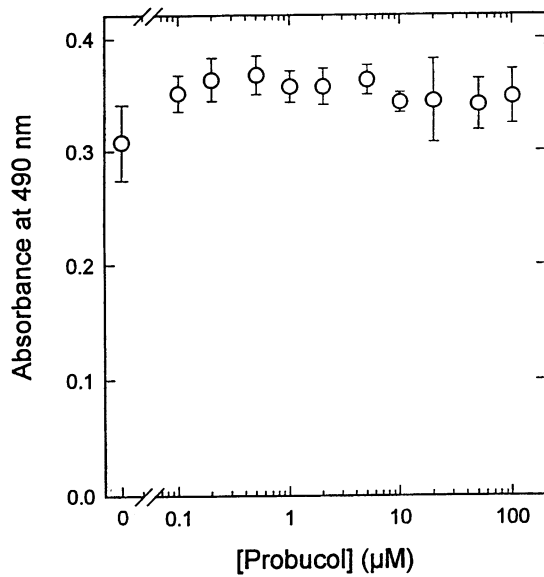


Fig. 1. The effect of probucol on CHO cell growth. Probucol dissolved in DMSO was added to the attached cells. The CHO cells were continuously incubated with probucol for 48 h and then assayed with MTT. The amount of DMSO in the cell medium was 0.5% (v/v) in all experiments. The absorbance values to the left of the axis break on a linear scale are controls that were obtained in the absence of adriamycin. The data points are averages ($\pm$ S.D.) of replicates in 6 wells.

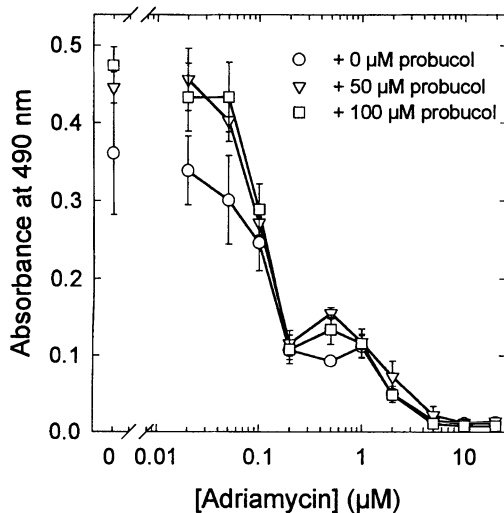


Fig. 2. The effect of probucol on adriamycin growth inhibition with continuous exposure to both drugs. Probucol was preincubated with the cells for 20 min before the addition of adriamycin. The CHO cells were continuously incubated with both drugs for 48 h and then assayed with MTT. Inhibition of growth of CHO cells: (○) doxorubicin alone; (▽) adriamycin + probucol (50 μ M); and (□) adriamycin + probucol (100 μ M). The absorbance values to the left of the axis break on a linear scale are controls that were obtained in the absence of adriamycin. The data points are averages ($\pm$ S.D.) of replicates in 6 wells.

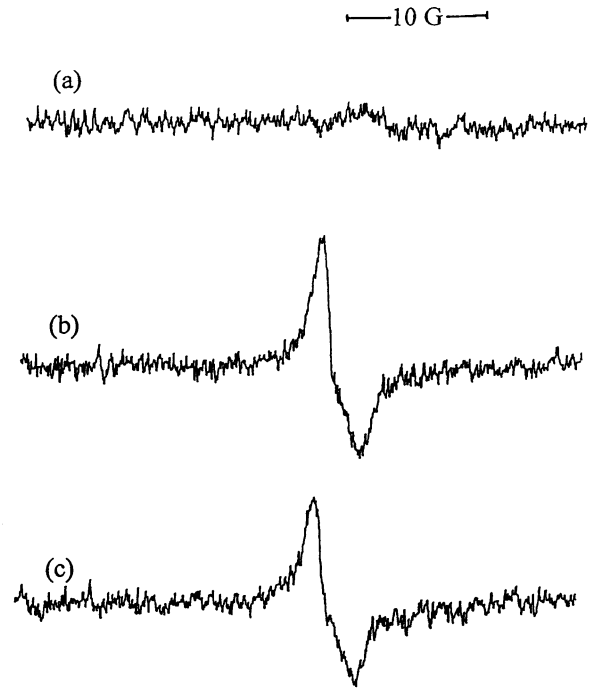


Fig. 3. Semiquinone free radical EPR signals produced in hypoxic Chinese hamster ovary cell suspensions (10^8 cells/ml in PBS at 37°C) in the presence of (a) 100 μ M probucol; (b) 1 mM adriamycin plus 100 μ M probucol; (c) 1 mM adriamycin. A total of 16 spectra were accumulated over 20 min and averaged.

concentration to 300 μ M probucol did not have any new effect (data not shown). With adriamycin, an asymmetric ESR spectrum with a ΔH_{pp} (peak-to-peak line width) of 3.2 G was seen (Fig. 3c), as has also been reported before [33]. This compares to a previously measured ΔH_{pp} of 3.0 G with CHO cells under the same conditions [33]. These values are similar to previously reported ΔH_{pp} of 4.5 G [34] and 3.25 G [35]. Addition of 100 μ M probucol to the cell suspension (Fig. 3b) had no effect on the amount of adriamycin-induced semiquinone (Fig. 3c).

Discussion

Adriamycin cardiomyopathy is a very well documented phenomenon in humans [2, 36, 37], as well as in different animal models [21, 22, 38, 39]. For a number of years, physicians followed the idea that adriamycin cardiomyopathy is a dose-dependent phenomenon and the drug dosage should be limited to minimize the cardiotoxic effects. However, in the last few years, it has become obvious that cardiomyopathy could develop even if a low dose of adriamycin was given, and a therapy had been completed many years ago [40]. The combination therapy regimen using probucol, an antioxidant

and a lipid-lowering drug, and adriamycin successfully prevented development of adriamycin cardiomyopathy. Furthermore, probucol was found to promote endogenous antioxidants. These findings are in agreement with previous studies [21].

Adriamycin treatment was shown to induce massive hyperlipidemia and probucol had a lipid-lowering effect. Adriamycin is a nephrotoxic drug as well, and it has been identified as a cause of glomerulonephritis that leads to nephrotic syndrome [41]. One of the hallmarks of nephrotic syndrome is hyperlipidemia, which has been seen in this study and has also been described in previous reports [41–43]. Hyperlipidemia appears to affect heart function adversely, probably through increased concentrations of free fatty acids which could decrease cardiac contractility [44]. It appears that lipid-lowering also plays some role in the observed protection by probucol. Thus, a complete protection by probucol may be the result of an unique combination of its antioxidant and lipid-lowering properties.

Adriamycin caused a decrease in the activity of endogenous antioxidant GSHPx and caused an increase in lipid peroxidation, measured by TBARS production. These findings confirm previous reports by us [21, 23] as well as by others [45, 46]. Probuco treatment significantly increased SOD and GSHPx levels. Hence, probucol acted as a promoter of endogenous antioxidants [21] thus improving antioxidant reserve, which is important for maintenance of normal cardiac structure and function [47]. Reduction in lipid peroxidation by probucol further supports the concept of a decrease in oxidative stress.

It is well established that adriamycin generates free radicals [15, 16, 33] which are toxic to many different tissues, including myocardium [19]. In this study, formation of adriamycin semiquinone free radical was demonstrated. The asymmetry in the EPR spectrum seen in Figs 3b and 3c is possibly due to an immobilized semiquinone free radical. The semiquinone radical may be associated with the cell, because of its size it would be expected to rotate less freely than a semiquinone radical free in solution. The adriamycin semiquinone free radical produced in solution from the xanthine oxidase-hypoxanthine system displays no such asymmetry [33]. The large majority of the adriamycin semiquinone is located extracellularly as it was shown previously [33] that the cellular impermeant paramagnetic line-broadening agent chromium (III) oxalate completely eliminates the adriamycin semiquinone EPR signal under the conditions used in this study. Probuco phenoxyl radical, which has a characteristic EPR spectrum [48], was not produced under our experimental conditions. The probucol phenoxyl radical was also not observed under normoxic conditions (data not shown) in CHO cell suspensions. Formation of semiquinone free radical species was not modulated by probucol thus highlighting an important point that probucol neither produced nor quenched radicals.

Probuco in low concentrations slightly promoted cell growth. However, the drug had no effect on the adriamycin-mediated inhibition of cell growth in culture. In an earlier study, we reported that probucol did not interfere with the antitumor effect of adriamycin in tumor bearing mice [23]. These *in vivo* as well as *in vitro* studies show that cardiomyopathic effects of adriamycin can be dissociated from its growth inhibitory effects as has also been suggested before [49].

In conclusion, protection by probucol seen in this study is most likely a combination of its ability to act as an antioxidant, its positive influence on endogenous antioxidants and its lipid-lowering properties. Data in the present study also show a lack of the involvement of any direct radical scavenging effect of probucol.

Acknowledgements

Supported in part by the Natural Sciences and Engineering Research Council of Canada grants to B. Hasinoff, and Manitoba Heart and Stroke Foundation grant to P.K. Singal.

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Myocardial oxidative stress changes during compensated right heart failure in rats

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Abstract

The suggested role of oxidative stress in the pathogenesis of heart failure is largely based on utilizing left heart failure models. The present study on rats evaluated changes in antioxidants as well as oxidative stress in relation to hemodynamic function subsequent to the right heart failure induced by monocrotaline (50 mg/kg, i.p.). During the post-injection period, monocrotaline (MCT)-treated rats demonstrated a persistent growth depression. Two to three weeks after the injection, MCT-treated rats showed signs of fatigue, peripheral cyanosis and dyspnea. In these rats, right heart hypertrophy was confirmed by a significant increase in right ventricular weight as well as right ventricle to body weight ratio. In MCT-treated rats, there was also a significant increase in right ventricular systolic as well as end diastolic pressures. No change in lung and liver wet/dry weight ratios between MCT-treated and control animals was observed. Based on the hemodynamic data as well as other clinical observations, the functional stage achieved was compensated heart failure. Myocardial antioxidant enzymes, catalase, glutathione peroxidase and superoxide dismutase, in the MCT-treated rats were not different compared to control rats. Vitamin E levels were significantly depressed in the RV and there was no change in retinol levels. There was a significant increase in lipid hydroperoxide concentrations in MCT-treated rats as compared to the control group. These data provide evidence that right heart failure is associated with an increase in oxidative stress. (*Mol Cell Biochem* **196**: 51–57, 1999)

Key words: vitamin E, antioxidant enzymes, retinol, lipid hydroperoxides

Introduction

In spite of the remarkable decline in the deaths from cardiovascular disease over the past 30 years, both the morbidity and mortality from congestive heart failure (CHF) have shown a steady increase. There have been considerable advances in our understanding of the functional alterations associated with the early adaptive phases of myocardial hypertrophy and the terminal stages of heart failure. However, the precise mechanisms involved in the evolution of cardiac dysfunction and CHF remain unknown. In spite of major advances, none of the available therapeutic interventions have substantially improved the long term survival of patients with CHF. Treatment of right heart failure has yielded especially disappointing results, except when it is

caused by left heart failure, in which case therapy can be effective. Although 14–34% of heart failure cases are due to development of right heart failure, the mechanisms involved in its development are still not well understood. Since many of the biochemical and structural changes observed during left heart failure resemble the ones seen in right heart failure [1, 2], it is possible that the mechanisms involved in the pathogenesis of the right heart failure are similar to the ones found in the left heart failure.

Many laboratories, including ours, have now established that oxidative stress is an important contributor to the evolution of decompensated myocardium [3–6]. However, in almost all of these studies the left heart has been the subject of investigation. Thus available data, with respect to the role of oxidative stress in right heart failure, is scarce. In the

present study, we examined oxidative stress changes during right heart failure.

Materials and methods

Male Sprague-Dawley rats weighing 110 ± 8 g received a single intraperitoneal injection of monocrotaline (MCT, 50 mg/kg body weight) in 1 N HCl buffered to pH 7.0 with 1 M NaOH. Control animals received the same volume of a 0.9% saline solution per kg body weight. Animals were housed, one in each cage, to allow measurement of individual food and water intake. Treated as well as control animals were observed every other day for up to 3 weeks for their general appearance, body weight, food and water intake. Animals began to show early signs of heart failure including dyspnea between 2–3 weeks. Thus further studies were done at 3 weeks post-treatment duration. All of the animal treatment procedures were carried out with approval from, and according to, the regulations of the Canadian Council on Animal Care (CCAC) as monitored by the Animal Care Committee of the University of Manitoba.

Hemodynamic measurements

Three weeks after MCT injection, the animals were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) given intraperitoneally. When the rats no longer responded to external stimuli, the right jugular vein was exposed and a miniature pressure transducer-tip catheter was inserted through a small incision in the vein and advanced into the right atria and the ventricle. Right ventricular systolic pressure (RVSP) and end diastolic pressure (RVEDP) were recorded using axotape software (Axon Instruments, Inc., Foster City, CA, USA). Following the hemodynamic assessment, animals were killed and the heart, lung and liver were removed for further studies. Hearts were immediately washed in 0.033 M Na_2HPO_4 /0.9% KCl buffer for further analysis.

Tissue weights

The atria and other adhering tissue were removed and the right ventricle was separated from the left ventricle and septum. Weights for each portion were recorded and the right ventricles were stored at -75°C until further analysis. Samples of lung and liver tissue were weighed and cut into smaller pieces for drying in an oven at 55°C until a constant weight was reached. Wet to dry weight ratios were calculated to assess the degree of lung and liver congestion.

Biochemical assays

Antioxidants

Both enzymatic and non-enzymatic antioxidants were measured in the right ventricles of control and MCT-treated rats, as follows:

Enzymatic antioxidants

Catalase. Right ventricle was homogenized in 10 vol of 0.5 mmol/L potassium phosphate buffer (pH 7.4) and centrifuged at $40\,000 \times g$ for 45 min. Supernatant (50 μl) was added to a cuvette containing 2.95 ml of 19 mmol/L H_2O_2 solution prepared in potassium phosphate buffer [7]. Absorbance change was monitored at 240 nm on a Zeiss spectrophotometer every minute for 5 min. Commercially available catalase was used as standard. Specific activity of the enzyme was expressed as units per mg of protein.

Glutathione peroxidase (GSHPx). GSHPx activity in the right ventricle of control and MCT-treated rats was expressed as nanomoles of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized to nicotinamide adenine dinucleotide phosphate (NADP) per min per mg of protein, with a molar extinction coefficient for NADPH at 340 nm of 6.22×10^6 [8]. Cytosolic GSHPx was assayed in a 3 ml cuvette containing 2.0 ml of 75 mmol/L phosphate buffer (pH 7.0). The following solutions were then added: 50 μl of 60 mmol/L glutathione, 100 μl glutathione reductase solution (30 U/ml), 50 μl of 0.12 mol/L NaN_3 , 100 μl of 15 mmol/L Na_2EDTA , 100 μl of 3.0 mmol/L NADPH, and 100 μl of cytosolic fraction obtained after centrifugation at $20\,000 \times g$ for 45 min. Water was added to make a total volume of 2.9 ml. The reaction was started by the addition of 100 μl of 7.5 mmol/L H_2O_2 . Conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm at 1-min intervals for 5 min.

Superoxide Dismutase (SOD). Supernatant ($20\,000 \times g$ for 45 min) was assayed for superoxide dismutase (SOD) activity by following the inhibition of pyrogallol autooxidation [9]. Pyrogallol (24 mmol/L) was prepared in 10 mmol/L HCl and kept at 4°C before use. Catalase (30 $\mu\text{mol/L}$ stock solution) was prepared in an alkaline buffer (pH 9.0). Aliquots of supernatant (150 μg protein) were added to Tris-HCl buffer containing 25 μl pyrogallol and 10 μl catalase. The final volume of 3 ml was made up with the same buffer. Changes in absorbance at 420 nm were recorded at 1-min intervals for 5 min. SOD activity was determined from a standard curve of percent inhibition of pyrogallol autooxidation with commercially available SOD. The standard curve was linear up to 250 μg protein with a correlation coefficient of 0.990. Data was expressed as SOD units per mg protein compared with the standard.

Non-enzymatic antioxidants

The content of the lipid soluble non-enzymatic antioxidants tocopherol and retinol were measured in the right ventricles of control and MCT-treated rats using a modification of the extraction procedure and reverse phase HPLC detection method described earlier [10]. Briefly, a known amount of the myocardial tissue was homogenized in 20 vol of ice-cold, double-distilled water. Proteins in the homogenate were then precipitated by adding an equal volume of ice-cold ethanol containing 60 µg/ml tocopherol acetate as an internal recovery standard were added to each sample. After thorough mixing, 2 ml of ethyl acetate:hexane (3:2 v/v) was added to each tube and the tubes were again thoroughly vortexed and allowed to stand on ice for 10 min, shielded from light. The two phases were separated by centrifugation at $2500 \times g$ for 3 min and a 1 ml aliquot of the top ethyl acetate:hexane layer was retrieved. The aliquot was dried under vacuum in a rotary evaporator to complete dryness, and the remaining oily residue was reconstituted in 70 µl of HPLC mobile phase before injecting directly into the HPLC system. Peaks for each sample were corrected for extraction efficiency using the tocopherol acetate peak recovered in the sample against the peak obtained by injecting tocopherol acetate solution directly. Tocopherol and retinol concentrations in each sample were extrapolated from standard curves (tocopherol = 400–1600 ng, and retinol 10–40 ng in the 20 µl HPLC loop). The HPLC mobile phase contained 70:20:10 (v/v/v) acetonitrile: dichloromethane: methanol and was delivered at 1 ml/min through an Adsorbosphere HS C 18 (250 × 4.6 mm, 5 µm pore size) preceded by a 15 mm guard column with the same packing material. Ultraviolet detection of peaks was accomplished by monitoring at 325 nm until 7 min for retinol and then switching to 292 nm for detection of tocopherol. Total run time was 30 min, with typical retention times for retinol and tocopherol of 4.5 and 10.9 min, respectively.

Lipid hydroperoxides

Lipid hydroperoxides in the right ventricles of control and MCT-treated rats were assayed using a commercial kit that specifically detects lipid hydroperoxides (LPO-CC assay, Kamiya Biomed Co., Seattle, WA, USA). This kit uses a derivative of methylene blue (10-N-methylcarbamoyl- 3,7 dimethylamino-10 H phenothiazine) which is specifically cleaved by lipid hydroperoxides to yield methylene blue dye, which can be quantified spectrophotometrically at 675 nm and compared to standard curve based on the same reaction with cumene hydroperoxide.

Statistical analysis

Data from control and MCT groups were compared using Student's *t*-test. When data were not normally distributed or

had a non-homogenous variance, means were compared using the Mann-Whitney rank sum test. Significance for both types of data analysis was accepted at the $p < 0.05$ level.

Results

Body weight and general appearance

Four days after the administration of MCT, the rats showed a general loss of appetite. Water consumption by these animals was also decreased. This condition of lower food and water intake was seen throughout the post-injection period. Although animals in both control and MCT groups showed body weight gain, the MCT-treated rats gained significantly less than the control rats (Fig. 1). The difference became statistically significant ($p < 0.05$) four days after injection. The weight difference continued to increase throughout the post-treatment duration and at the end of 3 weeks, weight gain in the MCT-treated rats was about 20% less than control rats. Two weeks after the treatment, MCT-treated rats showed signs of fatigue and cyanosis of limbs and ear lobes. At three weeks after the treatment, MCT-treated rats showed signs of tachypnea and dyspnea.

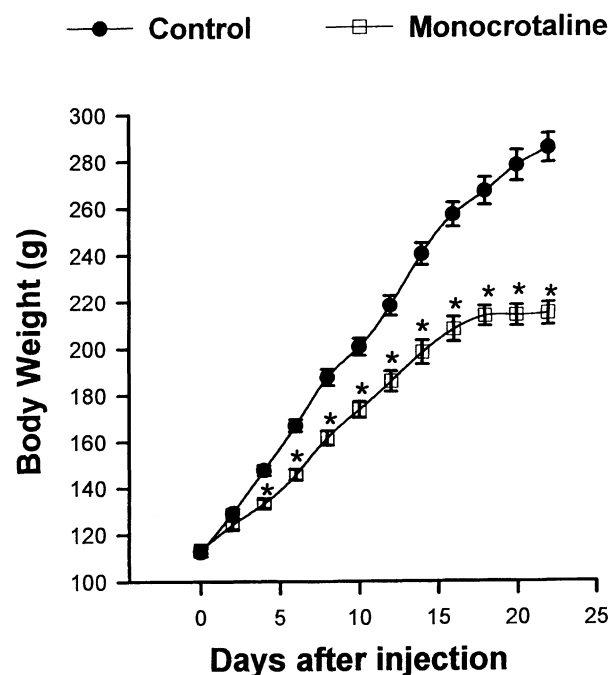


Fig. 1. Body weight gain in rats after monocrotaline (n = 25) or vehicle (n = 15) injection. Values are mean $\pm$ S.E. *Significantly different ($p < 0.05$) from the control.

Tissue weights

In order to assess the extent of heart hypertrophy developed by the rats injected with MCT, right and left ventricular weight as well as ventricle to body weight ratios were determined (Table 1). Right ventricular weight as well as right ventricle to body weight ratio were significantly higher in the MCT rats three weeks after MCT treatment. Left ventricular weight in the MCT group also declined but when these data were normalized for body weight, there was also an increase in the left ventricular to body weight ratio.

In order to estimate the extent of heart failure in the MCT-treated rats, wet to dry weight ratios of lung and liver were also determined (Table 2). Compared to control values, neither the lung nor the liver showed any changes in their wet to dry weight ratio.

Hemodynamics

After catheterization, a few minutes of stabilization were allowed prior to recording of the right ventricular pressures (Table 3). In MCT-treated rats, right ventricular systolic pressure (RVSP) was significantly increased compared to the control. A significant increase was also found in the right ventricular end diastolic pressure (RVEDP) of the MCT-treated rats compared to the controls.

Myocardial antioxidants and oxidative stress

Enzymatic antioxidants

Antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase were examined in MCT-treated and control rats and these data are shown in Table 4. At 3 weeks post-treatment, these enzyme activities in the MCT group were not different from the respective values in controls.

Non-enzymatic antioxidants

Vitamin E content was analyzed in the RV of both groups by quantitating α -tocopherol using an HPLC method. These data are shown in Fig. 2. Vitamin E levels in the right ventricle of rats from the MCT-treated rats were significantly lower than the control group. Retinol, the free alcohol form of vitamin A,

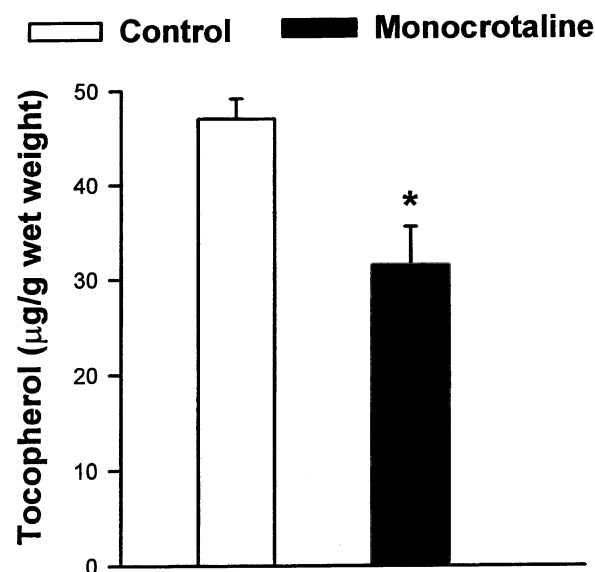


Fig. 2. Tocopherol concentration in right ventricle of control (n = 10) and monocrotaline (n = 15) treated rats. *Significantly different from the control ($p < 0.05$) (Student's *t*-test).

was not different in the MCT-treated animals as compared to the controls (Fig. 3).

Lipid peroxidation

In order to assess the levels of oxidative stress during heart failure, lipid hydroperoxide formation was determined. Lipid hydroperoxide content was significantly higher ($p < 0.05$) in the right ventricle of MCT-treated rats compared to the control (Fig. 4).

Discussion

Different models of right ventricular hypertrophy (RVH) have been created and characterized including the use of hypobaric chambers [11, 12], by performing right ventriculotomy and maintaining a pulmonary artery band [13, 14], tricuspid avulsion and progressive pulmonary artery constriction [15]. Recently, the pyrrolizidine alkaloid monocrotaline (MCT) has successfully been used to produce pulmonary hypertension

Table 1. Effects of monocrotaline on ventricular weights and ventricular to body weight ratios

Animal group	N	Right ventricle (mg)	Left ventricle (mg)	Right ventricle/body wt $\times 10^{-3}$	Left ventricle/body wt $\times 10^{-3}$
Control	15	288 $\pm$ 8.0	752 $\pm$ 30.7	0.707 $\pm$ 0.022	2.25 $\pm$ 0.07
Monocrotaline	25	374 $\pm$ 1.6*	614 $\pm$ 25.9*	1.824 $\pm$ 0.067*	3.22 $\pm$ 0.15*

Data are presented as mean $\pm$ S.E. Statistically different ($p < 0.05$) from the control group.

Table 2. Effects of monocrotaline on wet to dry weight ratio in lung and liver

Animal group	N	Lung	Liver
Control	12	4.66 ± 0.11	3.14 ± 0.08
Monocrotaline	14	4.04 ± 0.29	3.22 ± 0.14

Data are presented as mean ± S.E.

Table 3. Right ventricular pressure changes in rats three weeks after the monocrotaline injection

Animal group	N	Right ventricular pressure (mmHg)	
		Systolic	Diastolic
Control	23	30.6 ± 2.6	2.1 ± 1.5
Monocrotaline	10	50.0 ± 5.1	8.9 ± 2.0*

Data are presented as mean ± S.E. *Significantly different from control group ($p < 0.05$). Student's *t*-test followed by Mann-Whitney rank sum test.

Table 4. Antioxidant enzyme activities in control and monocrotaline-treated rats

Animal group	Catalase (U/mg prot)	GSHPx (nmol/mg prot)	SOD (U/mg prot)
Control	28.85 ± 2.14	31.94 ± 3.88	39.77 ± 4.93
Monocrotaline	30.79 ± 2.54	35.19 ± 3.07	45.59 ± 7.77

Data are presented as mean ± S.E of control ($n = 10$) and monocrotaline ($n = 20$) treated groups.

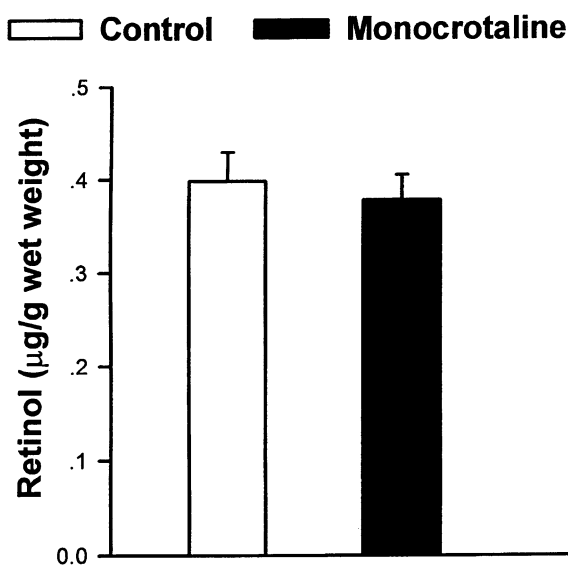


Fig. 3. Retinol concentration in right ventricle of control ($n = 6$) and monocrotaline ($n = 6$) treated rats.

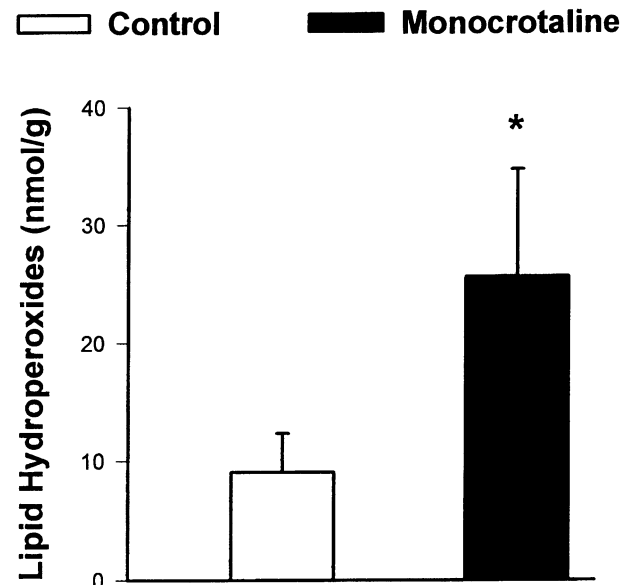


Fig. 4. Concentration of lipid hydroperoxides in right ventricle of control ($n = 4$) and monocrotaline ($n = 7$) treated rats. *Significantly different from the control ($p < 0.05$).

followed by right ventricle pressure overload, right ventricular hypertrophy and eventually CHF. This animal model is ideal because no surgical interventions are needed and right heart hypertrophy and right heart failure develop relatively quickly and reproducibly [16]. In addition, there appears to be intrinsic control in the unaffected left side of the heart [17].

In the present study, animals receiving MCT demonstrated a persistent growth depression as has also been shown by others [1, 18]. Differences in growth became apparent within days of MCT injection, and this difference significantly increased during the post-treatment period. This effect is probably due to gastrointestinal and/or hepatic disturbances induced by MCT intoxication. After MCT is bioactivated in the liver, a reactive intermediate, dehydromonocrotaline (MCTP), is produced which affects several tissues [19, 20].

Three weeks after MCT injection, RVSP and RVEDP were elevated, suggesting a compensatory phase during which cardiac output is maintained by pumping blood with an elevated pressure through the narrowed pulmonary circulation. In this regard, it has been reported that when MCTP is transported to the lung, it induces pulmonary vascular syndrome characterized by elevated pulmonary artery pressure [16, 21]. Such an increase in afterload may explain right ventricle hypertrophy indicated by a significant increase in right ventricular weight as well as right ventricular weight to body ratio in the present study. An increase in RVEDP is a hallmark of ventricular failure indicating that the ventricle functioning on the Frank-Starling curve had shifted to the

right. Thus increase in RVSP may be partly afforded by an increase in RVEDP as well as by RV hypertrophy.

In left heart failure, once heart failure is established there is edema of the lungs followed by edema of the liver manifested by an increase in wet to dry weight ratio [22, 23]. The same general phenomenon appears in right ventricular failure [1, 14] except that liver congestion appears before lung congestion. In this study, neither lung nor liver wet to dry weight ratios were different from the controls. It is likely that the hemodynamic compensations may not have been present for a long enough duration to influence these secondary tissues. Thus, increased RVSP as well as increased RVEDP without any change in lung and liver weight ratios suggest that the functional stage achieved in the present study was 'compensated heart failure.'

During the development of hypertrophy and heart failure there is a biphasic response to the change in oxidative stress. Left ventricular hypertrophy without heart failure is accompanied by a significant increase in antioxidant enzyme activities and reduction in lipid peroxidation, which suggests that this phase has a better protection against oxidative stress [5, 24]. In contrast, during the failure stage, there is a significant increase in oxidative stress and a decrease in antioxidant enzyme activities [4, 23, 25]. Results from the present study show that during compensated right heart failure, the activity of the enzymatic antioxidants was not different from the controls. It is possible that in the present study, the animals had gone past the early hypertrophic stage and thus the antioxidant enzymes changes were on a decline phase being recorded near the control values.

Previous studies of the non-enzymatic antioxidants in left heart failure, have shown that depleted concentrations of vitamin E and vitamin A are often correlated with increased lipid peroxidation [4, 26]. In this study of compensated heart failure, there was no change in retinol concentrations, but there was a significant decline in tocopherol concentrations in the RV of MCT-treated rats compared to the control. This decline in vitamin E indicates that oxidative stress is present during compensated RHF. Vitamin E is known to be a potent antioxidant and its presence in biological membranes represents a major defense system against membrane lipid peroxidation [3]. The decline in vitamin E content at this particular stage suggests that vitamin E is a more sensitive antioxidant compared to the other antioxidants measured in this study. Furthermore, lipid hydroperoxide concentrations, measured as an index of overall oxidative stress, were increased during the compensated right heart failure.

In conclusion, heart failure has been shown to be associated with a decrease in myocardial antioxidant enzymes [23, 25], a decrease in non-enzymatic antioxidants [4], and an increase in oxidative stress. In the present study, only 2 out of these 3 alterations were noted. This is likely due to the fact that the animals were in compensated heart failure stage,

a step before the establishment of overt heart failure. In order to resolve this issue, a time course study of these antioxidant changes in MCT-treated animals is needed. In particular, the decompensated phase of right heart failure, where further increases in lipid hydroperoxides production and antioxidant depletion are likely to take place, requires an in-depth analysis.

Acknowledgements

This study was supported by the Medical Research Council Group Grant in Experimental Cardiology (PKS). Dr. Palace is a post-doctoral fellow of the Manitoba Health Research Council. Ms. Pichardo was supported by an international fellowship awarded by the Government of Canada and Dr. Farahmand by a University of Manitoba Studentship Award.

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Reactive oxygen species function as second messenger during ischemic preconditioning of heart

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Abstract

Ischemic preconditioning has been shown to trigger a signaling pathway by potentiating tyrosine kinase phosphorylation leading to the activation of p38 MAP kinase and MAPKAP kinase 2. Recently, the nuclear transcription factor, NFκB, was found to play a role in the signaling process. Since NFκB is a target of oxygen free radicals, we hypothesized that reactive oxygen species might play a role in the signaling process. To test this hypothesis, isolated rat hearts were perfused in the absence or presence of either dimethyl thiourea (DMTU), a OH· radical scavenger, or SN 50 peptide, a NFκB blocker. Hearts were then subjected to ischemic preconditioning by four repeated episodes of 5 min ischemia each followed by 10 min reperfusion. All hearts were then made globally ischemic for 30 min followed by 2 h of reperfusion. The results of our study demonstrated enhanced tyrosine kinase phosphorylation during ischemic preconditioning which was blocked by DMTU. DMTU also inhibited preconditioning mediated increased phosphorylation of p38 MAP kinase and MAPKAP kinase 2 activity. However, DMTU had no effect on the translocation and activation of protein kinase C (PKC) resulting from preconditioning. Preconditioning reduced myocardial infarct size as expected. This cardioprotective effect of preconditioning was abolished by both DMTU and SN 50. Preconditioning resulted in the nuclear translocation and activation of NFκB. Increased NFκB binding was blocked by both DMTU and SN 50. The results of this study demonstrate that reactive oxygen species play a crucial role in signal transduction mediated by preconditioning. This signaling process appears to be potentiated by tyrosine kinase phosphorylation resulting in the activation of p38 MAP kinase and MAPKAP kinase 2 leading to the activation of NFκB suggesting a role of oxygen free radicals as second messenger. Free radical signaling seems to be independent of PKC although PKC is activated during preconditioning process suggesting the role of two separate signaling pathways in ischemic preconditioning. (*Mol Cell Biochem* **196**: 59–67, 1999)

Key words: oxygen free radicals, signal transduction, second messenger, NFκB, MAP kinases, MAPKAP kinase 2, protein kinase C, tyrosine kinase

Introduction

The heart possesses remarkable ability to adapt itself against any stressful situation by increasing resistance to the adverse consequences. Stress comprises the foundation of many degenerative heart diseases including atherosclerosis, spasm, thrombosis, cardiomyopathy, and congestive heart failure. The idea that excessive stress can play a key role in the pathogenesis of ischemic heart disease means that designing methods for the prevention of stress-induced myocardial injury is a fundamental process in the prophylaxis of such diseases. Indeed,

creating a stress reactions by repeated ischemia and reperfusion or subjecting the hearts to heat or oxidative stress can enable the heart to meet the future stress challenge. Repeated stress exposures adapt the heart to withstand more severe stress reactions probably by upregulating the cellular defence and direct accumulation of intracellular mediators which presumably constitute the material basis of increased adaptation to stress. Thus, the powerful cardioprotective effect of adaptation is likely to originate at the cellular and molecular levels.

Ischemic preconditioning is the manifestation of the earlier stress response that occurs during repeated episodes

of brief ischemia and reperfusion, and can render the myocardium more tolerant to a subsequent potential lethal ischemic injury [1]. This transient adaptive response has been demonstrated to be associated with decreased reperfusion-induced arrhythmias [2], increased recovery of postischemic contractile functions [3, 4], and reduction of infarct size [5, 6]. The adaptive protection has been found to be mediated by gene expression and their transcriptional regulation [7, 8].

Our laboratory demonstrated that ischemic preconditioning triggers a signaling pathway by potentiating tyrosine kinase phosphorylation [9, 10]. The signal transduction involves phospholipase D which subsequently transmits the signal via the activation of MAP kinases. Our results clearly indicated a role of tyrosine kinase, because inhibition of tyrosine kinase phosphorylation by genistein almost completely blocked the activation of protein kinase C, MAP kinases and MAPKAP kinase 2. Subsequently, we were able to identify p38 MAP kinase as one of the potential targets for tyrosine kinase phosphorylation [11].

Recently, oxygen-derived free radicals have been implicated in transmembrane signaling process [12]. In this study, the authors provided evidence that a tyrosine kinase inhibitor, herbimycin A and a free radical scavenger, N-acetyl-cysteine, inhibit free radical induced activation of NFκB indicating that activation triggered by reactive oxygen species is dependent on tyrosine kinase activity. A large number of studies from different laboratories including our own showed the induction of the expression of antioxidant genes during the preconditioning [13]. We recently demonstrated nuclear translocation and activation of NFκB in response to preconditioning [14]. Increased binding of NFκB was found to be dependent on both tyrosine kinase and p38 MAP kinase.

The present study was undertaken to further investigate the free radical signaling mechanism of preconditioning. Our results demonstrated that beneficial effects of preconditioning was abolished by preperfusing the hearts with dimethyl thiourea (DMTU), a hydroxyl radical (OH·) scavenger. DMTU also reduced the activation of NFκB suggesting that free radical signaling plays a crucial role in ischemic preconditioning.

Materials and methods

Isolated perfused heart preparation

Sprague Dawley rats weighing about 300 gm were anesthetized with pentobarbital (80 mg/kg, i.p.). After intravenous administration of heparin (500 IU/kg), the chests were opened, the hearts were rapidly excised and mounted on a nonrecirculating Langendorff perfusion apparatus [15]. Retrograde perfusion was established at a pressure of 100 cm H₂O with an oxygenated normothermic Krebs-Henseleit bicarbonate (KHB) buffer with the following ion con-

centrations (in mM): 118.0 NaCl, 24.0 NaHCO₃, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.7 CaCl₂, and 10.0 glucose. The KHB buffer had been previously equilibrated with 95% O₂/5% CO₂, pH 7.4 at 37°C. Hearts were randomly divided into four groups. Isolated hearts were perfused with : KHB buffer for 75 min (Group I); KHB buffer for 15 min in the absence (Group II) or presence of 10 mM DMTU (Group III) or 18 μM SN 50 peptide (Group IV). The hearts were then subjected to ischemic stress adaptation by repeated ischemia and reperfusion by inducing global ischemia for 5 min followed by 10 min of reperfusion, repeating the process four times as described previously [3]. All hearts were subjected to 30 min of ischemia followed by 2 h of reperfusion. A schematic diagram of the protocol is shown in Fig. 1. For NFκB binding activity, left ventricles from the control and experimental hearts were kept frozen at liquid nitrogen temperature. Myocardial infarct size, CK release and MDA formation were determined in the hearts as described below.

Evaluation of myocardial infarct size

For infarct size determination, the hearts were frozen and then sliced perpendicularly to the long axis from apex to base in 0.8 mm thick sections. Sections were then incubated for 20 min at 37°C in a 1% triphenyltetrazolium chloride in phosphate buffer (Na₂HPO₄ 88 mM, NaH₂PO₄ 1.8 mM). Thin heart cross sections were digitally imaged by using a McIntosh 7100/80 computer and a BP Scan Jet 5p scanner (Hewlett Packard) [17]. The image was adjusted with Abode Photoshop 4.0 (Abode System, Inc.). The areas of infarct (tetrazolium negative) and risk (tetrazolium positive) were determined by using NIH Image 1.61 (National Institute of Health, Bethesda) in pixels. Infarct size was expressed as percentage of the risk zone infarcted.

Estimation of CK release

CK was quantified from 0.5 ml of coronary effluent obtained prior to ischemic adaptation and at 30, 60 and 120 min during

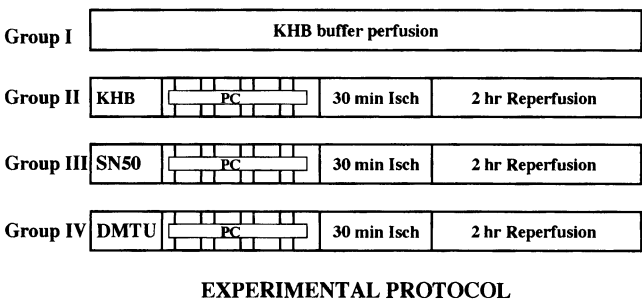


Fig. 1. Experimental protocol.

reperfusion. CK was analyzed by the enzymatic assay method using a CK assay kit (Sigma Diagnostics, St. Louis, MO, USA). The absorbance was read at 340 nm using a Beckman DU-8 spectrophotometer.

Measurement of MDA formation in heart

Malonaldehyde (MDA) was estimated in the perfusate to determine the development of oxidative stress and free radical generation as described previously [16]. In short, about 0.5 ml of coronary effluent was mixed with 2 ml of 20% trichloroacetic acid, 5.3 mM sodium bisulfite, kept on ice for 10 min, centrifuged at 3,000 g for 10 min, and then supernatants were collected, derivatized with 2,4-dinitrophenylhydrazine (DNPH), and extracted with pentane. Aliquots of 25 μ l in acetonitrile were injected onto a Beckman Ultrasphere C₁₈ (3 mm) column. The products were eluted isocratically with a mobile phase containing acetonitrile-water-acetic acid (40:60:0.1, v/v/v) and measured at three different wavelengths (307, 325 and 356 nm) using a Waters M-490 multichannel UV detector. The peak for malonaldehyde was identified by co-chromatography with DNPH derivative of the authentic standard, peak addition, UV pattern of absorption at the three wavelengths, and by GC-MS.

Estimation of protein C kinase, MAP kinase, and MAPKAP kinase

Approximately 0.3–0.4 gm of heart biopsy was placed in a measured volume of ice-cold Tris-sucrose buffer that contained 0.35 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT and 0.1 mM PMSF. The tissue was homogenized with a Polytron homogenizer, and the resulting homogenate was centrifuged at 15,000 g for 20 min. The supernatant was used for the estimation of Protein C kinase. Protein concentration was determined with a BCA protein assay kit (Pierce, Rockville, IL, USA). For protein C kinase assay, 15 μ g protein/15 μ l were used from each sample in duplicate and added to 30 μ l of final volume of kinase reaction mixture containing kinase buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM KCl and 1 mM DTT), synthetic peptide substrate (M.W. 2342) (Upstate Biotechnology Inc., Lake Placid, NY, USA), 40 μ M [γ -³²P] ATP (4.4×10^3 cpm/pmol), phosphatidylserine (20 μ g/ml), phorbol-12-myristate 13-acetate (2 μ g/ml) and 1.6 mM CaCl₂. The reaction was allowed to proceed for 20 min at 30°C. The ³²P incorporation into the peptide was analyzed using a liquid scintillation counter.

To quantify the protein phosphorylation of p38 MAP kinase, heart tissues were homogenized and suspended (5 mg/ml) in sample buffer [10 mM HEPES, pH 7.3, 11.5% sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM diisopropylfluorophosphate

(DFP), 0.7 mg/ml pepstatin A, 10 mg/ml leupeptin, 2 mg/ml aprotinin]. Proteins were then solubilized with the addition of same amount of 2 x Laemmli solution [9% (w/v) SDS, 6% (v/v) β -mercaptoethanol, 10% (v/v) glycerol and a trace amount of Bromophenol Blue dye in 0.196 M Tris/HCl (pH 6.7)]. The cellular proteins (50 μ l samples) were electrophoresed through 10% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore Corp.) using a semidry transfer system (Bio-Rad). Pre-stained protein standards (Bio-Rad) were run in each gel. The blots were blocked in Tris-buffered saline/Tween20 (TBS-T containing 20 mM Tris base, pH 7.6, 137 mM NaCl, 0.1% Tween-20) supplemented with 5% BSA for 1 h, incubated with 1:1,000 diluted primary rabbit antibodies specifically against Tyr182-phosphorylated p38 MAP kinase (NEB, Inc) for 2 h, and then with 1:10,000 diluted secondary antibodies of horseradish peroxidase-conjugated anti-rabbit IgG (Boehringer Mannheim Corp., Inc) for 1 h at room temperature. After three washes of 5 min each, blots were treated with Enhanced Chemi-Luminescence (ECL from Amersham) reagents and the phosphorylated MAP kinases were detected by autoradiography for variable lengths of time (15 sec to 3 min) with Kodak X-Omat film.

To evaluate enzymatic activities of tissue MAPKAP kinase 2, heart tissues were homogenized and suspended in sample buffer. After centrifugation at 5,000 x g for 10 min, the kinase activity in the resulting supernatant was examined by an *in vitro* protein phosphorylation assay using commercially available recombinant human HSP 27 (RHSP 27, StressGen Biotechnologies Corp.) as the specific substrate for MAPKAP kinase 2 [34]. The reaction was initiated by the addition of an equal volume (30 μ l) of freshly prepared phosphorylation reaction mixture containing 30 mM HEPES (pH 7.3), 20 mM MgCl₂, 2 mM EGTA, 10 μ M sodium orthovanadate, 5 μ M okadaic acid, 4 mM DTT, 30 μ M H⁺-7, 0.4 mM [γ -³²P]ATP (10^5 cpm/pmol), and 0.5 μ g RHSP 27, to 30 μ l tissue supernatant. The *in vitro* phosphorylation reaction was carried out at 30°C for 10 min and stopped by addition of 60 μ l 2 x Laemmli solution. Proteins were then separated on 11 % SD S-PAGE and the induced protein phosphorylation of rHSP 27 was detected by autoradiography.

Isolation of nuclear proteins

Nuclear proteins were isolated from the heart according to the method described previously by Schreiber [17] with slight modification. In short, about 150 mg of left ventricle from heart tissue was homogenized in ice-cold Tris-buffered saline (TBS) and centrifuged at 3,000 g for 5 min at 4°C. The pellet was resuspended by gentle pipetting in 1.5 ml of ice-cold hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1 μ M each of aprotinin, pepstatin and leupeptin.

The solution was allowed to swell on ice for 15 min. After addition of 100 μ l of 10% Nondit P-40, the tube was vigorously vortexed for 45 sec. This homogenate was centrifuged for 30 sec at 4°C in a microcentrifuge tube. The supernatant contained the cytoplasmic protein.

The nuclear pellet was resuspended in a solution containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 1 μ M each of aprotinin, pepstatin and leupeptin. The tubes were vigorously shaken at 4°C for 30 min on a shaking platform. The extracts were then centrifuged and the supernatants were stored at -70°C. Protein concentration was estimated by using Pierce Protein Assay kit (Pierce Chemical Co., Rockford, IL, USA).

Electrophoretic mobility assay (EMSA)

NF κ B oligonucleotide (AGTTGAGGGGACTTTCCCAGG) (2.5 μ l [20 ng/ μ l]) was labeled using T4 polynucleotide kinase as previously described [18]. The binding reaction mixture contained in a total volume of 20.2 μ l, 0.2 μ l DTT (0.2 M), 1 μ l BSA (20 mg/ml), 4 μ l PdI-dc (0.5 μ g/ μ l), 2 μ l Buffer D⁺, 4 μ l Buffer F, 2 μ l ³²P-oligo (0.5 ng/ μ l) and 7 μ l extract containing 10 μ g protein. Composition of Buffer D⁺ was 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP 40 while Buffer F contained 20% Ficoll 400, 100 mM HEPES, pH 7.9, and 300 mM KCl. Incubation was carried out for 20 min at room temperature. Ten μ l of the solution was loaded onto a 4% acrylamide gel and separated at 80 v until the dye hit the bottom. After electrophoresis, gels were dried up and exposed to Kodak X-ray film at -70°C.

Statistical analysis

For statistical analysis, a two-way analysis of variance (ANOVA) followed by Scheffe's test was first carried out using Primer Computer Program (McGraw-Hill, 1988) to test for any differences between groups. If differences were established, the values were compared using Student's *t*-test for paired data. The values were expressed as mean $\pm$ S.E.M. The results were considered significant if *p* was less than 0.05.

Results

Myocardial adaptation to ischemic stress by repeated ischemia and reperfusion and its inhibition by DMTU and SN 50 peptide

As expected, hearts subjected to repeated ischemia and reperfusion demonstrated significant reduction in infarct size compared to matched control (Fig. 2). Both DMTU and SN

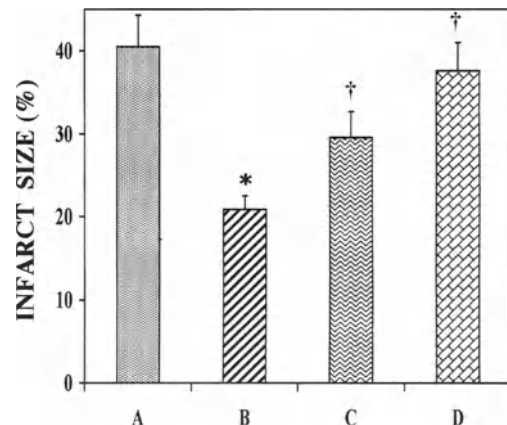


Fig. 2. Effects of ischemic adaptation, DMTU and SN 50 on the myocardial infarct size after ischemia and reperfusion. Normalized infarction was evaluated as described in the Materials and methods section. Results are expressed as mean $\pm$ S.E.M. for 10 animals in each group. **p* < 0.05 compared to control. †*p* < 0.05 compared to adapted group. A – control; B – adapted; C – SN 50; D – DMTU.

50 abolished this cardioprotective effect of ischemic preconditioning.

Effects of DMTU and SN 50 peptide on CK release from heart

Release of CK is considered as a presumptive marker for tissue necrosis and cell death. As shown in Fig. 3, CK release

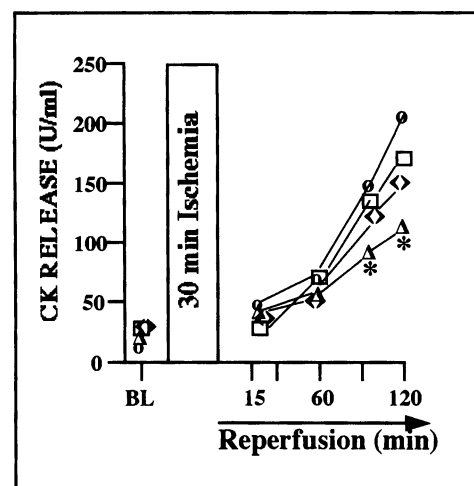


Fig. 3. Effects of ischemic adaptation, DMTU and SN 50 on the creatine kinase release from the coronary effluent. The level of CK was measured enzymatically as described in Materials and methods section. Results are expressed as mean $\pm$ S.E.M. for 10 animals in each group. Each assay was run in duplicate. **p* < 0.05 compared to control. ○ – control; Δ – adapted; ◇ – SN 50; □ – DMTU.

increased in all the three groups of hearts. However, a slight increase was noticed in the control heart, and only after 1.5 h of perfusion with SN 50 peptide. Increase in CK release from the non-adapted hearts were significantly higher compared to ischemically adapted group. Post-ischemic CK release was significantly elevated in both ischemically adapted and SN 50 peptide treated hearts, but it was significantly higher for SN 50 peptide treated hearts as compared to adapted hearts suggesting that SN 50 peptide blocked the beneficial effects of ischemic adaptation by increasing tissue injury. Amount of CK was not affected when hearts were perfused with SN 50 peptide only for 3.5 h (results not shown).

Effects of DMTU and SN 50 peptide on MDA formation in heart

MDA formation truly reflects the development of oxidative stress in biological system. MDA content of heart did not change even after 3.5 h of perfusion with SN 50 peptide only (results not shown). In all groups, MDA increased significantly compared to baseline except for DMTU during the early reperfusion (Fig. 4). Preconditioning initially resulted significant increase in the amount of oxidative stress as evidenced by increased MDA formation (not shown). However, the amount of MDA increased only slightly during subsequent ischemia and reperfusion compared to control group. DMTU decreased the amount of MDA formation to

the baseline level. SN 50 did not alter the amount of MDA content at any stage.

Effects of DMTU on tyrosine kinase phosphorylation

As shown in Fig. 5, tyrosine phosphorylation was enhanced after preconditioning (lane B). DMTU significantly blocked the phosphorylation of specific cellular proteins including 98, 80, 77, 70, 40, 38, 25 and 20 KDa proteins (lane C).

Effects of DMTU and SN 50 peptide on protein kinase C, p38 MAP kinase and MAPKAP kinase 2

To understand the regulation of p38 MAP kinase in myocardium, kinase activity was evaluated by using Western blot to detect the induced protein phosphorylation as described under Materials and methods. The antibodies specific against the tyrosine-phosphorylated p38 MAP kinase (NEB, Inc.) detected two proteins with molecular weight about 38 kDa. The antibodies against p38 MAP kinase (Santa Cruz Biotech, Inc.) also probed two protein bands in rat myocardium (unpublished observation). Enzymatic analysis showed that tissue p38 MAP kinase became phosphorylated and hence activated by ischemic adaptation and/or ischemic stress (Fig. 6). DMTU significantly inhibited the activation of p38 MAP kinase while SN 50 had no effect on the activity of p38 MAP kinase.

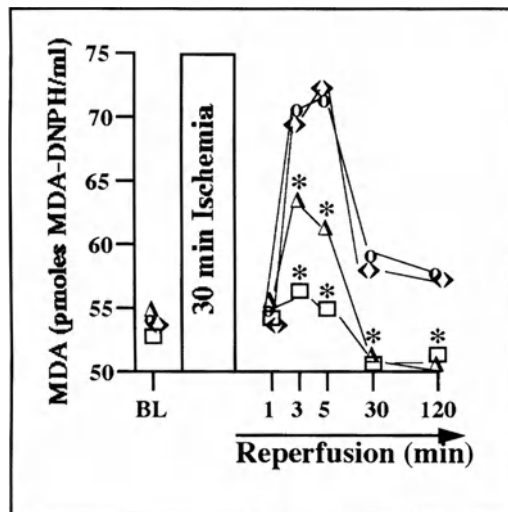


Fig. 4. Effects of ischemic adaptation, DMTU and SN 50 on the malonaldehyde content in the heart after ischemia and reperfusion. At the end of each experiment, hearts were excised and MDA was estimated by HPLC as described in Materials and methods section. Results are expressed as mean $\pm$ S.E.M. for 10 animals in each group. * $p < 0.05$ compared to control. $^{\dagger}p < 0.05$ compared to adapted group. ○ – control; Δ – adapted; ◇ – SN 50; □ – DMTU.

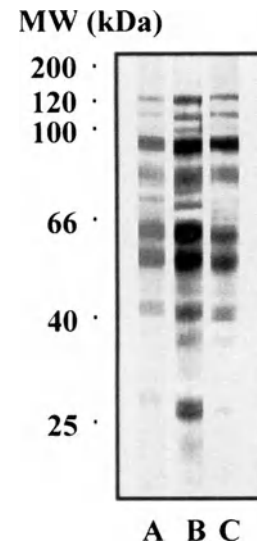


Fig. 5. Effects of ischemic adaptation, DMTU and adaptation on the tyrosine phosphorylation. Protein phosphorylation was evaluated as described in the Materials and methods section. Inhibition of protein phosphorylation by DMTU is clearly visible in lane C. Results are representative of five different experiments performed per group. Each experiment was repeated two times. A – control; B – adapted; C – DMTU.

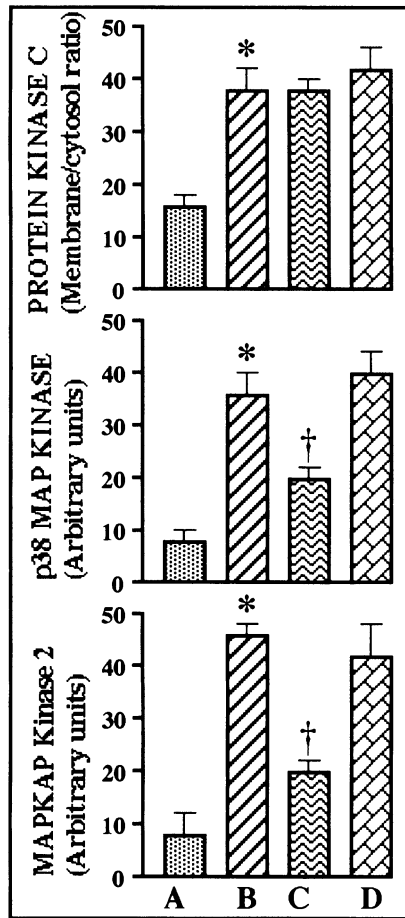


Fig. 6. Effects of ischemic adaptation, DMTU and SN 50 on PKC, p38 MAP kinase and MAPKAP kinase 2 activities in heart. Activities were determined as described in the Materials and methods section. Results are expressed as mean $\pm$ S.E.M. for six animals per group. Each experiment was run in duplicate. * $p < 0.05$ compared to adapted group. A – control; B – adapted; C – DMTU; D – SN 50.

MAPKAP kinase 2 has been demonstrated to be specific cellular substrate for p38 MAP kinase and responsible for the induced protein phosphorylation of small heat shock protein (HSP 25 and HSP 27). Enzymatic activity of tissue MAPKAP kinase 2 was examined with an *in vitro* protein phosphorylation assay using human recombinant HSP 27 as a specific substrate as described under Materials and methods. Adaptation and/or ischemic stress of isolated rat hearts induced the tissue MAPKAP kinase 2 activation (Fig. 6), which apparently resulted from the p38 MAP kinase activation. The induced activation of MAPKAP kinase 2 was completely inhibited by DMTU. Again, SN 50 had no effect on the MAPKAP kinase 2 activities.

PKC was also activated by preconditioning supporting the results of our previous studies. However, neither DMTU nor SN 50 had any effects on the induction of PKC as shown in Fig. 6.

Effects of DMTU and SN 50 on the nuclear translocation of NF κ B

NF κ B binding activity was found to be very low in non-ischemic control hearts (lane A), but it was clearly detected in the adapted hearts (Fig. 7). Significant increase in NF κ B binding was found in the hearts that were adapted by repeating this adaptation sequence for four times (lane B). NF κ B binding activity was significantly decreased in the hearts pretreated with either SN 50 (C) or DMTU (D) suggesting that NF κ B activation is regulated by the reactive oxygen species. To confirm the specificity of NF κ B binding activity, we performed supershift assays with polyclonal antibodies recognizing NF κ B p65 subunit proteins (results not shown).

Discussion

Mammalian heart can be adapted to ischemia by repeatedly subjecting it to short term reversible ischemia each followed by another short durations of reperfusion [1–6]. This phenomenon, known as ischemic preconditioning, causes the production of oxidative stress leading to the induction of gene expression which is subsequently translated into the develop-

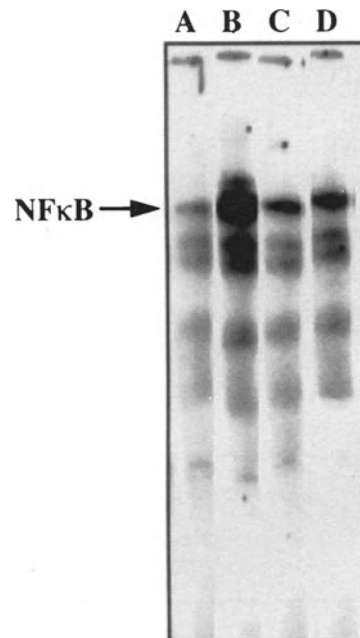


Fig. 7. Effects of ischemic adaptation, DMTU and SN 50 on the nuclear translocation of NF κ B. In perfused control heart (lane A) there is no sign of NF κ B translocation whereas in the adapted heart (lane B) significant amount of NF κ B translocation was noticed. The binding activities of NF κ B were significantly inhibited by SN 50 (lane C) and DMTU (lane D).

ment of several stress related proteins responsible for heart's defense [7, 8]. Although the beneficial effects of ischemic stress adaptation are well recognized, controversies exist regarding the mechanism of signal transduction by which ischemic stress builds up heart's defense. Myocardial adaptation to ischemia has recently been shown to be mediated through the activation of tyrosine kinase receptor protein. The signal transduction process appears to involve tyrosine kinase coupled phospholipase D and MAP kinases which leads to the activation of MAPKAP kinase 2 [19, 20]. Our recent study demonstrated that the ischemic stress specifically translocates and activates p38 MAP kinases which directly activates MAPKAP kinase 2 [21].

The present study shows that infarct size limiting effect of ischemic preconditioning were abolished by either DMTU or SN 50 peptide suggesting that both reactive oxygen species and nuclear transcription factor NFκB play crucial roles in preconditioning. Multiple kinases including PKC, p38 MAP kinase and MAPKAP kinase 2 were activated by preconditioning supporting a number of previous reports [19–21]. DMTU reduced preconditioning-mediated increase in the activation of p38 MAP kinase and MAPKAP kinase 2. However, DMTU had no effects on PKC suggesting that free radicals potentiate a signal transduction process independent of PKC.

The results of our study support the previous reports that free radical signaling involves p38 MAP kinase. A recent study showed potent activation of extracellular signal-regulated ERK2 kinase within 10 min of H₂O₂ treatment [22]. This study also showed that H₂O₂ also moderately activated other kinases including p38 MAP kinase. Lipopolysaccharides which can adapt the heart to oxidative stress [23] has been found to induce tyrosine phosphorylation of p38 protein [24]. Phosphorylation of p38 was blocked by treatment of cells with a protein tyrosine kinase inhibitor, herbimycin A suggesting that tyrosine phosphorylation is involved in LPS-induced oxygen free radical signaling.

Our results implicate that reactive oxygen species provokes a signal transduction pathway through the activation of tyrosine kinase phosphorylation and then function either independent of PKC or act downstream of PKC leading to the activation of p38 MAP kinase and MAPKAP kinase 2. MAP kinases-signal transduction pathway is likely to involve activation of Ras or Raf-1 which in turn induces mitogen-activated protein kinase kinase (MKK) and MAP kinases. It is also known that Raf-1 kinases possesses MAPKKK activity and lies upstream from MAPKK and MAP kinases in various cell types [25]. A recent study has demonstrated that hypoxia and hypoxia/reoxygenation activated raf-1, MKK as well as MAP kinases in cultured rat cardiomyocytes. Raf-1 operates downstream cell surface associated tyrosine kinases and upstream from MAP kinases. Raf is not strictly a member of the MEKK family, but it is functionally analogous. Ras is

part of the signal transduction chain extending from extracellular signals to transcriptional regulation in the nucleus. Upon activation, tyrosine kinase recruits a number of proteins including Ras-specific guanine nucleotide releasing proteins which then regulates the binding of Ras with GTP, thereby potentiating the Ras signal. Ras proteins then interact with Raf kinases to induce downstream signals activating MAP kinases and other protein kinases. Once Raf is activated, then Ras is no longer required. The precise mechanism by which Ras controls Raf-1 is poorly understood. The binding of Raf-1 to Ras is largely GTP dependent and requires the effector region of Ras and the regulatory region of Raf-1.

The nuclear factor kappa B (NFκB) appears to be a critical regulator for gene expression induced by diverse stress signals including mutagenic, oxidative and hypoxic stresses. NFκB is a ubiquitous transcription factor which is translocated in response to oxidative stress from its inactive cytoplasmic form by releasing the inhibitory subunit IκB from NFκB [26, 27]. Activation of NFκB is likely to be involved in the induction of gene expression associated with the ischemic adaptation, since this transcription factor has recently been found to play a crucial role in the regulation of ischemia/reperfusion-mediated gene expression [28].

Mounting evidence exists to support the notion that oxygen-derived free radicals are generated during the reperfusion of ischemic myocardium resulting in the development of oxidative stress [29, 30]. It seems logical to assume that ischemia/reperfusion-induced oxidative stress caused translocation of NFκB which might be involved in the regulation of the activation of MAP kinases observed in conjunction with myocardial stress adaptation. To examine the role of NFκB in tyrosine kinase signaling during myocardial adaptation to ischemia, isolated rat hearts were adapted to ischemic stress by repeated ischemia and reperfusion [31]. The adapted heart resulted in the nuclear translocation and activation of NFκB which was completely blocked by both DMTU and SN 50. In conjunction, the beneficial effects of ischemic adaptation was blocked by pretreating the hearts with SN 50 peptide or DMTU. These results support our previous observation [14] and further suggest that NFκB situating downstream of p38 MAP kinase, plays a crucial role in myocardial adaptation to ischemia.

Despite a great deal of research of the preconditioning phenomenon over the last decade, the pathophysiology and the mechanisms by which preconditioning exerts cardioprotection remain controversial. It is generally believed that one or more intracellular mediators rapidly released in response to stress are responsible for preconditioning. While there is a great deal of controversy regarding the exact pathway for signal transduction, protein kinase C (PKC) has been implicated as involved in signalling this adaptive response [32]. Recent findings indicate that multiple kinases including MAP kinases and MAPKAP kinase 2 are likely to

be involved in the preconditioning signaling process [19–21]. Additionally, receptor tyrosine kinase appears to play a role in transmembrane signaling of the stress response. This adaptive signal is proposed to be mediated through a phospholipase D-dependent MAPKAP kinase 2 pathway [19].

The acutely developing adaptive effect is short-lived, lasting for only up to 2–3 h. Hearts can subsequently undergo a secondary and delayed adaptation to stress presumably through the induction of the expression of new genes and their subsequent translation into proteins. A number of genes and proteins have been identified as possibly involved in the development of *second window* or delay preconditioning including heat shock proteins (HSP), superoxide dismutase (SOD), catalase, nitric oxide synthase as well as ATPase 6 and cytochrome b subunits. Such an adaptive response becomes evident only after approximately 24 h of stress-treatment and may include stress induced by heat shock, oxidant or other stress-inducible agents. MAPKAP kinase 2 appears to link the early preconditioning effect to the delayed adaptive response [19–21].

Evidence is rapidly accumulating to indicate that oxidative stress/free radicals lead to the activation of NFκB which in turn induces the expression of genes [33, 34]. Interestingly, H₂O₂ was found to activate DNA binding of NFκB *in vivo*, but not *in vitro* [35] suggesting that a by-product of H₂O₂ and not H₂O₂ by itself may be responsible for the activation of NFκB. Another related study using transient catalase over-expression in cos-1 cells showed that H₂O₂ may not serve as a messenger for TNFα or phorbol ester induced NFκB activation [36]. It is possible that OH· radical formed by transient metal-catalyzed Fenton reaction during the reperfusion of ischemic heart [37] can induce NFκB activation. Inhibition of NFκB induction by antioxidants further supports a role of free radicals in NFκB activation [38].

In summary, to the best of our knowledge, the results of our study provides evidence for the first time that tyrosine kinase and MAP kinases are the targets for reactive oxygen species generated in the preconditioned myocardium. The finding that p38 MAP kinase might be upstream of NFκB further supports our previous reports that MAPKAP kinase 2 could be the most likely link between the preconditioning and adaptation mediated by gene expression. p38 activation appears to be an important step in the translocation and activation of the nuclear transcription factor NFκB which in turn may be involved the induction of the expression of a variety of stress-inducible genes.

The results of our study indicate a vital role for the reactive oxygen species generated during preconditioning in mediating cellular responses through diverse intracellular and extracellular ligands. Stimulation of protein phosphorylation by oxygen free radicals as observed in the present study appears to be a feasible mechanism for subsequent signal transduction leading to the activation of the transcription factor NFκB. A

large number of reports exist in the literature to show the induction of antioxidant gene expression during preconditioning [13]. It seems, therefore, quite logical to assume that the reactive oxygen species function as ‘second messengers’ during ischemic preconditioning. This is substantiated from our observation that free radicals generated during preconditioning triggers a tyrosine kinase-dependent signal transduction resulting enhanced phosphorylation and activation of MAP kinase cascade leading to the activation of NFκB. It is interesting to note that PKC is not a potential target for oxygen free radicals in the preconditioning process. Activation of NFκB seems to be regulated by MAP kinases independent of PKC. The induction of the antioxidant genes and enzymes observed by many investigators is likely to be mediated by NFκB.

Acknowledgements

This study was supported by NIH HL 34360, HL 22559, HL 33889, HL 56803 as well as by a Grant-in-Aid from the American Heart Association.

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The role of oxidative stress in diseases caused by mineral dusts and fibres: Current status and future of prophylaxis and treatment

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Abstract

Inhalation of silica and asbestos fibres by humans can lead to fibrosis of the lung and cancer. Different mechanistic approaches, including oxidative stress, are used for prophylactic and therapeutic interventions to attenuate the fibrogenic and carcinogenic effects of these particles. Thus far, most of these therapeutic interventions have been only partly successful. A review of the mechanisms which are thought to be involved in mineral particle-induced toxicity and the relevant therapeutic interventions used to date to counteract these mechanisms, will help formulate better prophylactic and treatment intervention strategies in the future. (*Mol Cell Biochem* **196**: 69–77, 1999)

Key words: mineral particles, silica, asbestos fibres, inflammation, fibrosis

Introduction

Inhalation of mineral dusts and fibres produces a variety of pulmonary diseases including fibrosis (associated with excess collagen synthesis), and two types of cancer, bronchogenic carcinoma and mesothelioma (cancer of the mesothelial lining of the lung). The properties responsible for the pathogenicity of these minerals have been the subject of intense investigation over the last three decades. Early studies of exposed populations, animal experimentations, and *in vitro* cytotoxicity tests have suggested that the size distribution of mineral particles is a major determinant of their biological activity [1]. Later investigations have shown however, that particle morphology may not be the only important factor. Numerous important determinants of particle pathogenicity have been proposed. These include a variety of surface properties of mineral dusts and fibres and the generation of silica- and oxygen-centred radicals on these surfaces [2–9], as well as the activation of phagocytic cells and the increased generation of reactive oxygen species and inflammatory mediators from these cells [10–14].

Mechanistic approaches to counteract the toxicity of mineral particles

Due to multiple mechanisms of the pathogenicity of mineral particles, it is not surprising that the intervention by both prophylaxis and treatment to protect against silica dust- and asbestos fibre-induced toxicity, have included a wide range of mechanistic approaches to neutralise the properties which are thought to be responsible for the production of the observed pathological effects. In some cases, the agents used were found to exert their effects through more than one mechanism.

Masking surface active sites

Surface properties of mineral particles depend on their atomic structure, composition, microtopography and shape which, in turn, determine their biological activity [2]. Thus, surfaces which are in contact with the environment will be different for silica and for various types of asbestos fibres. Silica particles are composed entirely of silicon and oxygen (SiO₂)

and exist in different polymorphs [15]. Upon grinding, the Si-O covalent bonds may rupture by heterolytic cleavage, producing Si^+ and SiO^- surface charges [5].

In asbestos fibres, surface properties vary depending on the type of the fibre. The 1:1 layers of chrysotile, a serpentine asbestos, are tightly curled to partially compensate for the inherent misfit between the Mg octahedral sheet and the Si tetrahedral sheet [16]. With this curvature, the Mg-hydroxide surface is directed on the outward and the silicate sheet is directed inward, towards the axis of the fibril. The atomic layer of an ideal chrysotile fibre, which is directly in contact with its environment, is therefore an array of hydroxyl groups, $\text{Mg}(\text{OH})_2$. In aqueous fluid, the surface of chrysotile is positively charged.

The double-chain structure of crocidolite, an amphibole asbestos, has a layer of octahedrally coordinated cations sandwiched between the two tetrahedrally coordinated silica layers [16, 17]. These units are sometimes referred to as I-beams. The cations occupy different sites known as M_1 , M_2 , and M_3 (C-group cations), and M_4 (B-group cations) sites. Surface cleavage will therefore determine the surface structure which will be in direct contact with the environment. If the cleavage is to cut through the octahedral strip of half the I-beam, this will expose the C-cationic sites to the environment. In contrast to chrysotile, the net charge for crocidolite amphibole is negative.

Numerous prophylactic and/or therapeutic strategies have utilised compounds which would counteract and mask some of the surface sites on the silica and asbestos mineral particles.

Aluminium

In vitro and *in vivo* studies have shown that modifying the surface of silica particles by masking some active surface sites with Al compounds leads to a decrease in biological activity and an increase in clearance of quartz from the lung [18, 19]. One hypothesis to explain the reduction of activity of crystalline silica proposed that the insertion of $(\text{AlO}_4)^{5-}$ in the $(\text{SiO}_4)^{4-}$ tetrahedra network may take place after a surface treatment of silica by chemical compounds of Al^{3+} . This insertion is thought to modify the redox and acid-base properties of functionalities on the surface and reduce the siliceous radical species on the surface of crystalline silica.

A recent study [20] has shown however, that aluminum aerosol treatment of silicosis after radiographic recognition of disease did not alter the disease process and thus its use as a therapeutic intervention was excluded, even for the early stages of the disease.

Polyvinylpyridine N-oxide

Since Schlipkötter and Brockhaus [21] reported the inhibition of quartz-induced fibrosis in animals using the polymer

polyvinylpyridine N-oxide (PVPNO), many laboratories have confirmed its therapeutic efficacy in silicosis [22, 23]. The use of PVPNO as a prophylactic agent was also recommended in human silicosis [24]. However, PVPNO was reported as having no effects when used as a preventive agent with chrysotile asbestos [25].

Schlipkötter and Brockhaus [26] proposed that PVPNO action was due to the coating of the surface of the dust by the polymer. Marchisio and Comolli [27] concluded that the protection of peritoneal macrophages against quartz by PVPNO was either due to the protection of the ultrastructures of the cells by the polymer or the inactivation of silica by PVPNO. Nash *et al.* [3] believed that PVPNO formed hydrogen bonds with silicic acid and therefore prevented the permeability changes induced by the latter in biological membranes. Adsorption of PVPNO to the quartz surface was confirmed by UV spectrometry [28], while the ability of this polymer to scavenge hydroxyl radicals generated by mineral particles was shown by electron resonance spectrometry [29].

Amiodarone

Several *in vitro* studies have shown that coating of silica with phospholipids, especially dipalmitoyl lecithin, reduced its toxicity towards alveolar macrophages [30, 31]. Coating of chrysotile, on the other hand, did not result in such reduced toxicity [32], but was found to decrease chrysotile-stimulated superoxide anion production by alveolar macrophages by 90% [33]. *In vivo* experiments by Antonini and Reasor [34], on the other hand, could not confirm the protective effect of phospholipids against silica-induced toxicity despite measurable amounts of phospholipid bound to silica, isolated from animals. These authors suggested that the presence of a surfactant coating was inadequate to protect against damage; such a coating could delay, but not prevent, the cytotoxicity of silica. The same authors, however, reported a reduction in silica toxicity after phospholipidosis induced by amiodarone [35]. The protection observed was found to be due to the inhibition of microsomal phospholipases by amiodarone, leading to decreased digestion of the phospholipids in the coating of the particles [36]. However, due to the toxicities of this drug, its use in humans to treat or prevent silicosis could not be considered.

Organosilane compounds

As adsorbed materials on mineral particles are apt to dissociate, covalent derivatization of the surfaces was thought to be more resistant to subsequent surface alteration in biological surroundings. A variety of organosilane compounds were therefore tested to change the surfaces of silica and asbestos

fibres [37–40]. Results presented have shown a reduction in the toxicity and carcinogenicity of the surface-modified particles. These treatments were, however, aimed at confirming the involvement of surfaces in the pathogenicity and toxicity of particles, rather than identifying strategies as prophylactic and therapeutic interventions.

Protection from oxidative stress

Upon grinding, when the Si-O covalent bonds rupture by homolytic cleavage, a Si· (E' centre) and a SiO· radical are produced. These react with oxygen to produce SiO₂· (peroxyradical), SiO₃·, and SiO₂·⁻ (Superoxide radical). Some of these species may also react with hydrogen peroxide and produce the superoxide anion and the hydroxyl radicals [5]. Chrysotile and crocidolite fibres have been shown to catalyse the formation of both superoxide anion and hydroxyl radicals in the presence of hydrogen peroxide [6–8]. These minerals were also shown to generate the hydroxyl radical from oxygen [41–43]. The mechanism proposed involved the reduction of oxygen via the oxidation of iron on the mineral surfaces.

Another potential pathway for free radical generation has been shown to involve the interaction of mineral dusts and fibres with phagocytic and other cell types [9–11]. Active oxygen species, resulting from the surface activity of minerals and from the activated phagocytes, are known to peroxidise lipids [44] and attack DNA [45–47].

Tetrandrine

This bisbenzylisoquinoline alkaloid, isolated from the root of *Stephania tetrandra*, has been tested for its antisilicotic effects [48, 49] and is currently used in China for silica-induced fibrosis [50].

Mechanisms proposed to explain the efficacy of tetrandrine against silicosis include, its binding affinity for lipid vesicles, alveolar macrophages [51–53], and microtubules [54, 55]; its ability to inhibit respiratory burst activity of pulmonary phagocytes and to scavenge active oxygen radicals and act as an antioxidant [56, 57], as well as inhibit nitric oxide production in macrophages activated by bacterial lipopolysaccharide [58]; its cytotoxicity to macrophages with an accompanied overproduction of prostaglandins [58]; and its ability to affect collagen gene expression [59]. However, tetrandrine has been found to have no preventive effect on the inflammatory response induced by silica exposure [51].

Ascorbic acid

Preincubation of microsomal and cytosolic fractions with physiological concentrations of ascorbic acid ameliorated the

asbestos-induced decreases in the activities of benzo(a)pyrene hydroxylase and glutathione S-transferase, as well as the stimulation of the NADPH- and Fe²⁺-induced lipid peroxidation in microsomal fractions [60].

Long term administration of ascorbic acid (10 mg/rat or 40 mg/kg) to rats with silicosis retarded lung fibrosis development during both the early steps of silicosis (3–6 weeks) and the developed pneumofibrosis (14 weeks) [61]. Ascorbic acid also inhibited the squamous metaplasia in tracheal explants resulting from exposure to crocidolite asbestos [62].

21-aminosteroids (21-ASs)

The antioxidant properties of the 21-ASs have been well-documented. These drugs have been shown to scavenge lipid peroxy radicals and to block lipid radical chain reactions, thus inhibiting lipid peroxidation [63]. The treatment of silica-exposed animals with U74389G, a 21-AS, is shown to decrease significantly the acute inflammatory response in silica-exposed animals by reducing the infiltration of polymorphonuclear cells into the airspace of the lungs. It was also demonstrated that the treatment led to reductions in cellular and tissue chemiluminescence resulting from reactive oxygen species and nitric oxide. It was concluded therefore, that the anti peroxidative and antioxidative effects of U74389G may provide the basis for some of the protection offered by this 21-AS against silica injury. This led to the hypothesis that the 21-AS may be useful in the treatment of silica-induced pneumotoxicity [64].

Antioxidant enzymes

Dextran-conjugated superoxide dismutase administered to silica-exposed mice inhibited the development of pulmonary fibrosis [65]. Similarly, polyethylene-glycol-conjugated-catalase [66] decreased the numbers of alveolar macrophages, polymorphonuclear leukocytes and lymphocytes; furthermore, it reduced the fibrotic lesions in the exposed animals. A recent study [67] has shown that transfection of manganese-containing superoxide dismutase into hamster tracheal epithelial cells ameliorated asbestos-mediated cytotoxicity, suggesting a possible new protective and therapeutic strategy to alleviate or treat inflammatory or fibrotic lung diseases.

Carotene and vitamin A

The electron-scavenging property of β-carotene and the effect of vitamin A on cell differentiation and proliferation [68] and on the immune system [69] are some of the properties which

are thought to be of relevance in the antioxidant protection against cancer. These and other properties prompted the initiation of the β -carotene and retinol efficacy trial (CARET) in 1994, for chemoprevention of lung cancer in high risk populations such as smokers and asbestos-exposed workers [70]. Unfortunately, after four years of supplementation, the combination therapy of these two vitamins had no beneficial effect; on the contrary, it may have had an adverse effect on the incidence of lung cancer. As a result, the trial's active intervention was stopped officially in January 1996 [71]. This treatment, however, was found to have a protective effect on the ventilatory function in asbestos-exposed subjects [72].

Dietary intake

Despite the lack of protection of dietary supplementation of two previous vitamins in decreasing cancer, some evidence was presented on the ability of diets rich in vegetables, fruits, and some vegetable-related constituents to induce a protective effect on developing mesothelioma in humans [73]. Similarly, consumption of diets rich in carotene fruit were found to be protective for mesothelioma [74].

In animal experiments, excess availability of choline (beef liver, eggs, soybeans) and fat enhanced the fibrogenic and carcinogenic lesions induced by mica particles and asbestos fibres. On the other hand, the high consumption of natural Whole sugar-Jaggery (a solidified product of sugarcane juice), through its ability to reduce the consumption of fat, was demonstrated to have an antifibrotic effect [75].

Dibutyl cyclic AMP (dbcAMP)

There is evidence suggesting that cyclic adenine nucleotides can attenuate oxidant-induced lung injury caused by a variety of toxic substances. Adenosine 3',5'-cyclicmonophosphate (cAMP) or its analogues are shown to reduce pulmonary edema and lung toxicity caused by endotoxin [76], bleomycin [77], phosgene [78], and phorbol 12-myristate β -acetate-activated polymorphonuclear leukocytes (PMN) [79] and prevent oxidant-induced increased endothelial permeability [80].

Results presented have demonstrated that agents capable of raising intracellular cAMP (dbcAMP, aminophylline, and terbutaline) diminished the toxic effects of asbestos on cultured WI-26 cells by maintaining the levels of cytosolic ATP and by inhibition of cell growth [81]. These results suggest that cAMP analogues offer a novel strategy to limit asbestos-induced lung injury.

Similarly, the increased resistance offered by glutamate to macrophages against silica and the decreased fibrosis in the exposed animals were attributed to the ability of glutamate to increase the ATP/ADP ratio in these phagocytic cells [82, 83].

Oxidation state of iron

Precipitation of ferric iron salts [84] on the surfaces of asbestos fibres is shown to decrease the ability of the fibres to generate free radicals [85] and to induce lipid peroxidation [86], as well as the toxicity of the fibres to cells in culture [87]. Similarly, the conversion of ferrous ions into ferric ions by microwave radiation in crocidolite fibres decreased the ability of these particles to induce lipid peroxidation [88].

Chelation of iron

Depending on the type of chelating agents used, an increase or a decrease in the activity of mineral particles to generate free radicals was reported. An example, is phytic acid, an iron chelator, which is found to reduce amosite asbestos-induced hydroxyl radical generation, DNA strand break formation, and injury to cultured pulmonary epithelial cells [89]. It is also found to diminish pulmonary inflammation and fibrosis in rats after a single intratracheal instillation of amosite asbestos [90]. Similarly, desferrioxamine is reported to diminish the ability of mineral particles to generate free radicals and peroxidize lipids [91] and its possible use for the prevention of asbestos-induced diseases has been suggested [92]. However, a cautionary note was presented by other investigators, indicating that even though desferrioxamine treatment of fibres may reduce their toxicity, it does not necessarily eliminate it. This was explained by the fact that biologically available low molecular weight chelating agents were also able to bind iron but, in contrast to desferrioxamine, they increased the generation of free radicals [93].

Inflammatory response and inflammatory mediators

Particle-induced activation of macrophages produces inflammatory cytokines which recruit blood PMNs and monocytes and growth-associated cytokines which increase proliferation of fibroblasts and increase collagen synthesis. These cytokines include interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1ra), IL-8, IL-10, platelet-derived growth factor (PDGF), and tumor necrosis factor- α (TNF- α) [94, 95]. Due to the fact that inflammatory cytokines are believed to be central to the development of silica-induced pulmonary toxicities, prophylactic and treatment intervention strategies have been proposed to inhibit the production of these cytokines.

IL-1 receptor antagonist

Piguet *et al.* [96] reported that the continuous administration of IL-1ra inhibited the development of pulmonary fibrosis in

mice resulting from intratracheal instillation of either silica or bleomycin. In fact, IL-1ra caused reduction of fibrosis when administered following fibrosis development.

Anti-rat PMN anti-serum

Using a rabbit anti-rat PMN anti-serum to deplete blood PMN, Henderson *et al.* [97] demonstrated a decrease in the bronchoalveolar (BAL) fluid markers of acute silica lung damage.

Dexamethasone-containing liposomes

Partial protection from silica-induced pulmonary inflammation and fibrosis by the administration of dexamethasone-containing liposomes has been observed [98], thus suggesting a novel drug delivery system with potential to be used in the treatment of silica-induced pulmonary inflammation and fibrosis.

Basic fibroblast growth factor (bFGF)

Anti-bFGF serum was used to treat rabbit experimental silicosis [99]. The results showed that bFGF stimulated

proliferation of fibroblasts, whereas anti-bFGF serum inhibited fibroblast growth. It would appear that anti-bFGF serum blocked proliferation of fibroblasts and fibrosis development in rabbit experimental silicosis.

Inhibition of cell adhesion

Treatment with monoclonal antibodies (mAb) specific for the leukocyte integrins CD-11a or CD-11b, to control the state of activation and traffic of leukocytes, completely prevented collagen deposition and attenuated the fibrosing alveolitis induced by silica. Furthermore, anti CD-11a mAb were also effective in the treatment of an established pulmonary fibrosis, and thus suggesting the possibility of new therapies for pulmonary fibrosis [100].

At this stage, it is worthwhile mentioning other prophylactic and therapeutic strategies which appeared in the literature for protection against silicosis and asbestosis. These ranged from co-administration of anhydrite with quartz [101] and 'iodine bath' [102], to treatment with hydroxypiperquin phosphate [103] and rutin [104]. Studies are therefore needed to further investigate the efficacy of these treatments.

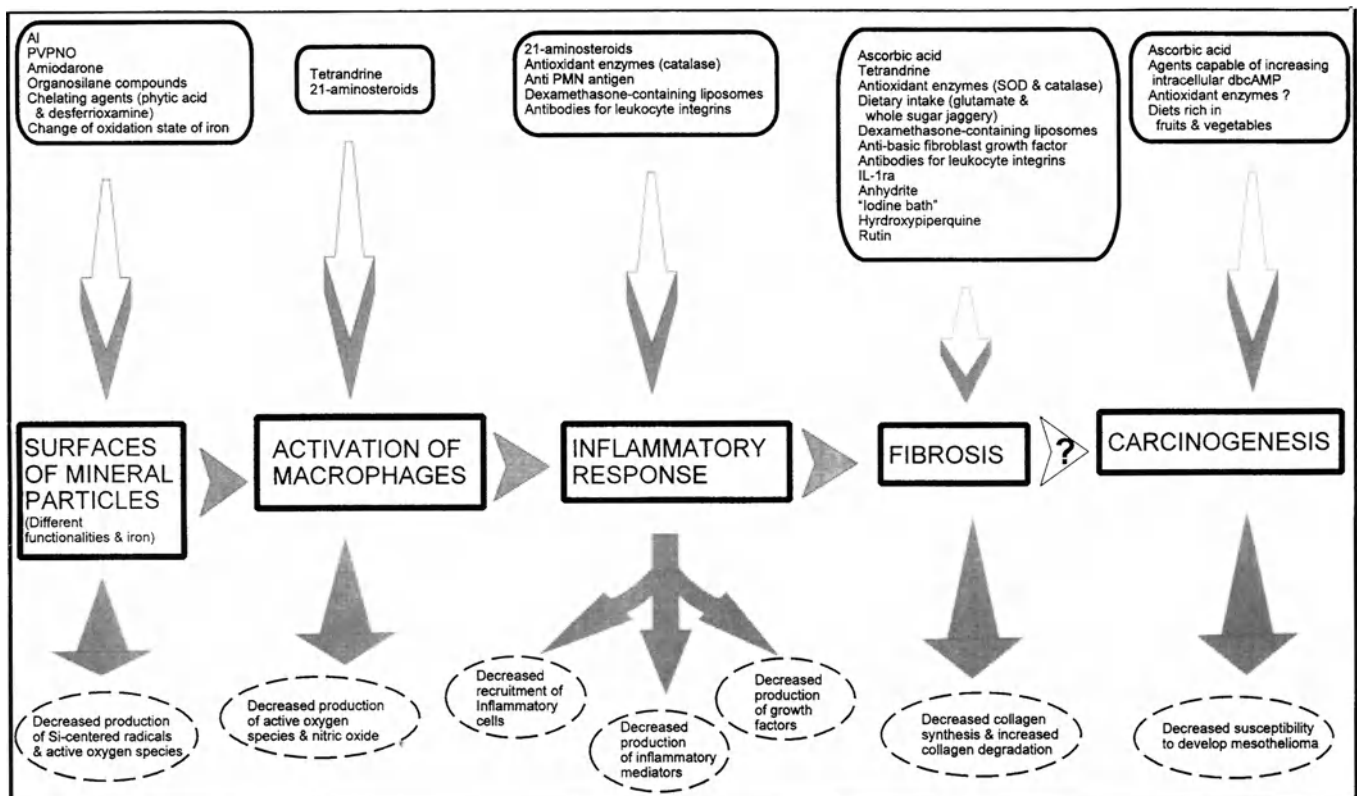


Figure 1. Prophylactic and therapeutic points of intervention by agents on mineral particle-induced pulmonary pathogenicity.

Discussion

Recent progress in the understanding of the mechanisms which mediate mineral particle-induced lung injury, fibrosis and carcinogenesis has produced some rational and specific prophylactic and therapeutic strategies. The mechanisms which are amenable to control include (a) changes in the surface activity of mineral particles by either coating their surfaces or by reducing the availability of iron to participate in the generation of active oxygen species, (b) increase in particle clearance, (c) decrease in the activation of macrophages and the generation of oxidants, (d) decrease in the recruitment of inflammatory cells, (e) decrease in the secretion of inflammatory mediators, (f) decrease in the secretion of growth factors, (g) decrease in the collagen synthesis, and (h) decrease in the cellular proliferation.

Figure 1 shows the agents proposed thus far for intervention at certain mechanistic points to change the pathogenicity of mineral particle. Some of these intervention strategies are already in use and others are still in experimental stages. The important observation, however, is the fact that most of these agents have effects, which, in one way or another, reduce the oxidative stress induced by mineral particles.

Based on the updated use of prophylactic and therapeutic strategies, it can also be seen that it is unlikely that a single strategic approach will suffice for adequate treatment of the whole spectrum of mineral particle-induced pulmonary inflammation, fibrosis, and carcinogenesis. Hence, additive and possibly synergistic effects between a number of intervention strategies need to be considered, in the hope of finding the best possible intervention strategy for lung fibrosis and cancer. Perhaps the future of prophylactic and therapeutic interventions lies in the combination of treatments with different agents which act by various mechanisms. Such a combination is presented in the literature as tetrandrine and PVPNO to treat silicosis [105]. Furthermore, diets rich in fruits and vegetables combined with pharmaceutical interventions with drugs which counteract inflammation, oxidative stress and collagen synthesis may offer the greatest promise in counteracting particle induced pathogenicity. These treatments should be on an ongoing basis, since mineral particles which are not cleared are durable and persist within the lung for long periods of time [106].

Acknowledgement

The author would like to thank Miss Elsa Casimiro for preparing Fig. 1 and Miss Claudina Nogueira for proofreading the article.

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Endotoxin-induced alterations in hepatic glucose-6-phosphatase activity and gene expression

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Abstract

The mechanisms responsible for the glycemic changes associated with endotoxic shock are not fully understood, but are known to involve the ability of the liver to produce glucose. The purpose of the present study was to determine whether endotoxin (LPS) influences the expression and activity of glucose-6-phosphatase (Glu-6-Pase) during the early hyperglycemic phase and the later hypoglycemic phase. Rats were injected with a relatively large dose of LPS (20 mg/kg) or saline (control), and sacrificed at 1 or 5 h post-injection. Both the plasma glucose concentration and glucose production were elevated 1 h post-LPS (2-fold) and both decreased at 5 h postinjection (50%). Compared to time-matched control values, hepatic glucose-6-phosphate and fructose-6-phosphate levels were significantly decreased at both 1 and 5 h. Hepatic Glu-6-Pase activity and mRNA levels were moderately increased, 1 h after injection of LPS. At 5 h, an 88% decrease in mRNA abundance for Glu-6-Pase was associated with a 30% decrease in activity of this enzyme. Plasma insulin concentrations were not different 1 h after LPS and were elevated 2-fold from control values at 5 h. Circulating levels of glucagon and corticosterone were elevated at both time points following LPS. Our data indicate that the LPS-induced hypoglycemia and reduction in hepatic glucose production were accompanied by a depression in Glu-6-Pase activity and gene expression. (*Mol Cell Biochem* **196**: 79–83, 1999)

Key words: phosphoenolpyruvate carboxykinase, hypoglycemia, glucose production, insulin, rats

Introduction

The glycemic response to lipopolysaccharide (LPS; endotoxin) is largely, but not exclusively, the result of changes in the rate of glucose production by the liver. The hypoglycemia that is a hallmark of severe endotoxic shock has been linked to gluconeogenic failure by the liver [1–3]. Although there is a relatively sustained increase in glucose uptake by some tissues after injection of LPS [3], the increase in glucose production that occurs during the early phase of endotoxemia is not maintained and the hypoglycemic phase is characterized by a depression in gluconeogenesis [1, 2]. The mechanism for this impairment, however, is not well defined.

Glucose-6-phosphatase (Glu-6-Pase; EC 3.1.3.9) is a key regulator and the terminal enzyme of gluconeogenesis and

glycogenolysis [4]. Recently, we have isolated a cDNA encoding rat liver Glu-6-Pase [5] so that studies may now investigate the regulation of the expression of this enzyme in various pathophysiological conditions. In previous studies, we demonstrated that the elevated gluconeogenesis observed during the early hyperglycemic phase of hemorrhagic shock is associated with increases in both Glu-6-Pase mRNA levels and enzymatic activity [6]. In addition, *in vitro* studies by our laboratory have shown that Glu-6-Pase gene expression in hepatoma cells can be induced by glucocorticoids and suppressed by insulin [5], two hormones whose concentration changes markedly after LPS administration. Other investigators have reported a decreased Glu-6-Pase activity during the hypoglycemic phase of septic shock [7]. The data from these and other studies indicate that Glu-6-Pase activity and gene expression are acutely regulated in shock. However,

there is a lack of information regarding changes in the expression of this enzyme following LPS administration.

The purpose of the present study was to determine whether LPS influences the expression and activity of hepatic Glu-6-Pase during the early hyperglycemic phase and during the late hypoglycemic phase, and whether these alterations are associated with concomitant changes in the concentrations of glucose and glucoregulatory hormones in the blood.

Materials and methods

Animal model

Male Holtzman rats (250–300 g; Charles River, Wilmington, MA, USA) were used in all experiments. In the first series of experiments, experimental rats ($n = 10$) were lightly anesthetized with ether and injected via the tail vein with saline containing LPS from *Salmonella enteritis* (20 mg/kg; lipopolysaccharide B; Difco Laboratories, Detroit, MI, USA). Time-matched control rats ($n = 10$) were similarly anesthetized and injected intravenously (IV) with an equal volume (1 ml/rat) of saline. LPS produces dynamic hormonal and metabolic changes acutely after *in vivo* administration. In the present study, we examined two time points during this continuum.

At the 1 h time point which is the peak of the hyperglycemic phase, half of the animals from each group were anesthetized with pentobarbital sodium (20 mg/kg, intraperitoneal) and blood and tissue obtained. The remainder of the rats in each group were sacrificed 5 h post-LPS administration, which is representative of the hypoglycemic phase. This model of endotoxemia has been previously described and extensively characterized [8–10]. A portion of the liver from control and LPS-treated rats was freeze-clamped and stored at -70°C for mRNA preparation and metabolite determinations. A second portion of liver was used for the preparation of fresh microsomes which were then stored at -70°C for Glu-6-Pase assay.

In a second series of experiments, rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (90 mg/kg and 9 mg/kg, respectively), and vascular catheters implanted in the carotid artery and jugular vein [3]. The next morning, rats received a primed constant IV infusion of [$3\text{-}^3\text{H}$]glucose (Du Pont-New England Nuclear, Boston, MA, USA) to determine the rate of *in vivo* glucose production. A 7- μCi bolus injection of labeled glucose was administered followed by an infusion of tracer at a rate of 0.083 $\mu\text{Ci}/\text{min}$, which was continued for the remainder of the experimental protocol. Blood samples (350 μl) were collected 120 and 140 min after the start of the tracer infusion to determine the basal glucose concentration and glucose specific activity. Thereafter, rats were injected IV with either LPS or an equal volume (0.5 ml/100 g BW) of sterile 0.9% saline, and serial arterial blood samples (350 μl each) obtained for up to 5 h.

Additional blood (500 μl) was obtained at 1 and 5 h after injection of LPS for the determination of plasma hormone concentrations. After each blood withdrawal, an equal volume of saline was given IV as volume replacement.

Determination of metabolite concentrations

Glucose-6-phosphate (Glu-6-P) and fructose-6-phosphate (Fru-6-P) concentrations in extracts of liver, obtained from the first series of experiments, were determined spectrophotometrically by standard enzymatic methods [11].

Preparation of liver microsomes and Glu-6-Pase activity

Liver microsomes were prepared from LPS-treated and control rats for the assay of Glu-6-Pase activity [6]. The homogenate was prepared using 0.25 M sucrose, 5 mM Tris/acetate, and 0.5 mM sodium EDTA (STE) (pH 7.4). Liver microsomes were isolated by sedimentation at $100,000 \times g$ and resuspended in STE (1/10 volume of homogenate). Glu-6-Pase was assayed by measuring the rate of Pi production colorimetrically [12]. Microsomes were added to Glu-6-P in 50 mM Tris cacodylate buffer (pH 6.5) in a volume of 0.1 ml. Incubations were for 10 min at 30°C . Reactions were terminated by addition of 0.9 ml of working solution (acid molybdate reagent, 10% sodium dodecyl sulphate, 10% ascorbic acid). Color was developed by incubating for 20 min at 45°C , and the absorbance read at 820 nm.

Preparation of mRNA and Northern blot analysis

Total RNA was prepared from liver by extraction with STAT-60, following the protocol provided by the manufacturer (Tel-Test 'B', Freidwood, TX, USA). Total RNA (20 μg per lane) was electrophoresed in a 0.85% agarose, 2.2 M formaldehyde gel using a buffer containing 50 mM boric acid, 5 mM sodium borate, 1 mM sodium citrate, pH 8.0. RNA was denatured with 20 mM methyl mercury hydroxide for 10 min at room temperature before loading. RNA was transferred overnight to a nylon membrane. Membranes were pre-hybridized overnight in a buffer containing 7% polyethylene glycol, 100 mg/ml Salmon Sperm DNA, 50% formamide, and 7% sodium dodecyl sulphate (SDS) at 42°C and hybridized with radiolabeled probe under the same conditions. Membranes were then washed three times for 20 min each with a $0.1 \times \text{SSC}$ and 0.1% SDS at 55°C . The probes were a 1.1 kb fragment of rat liver Glu-6-Pase cDNA [5], a 1.0 kb fragment of the PEPCK cDNA [13], and a 1.4 kb fragment of 18 S ribosomal RNA cDNA labeled by the random primer method [14; Boehringer Mannheim, Indianapolis, IN, USA]. Blots

were subjected to autoradiography and signals quantitated by using a GS 300 scanning densitometer (BioRad, Cambridge, MA, USA). The abundance of Glu-6-Pase and PEPCK were normalized relative to the intensity of the signal measured using the 18 S rRNA probe.

Plasma glucose and hormone concentrations

Plasma glucose concentration was determined by a hexokinase reagent system (Sigma, St. Louis, MO, USA). Glucose specific activity was determined on neutralized supernatants of deproteinized plasma, as previously described [3]. An aliquot of the supernatant was used to determine [^3H]glucose specific activity after tritiated water was evaporated. Glucose production, which estimates hepatic glucose output during the fasted state, was calculated using the non-steady-state equations [3]. Concentrations of immunoreactive insulin (Linco, St. Louis, MO, USA), glucagon and corticosterone (DPC, Los Angeles, CA, USA) in each plasma sample were also determined.

Statistical analysis

All values shown are means $\pm$ S.E. Data were analyzed by Student's *t* test, or ANOVA followed by a Student-Newman-Keuls test to determine treatment effect. Statistical significance was set at $p < 0.05$.

Results

Glucose production and metabolite concentrations

Figure 1 (top) illustrates that the plasma glucose concentration was increased 110% at 1 h after LPS, but had decreased by 50% 5 h after LPS, compared to values from time-matched control animals. Glucose production was elevated 30 min after LPS administration and peaked at 1 h (105%; Fig. 1, bottom panel). The rate of glucose production in LPS-treated rats was not different from basal values at 3 h, but was depressed by an average of 55% at 4–5 h after LPS.

Plasma insulin levels were not different 1 h after LPS administration (Table 1). However, by 5 h post-LPS, the insulin concentrations were increased significantly compared to values from control animals. Plasma glucagon concentrations were increased 1.5-fold at 1 h after LPS and were even further elevated (4-fold) at the conclusion of the experiment (Table 1). Likewise, LPS increased the circulating concentration of corticosterone by 5-fold at 1 h and 2-fold at 5 h after LPS (Table 1).

The alterations in glucose flux were associated with an 80–85% decrease in the hepatic content of Gluc-6-P and Fru-6-P at both 1 h and 5 h post-LPS (Table 2). Glu-6-Pase activity

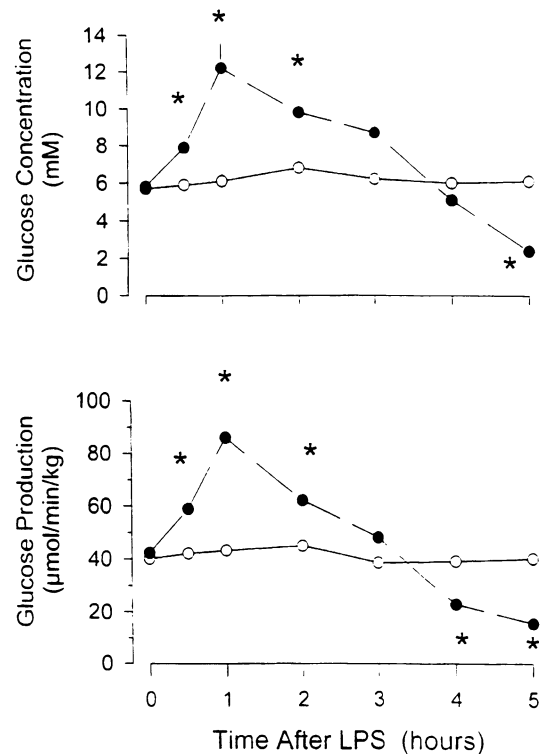


Fig. 1. The arterial plasma glucose concentration (top panel) and the rate of glucose production (bottom panel) in control and LPS-treated rats. Symbols represent saline-injected control (○) and LPS-treated (●) rats. Values are means $\pm$ S.E.M.; $n = 6-7$ rats per group. Where absent, standard error bars are within the symbol. * $p < 0.05$ compared to time-matched control value.

Table 1. Arterial plasma hormone concentrations in control and LPS-treated rats

	Control-1 h	LPS-1 h	Control-5 h	LPS-5 h
Insulin, $\mu\text{U/ml}$	9 ± 2	9 ± 3	7 ± 1	$15 \pm 2^*$
Glucagon, pg/ml	85 ± 2	$238 \pm 52^*$	97 ± 3	$517 \pm 117^*$
Corticosterone, ng/ml	90 ± 46	$567 \pm 36^*$	187 ± 50	$598 \pm 64^*$

Values are means $\pm$ S.E.M. $n = 5$ per group*. $p < 0.05$ compared to control.

Table 2. Glucose-6-Phosphate (Glu-6-P), Fructose-6-phosphate (Fru-6-P) concentration and Glucose-6-phosphatase (Glu-6-Pase) activity in control and LPS-treated rats

	Control-1 h	LPS-1 h	Control-5 h	LPS-5 h
Glu-6-P, $\mu\text{mol/g}$	0.19 ± 0.035	$0.032 \pm 0.004^*$	0.233 ± 0.039	$0.034 \pm 0.006^*$
Fru-6-P, $\mu\text{mol/g}$	0.052 ± 0.008	$0.013 \pm 0.002^*$	0.058 ± 0.009	$0.013 \pm 0.001^*$
Glu-6-Pase, $\mu\text{mol/g/min}$	10.12 ± 1.2	12.98 ± 1.2	10.8 ± 0.68	$7.68 \pm 0.35^*$

Values are means $\pm$ S.E.M.; $n = 5$ per group. * $p < 0.05$ compared to control.

in liver was increased by 28% at 1 h, compared to control values, but was significantly decreased by 30% at 5 h after injection of LPS (Table 2).

Glu-6-Pase and PEPCK mRNA expression

Figure 2 is a representative Northern blot analysis of liver Glu-6-Pase and PEPCK mRNA from control and LPS-treated rats. There was approximately 50% increase between the abundance of Glu-6-Pase mRNA in liver 1 h after LPS, compared to control values and normalized to 18 sRNA (Fig. 2A; LPS = 67 ± 8 AU vs Control = 44 ± 9 AU). However, at 5 h post-LPS, mRNA abundance for Glu-6-Pase was decreased by 88% (LPS = 3 ± 1 AU vs Control 29 ± 2 AU; $p < 0.05$) and PEPCK expression decreased by 70% (LPS = 15 ± 3 AU vs Control = 50 ± 11 AU; $p < 0.05$).

Discussion

The multifunctional enzyme Glu-6-Pase plays a unique role in liver by catalyzing the hydrolysis of Glu-6-P derived from two major glucose producing pathways, namely gluconeogenesis and glycogenolysis. This terminal step produces glucose which is released from the liver and which is central to glucose homeostasis. In order to study the regulation of Glu-6-Pase gene expression following LPS, we have used the recently isolated cDNA for the catalytic subunit of rat liver Glu-6-Pase [5]. Our data demonstrate that there was a slight increase in Glu-6-Pase activity in liver during the early hyperglycemic phase of endotoxemia with a moderate increase in mRNA levels. Other insults, such as hepatectomy [15], and hemorrhagic shock [6], have been shown to upregulate Glu-6-Pase expression within 30 min. Moreover, increase in mRNA abundance is also observed in streptozotocin-induced diabetes in which hepatic glucose production is elevated [15–17]. Although, the data suggest that increased Glu-6-Pase may contribute to hepatic glucose production during the hyperglycemic phase of endotoxemia, other factors, such as a marked decrease in tissue glycolytic rates as a result of decreased fructose 2,6-bisphosphate levels, may have to be considered [6, 18].

In contrast, during the subsequent hypoglycemic phase, both hepatic Glu-6-Pase mRNA expression and activity were decreased. A reduction in Glu-6-Pase enzyme activity has been previously reported [7, 19]. This response is comparable to the drop observed following chronic administration of the proinflammatory cytokine TNF [20]. The decrease in Glu-6-Pase may be responsible, at least in part, for the impaired rate of glucose production manifested in these conditions. However, a decrease in gluconeogenesis mediated solely by a reduction in Glu-6-Pase would be expected to result in the accumulation of Glu-6-P, and hence an increase in the level of Fru-6-P within the liver. Our results clearly demonstrate a marked reduction in the concentration of these hexose monophosphates. This decrease suggests that there is a coordinated decrease in the activity of a more proximal gluconeogenic enzyme, such as PEPCK which catalyzes the rate controlling step in gluconeogenesis from lactate/pyruvate to Fru-6-P. Other investigators have reported that hepatic PEPCK activity and gene expression are inhibited following endotoxic or septic shock [21, 22]. An LPS-induced decrease in PEPCK mRNA abundance was confirmed in the present study. Wang and colleagues [22] have characterized the changes in the gene expression of this enzyme following LPS. Steady-state mRNA levels of PEPCK were decreased 3–6 h after injection of LPS. Therefore, because endotoxic shock alters the expression and activity of several gluconeogenic enzymes, our data cannot provide a basis on which to evaluate the relative contribution of the decrease in Glu-6-Pase in mediating the fall in glucose production. Alternatively, or in addition to, the decrease in hepatic hexose monophosphates may have resulted from a stimulation in the glycolytic rate mediated by an increase in the concentration of fructose-2,6-bisphosphate within the liver [7].

Following LPS administration, there are changes in the circulating concentrations of insulin, glucagon and cortico-

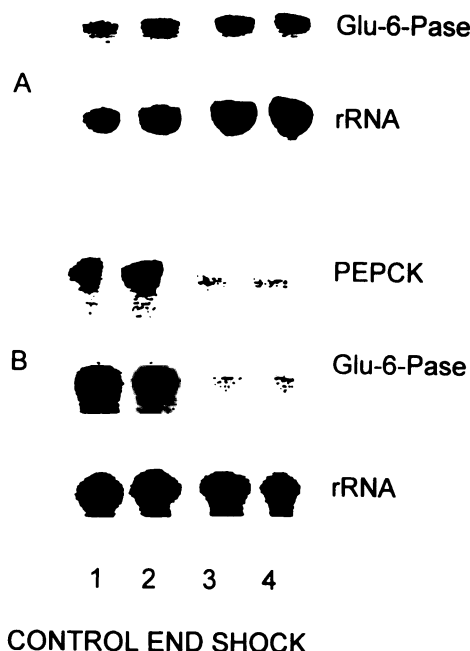


Fig. 2. (A) Representative Northern blot of rat glucose-6-phosphatase (Glu-6-Pase) mRNA in liver from control animals (lanes 1–2) and in animals 1 h after injection of LPS (lanes 3–4). (B) Representative Northern blot of rat phosphoenolpyruvate carboxykinase (PEPCK) and Glu-6-Pase mRNA in liver from control animals (lanes 1–2) and in animals 5 h after LPS (lanes 3–4).

sterone. All of these hormones have been demonstrated to influence Glu-6-Pase activity and/or mRNA expression [5, 18]. We have previously demonstrated that in FAO hepatoma cells, dexamethasone increase Glu-6-Pase mRNA over basal level and insulin greatly decreased mRNA levels [5]. In cells incubated with both dexamethasone and insulin, insulin completely blocked the increase seen with dexamethasone alone [5]. It is reasonable to assume, therefore, that the increase in Glu-6-Pase gene expression following 1 h LPS could be due to the increase in corticosterone level, and the subsequent decrease in Glu-6-Pase gene expression following 5 h LPS could be the result of increase in insulin concentration. However, to better define the role of insulin and glucocorticoids (and other hormones) in mediating the LPS-induced changes in Glu-6-Pase mRNA, subsequent experiments must be designed to prevent or minimize the changes in a single hormone.

Acknowledgements

The authors would like to dedicate this paper to the memory of Wansong Pan. This work was supported by National Institutes of Health Grant R01GM52025 (S.R.M.).

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Oxidative stress in Systemic Sclerosis

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Abstract

In 63 patients affected by Systemic Sclerosis (SSc) (limited subset, 40; diffuse subset: 23; early: 30; advanced: 33) the peroxidation product diene-conjugates (DC) and antibodies against oxidised low density lipoproteins (Ab oxLDL) were tested in serum by a spectrophotometer (absorbance 234 nm) and by a standard ELISA respectively. The data were compared with those obtained by 21 healthy subjects. DC was significantly higher in patients ($73.3 \pm 37.2 \mu\text{M/l}$; $p < 0.0001$) than in controls (48.4 ± 16.7) as well as in the limited (80 ± 48.8 ; $p < 0.05$) than in the diffuse subset (64.5 ± 36.4); and in early (84.1 ± 31.4 ; $p < 0.05$) than in advanced stage of the disease (67.9 ± 42.5). The levels of Ab oxLDL were significantly higher in SSc patients ($309.5 \pm 367.2 \text{ mU/ml}$; $p < 0.0001$) in all its subsets (limited: 351.9 ± 351.1 , $p < 0.0001$; diffuse: 207.7 ± 316.1 , $p < 0.05$; early: 428.9 ± 417.1 , $p < 0.001$; advanced: 302.7 ± 89.9 , $p < 0.0001$) than in controls (89.3 ± 29.1). These antibodies levels were higher in limited subset than in diffuse ($p < 0.05$) and in early SSc than in advanced SSc ($p < 0.05$). The highest values of parameters of oxidative stress are found in the early stages, when the episodes of reperfusion after ischemic episodes (Raynaud's phenomenon) are very frequent. Moreover, the damage is higher in the early stages of SSc, with intact microvessels, than in late stages, when microvessels are very reduced in number, destroyed by the worsening of the disease. These data show that the respiratory burst deduced by the lipoperoxidation is higher in SSc than in controls, and may be an important pathogenetic factors involved in tissue changes in SSc. (Mol Cell Biochem **196**: 85–91, 1999)

Key words: oxidative stress, Systemic Sclerosis, diene-conjugates, antibodies against oxidised low density lipoproteins, reperfusion injury

Introduction

Systemic Sclerosis (SSc) affects the whole organism (skin, lungs, heart, kidney and gut) and involves microvessels, immune system, fibroblasts and extracellular matrix leading eventually to fibrosis. Vessels are the target of the disease in the early phase, when dysfunction of the vascular tone and tissue edema are the predominant clinical signs. It has been demonstrated that different factors may contribute to the inflammation and derangement of the endothelium, thus favoring the adhesion and homing of immune cells and alteration of the coagulative/fibrinolytic system [1]. One of these factors is the stress induced on the endothelium by

oxygen free radicals (OFR) and reactive nitrogen species, generated locally by the inflammatory process and by frequent episodes of reperfusion injury (Raynaud's phenomenon). This has led to hypothesize that free radicals – that are highly reactive chemical species because of one or more unpaired electrons – are deeply involved in the pathogenesis of SSc [2]. The most important free radicals are superoxide anion ($\text{O}_2^{\cdot-}$), reactive nitrogen species as nitric oxide ($\text{NO}\cdot$) and hydroxyl radicals ($\text{OH}\cdot$) which derives from the reaction of hydrogen peroxide (H_2O_2) with $\text{O}_2^{\cdot-}$ [3, 4].

Phagocytic cells (e.g., neutrophils and macrophages) generate free radicals, through nicotinamide adenine dinucleotide phosphate (NADPH) dependent oxidoreductase

systems [5] and are used by phagocytic cells as microbicidal agents and for digestion of foreign material or immune complex [6, 7]. Also other cells with NADPH-oxidase, such as endothelial cells, chondrocytes, lymphocytes and fibroblasts produce free radicals [8, 9]. In humans the other endogenous generator of free radicals is xanthine-oxidase (XO), an enzyme involved in purine breakdown [11] and present predominantly in endothelial cells of small blood vessels [10]. Under ischemic conditions, xanthine dehydrogenase is converted to XO [12], which, in presence of purine substrates (Hypoxanthine and Xanthine), produces large amounts of O_2^- , $OH\cdot$ and H_2O_2 [13]. The targets of free radicals damage are lipids [14], DNA, proteins, proteoglycans, collages [15] and immune system [16]. Thus, microvascular endothelial cells are susceptible to free radicals injury during reperfusion because they are deficient in the synthesis of catalase, a critical enzyme in the natural defense against superoxide damage [17]. Free radicals may damage lipids through lipid peroxidation, an oxygen radical mediated process [3]. Polyunsaturated fatty acids are susceptible to free radicals attack and, as polyunsaturated fatty acids are fundamental elements of cellular membranes, their alteration may give rise to cellular dysfunction and death [18]. Lipid peroxidation of cellular membranes yields a variety of end products including saturated and unsaturated hydrocarbons, aldehydes, (as malonal-dialdehyde, a thiobarbituric acid reactive substance), and diene conjugates. These lipid peroxidation end products may themselves be cytotoxic and alter T cell/macrophage interactions [14]. The polyunsaturated fatty acids in plasma low density lipoproteins can be oxidised by endothelial cells and macrophages [19]. Such oxidised low density lipoproteins (LDL) show chemotactic properties for monocytes, are cytotoxic for endothelial as well as smooth muscle cells [20], bind to collagens [21], stimulate connective tissue formation [22] and monocyte endothelial cell interaction [23]. Thus, free radicals can induce increased permeability of endothelial cells [24], the deposition of extra layers of basal lamina and the further compensatory regeneration of intimal cells within the old basal lamina [25]. These events result in a progressively narrowing of the microvessel lumen, causing either proliferation of surrounding fibroblasts or localized and distal ischemia with the latter generation of more OFR and reactive nitrogen species. The further damage to endothelial cells may lead to a positive feedback effect of luminal narrowing and ischemia and therefore to the birth of a vicious cycle of Xanthine-Oxidase-mediated OFR generation, leading to endothelial cell damage, intimal thickening and fibrosis [2]. The exposure of endothelial-damaged nuclear contents may provoke an autoimmune response to various nuclear antigen leading to autoantibody formation and further inflammatory cells presence which themselves release large amounts of superoxide radicals during the respiratory burst by NADPH oxidase [26, 27]. Even at lower concentrations, free radicals

stimulate fibroblasts [28] to proliferate and produce collagen (Fig. 1).

During acute ischemia, xanthine-oxidase can generate OFR [2]: in SSc, Raynaud's phenomenon (RP) produces frequent daily episodes of reperfusion injury and several events of free radicals-mediated endothelial derangement. In SSc, free radicals damage capillary walls through peroxidation of lipid components of the endothelial cell membrane and degradation of collages and hyaluronic acid of the basement membrane [29]. The possibility that oxidative stress may be a pivotal factor in the pathogenesis of SSc prompted us to investigate, in different phases of the disease, the products of lipid peroxidation and the formation of antibodies against oxidized lipoproteins.

Patients

Sixty-three patients (60 female and 3 male, mean age $\pm$ S.E.M.: 53 years $\pm$ 3.8) affected with SSc were classified according to LeRoy *et al.* [30] and divided in the limited subset (LSSc) (40 patients) and the diffuse subset (DSSc) (23 patients), that were then subdivided in the early (30 patients) and advanced (33 patients) phase of the disease. The four groups of patients were subdivided as follows: 21 early-limited; 19 advanced-limited; 9 early-diffuse; 14 advanced-diffuse.

Twenty-one control subjects (mean age $\pm$ S.E.M.: 51 years $\pm$ 2.1, 17 female and 4 male) matched for age and sex were recruited from healthy volunteer hospital workers.

Materials and methods

Diene-conjugates

Diene-conjugates were measured in serum with a spectrophotometer by the increase of the absorbance at 234 nm [31].

Antibodies against oxidised LDL

Ca^{2+} -oxidised LDL is coated onto microtiter strips as antigen. Autoantibodies, if present in prediluted serum (1:50), bind specifically to the antigen. After a washing step (4 following washings with 300 μ l diluted washing buffer), a specific peroxidase-conjugated anti human IgG antibody detects the presence of bound antibodies. After removal of unbound conjugate through washing, tetramethylbenzidine is added to the wells as a non toxic chromogenic substrate. The concentration of specific IgG in the sample is quantified by an enzyme catalyzed color change detectable on a standard ELISA reader. The amount of color developed is directly proportional to the concentration of antibodies in

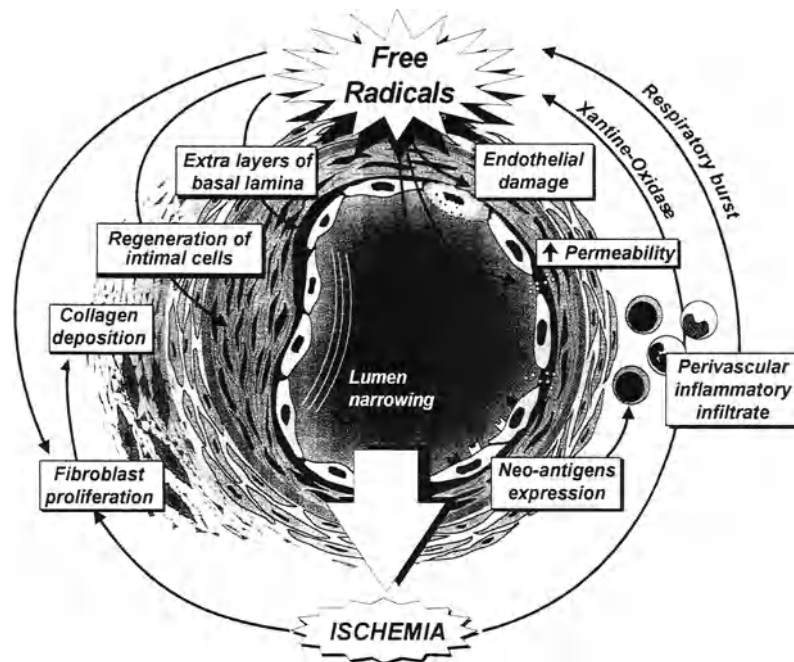


Fig. 1. Representation of potential role played by oxygen free radicals in the pathogenesis of Systemic Sclerosis.

the sample. The assay is standardized with defined amounts of antibodies against oxidised LDL in a serum matrix. The IgG concentration in the samples is quantified in mU based on a characterized serum with high titers of antibodies against oxidised LDL titer and a low cross reactivity with native LDL (< 5%) [32, 33].

Statistical analysis

Assessment of significant variations between the control group and SSc patients and between several disease groups was made using unpaired Student's *t*-test; the level of significance was taken as $p < 0.05$.

Results

Diene-conjugates

Serum levels of DC ($73.3 \pm 37.2 \mu\text{M/l}$; $p < 0.0001$) were significantly increased in 63 SSc patients vs. controls (48.4 ± 16.7) and both in LSSc (80.0 ± 48.8 ; $p < 0.0001$) and DSSc (64.5 ± 36.4 ; $p < 0.01$), being higher in limited than in diffuse subset ($p < 0.05$) (Fig. 2A).

DC were increased in the early (84.1 ± 31.4 ; $p < 0.0001$) as well as in the advanced phase of SSc (67.9 ± 42.5 ; $p <$

0.01), being higher in early edematous phase than in the advanced atrophic phase ($p < 0.05$).

When patients with LSSc or DSSc were subdivided in the early and advanced phase, DC resulted significantly higher in early-LSSc (83.4 ± 34.1 ; $p < 0.05$) than in early-DSSc (67.6 ± 30.9). No statistical difference was found between the levels of DC in advanced-LSSc and in advanced-DSSc. In early-LSSc, DC were significantly higher (83.4 ± 34.1 ; $p < 0.05$) when compared to advanced-LSSc (62.5 ± 12.3). No difference was found between advanced-DSSc and early-DSSc (Fig. 2B).

Antibodies against oxidised LDL

The levels of Ab oxLDL ($309.5 \pm 367.2 \text{ mU/ml}$; $p < 0.0001$) were significantly higher in the 63 SSc patients, as well as in LSSc (351.9 ± 351.1 ; $p < 0.0001$), in DSSc (207.7 ± 316.1 ; $p < 0.05$), in early SSc patients (428.9 ± 417.1 ; $p < 0.001$) and in advanced SSc patients (302.7 ± 89.9 ; $p < 0.0001$) than in controls (89.3 ± 29.1) (Fig. 3A). The antibody levels in early SSc were higher than advanced SSc ($p < 0.05$) and were higher in LSSc when compared to DSSc ($p < 0.05$). Moreover Ab oxLDL resulted higher in early-LSSc (376.1 ± 269.6 ; $p < 0.005$) when compared to early-DSSc (186.3 ± 50.1), while no statistical difference between advanced-LSSc and advanced-DSSc was found. At last, Ab oxLDL were significantly higher in early-LSSc than in advanced-LSSc

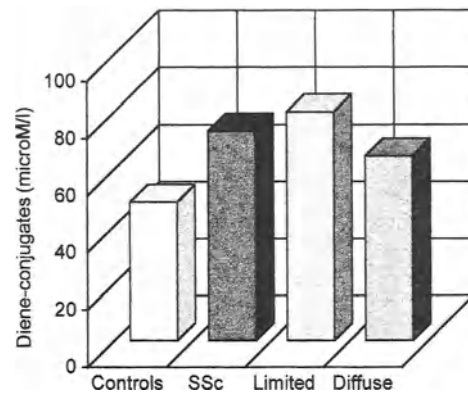
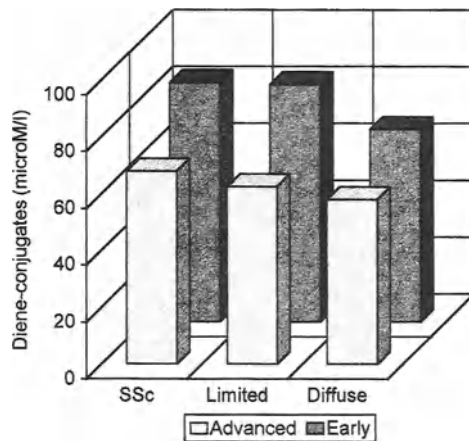
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Fig. 2. (A) Diene-conjugates serum levels in controls, in whole group of Systemic Sclerosis and in different subset of cutaneous involvement, limited and diffuse are shown; (B) The serum levels of Diene-conjugates in Systemic Sclerosis (limited and diffuse subset) are shown in the different phases of disease (early and diffuse).

(253.3 ± 216.1 ; $p < 0.01$), while no difference was found between early-DSSc and advanced-DSSc (Fig. 3B).

Discussion

In SSc, free radical-induced oxidative stress may occur either as a result of hypoxic/ischemic episodes and upon reperfusion of tissue, or because of altered immune process [2–4]. Thus, the formation of free radicals and lipid peroxidation seem to be a pivotal factor in the initiation of tissue damage in RP and SSc, and may be directly or indirectly involved in most of the other pathogenetic mechanisms of the disease. Increased levels of urinary F2-isoprostane, a measure of oxidative stress, provide further evidence that free radical-induced

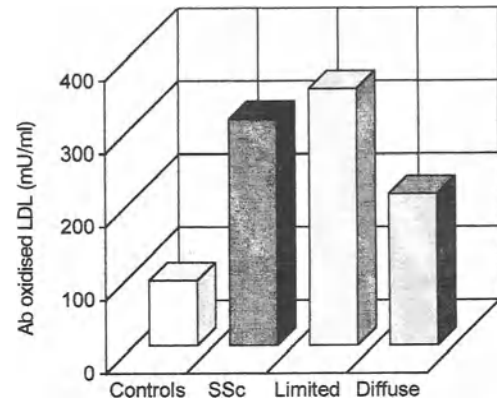
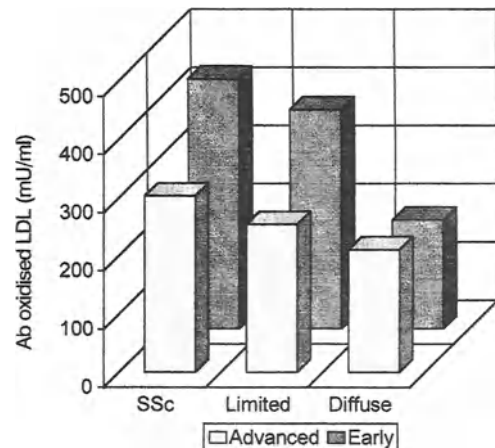
**A****B**

Fig. 3. (A) Antibodies against oxidised LDL in controls, in whole group of Systemic Sclerosis and in different subset of cutaneous involvement, limited and diffuse are shown; (B) The serum levels of antibodies against oxidised LDL in Systemic Sclerosis (limited and diffuse subset) are shown in the different phases of disease (early and diffuse).

oxidative injury occurs in SSc [35]. The presence of increased numbers of free radicals in RP and SSc patients has also been suggested based on the high rate of chromosomal instability noted in these patients relative to healthy individuals [36]. The importance of free radicals in SSc pathogenesis is stressed by the evidence that scleroderma antigens are sensitive to reactive oxygen species and result in fragmentation: autoantigens are cleaved at highly specific sites in a reaction that requires metal binding and the presence of OFR [37]. These data suggest that autoantibodies may reflect protein fragmentation provoked by ischemia reperfusion when specific metals (e.g. iron or copper), of critical pathogenic importance, are present.

In patients with SSc and RP, deficient levels of the antioxidants selenium, ascorbic acid [38], vitamin E and B-

carotene have been detected suggesting abnormalities of oxidative potential, favoring the development of injury mediated by oxygen radicals [39]. Herrick *et al.* documented the abtural intake of micronutrient antioxidants in patients with RP and SSc and found a reduced level of selenium and ascorbic acid not depending on a dietary deficiency. The authors suggested that malabsorption or altered renal clearance of antioxidants might explain their abnormal low level in plasma [40]. Furthermore, the antioxidants decrease was accompanied by increased levels of 9-cis, 11-trans-linolenic acid, an indirect marker of free radical activity on plasma unsaturated fatty acids, suggesting that free radicals are generated in SSc [38]. After an accurate analysis, the same authors found that the measurement of usual circulating markers of oxidative stress (lipid isomerization and lipid peroxidation products) are inappropriate because they are not the earliest target of reperfusion injury or are insufficiently sensitive or do not reflect leukocyte activation [41]. The levels of plasma superoxide dismutase, a main component of the antioxidant defenses, have been found higher in SSc than in healthy subjects and correlated with RP and skin involvement: the authors suggested that the enzyme activity may be a useful parameter for assessment of sclerotic progression [42].

Our data show clearly that oxidative stress takes place in the early phase of the disease and that the immune reaction against the products of lipid peroxidation is greater during this phase than in advanced phase of the disease. In advanced phase, the generation of oxidative stress products is less pronounced, probably because the episodes of reperfusion injury, neutrophil accumulation and endothelial damage are significantly reduced.

The highest levels of DC are found in the early edematous phase, when the episodes of RP-reperfusion injury are frequent and the endothelial activation and injury is in progress. In this phase of the disease, the increased formation of OFR and reactive nitrogen species contributes strongly to disease pathogenesis [2].

Also the products derived from free radicals, like DC, are chemiotactic and noxious to endothelial cells. This suggests that not only free radicals but also their end products may maintain the disease process triggered by OFR and reactive nitrogen species. Probably, the significant reduction of DC later in the disease indicates that the strength of oxidative stress is reduced proportionally to the reduction of the disease activity.

It is well known that oxidative stress can damage proteins, DNA and eventually lipids, but it is very difficult to evaluate their damage, that occur early in many diseases. Instead, it is easier to assess lipid peroxidation, that often occurs late in the injury process. In SSc, an increase in oxidized lipoproteins [43] as well as an increased susceptibility to oxidation of LDL [44] has been detected. However, it is likely that increased

oxidative damage occurs in most, if not all, human diseases but plays a significant pathological role only in some of them. Peroxidation is important in atherosclerosis, in patients with idiopathic pulmonary fibrosis and in early tissue injury caused by ischemic or traumatic brain damage [45]. Oxidation of LDL may be implicated in the development of atherosclerosis disease and its progression [46] and high titer of oxLDL is related to increased risk of ischemic heart disease [47].

Cell-mediated LDL-oxidation can cause endothelial cell dysfunction promoting atherogenesis, increased adhesion molecule expression, monocyte recruitment, impaired production or release of nitric oxide, and proliferation of smooth muscle cells [48]. Stimulation of both outgrowth of smooth muscle cells from vascular tissue and their proliferation induced by oxidised-LDL are significant pathological events in atherogenesis and restenosis [49, 50]. While native LDL have no cytotoxic effect, free radical mediated oxidation of LDL during atherosclerotic and ischemic conditions creates a molecule with a great potency for altering cell calcium and ultimately cell function and viability of a wide variety of cell types [51]. High titer of Ab oxLDL is present in plasma of patients with coronary atherosclerosis: in these patients, LDL oxidation takes place *in vivo* and plays a critical role in the development and progression of atherosclerosis [52]. In relation to SSc, Blake *et al.* demonstrated that in stored plasma, where the LDL fraction may become oxidized, samples from patients with SSc were much more cytotoxic than those from controls and this property was attributed to the oxidised LDL fraction [53].

In our patients the highest levels of Ab oxLDL are predominant in the early phase of both subsets, limited and diffuse, but more increased in the limited form. This may be linked to the pathophysiology of the limited form, where RP-reperfusion injury is predominant and lasts for a longer period than in diffuse SSc, where the loss of capillaries may significantly lessen the episodes of reperfusion injury.

In conclusion, our work shows that oxidative stress may have a pivotal role in SSc in particular in the early phase of the disease. Further studies are needed to verify the capacity of a single or combined antioxidant therapy to lower oxidative stress and the damage to the vessels.

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Signal transduction and transcriptional adaptation in embryonic heart development and during myocardial hypertrophy

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Abstract

In comparing the pathological state of cardiac hypertrophy with early embryonic growth and development of the primitive heart, important and informative aspects of mechanisms that underlie activation of the gene expression pattern become apparent. Interestingly, in both cases the muscle phenotypes share the expression of a 'fetal' gene expression program, raising the question whether the same genetic mechanism is being called upon by signals associated with the onsets of cardiogenesis and myocardial hypertrophy. A cell specific transcription factor, CLP-1, was recently identified in our laboratory that is likely to play a crucial role, in conjunction with other known regulatory factors, in early cardiac events leading to cardiogenic cell specification and differentiation. We have also identified a novel mechanism that involves activation of the Jak/Stat signaling pathway that is linked to the autocrine angiotensin-II loop associated with the hypertrophic response in cardiomyocytes. Since early cardiac cell development and the hypertrophic state involve the expression of the same battery of genes, one may speculate that common transcription factors may account for assembling a competent apparatus responsible for transcribing the genes. Our present studies are designed to investigate the potential role of these factors in control of both processes. (*Mol Cell Biochem* **196**: 93–97, 1999)

Key words: signal transduction, myocardial hypertrophy, cardiogenesis, transcriptional adaptation

Introduction

Development of the heart is a complex process involving coordinated evolution of various cell types and their organization leading to a four chamber specialized heart pump. Perturbation of this process in fetal development leads to congenital heart defects causing various syndromes and disorders in prenatal, postnatal and adult life. In addition, under various pathophysiological stimuli, the adult heart undergoes a heterogeneous adaptive process leading to its hypertrophic growth. Cardiac hypertrophy is of particular interest to developmental biologists as it involves revival of the embryonic gene expression program and although, in virtually all cases, the cause and effect relationship is well

established, the underlying mechanism(s) is not known [1]. Recent advances in molecular techniques and genetic manipulations have considerably enhanced our understanding of the embryonic heart development, which is the net result of coordinated expression of early genes for regulatory proteins and signalling molecules and that of the downstream genes for structural and functional proteins. Interestingly, the embryonic gene expression pattern resurges in the cardiac hypertrophy, which is characterized at the molecular level, among others, by an increase in protein synthesis, up-regulation of the embryonic genes and induction of the immediate-early response genes [1]. Thus, unravelling the mechanism underlying the control of embryonic heart gene expression would bear relevance to that of the resurgence of

the embryonic gene activation pathway during the onset of hypertrophy in the myocardium.

Gene control in cardiac specification and differentiation

The acquisition of cell specification in early embryos is believed to be determined by the appearance of region-restricted and lineage-specific transcription factors which influence the pattern of the downstream tissue specific gene expression. Cardiogenic cell lineage assignment is one of the first functional specifications in the developing embryos [2–4]. The cardiac cell commitment itself is perceived as a progressive process dictated by inductive signals originating from the axis and subsequently from the anterior endoderm [5–9]. Cells derived from chicken embryo stage 4, but not of stage 3, differentiate into myocytes, suggesting that cardiac specification occurs in chicken at stage 4, even though cardiogenic origin is traceable to the mesoderm of earlier stages [8, 10]. This would suggest that despite some level of commitment of the early mesodermal cells, a second inductive state is required permitting the cells to enter cardiac differentiation. Therefore, in considering the complex and progressive nature of inductive interactions that specify cardiogenesis [6–8], it is likely that multiple regulatory factors participate in mesodermal cell commitment.

Growth factors in early cardiogenesis

Several growth factors are known to play a role in early cardiac events which lead to specification of cell types and tissue differentiation. Although there has been a significant progress in understanding the role of polypeptide growth factors in establishment of the body plan [11, 12], their role in cardiac organogenesis is largely unknown [13, 14, 16]. In developing heart, the proliferative cardiac myocytes produce AFGF and BFGF which stimulate cardiac growth [13]. TGF- β is also involved in cardiac muscle growth and differentiation [14] and has been shown to induce differentiation of the presumptive cardiac mesoderm explanted from axolotl [15]. Other growth factors, such as FGF, TGF, NGF, also act as positive inducers of cardiac differentiation in uncommitted chicken embryonic cells of posterior origin [17]. Bone morphogenetic proteins, which express in peculiar pattern at gastrula stage chicken embryo induce cardiac differentiation [18]. Antisense oligonucleotides complementary to FGF-2 mRNA inhibit proliferation and contractility of cultured precardiac mesoderm cells [19]. The signal transduction pathways induced by these growth factors have been well

investigated in the context of their effect on stimulating hypertrophic growth in cultured cardiac myocytes [20] and it is expected that further understanding of the precise role could help delineate the contribution of those factors in heart development during embryogenesis.

Genes involved in early embryonic heart development

As in the case of other organs, the development of cardiovascular involves coordinated induction of various cell lineages, their differentiation, morphogenesis, innervation and attachment with other body structure [9, 21]. Since this results from the programmed expression of numerous genes, the involvement of cell type specific transcriptional apparatus underlies the onset of events in the entire process. In the developing embryo, a number of genes encoding transcription factors have been implicated in heart development [22–24]. Myocyte enhancer factors (MEF-2) constitute a sub-family of proteins which belongs to the MADS (MCMI, agamous, deficiens and serum response factor) family of transcription factors. MEF-2 proteins bind to the A/T-rich region of muscle promoters [24] and have been documented to play a crucial role in control of myogenesis [23, 25, 26]. The fact that multiple isoform of MEF-2 exist which bind to functional DNA sites points to their potential in execution of diverse functions in the regulation of gene transcription in various muscle types. During early mouse embryogenesis, MEF-2 expression marks the skeletal as well as cardiac cell lineages [26, 27]. Since the bHLH-MRFs are absent in cardiac muscle cells, we have proposed earlier that MEF-2s play a role analogous to bHLH muscle regulatory factors (MRFS) in cardiac muscle formation [25], possibly via the recruitment of other non-HLH regulatory co-factors. This is based on our findings that a member of the MEF-2 family, CLP-1 (see below), appears very early in chicken cardiogenic development, prior to cardiac fate assignment in the embryos [28]. In *Drosophila*, a homeodomain protein encoded by the *tinman* gene was shown to be essential for heart and visceral muscle formation [29]. A murine homologue of *tinman*, Nkx-2.5, is expressed in early cardiac progenitor cells prior to activation of the myogenic program [22]. Nkx-2.5 transcripts are subsequently found in the myocardial layer of the embryonic and adult heart and other non-cardiac tissues [30]. The homology between Nkx-2.5, XNkx-2.5 (amphibian homologue of Nkx-2.5) and *tinman* and the similarity of their expression pattern suggest a conserved role of these proteins in heart development [31]. Targeted inactivation of Nkx-2.5 resulted in heart morphogenic defects at embryonic day 8.5. A beating linear heart tube developed but subsequent looping was impaired [32]. In addition, there was a selective inactivation of MLC-2 gene expression even though the DNA element, CTNNAGTG, targeted by Nkx-2.5 is absent in the

MLC-2 promoter [33, 34]. One possibility is that Nkx-2.5 regulates one chain of events during heart development and it affects the expression of MLC-2 via the regulation of other transcription factors located further downstream in that cascade. This concept is supported by recent studies on cooperative interactions of Nkx with SRF and GATA-4 in cardiac muscle specific gene expression [33, 35]. GATA-4, a member of the GATA family of zinc finger domain proteins is also implicated in cardiac muscle specific transcription [36]. GATA-4 is expressed in the precardiogenic splanchnic mesoderm and activates the expression of a number of cardiac specific genes. Antisense inactivation of GATA-4 transcripts in embryonal carcinoma P19 cells inhibits contractile protein gene expression and cardiac muscle differentiation *in vitro* [37]. Two other members of the GATA family, GATA-5 and GATA6, also have similar expression pattern and are capable of activating cardiac specific genes [38, 39]. Targeted inactivation of GATA-4 gene causes morphogenetic defects but does not result in inactivation of marker genes, such as cardiac myosin heavy chain, troponin C, myosin light chain-2 [25, 40]. Therefore, it appears that both GATA-4 and Nkx-2.5 are essential for heart formation but none is required for initiation of cardiogenesis.

In addition to NKx and GATA family of transcription factors, several basic helix-loop-helix (bHLH) domain proteins have also been identified in the developing myocardium. Two such proteins, eHAND and dHAND, are expressed in mesodermal and neural crest-derived structures of the heart. Interestingly, in contrast to their homogenous expression in avian heart [41], in developing mouse heart, they are expressed in a complementary fashion and are restricted to segments of the heart tube fated to form the right and left ventricles [42]. Targeted inactivation of dHAND in mouse embryo results in embryonic lethality due to heart failure [43]. Several other transcriptional regulators such as Aryl hydrocarbon receptor, Aryl hydrocarbon receptor translocator and TEF-1, have also been implicated in heart development [36, 44], however, their precise biological role is not known.

CLP-1 (Cardiac Lineage Protein) is a candidate regulator of cardiogenesis

Despite the wealth of information on the putative cardiac regulatory factors described above, none of them were detected, at least in avian embryos, in cardiac progenitor cells in stages of development prior to cardiac fate assignment (stage 4), a criterion in accord with the expected role of the putative regulator(s) of cardiac cell lineage. We have previously reported that the binding activity of a cardiac specific transcription factor, BBF-1 (subsequently called CLP-1), which recognizes the MEF2 site in the cardiac MLC-2

promoter [45], appears in chicken blastoderms preferentially early at stage 4 and prior to the onset of cardiogenic gene expression [28]. The expression patterns of BBF-1 and CMD-1, the chicken homolog of MyoD [46], in early avian development are consistent with the established patterns of cardiac and skeletal morphogenetic development respectively and provide a compelling argument in favor of the role of BBF-1/CLP-1 in regulation of early cardiac development [27, 47]. The isolation of the BBF-1 CDNA from the early (stage 6) chick embryonic CDNA library and its characterization has demonstrated it to be a novel gene involved in early steps of cardiogenic regulation. The bacterially expressed CLP-1 protein binds with specificity to element B (MEF2 site) in cardiac MLC-2 promoter, and is antigenically distinguished from MEF2 proteins. The CLP-1 specific mRNA transcripts were detected as early as stage 1/2 of development, making CLP-1 expression the earliest marker of cardiogenesis to our knowledge. The expression pattern of CLP-1 based on the whole mount *in situ* hybridization assay coincides remarkably with the established pattern of appearance of the heartforming region in early embryos and is restricted in the late development primarily to the embryonic heart. Interference of CLP-1 mRNA by use of antisense CLP-1 oligonucleotides in cultured whole embryos caused impairment of the developing heart and arrested the fusion of the cardiac primordia. Taken together, CLP-1 is a novel transcription factor whose expression pattern is strikingly in agreement with the appearance of cardiogenic morphogenetic field. Since the CLP-1 mRNA is detectable prior to stage 4, it appears to be a powerful candidate regulator likely to play a role in avian cardiac muscle cell specification.

Cardiac hypertrophy is a revival of the embryonic gene expression program

Cardiac hypertrophy is a general adaptive response of the heart to work-overload and to many cardiac disorders. Studies on various models of hypertrophy, both *in vivo* and *in vitro*, have revealed that the molecular manifestations of hypertrophic response can be as divergent as its etiology (see [48] for review). However, one common feature of various hypertrophic responses is a partial revival of the embryonic gene expression program such as the reexpression of ANF (atrial natriuretic factor) and skeletal alpha actin genes in the left ventricle. In addition, expression of several other sarcomeric genes, such as MHC, vMLC2, is upregulated during hypertrophy [49]. At the early phase heart development, differentiated cardiac myocytes retain the ability to proliferate and following birth, they increase in size but not in number (physiological hypertrophy). It is believed that in pathological hypertrophy, the cardiac myocytes make an abortive attempt to achieve its proliferative capacity by revival of the embryonic

program. Therefore, identification of the various signal transduction pathways that lead to hypertrophic growth in myocardium is likely to shed light on the myogenic factors that respond to extracellular cues and activate early embryonic gene expression in the heart.

Renin-Angiotensin system and sympathetic nervous system both have central roles in vascular adaptive processes. Angiotensin II (Ang II) is a key factor in local Renin-Angiotensin system (RAS), intimately involved in a number of physiological processes important in heart development and disease. In pursuit of understanding the molecular mechanism of initiation of hypertrophic growth of cardiac myocytes, we have identified a novel mechanism wherein an Ang II autocrine loop amplifies its own expression via the JAK/STAT signal transduction pathway [50]. We documented that following Ang II treatment of cardiac myocytes, specific isoforms of STAT proteins are activated via phosphorylation which then target the STAT-binding domain of the prohormone angiotensinogen promoter to activate its transcription. In the cardiovascular system, abnormalities of Ang II production have been linked to progressive fibrosis, endothelial cell dysfunction, thrombosis, and atherogenesis.

We observed a dramatic increase in STAT-binding activity in the hypertrophied heart of the spontaneously hypertensive rats (SHR) relative to that of the age-matched normotensive strain WKY. STATs are also activated in normal rat strain WKY in response to exogenous administration of Ang II. These findings represent a significant step in our understanding of the regulatory pathway that controls the tissue autocrine Ang II loop and provide a compelling argument in favor of the linkage of the JAK/STAT pathway to the RAS-mediated hypertrophic response. We are currently investigating the potential involvement of the signaling pathway in cardiac development in early embryos and their link to hypertrophy.

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The cytotoxic effects of a novel IH636 grape seed proanthocyanidin extract on cultured human cancer cells

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Abstract

Grape seed proanthocyanidins are natural antioxidants which possess a broad spectrum of chemoprotective properties against free radicals and oxidative stress. In this study, we have assessed the cytotoxicity of a novel IH636 grape seed proanthocyanidin extract (GSPE) against MCF-7 human breast cancer cells, A-427 human lung cancer cells, CRL-1739 human gastric adenocarcinoma cells and K562 chronic myelogenous leukemic cells at 25 and 50 mg/lit concentrations for 0–72 h using cytomorphology and MTT cytotoxicity assay. In addition, we compared the effects on normal human gastric mucosal cells and normal J774A.1 murine macrophage cells with the effects on the cancer cell lines. Concentration- and time-dependent cytotoxic effects of GSPE were observed on the MCF-7 breast cancer, A-427 lung cancer and gastric adenocarcinoma cells. Following incubation of the MCF-7 cells with 25 mg/lit of the GSPE approximately 6.5, 30 and 43% inhibitions in cell growth were observed at 24, 48 and 72 h of incubation, respectively, while incubation of the MCF-7 cells with 50 mg/lit of the GSPE resulted in 11, 35 and 47% inhibition in cell growth at these same points, respectively. Similar results were observed in the A-427 and gastric adenocarcinoma cells. GSPE exhibited no cytotoxicity toward the neoplastic K562 myelogenous leukemic cells. However, GSPE enhanced the growth and viability of the normal human gastric mucosal cells and J774A.1 murine macrophage cells. These data demonstrate that GSPE exhibited cytotoxicity towards some cancer cells, while enhancing the growth and viability of the normal cells which were examined. (Mol Cell Biochem **196**: 99–108, 1999)

Key words: grape seed proanthocyanidin extract (GSPE), MCF-7 human breast cancer cells, A-427 human lung cancer cells, CRL-1739 human gastric adenocarcinoma cells, K562 human chronic myelogenous leukemic cells, normal human gastric mucosal cells, J774A-1 murine macrophage cells, cytomorphology, MTT assay

Introduction

Proanthocyanidins are naturally occurring polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics, and widely available in fruits, vegetables, nuts, seeds, flowers and bark [1, 2]. Proanthocyanidins are natural antioxidants which are known to possess a broad spectrum of biological, pharmacological and chemoprotective properties against free radicals and oxidative stress. Proanthocyanidins have been reported to exhibit antibacterial, antiviral, antiinflammatory, antiallergic and

vasodilatory actions [3–5], as well as reported to inhibit lipid peroxidation, platelet aggregation and capillary permeability and fragility and to modulate the activity of the enzyme systems including cyclooxygenase and lipooxygenase [5, 6].

We have previously assessed the concentration- or dose-dependent free radical scavenging abilities of a novel IH636 grape seed proanthocyanidin extract (GSPE), in both *in vitro* and *in vivo* models, and compared the free radical scavenging ability of GSPE with vitamin C, vitamin E and β -carotene [7, 8]. Excellent concentration/dose-response inhibitions were demonstrated by GSPE in both *in vitro* and *in vivo*

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experiments [7, 8]. These experiments demonstrated that GSPE is highly bioavailable and provides significantly greater protection against biochemically generated oxygen free radicals and free radical-induced lipid peroxidation and DNA damage than vitamin C, vitamin E, a combination of vitamins C plus E, and β -carotene [7, 8].

A novel laser scanning confocal microscopic technique was also used to assess the concentration-dependent protective ability of GSPE *in vitro* against hydrogen peroxide-induced oxidative stress in cultured macrophage J774A.1 and neuroactive PC-12 adrenal pheochromocytoma cells. The overall intracellular oxidized states of these cells were measured following incubation with hydrogen peroxide and/or GSPE at an excitation wavelength of 513 nm using 2,7-dichlorofluorescein diacetate as the probe. These results indicate that GSPE can significantly protect against hydrogen peroxide-induced oxidative stress in cultured macrophage J774A.1 and neuroactive PC-12 cells [9].

In a separate set of experiment, we have demonstrated the protective ability of GSPE against smokeless tobacco extract (STE)-induced oxidative stress, DNA fragmentation and apoptotic cell death in a primary culture of human oral keratinocyte cells [10]. These results demonstrated that pretreatment of the 300 μ g/ml STE-treated cells with 100 μ g GSPE/ml reduced STE-induced apoptotic cell death by approximately 82% in human oral keratinocyte cells, while pretreatment of the 300 μ g/ml STE-treated cells with a combination of vitamins E and C (75 μ M each) reduced STE-induced apoptotic cell death by 46% [10].

These data demonstrate the excellent free radical scavenging ability of GSPE in both *in vitro* and *in vivo* models. However, the mechanistic pathway of cytoprotection by GSPE is not well understood.

In the present study, the concentration- and time-dependent cytotoxicity of GSPE was assessed against selected human cancer cells including MCF-7 human breast cancer cells, A-427 human lung cancer cells, CRL-1739 human gastric adenocarcinoma cells and K562 chronic myelogenous leukemic cells using cytomorphology and the tetrazolium salt MTT cytotoxicity assay. In addition, we compared the effects on two normal cultured cells, namely, normal human gastric mucosal cells and J774A.1 murine macrophage cells, with the effects on the cancer cell lines.

Materials and methods

Chemicals

A commercially available dried, powdered IH636 grape seed proanthocyanidin extract (batch no. AV 609016) was obtained from InterHealth Nutraceuticals Incorporated (Concord, CA, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide; thiazolyl blue] (Catalog #M-2128) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, Dulbecco's Modified Eagle medium (DMEM) and fetal bovine serum were obtained from GIBCO BRL (Gaithersburg, MD, USA). Ham's F12 medium was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Keratinocyte growth medium (KGM) was obtained from Clonetics Corporation (La Jolla, CA, USA). Unless otherwise stated all other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

Phase contrast microscopy or cytomorphology was performed using a Nikon TMS-F inverted microscope equipped with a Nikon N2000 camera.

Cell cultures and treatment

MCF-7 Human breast cancer cells, A-427 human lung cancer cells, K562 human chronic myelogenous leukemic cells, CRL-1739 human gastric adenocarcinoma cells and J-774A.1 murine macrophage cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). MCF-7 human breast cancer cells, A-427 human lung cancer cells and K562 human chronic myelogenous leukemic cells were maintained and grown in culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum. CRL-1739 human gastric adenocarcinoma cells were maintained and grown in Ham's F12 medium containing 10% fetal bovine serum. J774A.1 Murine macrophage cells were maintained and grown in culture flasks in Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine serum. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ with regular passage. Trypsin solution was used to split cultures whenever they were grown to confluence.

Normal human gastric mucosal cells were cultured from *Helicobacter pylori*-negative endoscopic biopsies from human subjects. An approval was obtained from Creighton University Institutional Review Board [IRB #94-11121; Health and Human Services Assurance Identification #M-1117] for culturing gastric mucosal cells from human gastric biopsies. These biopsies were pre-digested in Medium A (70 mmol/L NaCl, 20 mmol/L NaHCO₃, 1.5 mmol/L Na₂HPO₄, 5 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L CaCl₂, 11 mmol/L glucose and 50 mmol/L HEPES) containing pronase. The absence of *H. pylori* in these biopsies was determined using a commercial CLO-Test strip (Tri-Med Specialties Inc., Lenexa, KS, USA). The gastric cells were subsequently digested for 45 min with collagenase in Medium A containing 0.2% bovine serum albumin. The cell suspensions were filtered through 100- and 60- μ M nylon filters and suspended in KGM growth medium (Clonetics Corporation, La Jolla, CA, USA). Gastric cells were finally

incubated at 37°C in collagen coated (200 µg rat tail collagen/well) microplates in KGM growth medium supplemented with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO₂ in 100% humidity and the culture media was changed daily. The parietal and chief cells did not attach to the collagen coated culture plates and were removed during change of medium. Periodic acid-Schiff staining was used to determine the integrity of mucosal cells.

The number of cells was determined using a Coulter counter. Viability was checked using the Trypan blue exclusion technique. Cultured cells were individually incubated with 25 and 50 mg/lit concentrations of GSPE for 0, 24, 48 and 72 h at 37°C. GSPE was freshly prepared by dissolving in sterile media prior to each experiment.

Phase contrast microscopy

Changes in cell morphology or cytomorphology following treatment with GSPE were detected using phase contrast microscopy. The concentration- and time-dependent detachment of dead or dying cells following exposure to GSPE was monitored using this technique. Healthy cells remain elongated while dead or dying cells become rounded and lose their adhesion to the culture plate.

Colorimetric MTT (tetrazolium) assay

The cleavage of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into a blue colored formazan by the mitochondrial enzyme succinate dehydrogenase [11, 12] is potentially very useful for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of cells present. Thus, the MTT assay detects living but not dead cells and the signal generated is dependent on the degree of activation of the cells.

A 5 mg/ml stock solution of MTT was prepared in sterile phosphate-buffered saline (PBS), and filtered through a 0.22 µm filter to sterilize and remove any small amount of insoluble residue or blue formazan product. The stock solution was stored for no more than 3–4 days in the dark at 4°C. The MTT assay was conducted as described earlier by Mosmann [11] and us [12]. Cultured cells were incubated with 0, 25 and 50 mg/lit of GSPE for 0, 24, 48 and 72 h. At the desired time point of assay a 25 µl aliquot of the stock solution of MTT was added and incubated at 37°C for 2 h [12]. Following incubation, extraction/lysing buffer was added to each culture well and mixed thoroughly to absorb all formazan crystals [12]. Optical densities were measured at 570 nm.

Results

GSPE-induced changes in cell morphology

Phase contrast microscopy or cytomorphology demonstrated that GSPE induces concentration-dependent changes in cell morphology in the human cancer and normal cells. With increasing concentration of GSPE and time, the MCF-7 human breast cancer cells, A-427 human lung cancer cells and CRL 1739 human gastric adenocarcinoma cells lost their adhesion to the culture plates and the cells became rounded in appearance. No concentration- and time-dependent cytotoxic effect of GSPE on cell morphology was observed on K562 chronic myelogenous leukemic cells, normal human gastric mucosal cells and J774A.1 murine macrophage cells. In fact, GSPE enhanced the growth and viability of normal human gastric mucosal cells and J774A.1 murine macrophage cells. Figures 1 and 2 demonstrate the representative comparative concentration- and time-dependent effect of GSPE on CRL-1739 human gastric adenocarcinoma cells and on a primary culture of normal human gastric mucosal cells. Figure 1 demonstrates the representative time-dependent cytotoxic effect of 50 mg/lit concentration of GSPE on CRL-1739 human gastric adenocarcinoma cells at 0, 24, 48 and 72 h of treatment based on the numbers of detached and rounded cells. Figure 2 demonstrates the effect of 50 mg/lit concentration of GSPE on normal human gastric mucosal cells at 0, 24, 48 and 72 h of treatment. Figure 2 clearly showed that GSPE exhibits no cytotoxicity towards normal human gastric mucosal cells. The concentration- and time-dependent growth inhibitory effect of these cultured cells by GSPE *in vitro* was quantitated using MTT assay, a method for measuring cellular respiration that entails the conversion of MTT to colored formazan.

Concentration- and time-dependent cytotoxicity of GSPE against MCF-7 human breast cancer cells

Following incubation of the cultured MCF-7 human breast cancer cells at 37°C without GSPE over a period of 0–72 h, a steady growth of these cells was observed. Figure 3 demonstrates the growth inhibitory effect of GSPE on MCF-7 breast cancer cells *in vitro*. Following incubation of MCF-7 human breast cancer cells with 25 mg/lit of GSPE, approximately 6.5, 30 and 43% inhibition in cell growth was observed at 24, 48 and 72 h of incubation, respectively, while incubation of the breast cancer cells with 50 mg/lit of GSPE resulted in 11, 35 and 47% inhibition in cell growth at these same time points, respectively.

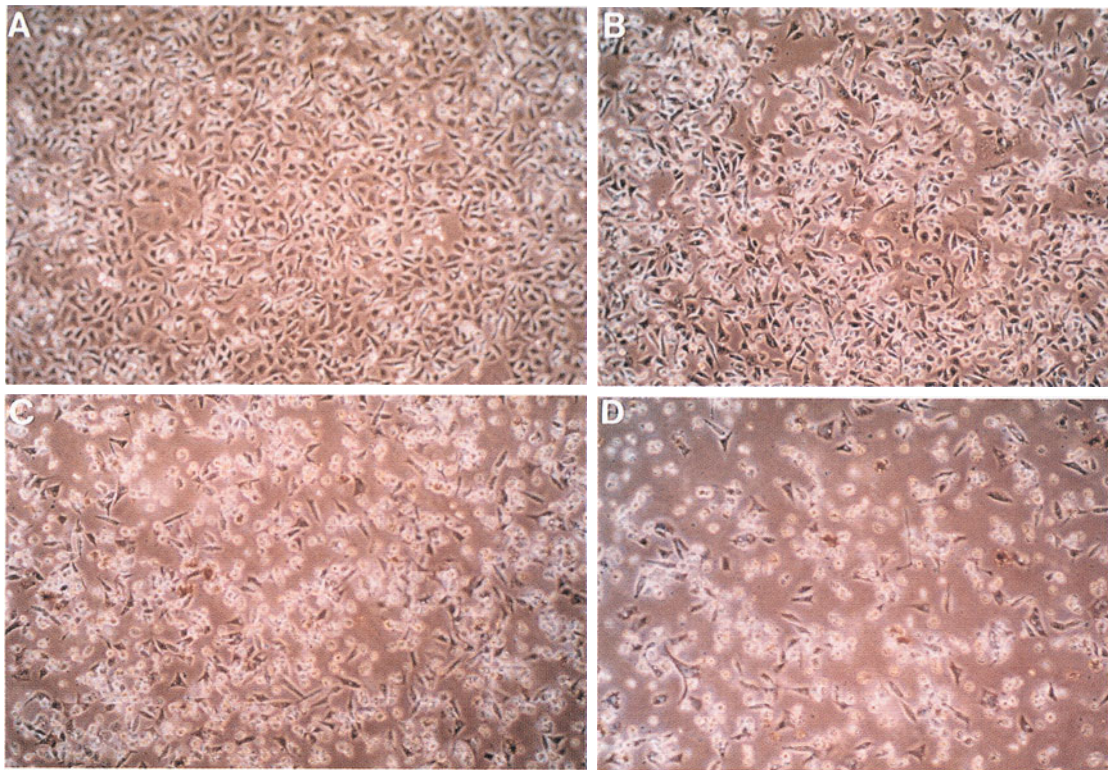


Fig. 1. Time-dependent effect of GSPE on cultured CRL-1739 human gastric adenocarcinoma cells as determined by phase contrast microscopy. Cultured gastric adenocarcinoma cells were incubated with 50 mg/lit concentration of GSPE for 0, 24, 48 and 72 h of treatment at 37°C. A – control; B – 24 h; C – 48 h; D – 72 h.

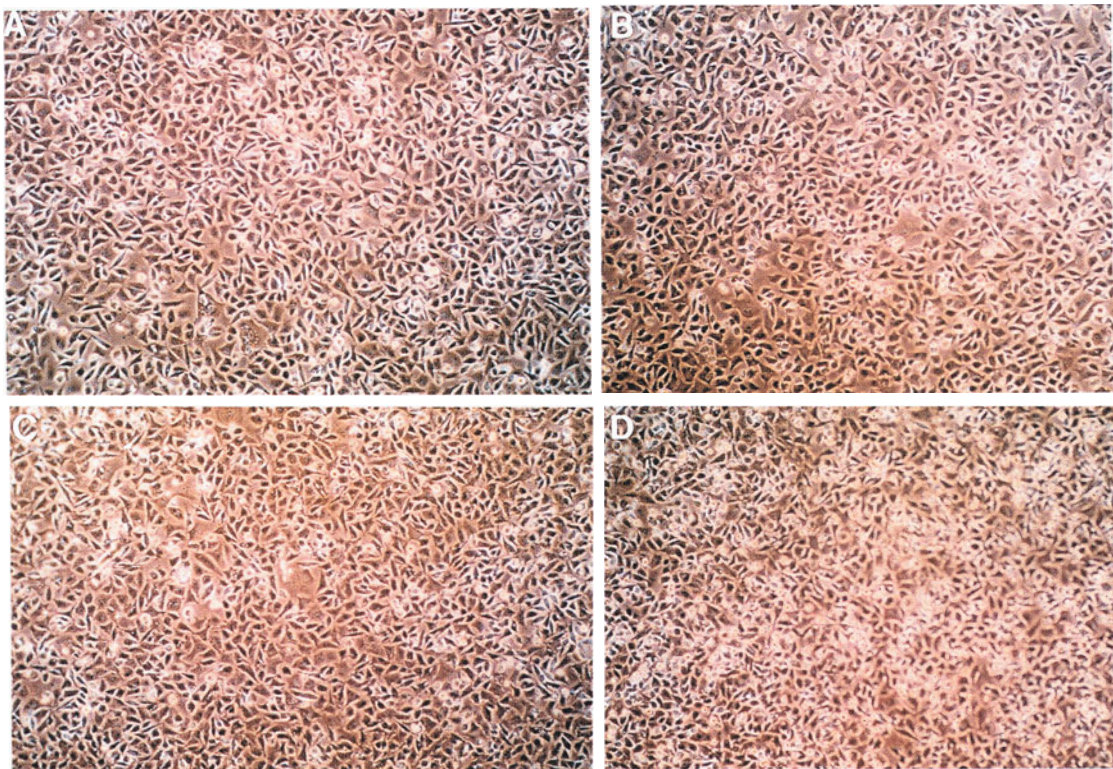


Fig. 2. Time-dependent effect of GSPE on a primary culture of normal human gastric mucosal cells. Cultured gastric adenocarcinoma cells were incubated with 50 mg/lit concentration of GSPE for 0, 24, 48 and 72 h of treatment at 37°C. A – control; B – 24 h; C – 48 h; D – 72 h.

Concentration- and time-dependent cytotoxicity of GSPE against A-427 human lung cancer cells

The concentration- and time-dependent cytotoxic or growth inhibitory effect of GSPE on cultured A-427 human lung cancer cells is shown in Fig. 4. Following incubation of the cultured human lung cancer cells without GSPE over a period of 0–72 h, a steady growth of these cells were observed. Following incubation of A-427 human lung cancer cells with 25 mg/lit of GSPE, approximately 2, 20 and 37% inhibition in cell growth was observed at 24, 48 and 72 h of incubation, respectively, while incubation of the lung cancer cells with 50 mg/lit of GSPE resulted in 14, 32 and 48% inhibition in cell growth at these same time points, respectively.

Concentration- and time-dependent cytotoxicity of GSPE against CRL 1739 human gastric adenocarcinoma cells

The concentration- and time-dependent cytotoxic or growth inhibitory effect of GSPE on cultured CRL-1739 human gastric adenocarcinoma cells is shown in Fig. 5. Following incubation of the cultured human gastric adenocarcinoma cells without GSPE over a period of 0–72 h, a steady growth of these cells was observed. Following incubation of the human gastric adenocarcinoma cells with 25 mg/lit of GSPE, approximately 2, 18 and 34% inhibition in cell growth was observed at 24, 48 and 72 h of incubation, respectively, while incubation of the human gastric adenocarcinoma cells with 50 mg/lit of GSPE resulted in 5, 22 and 41% inhibition in cell growth at these same time points, respectively.

Concentration - and time-dependent cytotoxic effect of GSPE on K562 human chronic myelogenous leukemic cells

The concentration- and time-dependent growth inhibitory effect of GSPE on cultured K562 human chronic myelogenous leukemic cells is shown in Fig. 6. Following incubation of these K562 myelogenous leukemic cells over a period of 0–72 h, a steady growth of these cultured cells was observed. Following treatment of these cells with 25 or 50 mg/lit concentrations of GSPE, no significant changes in the growth of these cultured cells were observed at these same time points.

Concentration- and time-dependent effect of GSPE on normal human gastric mucosal cells

The *in vitro* concentration- and time-dependent effects of GSPE on cultured normal human gastric mucosal cells are shown in Fig. 7. Following incubation of the normal human gastric mucosal cells at 37°C over a period of 0–72 h, a steady growth of these cultured cells was observed. Following incubation of these cells with 25 mg/lit of GSPE, approximately 8, 13 and 9% increases in cell growth were observed at 24, 48 and 72 h of incubation, respectively, as compared to the control cells, while under these same conditions incubation of these cells with 50 mg/lit of GSPE resulted in 32, 26 and 18% increases in cell growth at these same time points, respectively.

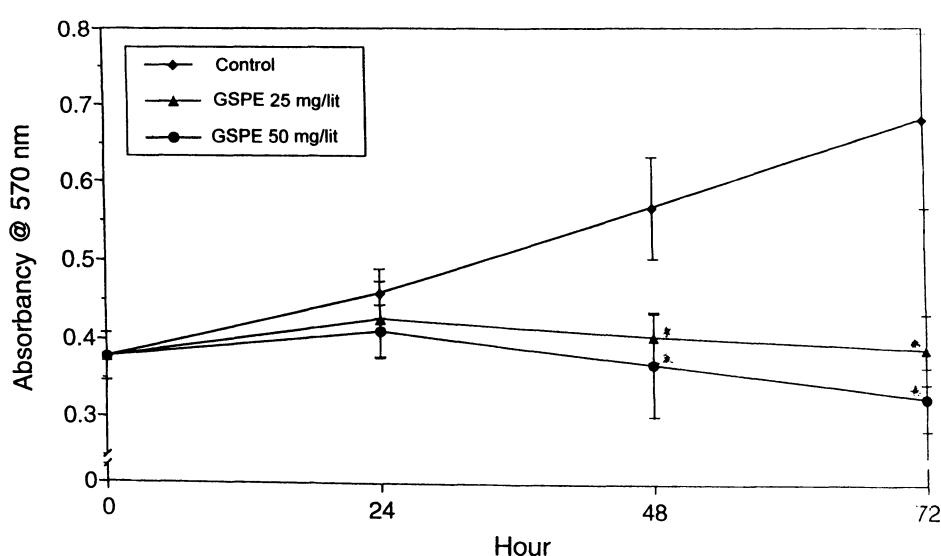


Fig. 3. Growth inhibition of MCF-7 human breast cancer cells by 0, 25 and 50 mg/lit concentrations of GSPE at 0, 24, 48 and 72 h of treatment as determined by tetrazolium dye MTT assay. Each value represents the mean $\pm$ S.D. of 4–6 replicates. * $p < 0.05$ compared to nontreated group.

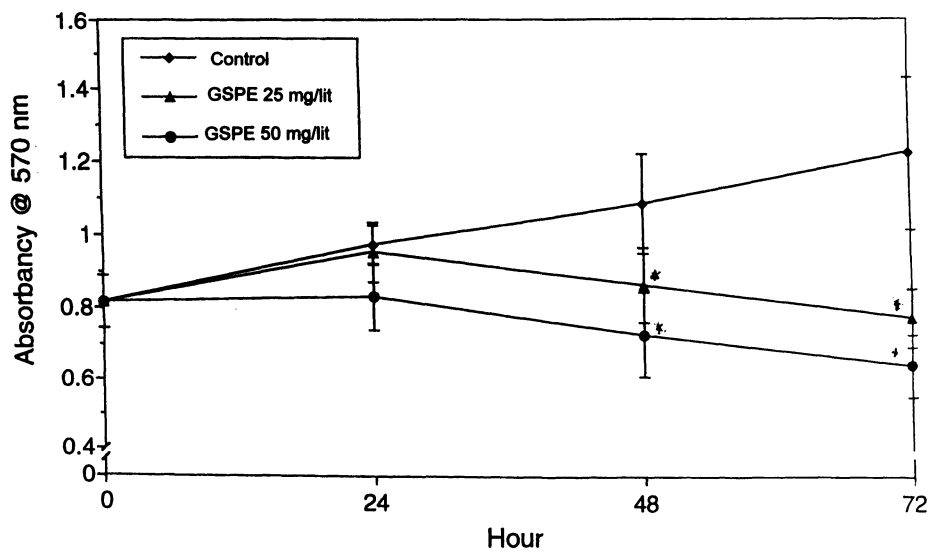


Fig. 4. Growth inhibition of A-427 human lung cancer cells by 0, 25 and 50 mg/lit concentrations of GSPE at 0, 24, 48 and 72 h of treatment as determined by tetrazolium dye MTT assay. Each value represents the mean $\pm$ S.D. of 4–6 replicates. * $p < 0.05$ compared to nontreated group.

Concentration- and time-dependent effect of GSPE on J774A.1 murine macrophage cells

The concentration- and time-dependent effect of GSPE on cultured J774A.1 murine macrophage cells are shown in Fig. 8. Following incubation of the cultured murine macrophage cells without GSPE over a period of 0–72 h, a steady growth of these cultured cells was observed. Following incubation of these cells with 25 mg/lit of GSPE, approximately 8, 10 and 8% increases in cell growth were observed

at 24, 48 and 72 h of incubation, respectively, as compared to the control cells, while under these same conditions incubation of these cells with 50 mg/lit of GSPE resulted in 9, 7 and 12% increases in cell growth at these same time points, respectively.

Discussion

Proanthocyanidins are a group of polyphenolic bioflavonoids

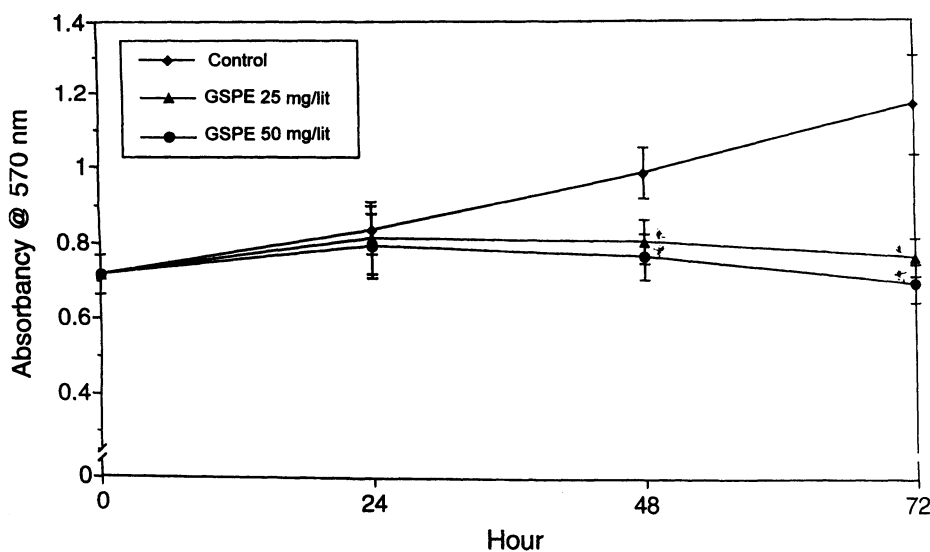


Fig. 5. Growth inhibition of CRL-1739 human gastric adenocarcinoma cells by 0, 25 and 50 mg/lit concentrations of GSPE at 0, 24, 48 and 72 h of treatment as determined by tetrazolium dye MTT assay. Each value represents the mean $\pm$ S.D. of 4–6 replicates. * $p < 0.05$ compared to nontreated group.

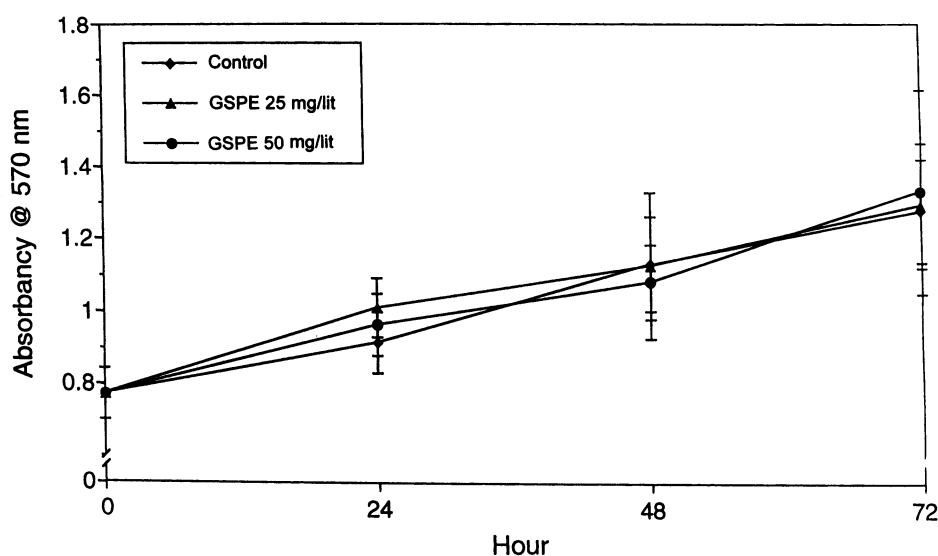


Fig. 6. Effect of 0, 25 and 50 mg/lit concentrations of GSPE on K562 human chronic myelogenous leukemic cells at 0, 24, 48 and 72 h of treatment as determined by tetrazolium dye MTT assay. Each value represents the mean $\pm$ S.D. of 4–6 replicates. * $p < 0.05$ compared to nontreated group.

found ubiquitously in fruits and vegetables. There is an increasing interest in proanthocyanidins due to their potential involvement in biological and pharmacological processes including therapeutic strategies. Some of their remarkable properties include inhibition of DNA topoisomerase II, and modulation of protein kinase C, angiotensin-converting enzyme, and hyaluronidase enzyme activities [13–19]. Proanthocyanidins also possess potent antibacterial [20], antiviral [21] and anti-HIV activities [22]. In addition these plant pigments demonstrate powerful anti-hypertensive

effects [1, 2], anti-peptic activity [23], monocyte stimulating ability [24], and anti-hepatotoxic activity [25]. The biological, pharmacological and medicinal properties of bioflavonoids have been reviewed extensively [1, 2, 26]. However, much less attention has been paid to the cytotoxic and antitumor activities, and their role in mechanistic pathways of cytoprotection of proanthocyanidins. Based on these previous studies, we hypothesized that proanthocyanidins may possess selective cytotoxic activity towards human cancer cells as compared to normal cells.

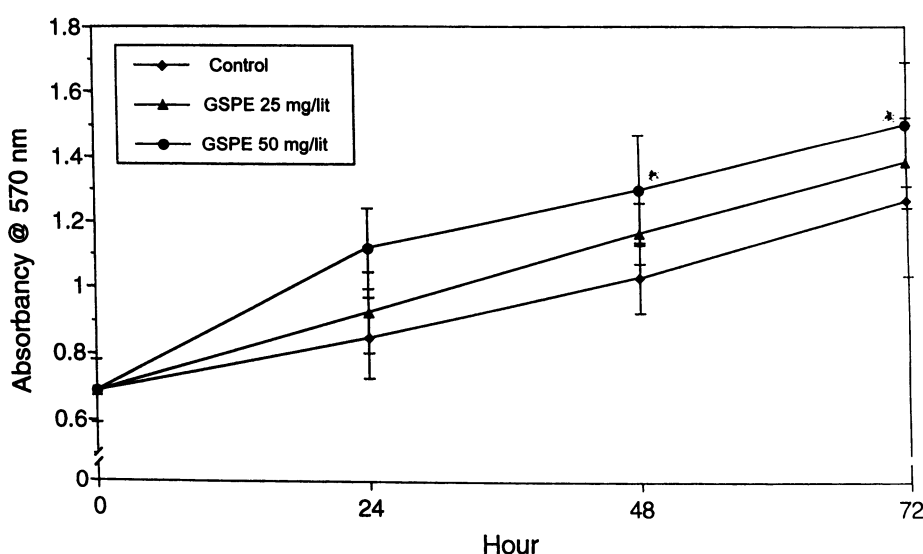


Fig. 7. Effect of 0, 25 and 50 mg/lit concentrations of GSPE on a primary culture of human gastric mucosal cells at 0, 24, 48 and 72 h of treatment as determined by tetrazolium dye MTT assay. Each value represents the mean $\pm$ S.D. of 4–6 replicates. * $p < 0.05$ compared to nontreated group.

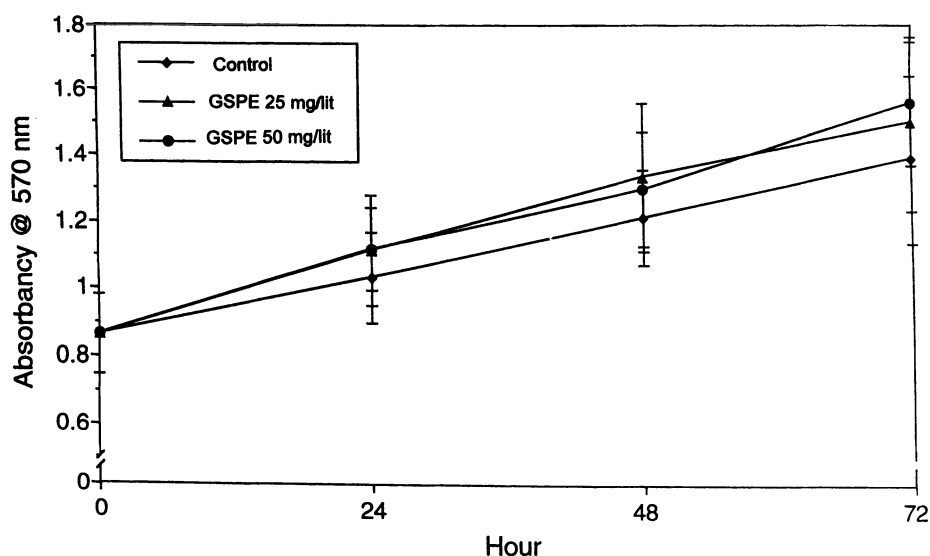


Fig. 8. Effect of 0, 25 and 50 mg/lit concentrations of GSPE on J774A.1 murine macrophage cells at 0, 24, 48 and 72 h of treatment as determined by tetrazolium dye MTT assay. Each value represents the mean $\pm$ S.D. of 4–6 replicates. * $p < 0.05$ compared to nontreated group.

The chemical properties of bioflavonoids in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers and singlet oxygen quenchers predicts their antioxidant activity [1, 13, 26]. Bioflavonoids have also been demonstrated as potent inhibitors of the enzymes phospholipase A₂, cyclooxygenase and lipoxygenase [5, 6, 26, 27]. For a proanthocyanidin or a bioflavonoid to be defined as an antioxidant it must satisfy two basic conditions: (i) when present in low concentrations relative to the substrate to be oxidized it can delay, retard, or prevent autooxidation or free radical-mediated oxidative injury; and (ii) the resulting product formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation [28].

The presence of various phenolic compounds including phenoldienones, epicatechin, epigallocatechin, epigallocatechin gallate, ferulic acid, caffeic acid, p-coumaric acid, kaempferol, quercetin and myricetin have been well established in proanthocyanidin extracts [1, 2, 26]. It is well known that diets rich in fresh fruits and vegetables are protective against cardiovascular diseases and other oxidative stress-induced diseases and disorders including cancer [2, 29]. These chemoprotective properties have been attributed, for the most part, to the presence of antioxidant nutrients vitamin C, vitamin E, β -carotene and mineral micronutrients. However, plant phenolics such as the bioflavonoids, proanthocyanidins and phenylpropanoids may also play a significant role. The proanthocyanidins or polyphenolic bioflavonoids may act as antioxidants or by other mechanisms, contributing to anticarcinogenic and/or chemoprotective actions. However, no direct investigation has been conducted so far.

In our previous studies, we have demonstrated excellent concentration- or dose-dependent free radical scavenging abilities of GSPE, in both *in vitro* and *in vivo* models, and compared the free radical scavenging ability of GSPE with vitamin C, vitamin E and β -carotene [7–9]. GSPE provided significantly greater protection against biochemically generated oxygen free radicals *in vitro* as compared to vitamins C and E [7]. In a separate set of *in vitro* experiments, we have assessed the concentration-dependent protective ability of GSPE against hydrogen peroxide-induced oxidative damage in cultured murine macrophage J774A.1 cells and adrenal pheochromocytoma PC-12 cells using laser scanning confocal microscopy [9]. GSPE demonstrated significant protection against hydrogen peroxide-induced oxidative damage in these cultured cells as demonstrated by fluorescent intensity [9]. Our *in vivo* experiments demonstrated that GSPE is highly bioavailable to the hepatic and brain tissues, as well as to the peritoneal macrophage cells [8]. Three oral doses of GSPE (25, 50 and 100 mg/kg p.o.) were assessed in this study. In these *in vivo* experiments, GSPE demonstrated significantly greater protection against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced increased lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as activation of peritoneal macrophage cells as compared to vitamin C, vitamin E, a combination of vitamins C plus E, and β -carotene [8]. We have also demonstrated the protective ability of GSPE against smokeless tobacco extract (STE)-induced oxidative stress, DNA fragmentation and apoptotic cell death in a primary culture of human oral keratinocyte cells [10]. The results demonstrate that STE produces oxidative tissue damage and apoptosis, which can be protected by antioxidants. The GSPE

demonstrated significantly better protection as compared to vitamins C and E, singly and in combination [10].

The objective of the present study was to assess the concentration- and time-dependent cytotoxicity of GSPE on selected human cancer cells including MCF-7 human breast cancer cells, A-427 human lung cancer cells, CRL 1739 human gastric adenocarcinoma cells and K562 chronic myelogenous leukemic cells using phase contrast microscopy and the tetrazolium salt MTT cytotoxicity assay, and to compare the effects of GSPE (25 and 50 mg/lit) on a primary culture of normal human gastric mucosal cells and cultured J774A.1 murine macrophage cells. Concentration- and time-dependent cytotoxicity was observed following incubation of the MCF-7 human breast cancer cells, A-427 human lung cancer cells and CRL 1739 human gastric adenocarcinoma cells with GSPE. However, no cytotoxic or growth inhibitory effects of GSPE were observed on human K562 chronic myelogenous leukemic cells, normal human gastric mucosal cells and J774A.1 murine macrophage cells. In fact, GSPE enhanced the growth and viability of the normal human gastric mucosal cells and J774A.1 macrophage cells. These data demonstrate that GSPE exhibited selective cytotoxicity towards some human cancer cells, while enhancing the growth and viability of the normal cells which were examined. It seems likely to suggest that GSPE may induce selective apoptotic cell death in human MCF-7 breast cancer, A-427 lung cancer and CRL 1739 gastric adenocarcinoma cells, while enhancing the growth and viability of normal cells. The other possibility may include the upregulation of *bcl-X_s* (death promoter) and downregulation of *bcl-X_L* (death inhibitor) genes in these human cancer cells following incubation with GSPE, while no such effect may happen in the normal cells. Further investigation is warranted to unveil the mechanism.

Acknowledgements

These studies were supported in part by a grant from InterHealth Nutritionals Incorporated (Concord, CA, USA). The corresponding author gratefully acknowledges the funding from the National Cancer Institute (NIH #CA70976-01A2) and from the Procter & Gamble Company (Mason, OH, USA) for the establishment of a primary culture of normal human gastric mucosal cells from *Helicobacter pylori*-negative endoscopic biopsies from human subjects (IRB #94-11121).

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Acute and chronic stress-induced oxidative gastrointestinal mucosal injury in rats and protection by bismuth subsalicylate

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Abstract

Reactive oxygen species (ROS) are implicated in the pathogenesis of stress-induced gastrointestinal mucosal injury. In the present study, we have investigated the effects of acute and chronic stress on the enhanced production of ROS including superoxide anion [SA; as determined by cytochrome *c* reduction (CCR)] and hydroxyl radicals (OH), and correlated the enhanced production of these free radicals with increased lipid peroxidation, membrane microviscosity and DNA fragmentation, indices of oxidative tissue damage, in the gastric and intestinal mucosa of female Sprague-Dawley rats. Furthermore, the protective ability of bismuth subsalicylate (BSS) against the gastrointestinal mucosal injury induced by acute and chronic stress was determined. Acute stress was induced for a period of 90 min, while chronic stress was induced for 15 min/day for 15 consecutive days. Half of the animals exposed to acute stress were pretreated orally with 15 mg BSS/kg 30 min prior to the exposure to acute stress. Similarly, half of the animals exposed to water-immersion restraint chronic stress were pretreated orally with 7.5 mg BSS/kg/day for 15 consecutive days 30 min prior to the exposure to chronic stress. Acute stress produced greater injury to both gastric and intestinal mucosa as compared to chronic stress. Acute stress increased CCR and OH production by 10.0- and 14.3-fold, respectively, in the gastric mucosa, and 10.4- and 17.0-fold, respectively, in the intestinal mucosa. Pretreatment with BSS prevented the acute stress-induced increase in CCR and OH production. Acute stress increased lipid peroxidation, DNA fragmentation and membrane microviscosity by 3.6-, 4.0- and 11.6-fold, respectively, in gastric mucosa, and 4.1-, 5.0- and 16.2-fold, respectively, in intestinal mucosa. BSS decreased acute stress-induced lipid peroxidation, DNA fragmentation and membrane microviscosity by approximately 26, 35 and 30%, respectively, in gastric mucosa, and by 20, 36 and 30%, respectively, in the intestinal mucosa. Chronic stress increased CCR and OH production by 4.8- and 6.3-fold, respectively, in gastric mucosa, and 4.6- and 6.9-fold, respectively, in intestinal mucosa. Chronic stress increased lipid peroxidation and DNA fragmentation by 2.9- and 3.3-fold, respectively, in gastric mucosa, and 3.3- and 4.2-fold, respectively, in intestinal mucosa. BSS decreased chronic stress-induced lipid peroxidation, DNA fragmentation and membrane microviscosity by approximately 41, 44 and 45%, respectively, in gastric mucosa, and by 39, 52 and 51%, respectively, in the intestinal mucosa. Daily administration of BSS provided greater protection against chronic stress-induced oxidative gastrointestinal injury as compared to the acute stress. These results demonstrate that both acute and chronic stress can induce gastrointestinal mucosal injury through enhanced production of ROS, and that BSS can significantly protect against gastrointestinal mucosal injury. (Mol Cell Biochem **196**: 109–116, 1999)

Key words: acute and chronic stress, rats, gastric mucosa, intestinal mucosa, oxygen free radicals, lipid peroxidation, DNA fragmentation, membrane microviscosity, bismuth subsalicylate

Introduction

An effective balance exists in the healthy human stomach between the potential for gastric acid and pepsin to damage gastric mucosal cells and the ability of these cells to protect themselves from injury [1–4]. Disruption of this balance has been attributed to several factors including environmental and emotional stress, age, diet, genetics and individual behaviour, and is evidenced as a burning, aching, or gnawing pain that may be perceived as abdominal pressure or fullness [1, 3]. Most of the symptoms experienced by patients under such conditions result from a breakdown of the normal mucosal defense mechanisms [3]. It is well documented that gastric acid and pepsin are important in the pathogenesis of dyspepsia, stomach upset, gastroesophageal reflux disease, and peptic ulcer disease [1, 3, 4]. Several mechanisms are believed to be important in protecting the gastric and duodenal mucosa from damage by acid, pepsin, bile, and pancreatic enzymes as well as external stressors/factors [1, 2, 4]. These defense mechanisms include mucus, mucosal blood flow, cell renewal, and bicarbonate [4]. The aggressive and defensive factors act in balance help maintain mucosal integrity. It has been suggested that oxygen free radicals are greatly involved in the pathogenesis of gastric injury [1]. However, the detailed mechanistic pathways of these events are still unknown.

Physical stress has been shown to induce significant gastrointestinal mucosal injury in animals [5]. Kuyawama *et al.* [5] demonstrated that water-immersion restraint stress results in an increase in gastric epithelial cell loss and macroscopic mucosal injury, which is accompanied by compensated acceleration of cell migration in fundic mucosa of rats before the development of gastric erosions. Cell migration was found to be accelerated in fundic mucosa after 90 min of stress [5]. The combination of increased cell loss with depressed epithelial proliferation may play a role in stress-related gastric lesions and injury. However, no mechanistic interpretation of stress-induced gastrointestinal injury was investigated.

Previous studies in our laboratories have demonstrated that oral administration of necrotizing agents including 0.6 M HCl, 0.2 M NaOH, 80% ethanol, and aspirin (200 mg/kg) induce increased lipid peroxidation and membrane microviscosities in gastric and intestinal mucosa as well as cause gastric and intestinal mucosal DNA fragmentation in female Sprague-Dawley rats [6]. Pretreatment and post-treatment of the rats with bismuth salts provided significant protection against the mucosal injury induced by these necrotizing agents [6]. No comparative studies have been conducted to investigate the effects of acute and chronic stress on the enhanced production of reactive oxygen species (ROS) and oxidative tissue, and membrane and DNA damage in rats.

In the present study, we have investigated the effects of acute and chronic restraint stress on the enhanced production of superoxide anion and hydroxyl radicals, and DNA frag-

mentation and lipid peroxidation, indices of oxidative tissue damage, in the gastric and intestinal mucosa of female Sprague-Dawley rats. Furthermore, the protective ability of bismuth subsalicylate (BSS) was determined against the gastrointestinal mucosal injury induced by acute and chronic stress. The objectives of this project were to assess the relationship between gastrointestinal oxidative stress induced by acute and chronic stress, and the protective ability of BSS.

Materials and methods

Animals and treatment

Female Sprague-Dawley rats (160–180 gms) were obtained from Sasco (Omaha, NE, USA). The animals were housed in a controlled environment at 25°C with a 12 h light and 12 h dark cycle, and were acclimated for at least 3–5 days before use. Unless otherwise stated, all animals were allowed free access to food (Purina Rodent Lab Chow No. 5001) and tap water. The study protocol was approved by the Creighton University Animal Research Committee for this project (ARC #0295). BSS solution was freshly prepared daily for this study.

Acute stress was induced in rats for 90 min by water immersion as described by Kuwayama *et al.* [5]. Half of these animals were supplemented with BSS (15 mg/kg) 30 min prior to the exposure to acute stress [7]. Animals were sacrificed after acute stress exposure.

After an overnight fast, chronic stress was induced as described by Kuwayama *et al.* [5] for 15 min/day for 15 consecutive days. Animals were pretreated with BSS (7.5 mg/kg/day) or vehicle 30 min prior to the exposure to chronic stress. Animals were sacrificed on day 15 following 15 min exposure to restraint stress.

The restraint stress water tank was 12 × 18 inches with a water depth of approximately 4.5 inches. The temperature of the water was 23°C. All animals were killed by decapitation. The gastric and intestinal mucosa were isolated from these animals as described by Heylings [8].

Chemicals

Bismuth subsalicylate (BSS) (Lot #4; Mfg. Date 3-18-96; Trash Date 11-97; Moisture content 53%) was generously provided by the Procter & Gamble Company (Cincinnati, OH, USA). Sodium acetate trihydrate and citric acid used for HPLC were purchased from Fluka (Buchs, Switzerland). 2,3-Dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, salicylic acid and HPLC grade water were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals used in this study were obtained from Sigma Chemical Co.

(St. Louis, MO, USA) and were of analytical grade or the highest commercial grade available.

Lipid peroxidation

The formation of thiobarbituric acid reactive substances (TBARS) associated with gastric and intestinal mucosa from control and treated animals was determined as an index of lipid peroxidation according to the method of Buege and Aust [9], and as previously published by us [10]. Malondialdehyde was used as the standard. Absorbance values were measured at 535 nm and an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ was used.

DNA fragmentation

Frozen gastric and intestinal mucosa samples were homogenized in lysis buffer (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were centrifuged at $27,000 \times g$ for 20 min to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 N perchloric acid and 5.5 N perchloric acid was added to supernatant fractions to reach a concentration of 0.5 N. Samples were heated at 90°C for 15 min and centrifuged at $1,500 \times g$ for 10 min to remove protein. Resulting supernatant fractions were reacted with diphenylamine for 16–20 h at room temperature. Absorbance was measured at 600 nm. DNA fragmentation in control samples is expressed as percent of total DNA appearing in the supernatant fraction [6]. Treatment effects are reported as percent of control fragmentation [6].

Membrane microviscosity

The microviscosity of the gastric and intestinal mucosa from control and treated animals was determined as described previously [6]. The samples were treated with 0.5 mM diphenyl hexatriene (DPH) in tetrahydrofuran as the fluorescent probe and incubated for 2 h at 37°C as described by Mukherjee *et al.* [11]. Fluorescence polarization as a measure of membrane microviscosity was determined at 25°C using a thermostated cuvette holder in a Perkin-Elmer spectrofluorometer equipped with perpendicular and parallel polarizers and an excitation wavelength of 365 nm and an emission wavelength of 430 nm. Fluorescence polarization and the apparent microviscosity were calculated as described by Shintzky and Barrenholz [12].

Cytochrome c reduction

Superoxide anion production was measured by the assay procedure of Babior *et al.* [13] and as previously described by

us [10], which is based on the reduction of cytochrome *c* reduction. In 1.0 ml, the reaction mixtures contained 1 mg protein and 0.05 mM cytochrome *c* in the incubation buffer. The incubation mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the reaction mixtures on ice. The mixtures were centrifuged for 10 min, and the supernatant fractions were transferred to clean tubes for spectrophotometric measurement at 550 nm. Absorbance values were converted to nmol of cytochrome *c* reduced/15 min/mg of protein using the extinction coefficient $2.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

Detection and quantitation of hydroxyl radicals

Hydroxyl radicals, based on the formation of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acids, were determined in an HPLC equipped with a Waters 460 electrochemical detector as previously described by us [14]. The mucosal samples were incubated in the presence of 1.0 mmol/l of salicylic acid. The samples were filtered through a Rainin 0.22 μm pore size Nylon-66 sample filter, and 25 μl volumes of the samples were injected onto an Altex Ultrasphere (3 μm ODS, $75 \times 4.6 \text{ mm}$) column (Rainin) protected by a Rainin Brownlee Spheri-5 polyfunctional C18 ODS-GU precolumn ($30 \times 4.6 \text{ mm}$). The hydroxylated products of salicylic acid after interaction with hydroxyl radicals, 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid, were eluted with a mobile phase containing 0.03 mol/l of sodium acetate and 0.03 mol/l of citric acid (pH = 3.6) at a flow rate of 1 ml/min. The detection potential was maintained at + 0.6 V, employing a glassy carbon working electrode and an Ag/AgCl reference electrode. Retention times for the peaks of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were verified by injecting authentic standards.

Statistical methods

The presence of significant differences between mean values was determined by Analysis of Variance (ANOVA) followed by Scheffe's S method as the post hoc test. Each value is the mean $\pm$ S.D. from at least 4–6 experiments. The level of statistical significance in all cases was $p < 0.05$.

Results

Gastrointestinal mucosal lipid peroxidation

Since lipid peroxidation occurs in response to free radicals and oxidative stress, the *in vivo* effects of acute and chronic stress were assessed on lipid peroxidation as an index of oxidative lipid degradation in both gastric and intestinal

mucosa. The production of thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation is presented in Table 1. The results demonstrate that acute and chronic stress increased mucosal lipid peroxidation by approximately 3.6- and 3.2-fold, respectively, in the gastric mucosa, and 4.1- and 3.1-fold in the intestinal mucosa, respectively, as compared to the control animals (Table 1). Following administration of BSS alone for 15 days to the rats gastric and intestinal mucosal lipid peroxidation increased by approximately 1.5- and 1.7-fold, respectively, as compared to control animals. Similar increases were observed following treatment with BSS for 1 hr. Gastrointestinal lesions were observed in animals exposed to both acute and chronic stress. No lesions occurred in the control animals which received BSS.

Pretreatment of the rats with BSS decreased acute and chronic stress-induced lipid peroxidation by approximately 26 and 41% in the gastric mucosa, and 20 and 39% in the intestinal mucosa, respectively, as compared to the control rats (Table 1). These results indicate that administration of BSS can significantly reduce the mucosal lipid peroxidation induced by acute and chronic stress.

DNA fragmentation

Apoptosis (programmed cell death) is a selective process of physiological cell deletion. It plays a major role in developmental biology and in maintenance of homeostasis in vertebrates. Apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, condensation and fragmentation of nuclei, and extensive degradation of chromosomal DNA. Fragmentation of nuclear DNA is a biochemical hallmark of apoptosis. DNA fragmentation in the gastric and intestinal mucosa induced by these diverse stressors was determined as an index of oxidative DNA damage, and is shown in Table 2.

Approximately 4.0- and 3.2-fold increases in DNA fragmentation were observed in the gastric mucosa following

exposure to acute and chronic stress, respectively, while under these same conditions approximately 4.9- and 4.0-fold increases in DNA fragmentation were observed in the intestinal mucosa, respectively, as compared to control animals. Following administration of BSS alone for 15 days DNA fragmentation increased by 1.3- and 1.6-fold in the gastric and intestinal mucosa, respectively, as compared to control animals.

Pretreatment of rats with BSS decreased acute and chronic stress-induced DNA fragmentation by 35 and 44% in the gastric mucosa, respectively, and by approximately 35 and 52% in the intestinal mucosa, respectively, as compared to control animals.

Modulation of membrane microviscosities

Membrane microviscosity was determined as an index of membrane integrity. Steady-state fluorescence spectroscopy was utilized to evaluate changes in membrane microviscosity as a result of the induction of chronic stress to rats. Membrane microviscosity is inversely proportional to the membrane fluidity. The changes in membrane fluidity occur due to the direct effect of these stressors on the structural integrity of the gastric and intestinal mucosa, including the changes in lipid peroxidation. The effects of acute and chronic stress on the membrane microviscosity of gastric and intestinal mucosa are shown in Table 3.

Acute and chronic stress increased membrane microviscosities by 11.6- and 6.3-fold in the gastric mucosa, respectively, and approximately 16.6- and 9.3-fold in the intestinal mucosa, respectively, as compared to control animals. Following administration of BSS alone for 15 days membrane microviscosities increased in the gastric and intestinal mucosa by approximately 2.3- and 1.5-fold, respectively, as compared to control animals.

Pretreatment of the animals with BSS decreased acute and chronic stress-induced membrane microviscosities by 30 and

Table 1. Acute and chronic stress-induced lipid peroxidation (TBARS content nmol/mg protein) in the gastric and intestinal mucosa, and the protective ability of bismuth subsalicylate (BSS)

	Lipid Peroxidation (TBARS Content nmol/mg protein)	
	Gastric Mucosa	Intestinal Mucosa
1. Control group	1.64 ± 0.20 ^a	1.27 ± 0.28 ^a
2. BSS-treated group (1 h)	2.40 ± 0.37 ^b	1.72 ± 0.20 ^b
3. BSS-treated group (15 days)	2.54 ± 0.22 ^b	2.15 ± 0.34 ^{b,c}
4. Acute Stress	5.95 ± 0.25 ^c	5.22 ± 0.33 ^c
5. Acute Stress +BSS	4.39 ± 0.27 ^d	4.17 ± 0.30 ^d
6. Chronic Stress	5.22 ± 0.30 ^e	3.95 ± 0.29 ^d
7. Chronic Stress +BSS	3.08 ± 0.22 ^f	2.41 ± 0.51 ^e

Acute and chronic stress were induced in female Sprague-Dawley rats in the presence and absence of BSS. Each value is the mean ± S.D. of 4 animals. Malondialdehyde was used as the standard. Values with non-identical superscripts are significantly different ($p < 0.05$).

Table 2. Acute and chronic stress-induced DNA fragmentation in the gastric and intestinal mucosa, and the protective ability of bismuth subsalicylate (BSS)

	Gastric Mucosa	Intestinal Mucosa
1. Control group	4.47 ± 0.54 ^a	3.65 ± 0.67 ^a
2. BSS-treated group (1 h)	4.92 ± 0.43 ^a	4.73 ± 0.59 ^b
3. BSS-treated group (15 days)	5.65 ± 0.48 ^b	5.79 ± 0.41 ^c
4. Acute Stress	17.66 ± 1.57 ^c	18.01 ± 1.39 ^d
5. Acute Stress + BSS	11.54 ± 0.89 ^d	11.77 ± 1.12 ^e
6. Chronic Stress	14.36 ± 2.42 ^c	14.62 ± 1.80 ^f
7. Chronic Stress + BSS	8.09 ± 1.09 ^f	7.05 ± 0.87 ^g

Acute and chronic stress were induced in female Sprague-Dawley rats in the presence and absence of BSS. Each value is the mean ± S.D. of 4 animals. Values with non-identical superscripts are significantly different ($p < 0.05$).

Table 3. Acute and chronic stress-induced modulation of membrane microviscosity (in poise) in the gastric and intestinal mucosa, and the protective ability of bismuth subsalicylate (BSS)

	Membrane Microviscosity (in poise)	
	Gastric Mucosa	Intestinal Mucosa
1. Control group	0.16 ± 0.03 ^a	0.11 ± 0.03 ^a
2. BSS-treated group (1 h)	0.25 ± 0.04 ^b	0.18 ± 0.03 ^b
3. BSS-treated group (15 days)	0.36 ± 0.08 ^c	0.17 ± 0.02 ^b
4. Acute Stress	1.86 ± 0.22 ^d	1.83 ± 0.40 ^e
5. Acute Stress + BSS	1.31 ± 0.20 ^e	1.24 ± 0.17 ^d
6. Chronic Stress	1.00 ± 0.13 ^e	1.02 ± 0.18 ^d
7. Chronic Stress + BSS	0.55 ± 0.07 ^f	0.50 ± 0.11 ^e

Acute and chronic stress was induced to female Sprague-Dawley rats in the presence and absence of BSS. Each value is the mean ± S.D. of 4 animals. Values with non-identical superscripts are significantly different ($p < 0.05$).

45% in the gastric mucosa, respectively, and approximately by 32 and 51% in the intestinal mucosa, respectively, as compared to the control animals (Table 3).

Reactive oxygen species production

The production of reactive oxygen species (ROS) in gastric and intestinal mucosa following induction of acute and chronic stress was assessed by superoxide anion and hydroxyl radical production. Cytochrome *c* reduction was used to assess superoxide anion production, which is a relatively specific test for superoxide anion production. Production of hydroxyl radicals was determined based on the formation of hydroxylated products of salicylic acids i.e. 2,5-dihydroxybenzoic acid (2,5-DHBA) and 2,3-dihydroxybenzoic acid (2,3-DHBA), following interaction with hydroxyl radicals. These hydroxylated products were determined using a high-performance liquid chromatograph equipped with an electrochemical detector.

The results of cytochrome *c* reduction in gastric and intestinal mucosa are presented in Table 4. Approximately 10.0- and 4.8-fold increases in cytochrome *c* reduction were observed in gastric mucosa, following exposure to acute and chronic stress, respectively, while under these same conditions

10.4- and 4.6-fold increases in cytochrome *c* reduction were observed in the intestinal mucosa, respectively, as compared to the control animals (Table 4). Following administration of BSS (7.5 mg/kg/day) for 15 days, approximately 1.9- and 1.8-fold increases in cytochrome *c* reduction were observed in the gastric and intestinal mucosa, respectively (Table 4).

Pretreatment of the rats with BSS decreased acute and chronic stress-induced cytochrome *c* reduction by approximately 32 and 52% in the gastric mucosa, and 22 and 55% in the intestinal mucosa, respectively, as compared to control animals (Table 4).

The results for hydroxyl radical production in the gastric and intestinal mucosa are shown in Table 5. Approximately 14.7- and 6.3-fold increases in hydroxyl radical production were observed in gastric mucosa, following exposure to acute and chronic stress, respectively, while under these same conditions 16.3- and 6.9-fold increases in hydroxyl radical production were observed in the intestinal mucosa, respectively, as compared to the control animals. Following administration of BSS (7.5 mg/kg/day) for 15 days, approximately 1.9- and 2.0-fold increases in hydroxyl radical production were observed in the gastric and intestinal mucosa, respectively (Table 5).

Pretreatment of the rats with BSS decreased acute and chronic stress-induced hydroxyl radical production by

Table 4. Acute and chronic stress-induced production of superoxide anion (cytochrome *c* reduction) in gastric and intestinal mucosa, and the protective ability of bismuth subsalicylate (BSS)

	nmol cytochrome <i>c</i> reduced/15 min/mg of protein	
	Gastric Mucosa	Intestinal Mucosa
1. Control group	22.6 ± 2.6 ^a	22.8 ± 1.4 ^a
2. BSS-treated group (1 h)	26.5 ± 3.7 ^a	27.9 ± 2.8 ^b
3. BSS-treated group (15 days)	42.8 ± 3.8 ^b	40.6 ± 7.2 ^c
4. Acute Stress	226.3 ± 11.2 ^c	237.9 ± 18.0 ^d
5. Acute Stress + BSS	154.5 ± 13.2 ^d	184.7 ± 15.3 ^e
6. Chronic Stress	108.7 ± 9.4 ^e	104.9 ± 11.0 ^f
7. Chronic Stress + BSS	52.3 ± 6.0 ^f	47.3 ± 9.2 ^e

Acute and chronic stress were induced to female Sprague-Dawley rats in the presence and absence of BSS. Each value is the mean ± S.D. of 4 animals. Values with non-identical superscripts are significantly different ($p < 0.05$).

approximately 37 and 42% in the gastric mucosa, and 49 and 63% in the intestinal mucosa, respectively, as compared to control animals (Table 5).

Discussion

The objective of this study was to assess the comparative effects of acute and chronic stress on the induction of oxidative gastrointestinal mucosal injury, and the protective ability of bismuth subsalicylate (BSS). Sprague-Dawley rats were subjected to acute and chronic stress after which the production of reactive oxygen species and the extent of membrane, lipid and DNA damage in the gastric and intestinal mucosa were assessed. Furthermore, the gastroprotective ability of BSS was determined.

Bismuth salts are commonly used to treat a variety of gastrointestinal disorders including peptic ulcers, dyspepsia, infectious diarrhea and parasitic infections [15, 16]. Agents which provide protection against gastric and intestinal mucosal injury by a mechanism other than inhibition or neutralization of gastric acid have been described as gastric cytoprotectants [4]. Several mechanisms of gastric cyto-

protection have been proposed [17]. However, the mechanism associated with cytoprotection by bismuth subsalicylate is not clearly understood.

Naganuma *et al.* [18] demonstrated that bismuth subnitrate significantly decreased lethal toxicity, cardiotoxicity and bone marrow toxicity of adriamycin. In a more recent study, Nakagawa *et al.* [19] have demonstrated that pretreatment of mice with bismuth subnitrate significantly prevented the clastogenicity of adriamycin, cyclophosphamide, cisplatin and L-phenylalanine mustard. Since a significant increase in tissue concentrations of metallothionein is observed following treatment with bismuth subnitrate, the authors concluded that the ability of bismuth subnitrate to reduce toxicity of adriamycin and the clastogenicity of various antineoplastic drugs may be ascribed to the induction of metallothionein. From these studies it can be concluded that the protective effect of metallothionein might be due to its ability to scavenge reactive oxygen species or inhibit their formation. However, recent studies by Klaassen (personal communication) have demonstrated that the toxicity of adriamycin is not altered in metallothionein transgenic mice or metallothionein null (knockout) mice. Furthermore, the effect of bismuth on adriamycin toxicity did not differ

Table 5. Stress induced production of hydroxyl radicals based on the formation of 2,3- and 2,5-dihydroxybenzoic (DHBA) in gastric and intestinal mucosa, and the protective ability of bismuth subsalicylate (BSS)

	Formation of 2,3- and 2,5- DHBA (nmoles/mg of protein)					
	Gastric Mucosa 2,5-DHBA	2,3-DHBA	Total	Intestinal Mucosa 2,5-DHBA	2,3-DHBA	Total
1. Control group	0.36 ± 0.09 ^a	0.45 ± 0.06 ^a	0.81	0.46 ± 0.05 ^a	0.48 ± 0.10 ^a	0.94
2. BSS (1 h)	0.36 ± 0.07 ^a	0.82 ± 0.18 ^b	1.18	0.53 ± 0.10 ^{a,b}	0.64 ± 0.12 ^a	1.17
3. BSS (15 days)	0.60 ± 0.11 ^b	0.95 ± 0.10 ^b	1.55	0.64 ± 0.12 ^b	1.24 ± 0.24 ^b	1.88
4. Acute Stress	3.84 ± 0.66 ^c	8.13 ± 2.21 ^c	11.97	3.56 ± 0.28 ^c	11.73 ± 0.41 ^c	15.29
5. Acute Stress + BSS	1.92 ± 0.42 ^d	5.63 ± 1.20 ^d	7.55	2.13 ± 0.51 ^d	5.73 ± 1.13 ^d	7.86
6. Chronic Stress	2.19 ± 0.30 ^d	2.94 ± 0.53 ^e	5.13	3.17 ± 0.38 ^c	3.35 ± 0.40 ^c	6.52
7. Chronic Stress + BSS	0.89 ± 0.19 ^e	1.25 ± 0.30 ^f	2.14	1.06 ± 0.24 ^c	1.35 ± 0.21 ^b	2.41

Chronic stress was induced to female Sprague-Dawley rats in the presence and absence of BSS. Each value is the mean ± S.D. of 4–6 animals. Values with non-identical superscripts are significantly different ($p < 0.05$).

between these genetically altered mice and control animals. Thus, it appears that metallothionein may not be involved in the chemoprotective effects of bismuth salts.

Forsell [3] proposed that bismuth acts as a mild irritant inducing liberation of endogenous mucosal prostaglandins which help to maintain blood flow and prevent vascular injury caused by irritants such as ethanol. This hypothesis has not been confirmed with respect to the chemoprotective abilities of bismuth salts. In addition, the mild irritant properties of BSS may explain the small but significant increases produced by BSS in lipid peroxidation, DNA fragmentation, membrane microviscosity and reactive oxygen species. Because bismuth does not undergo redox cycling, it may produce oxidative stress by binding to sulfhydryl groups.

Although bismuth salts are widely used to prevent gastric injury caused by diverse stressors, and reactive oxygen species are believed to participate in many gastrointestinal disorders [1–4], no studies have been conducted to determine if the cytoprotection by bismuth salts is associated with reactive oxygen species which are produced in conjunction with acute and chronic stress-induced oxidative stress. Previous studies in our laboratory [6] have demonstrated that BSS can induce a concentration-dependent inhibition in the biochemically generated production of superoxide anion, hydroxyl radical and hypochlorite radical plus hypochlorous acid *in vitro*. In these studies, the free radical scavengers superoxide dismutase plus catalase, mannitol and allopurinol inhibited the production of superoxide anion, hydroxyl radical and hypochlorite radical plus hypochlorous acid, respectively. Thus, these results suggest that BSS exhibits the ability to scavenge or quench various oxygen free radicals or prevent their formation.

In order to determine whether BSS could prevent oxidative tissue damage to gastric and intestinal mucosa, mucosal tissues were incubated with reactive oxygen species generating systems in the presence and absence of BSS [6]. The results clearly demonstrate that BSS induces a concentration-dependent inhibition of lipid peroxidation in gastric and intestinal mucosa which was produced by using a superoxide anion generating system, a hydroxyl radical generating system, and a hypochlorite radical plus hypochlorous acid generating system. Thus, our previous studies demonstrated that BSS can provide protection against oxygen free radical induced tissue injury in gastrointestinal mucosa *in vitro*. Furthermore, we have demonstrated that pre- and post-administration of BSS to rats can partially protect the gastric and intestinal mucosa against 0.2 M NaOH, 0.6 M HCl, 80% ethanol and aspirin (200 mg/kg)-induced increases in lipid peroxidation, membrane microviscosities and DNA fragmentation [6]. The results of these *in vitro* and *in vivo* studies support the hypothesis that the gastroprotective ability of BSS at least in part involves the ability of BSS to scavenge or prevent the formation of reactive oxygen species,

and prevent oxidative tissue damage by reactive forms of oxygen.

The present studies have assessed the protective ability of bismuth subsalicylate (BSS) against acute and chronic stress-induced gastrointestinal injury. Kuwayama *et al.* [5] have demonstrated that water-immersion restraint stress in rats resulted in an increase in cell loss in the gastrointestinal tract and macroscopic mucosal injury. This combination of increased cell loss with depressed epithelial proliferation may play a role in stress-related gastric lesions and injury in rats.

These studies demonstrated enhanced production of oxygen free radicals including superoxide anion and hydroxyl radicals in the gastric and intestinal mucosa *in vivo* following exposure to acute and chronic stress. Since lipid peroxidation occurs in response to free radicals and reactive oxygen species, the *in vivo* effects of acute and chronic stress were assessed on both gastric and intestinal mucosa. Lipid peroxidation was assessed as an index of oxidative lipid degradation induced by acute and chronic stress. Programmed cell death is a selective process of physiological cell deletion that plays a major role in developmental biology and in maintenance of homeostasis in vertebrates [20, 21]. Programmed cell death is morphologically known as apoptosis. Apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, condensation and fragmentation of nuclei, and extensive degradation of chromosomal DNA [20, 21]. Thus DNA fragmentation was quantitated and correlated with oxidative stress [20, 21]. Membrane microviscosity is inversely proportional to the membrane fluidity [11, 12]. The changes in membrane fluidity occur due to the direct effect of these stressors on the structural integrity of the gastric and intestinal mucosa, including the changes in lipid peroxidation [11, 12].

In summary, the results of these experiments indicate that oxygen free radicals, including superoxide anion and hydroxyl radicals, are involved in the pathogenesis of acute and chronic stress-induced gastrointestinal injury, and can result in the production of lipid peroxidation, DNA fragmentation and altered membrane fluidity. BSS demonstrated excellent protection against chronic stress-induced gastric injury, while some protection was observed against acute stress under the conditions that were employed.

Acknowledgement

These studies were supported in part by a grant from the Proctor & Gamble Company, Mason, OH, USA.

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Stress genes and species survival

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Abstract

Stress genes can be ascribed to have been generated by the organism for their intrinsic urge to survive against the changing environmental odds, during the evolutionary process. This concept has been supported by a large number of reports describing individual types of phenomena. These have been reconciled and globalised in terms of their relevance in this article. Supporting evidences have been drawn from the literature which indicated that by using different types of inducer one can express heat shock proteins. Similarly, several types of stress inducers, such as calorie restriction, LPS stimulation and Staphylococcal Protein-A stimulation, it was possible to induce a wide array of biological, biochemical and immunological reactions. Such biological reactions rendered protection against toxic, carcinogenic, metabolic, as well as biological stresses induced by microorganisms. Heat shock proteins have been implicated as having a role in providing resistance to the host against different types of stressors. In this article, some mechanistic schemes have been proposed as possible pathways globalising such phenomena. A minute amount of stress inducers has been observed to have helped expression of stress resistance genes, providing increased capability to the host to protect itself against myriads of both biotic and abiotic stressors. More understanding about such phenomena would help in keeping our physiological systems vigilant and our bodies healthy, fighting out the stress-related events effectively. (Mol Cell Biochem **196**: 117–123, 1999)

Key words: stress, resistance, protection, stress gene superfamily, protein A, lipopolysaccharide, heat shock protein, calorie restriction, metabolic stress, carcinogenic stress, chemical stress

Abbreviations: DNA – deoxyribonucleic acid; SGS – stress gene superfamily; BCG – bacillus calmette guerin; LPS – lipopolysaccharide; HSP – heat shock proteins; IL – interleukin; TNF – tumor necrosis factor; NK – natural killer; ADCC – antibody dependent cell-mediated cytotoxicity; LAK – lymphokine activated killer; Cyt – cytochrome.

Introduction

Evolutionary dogma revisited

Some three thousand million years ago, when the first living cell evolved on the surface of the earth, it had to survive in the face of seemingly insurmountable environmental odds. It had to traverse a long and arduous path on its way to become a more organised form suitable to cope with adverse circumstances. A series of biochemical manipulations of genomic organization were required to adapt to the changing circumstances. The result was the evolution of more organised life forms → from photosynthetic bacteria → blue green algae → aerobic bacteria → eucaryotic cells →

multicellular organisms – invertebrates – fishes – terrestrial plants – birds – mammals . . . to Man. In the evolutionary pathway, more than 99% of all species of living organisms, which existed at one time or the other, are now extinct. They have no present day descendants. They could not survive the potentially damaging environmental pressure and were eliminated. Throughout the entire process of biological evolution, the surviving organisms were better equipped with the ability to transform into non-lethal mutants as a result of chemically and physically induced changes in the genomic structure. Thus, it was possible to select the best suited to survive the contemporary environmental conditions of life [1]. It was because of the evolution of ‘Stress Response Genes’ (SRG), which appear to be one of the most highly conserved and abundant genomic sequences found in nature.

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Evolutionary Gains

A large number of reports are available in the literature which suggest that there were unique genomic sequences which imparted high efficiency for survival of the organism. They appear to have been 'conserved' in the genome, even across species barriers [2–6]. When required, the organisms may use them by allowing them to be expressed freely. These genes appear to be activated to cope with certain stressful events, such as heat shock [5–7], metabolic stress [8], exposure to microgravity [9], hydration [10], nicotine, ethanol or heat stress [11], genotoxic stress [12], oxidative stress [13], osmotic stress [14], radiation stress [15], carcinogenic stress [16, 17] etc. It is very interesting that even though the nature of stress inducers are different in terms of their both physical and chemical nature, the biological reactions they induce may be similar. They all induce Heat-Shock Proteins (HSPs). That is to say that those SRGs can be induced by altogether different and varied nature of stress inducers or stressors, as they will be termed throughout this article.

Usually, the urge of an organism is to adjust itself to the prevalent conditions to come back to a normal or steady state, conducive enough for perpetuation of life processes. We know that all organisms are continuously getting exposed to environmental, toxic or carcinogenic, physiological and metabolic stressors. How are they alive then? It is because they possess the intrinsic capabilities to withstand divergent types of stressors. Unless they would have had such built-in capacity, they would not have existed thus far. Those inherent mechanisms of stress-resistance can be considered, therefore, as our evolutionary gains.

Stress gene superfamily (SGS)

It is only logical to assume that a large number of SRGs may have organised themselves together in a cluster in the form of a Stress Gene Superfamily (SGS). Such genes may act and function in harmony as per the needs of the organism. For years, the scientists have been trying to elucidate such intrinsic mechanisms as are responsible for protecting the 'self'. A large number of such studies have been reported in the literature from time to time, which looked at this problem from one point or the other, studying one type of stressor or the other. It is pertinent therefore to take a note of a few of such interesting observations to find out if there exists any common mechanism which so far escaped the attention of the researchers.

First of all, it may be observed that although the nature of the stressors (inducers) used by various investigators are different, the reactions they induce appear to be similar in nature [5–7, 11]. On the other hand, a particular type of stressor (inducer) was also observed to be able to induce a

large array of reactions, which are often required to protect the host from the deleterious effects of different types of physical, chemical and biological stressors. These include also the toxic or carcinogenic insults caused by a wide variety of toxic or carcinogenic chemicals. These observations [9–34] provide us with the understanding that by using a specific stressor one may be able to activate a number of gene sequences of the 'SGS'. As a result of such activation a large number of biomolecules may be expressed, in a cascade type of reactions to abrogate the harmful effects of different stressors. This appears to be true in case of both biological and chemical stressors [19, 20–23].

DNA – still a puzzle

If we have to understand how such stress resistance mechanisms function, we have to look at the genomic organization. It is known that significant stretches of the genomic sequences still remain a great puzzle to us and their functions are yet to be elucidated. Because of our inability to understand the functional property of most parts of such sequences, they have wrongfully been labelled as 'Junk'. Interestingly, nature has not created anything without a meaning. There have been scores of examples in scientific literature that some of the same 'Junk' portion of the genome is made operative at times. Normally they remain dormant as reserve force and are used when there is a need. It is quite understandable that the organism keeps them on reserve, only to deal with unusual and abnormal (stressful) circumstances to safeguard the 'self' survival. SGS appears to be still a part of this hitherto unknown region of cellular DNA. It is also not known whether or not all cells process all the stretches of SRG or if they are distributed in different cell types. It is logical to assume that the latter may be the case. Further research will delineate the actual mechanism governing the expression of SGS.

Stress response genes versus stress resistance genes

When attempting to withstand the onslaughts of the abnormal stressful conditions the organisms may activate 'genes' to produce specific proteins, each responsible for counteracting specific or non-specific stress induced abnormalities [17–38]. Such proteins may have a variety of functions: as enzymes to catalyze biotransformation and detoxification reactions (Phase I and Phase II reactions), as hormones to potentiate cell proliferation and differentiation, as structural proteins to supply the building blocks to repair structural damages, as antibodies to nullify antigenic bacteria, fungus, virus, foreign cells and tissues etc., as cytokines to potentiate cell proliferation, differentiation and production of growth factors,

carrier or transport proteins (such as chaperones, heat shock proteins), as signal proteins transmitting molecular messages from membrane to the genomic sequences in the nucleus for expressions of gene products, and also as DNA binding proteins, transcription factors, transcription activating factors, transactivating factors etc., for proper activation and expression of gene products.

Studies carried out on heat-shock proteins [5–7, 11], and various other stress induced proteins [8, 11–40] have yielded a wealth of information in recent years. Genes dealing with the resistance mechanisms in microorganisms against the immune attack of the host, in addition to those which induce immunity against them in the host [18–20, 25–28, 34], added some important information regarding the existence of Stress Response Genes in microbes. Abrogating such mechanisms would help in controlling their growth and proliferation. Nature has evolved such unique gene sequences as to provide adequate resistance mechanisms against environmental odds of physical, chemical and biological nature in every species. The evolutionary dogma of ‘survival of the fittest’, therefore, lies in the ability of the organisms to perpetuate the Stress Response Genes vis-a-vis the Stress Resistance Genes.

Stress induction and cross tolerance phenomena

It is very intriguing to note that several immunomodulators when used in very small dose, which induces small stress, such as BCG vaccine, *S. aureus*, *S. faecalis*, *C. parvum*, Coley’s toxin, various types of viruses, bacterial lipopolysaccharides, lipid A, staphylococcal Protein A etc., not only potentiate non-specific immune responses against a variety of bacteria and viruses, but also have been observed to cause regression of various tumors [41]. As is well understood, the tumor is a major stress inducer so far as the host is concerned. One of the major outcomes of the progressive growth of a tumor is its profound suppressive effect on the immune system, causing lymphocytotoxicity, inhibition of phagocytic response, induction of humoral and cellular immunosuppressive factors etc. Thus, it gives rise to total anergy [41] in advanced cases of malignancy. It has been well established that sensitising the host with small amounts of various types of stressors, as are mentioned above, helps in activating the host resistance mechanism to fight out tumor growth [41].

The various compartments of the host immune system have been observed to be activated by such immunomodulators, resulting in the proliferation of the lymphoid cell populations, potentiation of the natural killer cell and lymphokine activated killer cell activity. It also stimulates the antibody dependent cell mediated cytotoxicity, phagocytic phenomena, increased antibody production, and an increased elicitation of growth factors and cytokines. These observed facts can only be explained if it is accepted that the activation of any

one of the gene(s) of the SGS may help in coactivation and coexpression of multiple gene products, perhaps in a cascade type of reaction. It is not known, however, what are those sequences of reactions and also how many gene products are liberated at a time, and also whether different set of SRGs are located in different cell types. Future research will throw more light in this direction to help us in developing strategy as to when and how to induce such SRGs for sufficient protection.

Stress-induced preconditioning

During the past decades, a tremendous volume of literature had indicated that ‘Calorie Restriction’ was able to provide the host with an increased ability to fight out major toxic or carcinogenic stresses [35]. Caloric restriction definitely causes various forms of physiological stresses in the body.

LPS-induced induction of minor oxidative stress has been reported to stimulate the anti-oxidant mechanisms of the body, such that the reperfusion-induced cardiac damages could be lessened [23].

It is now well established that the resistance of the myocardium to ischemia can be substantially enhanced both by preconditioning of the host and by upregulating the cytoprotective proteins, particularly heat shock proteins (HSPs).

Currie et al. [36] described the association between the heat shock proteins and myocardial protection. They showed that by raising the body temperature in rats, both cardiac HSPs and catalase activity were increased, and at this point hearts became resistant to ischemia/reperfusion injury.

Of greater pathological relevance was the observation that ischaemia itself could induce evolution of HSPs and involution of cardiac stress [37], by simultaneously increasing the concentration of myocardial antioxidant enzyme, superoxide dismutase [37, 38].

Thus, it is quite evident that a very minute amount of stress-induced shock may help potentiating the intrinsic ability of the host to absorb more shock as it might have to face later. Heat shock proteins appear to play a major role in this stress-induced resistance mechanism.

Diverse functions of Protein A of S. aureus

The above proposition is strengthened by our observations spanned across the past two decades. We observed that although Protein A of *S. aureus* Cowan-I was originally discovered for its Fc-binding affinity of IgG, and that such property was utilized for complexing both IgG and immune complexes; the same Protein A had been observed to possess multifarious properties, such as anti-toxic [18–23, 29–34, 28–36], anti-carcinogenic [21, 22] and anti-tumor [39–40]

properties. It renders protection to the host against a wide variety of stressors, such as cyclophosphamide, carbon tetrachloride, benzene, dimethyl benz-anthracene, Salmonella endotoxin, Aflatoxin, various tumors, and also intracellular parasites [18–23, 28–36, 39, 40]. Significant protection against the toxic, carcinogenic and immunosuppressive effects of those compounds were observed with prior sensitization of the host with a very small amount of Protein A [18, 23, 29, 34]. Further, Protein A has been found to cause increased phagocytic response, activate respiratory burst phenomena, increase production of IL1, IL2, Gamma-interferon, TNF-alpha, CFU-E and CFU-MG, erythropoietin, and also effect an increase in NK cell, ADCC and LAK cell activities. Our recent observations have indicated that Protein A can induce selective apoptosis to tumor cells as against spleen cells of the same tumor hosts (P.K. Ray et al., unpublished observation). In general, Protein A has been shown to activate Th1 response and depress Th2 response. These cytoprotective and immunostimulatory properties of Protein A, including its ability to elevate the Cyt-P450 dependent hepatic microsomal mixed function monooxygenases, Glutathione S-transferases Glutathione Content etc., simul-

taneously activating the bioelimination processes by activating the cells of monocytes and PMN lineages, have been implicated to be responsible for its anti-toxic, anti-carcinogenic, immunostimulatory, cytoprotective and cross-tolerance inducing properties.

The above observations provide supporting evidences to our contention that minute amount of stress induction by a stressor-inducer molecule might offer increased resistance to either the same or other stressors. These observations are indicative of the existence of SGS, expression of which would offer protection to the host against various environmental agents. One may be tempted to speculate that activation of SGS should be able to provide cross tolerance and protection against a wide variety of stressors [42]. Role of stress proteins in myocardial protection [43] also supports our hypothesis.

Autoregulation of stress

The phenomenon of induced enzyme synthesis has been well documented over the years. This happens due to activation of an otherwise dormant gene sequence, which is induced by

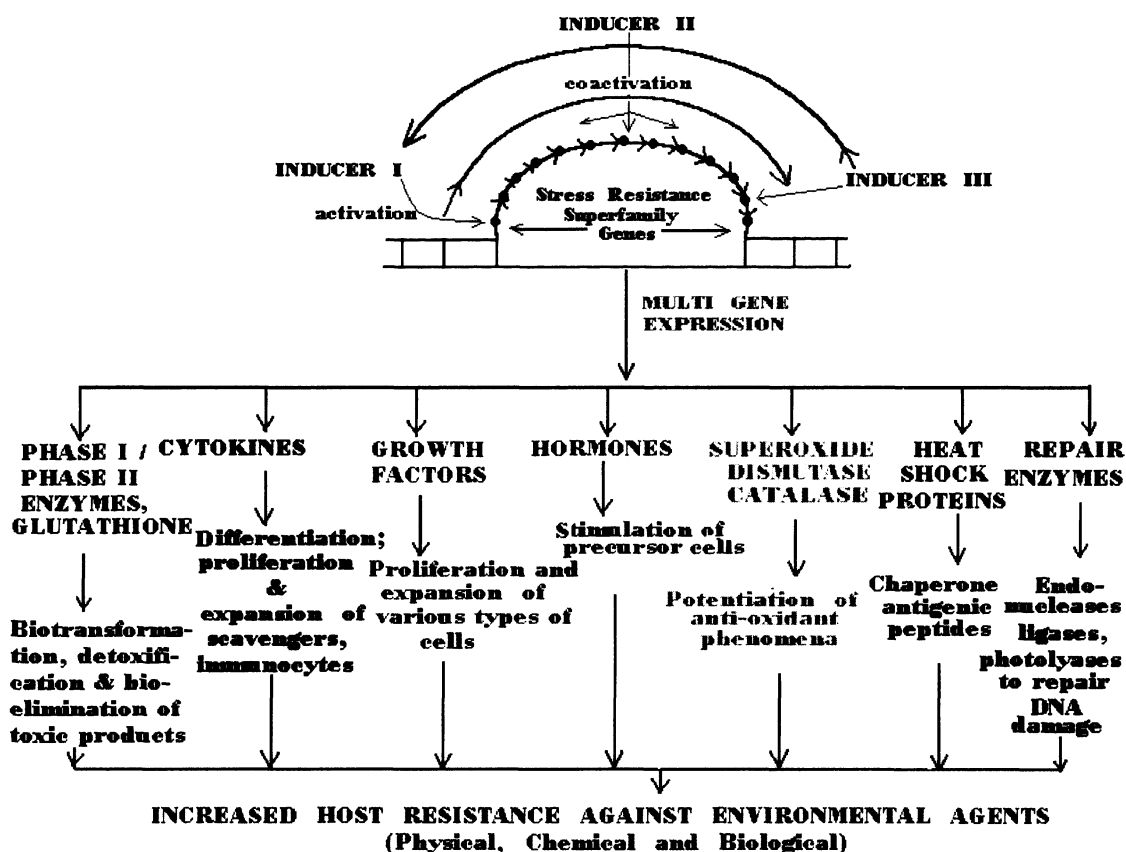


Fig. 1. Possible mechanism of expression/coexpression of stress resistance superfamily genes by varied nature of inducers (I/II/III). The mechanism shows only three different possibilities as an example. There could be many others. The direction of activation/coactivation is purely empirical and requires validation. Obviously, the sequences of events cannot be predicted, and may depend on the nature of inducers (stressors) on the one hand, and regulatory mechanisms of the host on the other.

the substrate itself. Also, the mechanisms of induced-drug resistance in microbes, parasites and tumour cells have been well established. Interestingly, there exists striking resemblances among such inductive processes. The evolution of heat shock proteins in plants, bacteria and mammalian cells is yet another unique phenomena. The HSPs are liberated as a part of the host resistance phenomena. Feedback mechanism to inhibit the production of unwanted excesses of a product is a well known control mechanism in biological systems. An elaborate control mechanism also exists to detoxify harmful chemicals in various species, which again are mostly inducible systems.

Further, it has been observed that when naturally occurring innate immune resistance fails, the induced immunoprotective mechanisms prevail. All these phenomena appear to be genomically linked. Normally they remain dormant or less functional under the normal conditions. Any stress inducer (physical, chemical and biological) which we encounter in

our everyday life from food, water, air or by deliberate ingestion of drugs may effect activation of such stress genes. Thus, such dormant genes are made operative to keep our physiological system vigilant to fight out the odds. Continuous search in this area might provide more insight into our understanding about what they are, and how they are, especially how they are regulated during both normal and abnormal circumstances. In the event of availability of suitable inducers it might be possible to boost the ability of our 'physiological system to render greater protection against toxic, carcinogenic, immunosuppressive and infectious agents than we would have otherwise experienced [42].

Our hypothesis

It appears more and more evident from the above that a large number of genes may have clustered together because of the

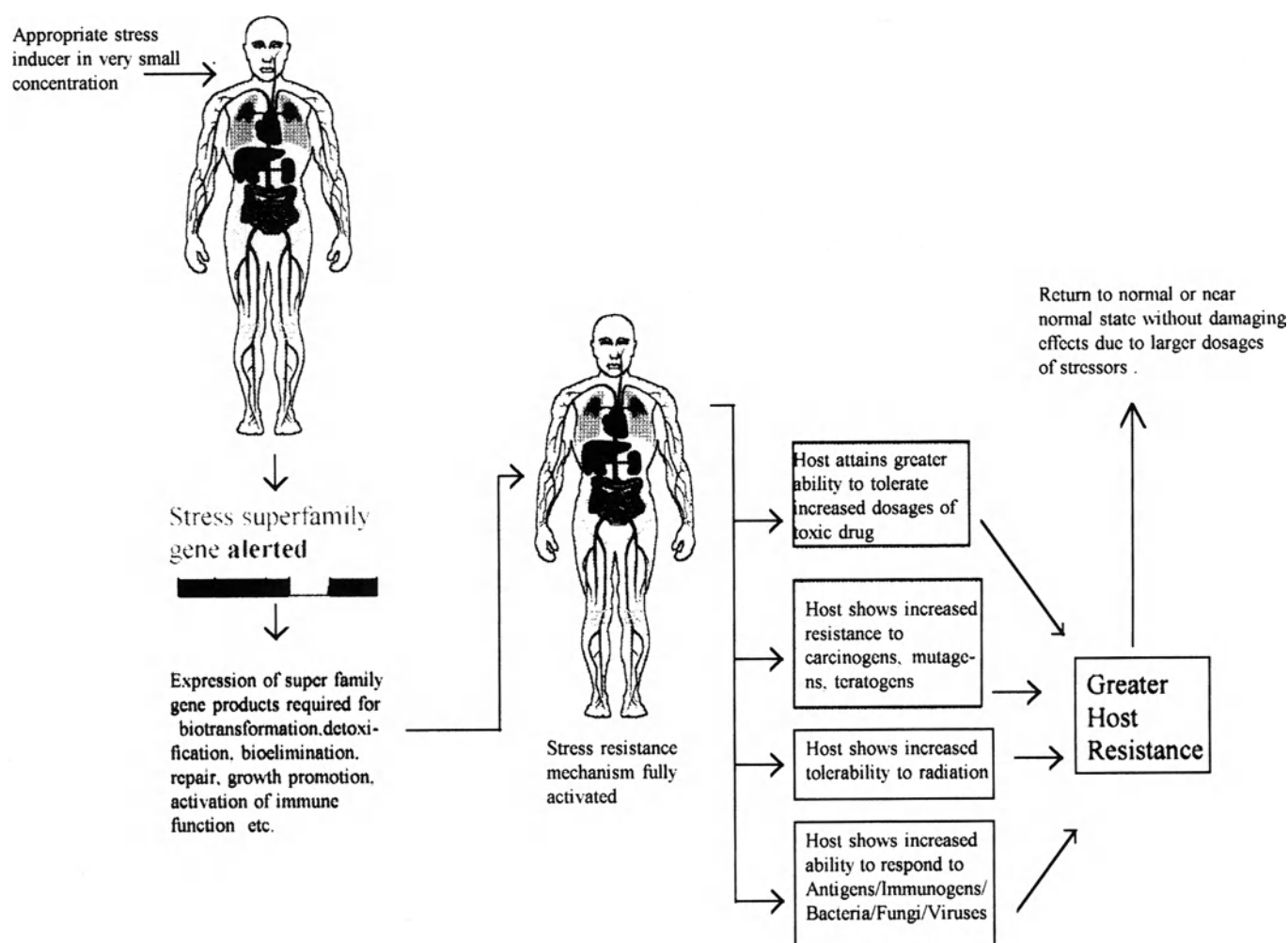


Fig. 2. A hypothesis regarding the mechanism of small stress induction in rendering greater capability to withstand larger dosages of stressors.

survival pressure in the form of a superfamily. Any one of them, following activation, may help triggering coexpression of the others in the form of a cascade type of reaction. A possible but hypothetical scheme is presented in Fig. 1.

It would have been a self-limiting mechanism if more than one such gene were not to be coexpressed. Since to repair various damages caused by any environmental agent, a multitude of biochemical reactions are necessary, such requirement would necessitate the expression of multiple genes. Thus, it indicates the existence of SGS and the necessity of their co-expression. The gene products may constitute repair enzymes, biotransformation and detoxification enzymes, cytokines, growth factors and hormones, antioxidants, heat-shock proteins, antibody molecules etc. It is not known, however, whether all such requirements are met with by one type of cell or if it is the collective efforts of a large number of different types of cells, located in different tissues and organs. It is logical to assume that the latter is the possibility. In such a case, the mechanisms of 'cross-talk' is required to be elucidated to have more understanding about it.

It seems likely that deliberate introduction of minutest amount of stress may sensitize the host, alerting it to switch on its SGS, thus rendering protection against secondary exposures to larger amount of the same and/or different stressors (Fig. 2).

This phenomenon appears to be quite similar to the development of immunity by vaccinating the host with very small amount of a sensitizing substance – an antigen or immunogen. In the immune system, the infectious substances are generally dealt with. The concept of repair and reconstruction is virtually lacking in the overall concept of the development of immunity.

It is known that to provide protection against toxic chemicals, mutagens, carcinogens, radiation etc., other physiological systems, such as Phase I and II biotransformation and detoxification mechanisms, together with the immuno-neuro-endocrine axis, antioxidant mechanisms, repair mechanisms, cytokine-growth factors, hormone mediated growth promotion and proliferation phenomena, the phenomena of heat shock protein chaperoning peptide/proteins etc., appear to be involved in rendering the overall protection to the self. All or some of such systems may have to function in harmony with one another to cope with the multitude of both biotic and abiotic stress-inducers to safeguard the 'self'.

Acknowledgements

The hard work of all my students and co-workers is thankfully acknowledged. Fruitful discussions and suggestions of Dr. Ronald W. Hart of National Center for Toxicological Re-

search, Arkansas, Prof. D.K. Das of Cardiovascular Division, University of Connecticut, Prof. P.K. Srivastava of the Cancer Center of University of Connecticut Health Sciences Center, USA, Prof. J.J. Ghosh of Calcutta University and Prof. R.K. Mandal of Bose Institute are gratefully acknowledged. The author is thankful to Mr. Arup Sengupta and Mr. Shankar Sinha for their secretarial assistance and to Parthasarathi Ray and Amartya K. Ray for their editorial assistance.

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Fever: An integrated response of the central nervous system to oxidative stress

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Abstract

Immediately after bacterial endotoxin (LPS) enters the circulatory system there is increased production of free oxygen radicals by cells of the reticulo-endothelial system, followed by the release of cytokines considered as putative endogenous pyrogens. Fever originates by central nervous system activities, but neither exogenous nor endogenous pyrogens are able to cross the blood–brain barrier and the true signal which is transmitted to structures inside the blood–brain barrier is still unknown. To study the role of oxygen radicals in fever, we pretreated rats with methylene blue, an inhibitor of superoxide and hydroxyl radical production and investigated the febrile response to LPS in conscious rats by measuring malondialdehyde formation as an index of lipid peroxidation by oxygen radicals. Methylene blue lowered resting malondialdehyde levels to near detection level and significantly suppressed its rise which was regularly found following LPS in the untreated state. Pretreatment with methylene blue completely blocked the febrile response. Since fever is a central nervous system-mediated response these results indicate that the brain is able to sense oxidative stress and vicinal thiol groups of the redox-modulatory site of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor-channel complex could function as a possible receptive structure. To test this hypothesis we injected rabbits with the disulfide reducing agent dithiothreitol (DTT), known to penetrate the blood–brain barrier, and monitored its effect on normal and febrile body temperatures. DTT induced, independently of ambient temperature, within minutes and dose-dependently the full pattern of heat loss responses causing a fall of core temperature, indicative of a lowered thermoregulatory setpoint. Pretreatment with a bolus dose of 5 mg/kg DTT, followed by a continuous infusion of 5 mg/kg/h for 3 h completely prevented LPS-induced fever. A bolus dose of 20 mg/kg DTT, starting 30 min after LPS, immediately reversed the febrile cold defense pattern and lowered body temperature. We conclude that DTT reduces in the central nervous system oxidized vicinal thiol groups of NMDA receptors, thereby augmenting glutamate-induced nitric oxide synthase activation, and, thus, enhanced formation of NO, which, in turn, lowers the thermoregulatory setpoint. Reduction of other disulfide-containing molecules, especially oxidized glutathione and thiol-containing enzymes, by DTT by might additionally contribute to preventing fever. (*Mol Cell Biochem* **196**: 125–132, 1999)

Key words: fever, *E. coli* lipopolysaccharide, dithiothreitol, methylene blue, nitric oxide, malondialdehyde, biothiols, glutamate, NMDA-receptor, oxidative stress

Introduction

Oxidative stress results when the balance between oxygen-derived species such as superoxide anion (O_2^-), hydrogen peroxide, hydroxyl radical, hypochlorous acid and singlet oxygen, and the cellular antioxidant defenses against them, is disturbed [1]. Most of the highly reactive oxygen species, although produced as side products of normal electron

transport processes, are potentially cytotoxic. To protect against tissue damage, a number of cellular antioxidant defenses such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase have developed. Additionally, several small molecules such as α -tocopherol, ascorbate, carotenoids, and glutathione act as scavengers of oxygen radicals.

Nitric oxide (NO), another highly reactive and unstable

free radical detected first in endothelial cells [2], but later in nearly all other mammalian cells including neurons, is produced by the action of the cytosolic enzyme NO synthase (NOS) which synthesizes NO by converting L-arginine to citrulline. The neuronal isoform of NOS has been allocated to discrete neuronal populations, most prominently to those containing the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. When glutamate binds to the NMDA receptor, it triggers the opening of a cation permeable channel. The Ca^{2+} entering the neuron binds to calmodulin, a cofactor for NOS and stimulates NOS activity [3]. NO then diffuses out to act on neighbouring presynaptic nerve endings and astrocyte processes, and, thus, functions as a neurotransmitter [3, 4]. Stimulation of glutamate receptors has also been implicated as a cause of neuronal injury by the calcium- and arachidonate-dependent superoxide anion (O_2^-) formation [5]. The simultaneous production and release of NO and O_2^- , which occurs in macrophages [6], but also in the brain [7] can rapidly lead to the formation of peroxynitrite anion (ONOO^-), which may exert its cytotoxic effects in part by oxidizing tissue sulfhydryls [8]. On the other hand, NO has been shown to mutually interact with and inactivate superoxide anion, and may thus exert a protective role in tissue injury [9].

Fever is the reaction of homeothermic animals and men when lipopolysaccharide (LPS) constituents of microbial cell walls enter the vascular space and interact with the cells of the reticulo-endothelial system (RES). LPS rapidly activates in mobile or sessile macrophages the membrane-bound respiratory burst (NADPH) oxidase, which causes, within minutes, the release of free oxygen radicals [10, 11]. A similarly fast response of the RES to LPS is the release of cytokines, among those tumor necrosis factor α ($\text{TNF}\alpha$), interleukin-1 (IL-1), interleukin-6 (IL-6), and the interferons $\alpha 2$ and γ have been considered as the putative endogenous mediators of fever [12, 13]. If NOS is stimulated by LPS then it is the constitutive (cNOS) isoform [14], because the inducible NOS is only activated with a latency of hours [15]. There is general agreement that fever originates by central nervous system (CNS) activities, and traditionally prostaglandin E_2 (PGE_2) is believed to act centrally as the obligatory mediator of fever [16, 17, 18]. Since neither the exogenous nor the endogenous pyrogens are able to cross the blood-brain barrier (BBB) it is hypothesized that circulating pyrogens act outside the BBB from where the signal is transmitted to structures inside the BBB resulting in the augmentation of IL-1 and IL-6. Alternatively circumventricular organs, which lack a blood-brain barrier, especially the organum vasculosum laminae terminalis (OVLT), have been implicated as brain sites where circulating pyrogens can directly contact microglial elements.

We have recently proposed a role of nitric oxide (NO) as a signal transducing agent in febrile response by showing an

early decrease of NO production following LPS, as evaluated by falling plasma nitrate levels, which inversely correlated with the height of fever [19]. Conversely, in rabbits pretreated with methylene blue, application of LPS was followed by rising plasma nitrate levels and abolition of oxygen radical formation, and fever was completely prevented despite elevated levels of PGE_2 and $\text{TNF}\alpha$ [20].

Methylene blue is known as a weak inhibitor of guanylate cyclase, but inhibits also purified NOS [21], and has been found to enhance extracellular glutamate accumulation within the brain [22]. An elevation of glutamate after treating cerebellar granular cells with the disulfide reducing agent dithiothreitol (DTT) was observed by Oh *et al.* [22]. We hypothesize that the fever preventing effect of methylene blue is in part due to its ability to prevent oxidation of vicinal thiol groups of the redox modulatory site of the NMDA receptor, resulting in the enhanced transduction efficiency of calcium entry with the consequence of NOS stimulation and, thus, enhanced NO formation.

We have now examined the potential of DTT as a modulator of glutamate release ascribed to its capability to reduce the redox modulatory site of the NMDA receptor-channel complex with regard to its influence on the febrile response of rabbits.

Materials and methods

Animals and experimental protocol

Samples of 20 female Chinchilla rabbits (Charles River, Kisslegg, Germany), weighing from 3.5 to 4.5 kg, and of 26 male Sprague-Dawley rats (Charles River, Kisslegg, Germany) of approximately 300 g body weight, were studied. The animals were housed individually in cages under artificial illumination from 0600 to 1800 h at 20°C in a temperature-controlled room, with water and food *ad libitum*. The animal protocol described here was approved by the bioethical committee of the District of Darmstadt, Germany. Experiments using conscious animals were performed with the animals sitting unrestrained in a box, adjustable to body size, with temperature controlled metal walls and bottom, while back skin and ears were exposed to room temperature of 25°C. Foot pad skin, ear skin, and rectal temperatures were continuously recorded using thermistors. Respiratory rate of rabbits was counted by means of a solid state strain gauge (Pneumotrace, ADInstruments, Milford, MA, USA) wrapped around the chest. Metabolic rate was determined by measuring oxygen consumption using open flow respirometric techniques with a two-channel oxygen analyser (Taylor Servomex, Ratingen, FRG). All electrical signals were monitored on a storage oscilloscope while being fed via a

MacLab/4s interface into a Macintosh 7500/100 PC. In rabbits, the central ear artery was cannulated on the day of the experiment, under local anesthesia, using 1% xylocaine, for recording blood pressure and heart rate, but also for blood sampling, while intravenous (i.v.) injection of drugs was performed after catheterization of an ear vein under local anesthesia. The experiments were started around 09.00 h, when core temperature and cardio-respiratory parameters had been stable for at least 45 min. In a first experimental session, each rabbit received 1 µg/kg LPS i.v. After an interval of 4–6 weeks the same dose of LPS was applied again, however, during a continuous infusion of 15 mg/kg/h dithiothreitol, which had been started 45 min earlier. Rats were placed in the temperature controlled box around 0800 h, and were injected intraperitoneally at 10.00 h either with saline or 10 µg/kg LPS. Paw skin and rectal temperatures were taken every hour. The same rats received, after an interval of 4–6 weeks, the same dose of LPS after i.p. injection of 10 mg/kg methylene blue given 1 h prior to LPS.

To investigate the effects of methylene blue on LPS-induced lipid peroxidation and DNA damage, two groups of rats were slightly anesthetized with intraperitoneal pentobarbital [30–35 mg/kg) and the femoral vein was cannulated. Six rats were injected 10 µg/kg LPS after a continuous infusion of saline for 1 h, the other 6 rats received LPS after infusion of 5 mg/kg/h methylene blue for 1 h. Blood samples were taken immediately before, and 1 h after the injection of LPS. The rats received thereafter 60 mg/kg pentobarbital, and brain, liver and heart were excised and frozen in liquid nitrogen.

Assay for malondialdehyde (MDA)

For quantitative evaluation of aldehydes in rabbits, blood was collected in chilled tubes containing 28.3 mM EDTA and 5.5 mM sodium bisulfite per ml and immediately centrifuged at 4°C at 5000 g. Deproteinization of plasma was performed with equal volumes of 20% TCA in 5.5 mM sodium bisulfite. After vortexing for 10 min at 4°C, the supernatant was derivatized with DNPH [23, 24], extracted with pentane, dried under nitrogen at 30°C and reconstituted in 200 µl acetonitrile. Ten µl of the acetonitrile extract were directly injected onto a Beckman Ultrasphere ODS C18 (3 µm particle size, 75 × 4.6 mm i.d.) HPLC column kept at 30°C. Chromatography was performed using a Waters Alliance 2690 Separations Module (Milford, MA, USA) controlled by Waters 2010 Millennium Chromatography Manager. The DNPH derivatives were detected with a Waters 996 photodiode array detector at either 307 or 356 nm at a flow rate of 1 ml/min with an isocratic gradient of acetonitrile-water-acetic acid (34:66:0.1, v/v/v) for 15 min, a linear gradient to acetonitrile-water-acetic acid (50:50:0.1, v/v/v) for 10 min,

an isocratic gradient of acetonitrile-water-acetic acid (50:50:0.1, v/v/v) for 5 min, then acetonitrile-water-acetic acid (95:5:0.1, v/v/v) for 10 min with a 20 min re-equilibration with the initial mobile phase. The amounts of the various aldehydes were quantitated by performing peak area analysis using Waters Millennium software program. The peaks were identified by co-chromatography with DNPH derivatives of authentic standards, prepared after Largillière and Mélançon [24], and peak area was calculated using Waters Millennium software. Detection limit was 15 pmol/ml.

Assay for 8-hydroxydeoxyguanosine (8-OHDG)

Tissues (1 gm) were homogenized in 10 ml solution containing 1% SDS and 1 mM EDTA using a Polytron homogenizer, and the homogenates incubated at 38°C for 30 min with proteinase K (500 mg/ml). After adding Tris-HCl buffer, pH 7.4, the resulting solution was extracted with 1 volume of phenol and Sevag (1:1 v/v). The DNA in the aqueous phase, after centrifugation, was precipitated with NaCl/ethanol at –20°C. After dissolving the DNA in NaCl, Na-citrate and EDTA, the solution was incubated for 30 min at 38°C with RNase T₁ (50 units/ml) and RNase A (100 µg/ml). The DNA solutions were extracted with Sevag and precipitated with NaCl/ethanol [25, 26]. The DNA pellets were dissolved in 0.5 ml of 20 mM sodium acetate, pH 4.8, and incubated for 30 min at 37°C with 63 mg of Nuclease P₁. After adding 50 µl Tris-HCl, pH 7.4, the nucleotides were incubated for 60 min at 37°C with 6.3 units of *E. coli* alkaline phosphatase [27]. A 5 µl of the filtered aliquot of the hydrolyzed DNA was injected onto a Beckman Ultrasphere ODS C18 (3 µm particle size, 75 × 4.6 mm I.D.) HPLC column. Chromatography was performed using a Waters Alliance 2690 Separations Module and a Water 996 photodiode array detector, controlled by Waters 2010 Millennium Chromatography Manager (Milford, MA, USA). The sample was run isocratically for 10 min using a mobile phase containing 4% acetonitrile and 0.1 acetic acid. The DNA-OH[•] conjugate, 8-OHDG, was detected at a retention time of 3.8 min using a wavelength of 297 nm [28]. Purity of 8-OHDG was determined by the UV-scan of the authentic standard with comparison to the sample 8-OHDG.

Drugs

Methylene blue was purchased from Neopharma (Aschau, FRG), and xylocaine was from Astra (Wedel, FRG). LPS from *Escherichia coli* (serotype O113, Ass. Cape Cod, Woods Hole, MA) was dissolved to 5 µg/ml in saline. Dithiothreitol, *E. coli* alkaline phosphatase, proteinase K, malonaldehyde bis(dimethyl acetal), formaldehyde, acetaldehyde, acetone, and propionaldehyde, 2'-deoxyguanosine, 2'-deoxycytidine,

2'-deoxyadenosine, and thymidine were purchased from Sigma-Aldrich, Deisenhofen, FRG. Dinitrophenylhydrazine (DNPH) was obtained from Fluka Chemie, Buchs, Switzerland. High purity DNPH standards were prepared by stoichiometric derivatization and repeated crystallization from methanol. 8-OHDG was synthesized by a Fenton reaction as described by Floyd *et al.* [26]. Acetonitrile, ethanol and methanol (HPLC-grade), was from Acros Chimica, Neuss, FRG.

Statistical analysis

All data are presented as means $\pm$ S.E.M. Statistical analysis was carried out by standard one- and two-way parametric analysis of variance (ANOVA) with repeated measures design coupled with paired Student's *t*-test using Sigmastat software (Jandel Scientific, Corte Madera, 1995). A value of $p < 0.05$ was considered to indicate a significant difference.

Results

Effects of methylene blue on LPS-fever in rats

The experiments were performed at ambient and box temperatures kept at 25°C. As shown by the data of Table 1 and in Fig. 1, LPS evoked in all rats with a latency of approximately 30 min cutaneous vasoconstriction, as deduced from the fall of paw temperature. The animals also stopped moving and took a crouched position. Core temperature fell by $0.9 \pm 0.2^\circ\text{C}$, together with a reduction of metabolic rate, 1 h after LPS. Thereafter core temperature rose, associated with a rise of metabolic rate and accompanied by visible shivering. Cutaneous vasoconstriction persisted until 4 h after LPS, and core temperature rose by more than 1°C , showing, thus, the typical febrile response of homeothermic mammals. Pretreatment with methylene blue given 1 h before reversed the

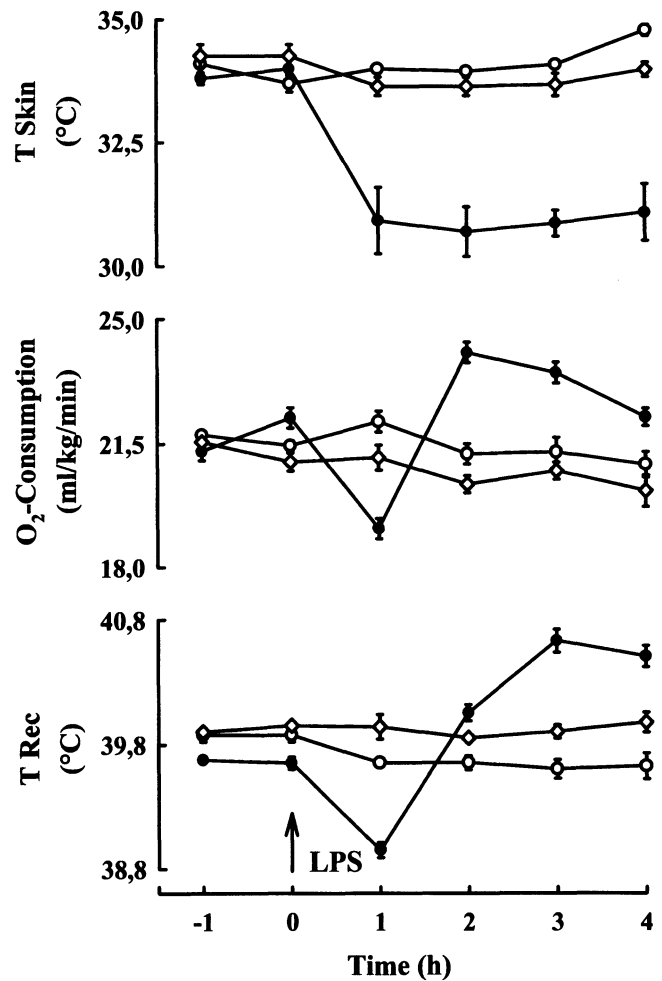


Fig. 1. Changes of paw skin temperature (T Skin), core temperature (T Rec), and oxygen consumption of saline-treated rats (open circles), LPS-treated rats (closed circles), and LPS-treated rats after pretreatment with methylene blue (open squares). Ambient temperature: 25°C. Mean values with S.E.M. of 8 rats. Error bars may be covered by symbols.

effects of LPS: the animals remained alert, paw skin temperature remained normal, and there were no significant changes in metabolic rate or core temperature. Thus, methylene blue totally blocked the febrile response to LPS.

Table 1. Effects of LPS on body temperatures and metabolic rate of saline treated (Group I) rats and methylene blue treated (Group II) rats

Group I (n = 8)	Control	1 h after LPS	4 h after LPS	
T skin (°C)	34.0 ± 0.2	31.4 ± 0.4*	31.2 ± 0.4*	
T rec (°C)	39.7 ± 0.1	38.8 ± 0.2*	40.5 ± 0.2*	
O ₂ (ml/kg/min)	22.2 ± 0.9	19.9 ± 0.6*	22.3 ± 0.8	
Group II (n = 8)	Control	1 h after MB	1 h after LPS	4 h after LPS
T skin (°C)	34.2 ± 0.6	34.0 ± 0.4	33.6 ± 0.4	34.2 ± 0.4
T rec (°C)	39.9 ± 0.2	39.7 ± 0.2	40.0 ± 0.1	39.7 ± 0.6
O ₂ (ml/kg/min)	21.5 ± 0.8	20.8 ± 0.9	21.1 ± 0.5	20.0 ± 0.4

* $p < 0.05$ compared to control.

Effects of LPS and methylene blue on lipid- and DNA- peroxidation in rats

As shown by the data of Table 2, LPS caused a substantial increase of plasma-MDA within 1 h, indicating that oxygen radicals were produced which reacted with lipids and, furthermore, that free oxygen radicals attacked nuclear structures as demonstrated by significantly increased levels of 8-OHDG in heart and liver tissue (Table 3). No evidence for nuclear attack by oxygen radicals of brain tissue following

Table 2. Effects of LPS on plasma aldehyde formation in saline treated rats ($n = 6$) and in rats pretreated with methylene blue ($n = 6$)

	Control (NaCl)	LPS	Control (MB)	LPS + MB
Malondialdehyde pmol/ml	240 $\pm$ 70	599 $\pm$ 138*	17 $\pm$ 6*	30 $\pm$ 19*
Formaldehyde μ mol/ml	53.25 $\pm$ 1.36	49.76 $\pm$ 3.59	49.93 $\pm$ 3.56	43.10 $\pm$ 3.90
Acetaldehyde μ mol/ml	260.13 $\pm$ 9.36	196.76 $\pm$ 10.58	252.35 $\pm$ 8.40	226.52 $\pm$ 9.30
Acetone μ mol/ml	151.87 $\pm$ 25.61	120.27 $\pm$ 22.12	192.54 $\pm$ 28.11	175.83 $\pm$ 26.30
Propionaldehyde μ mol/ml	366.01 $\pm$ 7.24	332.71 $\pm$ 8.34	343.66 $\pm$ 7.02	313.61 $\pm$ 6.83

* $p < 0.05$ compared to (NaCl) control.

Table 3. Tissue levels of 8-OHDG of control rats ($n = 6$), 1 h after LPS (LPS, $n = 6$), and 1 h after LPS in methylene blue pretreated rats (MB/LPS, $n = 6$)

	Brain (pmol/mg DNA)	Liver (pmol/mg DNA)	Heart (pmol/mg DNA)
Control	98 $\pm$ 18	612 $\pm$ 28	380 $\pm$ 14
LPS	117 $\pm$ 29	1078 $\pm$ 48*	790 $\pm$ 24*
MB/LPS	108 $\pm$ 21	681 $\pm$ 41	431 $\pm$ 19

* $p < 0.05$ compared to control.

LPS was found. Pretreatment with methylene blue given 1 h before caused a reduction of MDA to near detection level, and LPS did not significantly elevate these low levels within the following hour. The levels of 8-OHDG in brain, liver and heart were not significantly altered by combined methylene blue and LPS treatment.

Effects of DTT on body temperatures and fever in rabbits

These experiments were performed at ambient and box temperatures between 26 and 28°C. As shown by the data in Table 4, application of 20 mg/kg DTT within 15 min and followed by continuous infusion of 20 mg/kg within the next

Table 4. Effects of DTT on thermoregulatory activities in rabbits

	Control	30 min after DTT	60 min after DTT
T skin (°C)	35.4 $\pm$ 0.4	37.6 $\pm$ 0.4*	36.6 $\pm$ 0.4*
Resp. rate	185.0 $\pm$ 16	420.0 $\pm$ 22*	344.0 $\pm$ 33*
T rec (°C)	39.4 $\pm$ 0.1	39.1 $\pm$ 0.2*	38.9 $\pm$ 0.2*
O ₂ (ml/kg/min)	7.14 $\pm$ 0.57	6.47 $\pm$ 0.52*	6.73 $\pm$ 0.68*

* $p < 0.05$ compared to control.

Table 5. Effects of LPS in rabbits

	Control	30 min after LPS	30 min after DTT	180 min after LPS
T skin (°C)	36.6 $\pm$ 0.2	31.8 $\pm$ 0.2*	31.3 $\pm$ 0.2*	31.8 $\pm$ 0.3*
Resp. rate	228 $\pm$ 20	64 $\pm$ 12*	88 $\pm$ 16*	56 $\pm$ 32*
T rec (°C)	39.3 $\pm$ 0.1	40.1 $\pm$ 0.1*	40.6 $\pm$ 0.2*	41.5 $\pm$ 0.2*
O ₂ (ml/kg/min)	7.38 $\pm$ 0.22	9.46 $\pm$ 0.36*	8.12 $\pm$ 0.30*	7.68 $\pm$ 0.26

* $p < 0.05$ compared to control.

30 min, evoked in all rabbits an immediate heat loss effector response with cutaneous vasodilation and panting, and core temperature fell by $0.3 \pm 0.2^\circ\text{C}$ within 30 min. Oxygen consumption was significantly lowered 30 min after DTT. The heat loss effector responses remained activated after termination of DTT application, causing a further lowering of body temperature by $0.5 \pm 0.1^\circ\text{C}$ 2 h after application of DTT. As shown in Fig. 2 and by the data given in Table 5, all rabbits exhibited in response to LPS a biphasic fever, initiated with the typical cold defense response consisting of cutaneous vasoconstriction, a depression of respiratory rate and a rise of oxygen consumption. Pretreatment with DTT, starting 45 min before the injection of LPS, with an initial dose of 20

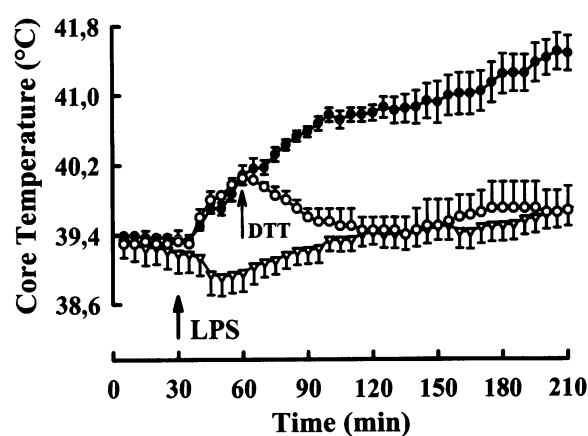


Fig. 2. Changes of core temperature of rabbits following LPS obtained in ambient temperature of 25–26°C. Closed circles: effect of 1 μ g/kg LPS in the untreated state ($n = 10$). Open circles: effect of 1 μ g/kg LPS followed by bolus injection of 20 mg/kg DTT (arrow) 30 min after LPS ($n = 5$). Open triangles: effect of 1 μ g/kg LPS injected 45 min after continuous DTT-treatment ($n = 5$). Values are means with S.E.M.

Table 6. Prevention of fever by DTT-pretreatment

	Control	30 min after DTT	30 min after LPS	180 min after LPS
T skin (°C)	37.9 ± 0.2	38.2 ± 0.1*	38.6 ± 0.1*	38.8 ± 0.2*
Resp. rate	317 ± 26	340 ± 22	373 ± 21*	256 ± 38
T rec (°C)	39.2 ± 0.1	39.0 ± 0.1*	39.3 ± 0.1	39.7 ± 0.2*
O ₂ (ml/kg/min)	7.19 ± 0.37	7.58 ± 0.32*	7.49 ± 0.48	7.43 ± 0.39

*p < 0.05 compared to control.

Table 7. Antipyretic effect of DTT in rabbits

	Control	30 min after LPS	30 min after DTT	180 min after LPS
T skin (°C)	37.2 ± 0.2	33.6 ± 0.3*	39.2 ± 0.2*§	39.0 ± 0.2*§
Resp. rate	228 ± 26	90 ± 17*	416 ± 24*§	366 ± 32*§
T rec (°C)	39.3 ± 0.1	40.1 ± 0.1*	39.6 ± 0.1§	39.7 ± 0.2*§
O ₂ (ml/kg/min)	7.22 ± 0.30	9.31 ± 0.42*	7.89 ± 0.34*§	7.56 ± 0.35

*p < 0.05 compared to control; §p < 0.05 compared with 30 min after LPS.

mg/kg DTT followed by a continuous infusion of 10 mg/kg/h and lasting about 2 h, completely prevented fever (Table 6). When DTT was given at a dose of 20 mg/kg 30 min following LPS, it induced an immediate heat loss effector response with cutaneous vasodilatation and panting, and the elevated core temperature returned to normal values within 45–60 min (Table 7). Thus, DTT totally prevented the febrile response to LPS or induced antipyresis when given during fever.

Discussion

It is generally accepted that bacterial endotoxin, after entering the circulatory system, stimulates in cells of the RES the release of cytokines, among which TNF α , IL-1, IL-6 and IFN γ have been considered to act within the CNS as the putative endogenous mediators of fever [12, 13, 29]. PGE₂ has been assumed as a further obligatory step in the fever cascade, because it elicits fever when microinjected into the hypothalamus [16, 30], while the cyclooxygenase-inhibitor aspirin prevents fever, or induces antipyresis when injected in the febrile state [31]. Recent experiments performed in rabbits have shown, however, that pretreatment with methylene blue totally prevented fever although the increase of TNF α following LPS was not significantly different from the untreated state, and circulating PGE₂ rose to even higher levels [20]. That methylene blue does not affect PGE₂ synthesis was recently shown by Salvemini *et al.* [32]. Similar as found in rabbits [20], methylene blue significantly reduced malondialdehyde production following LPS in the rat. This finding suggests that methylene blue is able to block under *in vivo* conditions the generation of oxygen radicals. McCord and Fridovich [33] and Kelner *et al.* [34] have shown that

methylene blue is a potent inhibitor of superoxide anion production. However, methylene blue has also been found to act as an inhibitor of soluble guanylate cyclase [35], although it might primarily inhibit NOS [21]. Concluding from plasma nitrate levels as a measure of NO metabolism under aerobic conditions [36] we found recently that cNOS is inhibited immediately following LPS and that this inhibition is reversed by methylene blue [19, 20]. That the fever-preventing action of methylene blue is unlikely to be primarily caused by a NO-cGMP-dependent pathway is deduced from results showing that L-NMMA-treated rabbits react with extremely high fevers which could be prevented with methylene blue as long as plasma nitrate levels remained elevated [19]. Taken these data together it becomes obvious that NO, if produced in normal or higher quantity within the CNS, is involved in heat loss activation and in blocking fever [37]. Methylene blue supports, thus, the maintenance of a normal NO production within the CNS under conditions of oxidative stress.

A considerable body of evidence indicates that the fever-preventing and antipyretic effects of DTT could also be associated with facilitated NO release within the CNS, and the most plausible structure concerned with this action of DTT seems to correlate with the NMDA subtype of neuronal glutamate receptors. This receptor has been found to contain vicinal thiol groups which in the oxidized state or after application of GSSG [38] prevent calcium entry, the physiological stimulus to activate NOS [39]. DTT has been shown to be a potent reducing agent of thiol groups and imitates thus the major intra- and extracellular reducing molecule glutathione (GSH) [40]. Its disulfide (GSSG) is produced from GSH during the destruction of reactive oxygen species and is present normally at much lower concentrations owing to the activity of glutathione reductase [39]. The present study shows that application of DTT elicits the full pattern of heat loss and lowers the thermoregulatory setpoint in warm, but

more prominently in cold environment [41], and under febrile conditions. We explain our results such that DTT by fully reducing GSSG of the redox-modulatory site of the NMDA receptor triggered the release of NO, which has been identified as an intercellular messenger within the brain [42]. It is possible that the effects of methylene blue and DTT in our studies were additionally exaggerated by their ability to release glutamate from neuronal tissue [22]. DTT, similar to GSH or cysteine, seems to play a dual role in supporting NO generation by regenerating nonenzymatically tetrahydrobiopterin (BH₄), an important cofactor for NOS, but also by activating BH₄-reductase, a thiol-containing enzyme [43]. Furthermore, DTT reverses the lowered cellular GSH level caused by exposure to glutamate [44].

Our results allow the conclusion that body temperature depends critically on a precisely regulated ratio of GSH/GSSG within the brain. This balance is lost after pyrogens enter the circulation and cause oxidative stress. We hypothesize that the redox modulatory site of the NMDA receptor is sensing this unbalance. Alternatively, glutamate released from neurons or glia following LPS stimulation could activate intraneuronally a calcium-dependent protease converting xanthine dehydrogenase to oxidase with subsequent production of reactive oxygen species [45], although such a conversion might occur by oxidation of enzyme thiol groups [46]. Generation of peroxides has therefore been considered to contribute importantly to glutamate toxicity and neuronal cell death [7, 47]. This is corroborated by findings of Atlante *et al.* [45], showing that DTT, and to a similar extent GSH, reduced superoxide anion production and cell necrosis in glutamate-exposed cerebellar granule cells by 67%, concomitant with a marked reduction of xanthine oxidase activity. A similar mechanism has been proposed for methylene blue by converting xanthine oxidase back to dehydrogenase [48], and Kelner *et al.* [34] have assumed that methylene blue interacts with xanthine oxidase at its iron-sulfide site. This site is known to serve as an electron-sink, and shunting of electrons to methylene blue at this site effectively diverts their flow away from the FAD site, where molecular oxygen is converted to superoxide radicals.

In summary, one of the earliest events after LPS has entered the circulatory system seems to be the production of oxygen radicals by phagocytosing cells of the RES. It seems that the combination of increased free oxygen radical formation and decreased formation of NO is causing fever. It appears that oxidative stress is sensed in the brain via alteration of the ratio of reduced vs. oxidized sulfhydryl groups of the redox modulatory site of the NMDA subtype of glutamate receptor. The consequent reduction of calcium entry and lowered NO release from these neurons contributes to the elevation of the setpoint of the thermoregulatory system, causing the rise of core temperature by releasing the full pattern of cold defence.

Acknowledgement

The authors wish to thank Prof. E. Simon for critically reading the manuscript. The study was supported in part by NATO Collaborative Research Grant CRG 971145 to W. Riedel.

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Enhanced expression and localization of heme oxygenase-1 during recovery phase of porcine stunned myocardium

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Abstract

Myocardial adaptation to ischemia involves up-regulated expression of a number of genes implicated in conferring cytoprotection. We have previously shown that myocardial ischemia followed by reperfusion leads to a co-ordinated expression of mRNAs encoding heme oxygenase-1 (HO-1) and ubiquitin in pigs. HO-1 participates in biological reaction leading to the formation of the antioxidant, bilirubin and the putative cellular messenger, carbon monoxide. In the present study, we examined the expression and cellular localization of HO-1 in the heart during myocardial stunning in anesthetized pigs. After thoracotomy, the LAD was occluded for 10 min and reperfused for 30 min (group I, n = 4), again occluded for 10 min and reperfused for 30 min (group II, n = 6), 90 min (group III, n = 4), 210 min (group IV, n = 5) and for 390 min (group V, n = 4). Myocardial tissue specimens were collected in 10% formalin as well as in liquid nitrogen and processed for immunohistochemistry and mRNA expression analysis, respectively. In the distribution territory of the LAD (experimental, E), systolic wall thickening was significantly decreased ($39 \pm 6\%$) as compared to that of the area perfused by left circumflex coronary artery (LCx, control) in group I and remained depressed in all subsequent groups. Northern blot analysis revealed that the expression of a single mRNA species of 1.8 kb encoding HO-1 was significantly induced in E as compared to control in groups II and III with maximum mRNA levels in group II (1.9 ± 0.4 fold vs. control). Immunoreactive HO-1 was localized in the cytoplasm of cardiomyocytes as well as in the perivascular regions in all groups. Semiquantitative analysis of HO-1 staining showed significantly enhanced levels of HO-1 in perivascular region in E as compared to respective controls derived from groups III and IV. These results suggest that myocardial adaptive response to ischemia involves up-regulation of HO-1 in cells of perivascular region indicating that this enzyme may participate in regulating vascular tone via CO and thereby, contributing in pathophysiologically important defense mechanism(s) in the heart. (Mol Cell Biochem **196**: 133–139, 1999)

Key words: ischemia, heme oxygenase, immunohistochemistry, heart, pig

Introduction

Heme oxygenase-1 (HO-1) is one of the two isoforms of the enzyme, heme oxygenase (EC 1.14.99.3) that catabolize cellular heme to biliverdin, free iron, and generate the putative cellular messenger carbon monoxide (CO) in mammalian cells [1–3]. Both isoforms of heme oxygenase, HO-1 and HO-2 are products of two different genes and they differ markedly in their tissue distribution and molecular properties [4–6]. Originally, HO-1 was identified as a metal

inducible enzyme in the cell and now a large number of stimuli have been implicated in the induced expression of HO-1 gene [6, 8–10]. Induction of HO-1 in human skin fibroblasts by UVA irradiation has been shown to lead to an increased level of intracellular ferritin, an iron storage protein that has been implicated in cellular protection against oxidative stress [8, 9]. Being a heat inducible protein, HO-1 is now also referred to as heat shock protein-32 (HSP32) and regarded as a member of HSP family; whereas, HO-2 is a constitutively expressed and non-inducible gene product [6,

10–12].

Carbon monoxide is produced endogenously by various cell types as a byproduct of heme catabolism through the HO system [1]. In general, CO is known as a highly toxic combustion product, its endogenous production in the body is believed to modulate blood flow under normal and pathophysiological conditions [3, 13, 14]. Recently, several groups have focused attention to unravel the role of HO-1 in the heart as an effector enzyme in regulating the cellular defence mechanisms and the vascular tone under different pathophysiological conditions including ischemia [14–16]. Interestingly, Poss and Tonegawa found reduced defence capacity to heavy metals as well as to hydrogen peroxide in cells lacking HO-1 [17]. Vascular smooth muscle cells when exposed to hypoxia produce drastically increased amounts of CO resulting in enhanced levels of cyclic guanosine 3',5'-monophosphate (cGMP) [10, 18, 19]. Binding sites for transcription factors NF- κ B and AP-2 as well as the presence of a functional heat shock element in the promoter region of the HO-1 gene have been identified [20]. Induction of HO-1 gene expression by lipopolysaccharides has been shown to be mediated by the activation of AP-1 transcription factor [21].

Several observations have established that one or more brief periods of myocardial ischemia increase cardiac tolerance to a subsequent sustained period of ischemia a phenomenon of ischemic preconditioning [22–24]. Such cardiac adaptation has also been associated with altered expression of a number of genes [25–27]. In order to examine whether myocardial ischemia and reperfusion influence the HO-1 system, an important enzyme involved in generating anti-oxidants under physiological conditions may contribute to the protective/adaptive processes in the ischemic heart, we studied the pattern of HO-1 expression and cellular localization in the porcine heart subjected to ischemia and reperfusion.

Materials and methods

Experimental model of myocardial stunning

The experimental protocol to induce myocardial stunning in pigs for the present study was almost same as has been described previously by our group [7, 25–27]. Briefly, cross-bred Landrace $\times$ Yorkshire pigs (average wt. 30 kg) were fasted overnight. Pigs were sedated with an intramuscular injection of azaperone (2–3 mg/kg) and then anesthetized with an intravenous injection of 20 mg/kg pentobarbitone followed by continuous infusion (15 mg/kg/h) and intubation for artificial ventilation. Pancuronium bromide was used for muscle relaxation (4 mg). Following midsternal thoracotomy the left anterior descending coronary artery was dissected free distal from its first diagonal branch for the placement of the arterial clamp (Fig. 1). A 7F Sensodyn micromanometer-

tipped catheter was inserted into the left ventricle by way of the left carotid artery, for the measurement of left ventricular blood pressure and its first derivative (LVdP/dt). An 8F Catheter was inserted into the aorta for the measurement of central aortic pressure. Regional myocardial function was estimated from recordings of myocardial wall thickness obtained with sonomicrometry by implanting two pairs of ultrasonic crystals one pair each in the area perfused by the left anterior descending coronary artery (LAD) and in the area perfused by the left circumflex coronary artery (LCx). After instrumentation and stabilization for at least 30 min, systemic hemodynamics and regional myocardial wall thickness were recorded. Subsequently, the LAD was completely occluded for 10 min followed by 30 min of reperfusion (group I). This cycle of occlusion and reperfusion was then repeated (group II) with additional 120 (group III), 210 min (group IV) and 390 min (group V) of reperfusions. At each time point, experiments were terminated and tissue from LAD region (ischemic, experimental, E) and LCx region (nonischemic, control, C) were excised, washed in 0.9% NaCl solution, cut into small pieces, snap frozen in liquid nitrogen and stored at -80°C until analysed by Northern hybridization and immunohistochemistry.

Isolation of total cellular RNA and Northern blot analysis

Total cellular RNA was isolated from the porcine stunned and normal myocardium by the method of Chomczynski and Sacchi [28]. For Northern hybridization, total RNA was denatured at 65°C in buffer containing formamide and ethidium bromide and electrophoresed on 1% agarose gel containing 2.2 M formaldehyde [7, 25]. RNA was transferred to hybond-N membrane, UV cross linked and hybridized with radiolabelled cDNA inserts encoding human HO-1. Membranes were washed under stringent conditions and exposed to Kodak X-OMAT^{AR} films. A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to rehybridize membranes for reference purposes. Autoradiographs were scanned using a laser densitometer and induction of HO-1 gene was calculated and expressed as fold induction of mRNA signal ($\pm$ S.E.M.) in E and compared with that of C.

Immunohistochemistry

In order to examine the cellular source of HO-1 in the porcine myocardium, immunohistochemical localization was performed. Formalin fixed and paraffin embedded tissue specimens were processed for staining. Paraffin sections (6 μm thickness) of the myocardial tissues were cut and mounted on 3-amino-propyl-trioxysilane (Sigma, St Louis, MO, USA)

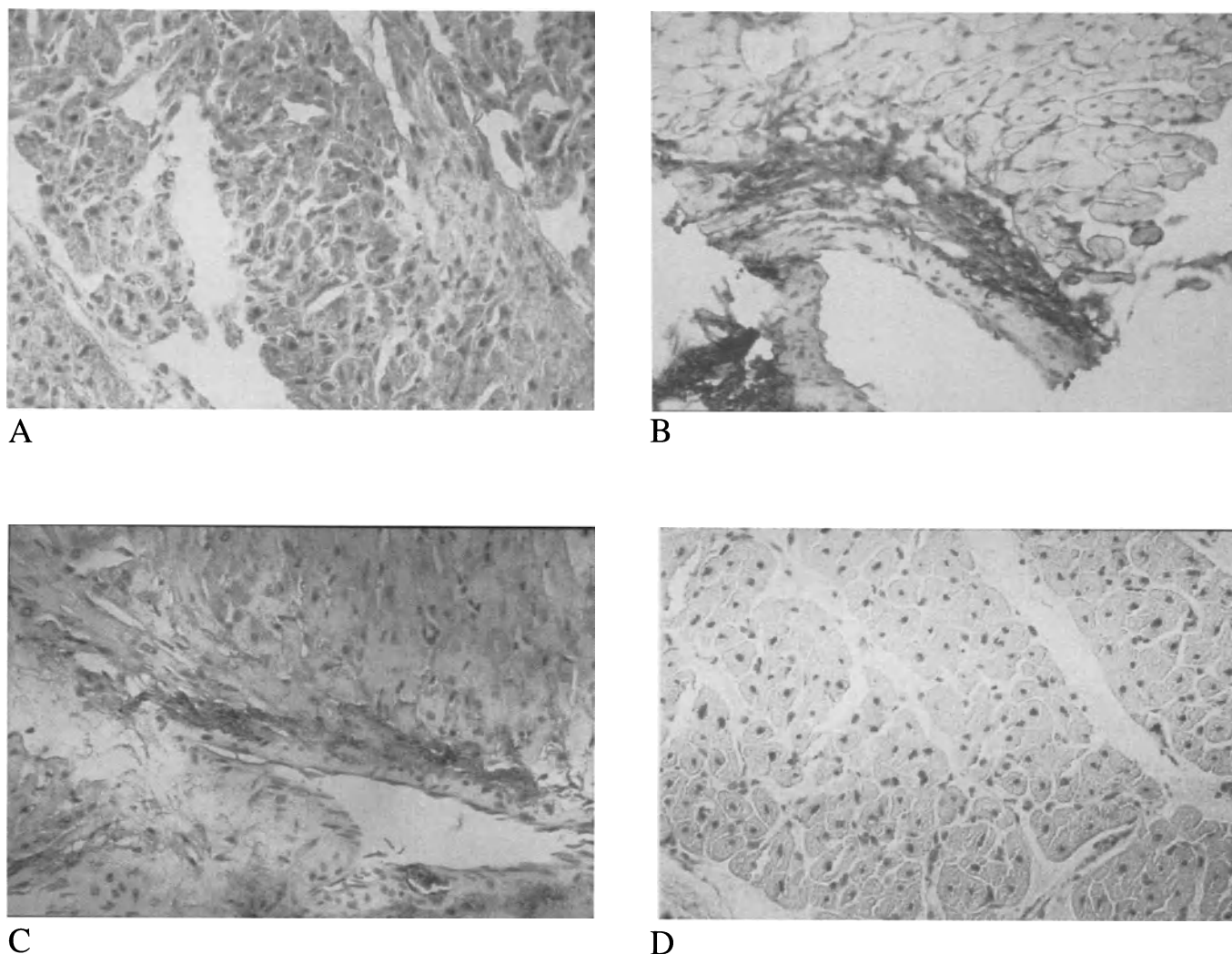


Fig. 1. Immunohistochemical localization of HO-1 in the porcine heart subjected to ischemia and reperfusion. Paraffin sections 5 μ m thick of porcine myocardial tissue obtained from LAD and Lcx regions were incubated with the polyclonal antibody against purified rat HO-1 followed by visualization after immunoperoxidase color reaction as described in Materials and methods. Panel A: shows immunoreactive HO-1 in the cardiomyocytes of tissue specimen obtained from group II; Panel B: shows intense perivascular expression in the tissue specimen obtained from group III; Panel C: shows HO-1 expression in the cardiomyocytes and in the perivascular region tissue specimen obtained from the control region of the group II; Panel D: depicts a negative control where anti-HO-1 antibody was replaced by rabbit antiserum.

coated glass slides. Immunohistochemistry using a multiple step avidin-biotin complex (ABC) method was followed as previously described [29, 30]. In brief, after deparaffinisation in xylene and rehydration through graded alcohol the slides were rinsed with water and phosphate buffered saline. The slides were placed in Sequeza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor, Runcorn). Slides were preincubated for 15 min with normal goat serum to block non specific binding, then incubated for 30 min at room temperature with a rabbit polyclonal antibody against purified rat liver HO-1 (OSA-100) obtained from Stress Gene Biotechnologies Corp., Victoria, Canada. The supra optimal dilution was obtained by examining the intensity of

staining obtained with a series of dilutions of the antisera from 1:100–1:750 and selecting the dilution that gave specific, weak but easily visible signals. After washing with phosphate buffered saline, the slides were incubated for 30 min with biotinylated secondary antibodies (Multilink, 1:75 dilution, Biogenex, San Ramon, MO, USA). The slides were washed again and incubated for 30 min with alkaline phosphatase conjugated streptavidin (Biogenex, San Ramon, MO, USA) in dilution of 1:50. Finally, the slides were rinsed with 0.2 M TRIS-HCL pH 8.0, Levamisole (Sigma, St Louis, MO, USA) used to block the endogenous alkaline phosphatase activity, and stained for 30 min with 0.3% New Fuchsin/TRIS-HCL (Sigma, St Louis, MO, USA) as colour enhancement

system. Negative controls were prepared by omission of the primary antiserum. Slides were counterstained with Mayer's hematoxylin for 10 sec.

Quantitative analysis of HO-1 localization

HO-1 expression was semi-quantitatively analyzed using an arbitrary visual scale with low inter-observer variability to study the differences. Specifically the whole surface area of the sections were quantified at the same magnification by two independent observers. Sections were graded from 0–4 for the localization of HO-1 in the cardiomyocytes as well as perivascular regions. Grade 0 represented no staining, grade 1 focal staining, grades 2, 3 and 4 diffuse, weak, moderate and strong staining respectively. Despite possible inter-observer differences, this method was even capable of detecting expression level differences as small as 1.5 times [31].

Statistical analysis

The expression of HO-1 staining score was calculated from two different groups and the results are expressed as means $\pm$ S.E.M. Significance of the results for probability value at or beyond 0.05 was evaluated and accepted.

Results

Hemodynamics

We used the formula $\{(SWT\% = 100 \times (EST-EDT)/EDT;$ where EST = end-systolic thickness and EDT = end-diastolic thickness) to calculate systolic wall thickening in percentage during ischemia and reperfusion. As reported earlier, two cycles of LAD occlusion (10 min) each followed by reperfusion (30 min) resulted swine myocardium stunned with decreased regional wall thickening [7, 25–27]. In the distribution territory of the LAD (E), systolic wall thickening was significantly decreased ($39 \pm 6\%$) as compared to that of the area perfused by left circumflex coronary artery (control) in group I and remained depressed in all subsequent groups. However, myocardial function as assessed above recovered partially during the longer period of reperfusion (group IV and V) and eventually it was observed that a period of up to 72 h was necessary to completely regain the function.

HO-1 gene expression

Using Northern blot hybridization technique, we demonstrated myocardial basal expression of a mRNA species of 1.8 kb

encoding HO-1. The steady state mRNA levels of HO-1 were significantly enhanced by repetitive coronary artery occlusion and reperfusion, i.e. in the stunned myocardium as compared to the control [7]. The time course of induction of the HSP32 gene showed maximal mRNA levels after two cycles of 10 min ischemia and 30 min of reperfusion. Thereafter, expression in the stunned myocardial tissue declined but remained slightly elevated as compared to control. These results indicate that the HO-1 gene responded towards ischemia, but significant myocardial accumulation of mRNA occurred only during the reperfusion phase. Densitometric analysis of blots revealed that myocardial ischemia followed by reperfusion induced the expression of HO-1. The expression of HO-1 mRNA was significantly induced in E as compared to control in groups II and III with maximum mRNA levels in group II (1.9 ± 0.4 fold vs. respective control) [7].

Immunohistochemical localization of HO-1

Using immunohistochemistry, we observed that HO-1 protein was localized in the cardiomyocytes and in the interstitium (Fig. 1). Increased immunoreactive HO-1 was found in the perivascular region (mainly in fibroblasts) around blood vessels in the myocardial tissue specimens obtained from stunned LAD area as compared to respective control. Semi-quantitative analysis using an inter-observer visualization score (0–4) method, we found a significantly enhanced staining of HO-1 in the perivascular region in E as compared to respective controls derived from group III and IV (Fig. 2).

Discussion

In the present study we have demonstrated that HO-1 expression is upregulated both at mRNA and protein levels in the myocardium during the reperfusion phase after a short repetitive ischemia. The presence of HO-1 in the cardiomyocytes indicates that these cells possess an endogenous protective mechanism thereby, producing bile pigments that act as radical scavengers particularly during the reperfusion phase after the ischemia. The perivascular existence of HO-1 may be attributed to its role in regulating the vascular tone by generating carbon monoxide as a result of haem metabolism. Therefore, it is conceivable, that HO-1 plays an important role in improving cardiac function after ischemia by dilating coronary arteries with carbon monoxide via endothelium independent mechanism. As stated earlier, HO-1 gene induction in hearts during reperfusion phase after ischemia and in OH $\cdot$ generating system perfused hearts may be one of the protective mechanisms against oxidative stress [16, 29]. Our results suggest that the myocardial adaptive response to ischemia involves up-regulation of HO-1 indicating a pivotal

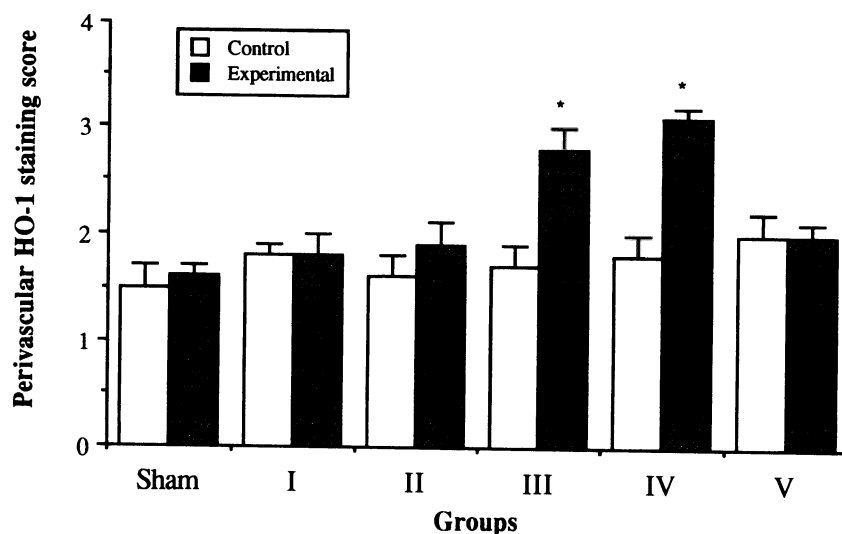


Fig. 2. Bar graph showing quantitative analysis of the HO-1 expression. Data are means $\pm$ S.E.M. ($n = 4$) and expressed as HO-1 staining score as described in Materials and methods. * $p < 0.05$ vs. control.

role for this enzyme in endogenous patho-physiologically important defense mechanisms in the reperfused heart. Furthermore, perivascular localization of HO-1 may be attributed to its role in regulating the vascular tone by generating, carbon monoxide.

Although, induction of the HSP32 gene was originally observed in cultured skin fibroblasts, this now holds true to different species and different cell types [32]. The induction is clearly related to the cellular redox state since lowering of cellular glutathione levels strongly enhances HO-1 mRNA accumulation [32, 33]. For these reasons enhanced expression of HO-1 gene appears to be a fairly sensitive marker of oxidative stress and may be applied as a diagnostic tool such as in cardiovascular system. It may be mentioned here that the promotor region of human HO-1 gene contains the binding sites for transcription factors NF- κ B and AP-2 [20]. The presence of regulatory sequences for binding of NF- κ B and AP-2, whose activation is associated with immediate response of the cell to an injury, may underlie the role for HO-1 in defense mechanisms against tissue injury [7, 24, 32].

CO is the diatomic gas molecule that shares some of the physicochemical properties of another physiological messenger called endothelium-derived relaxing factor or nitric oxide (NO). CO, like NO has the ability to bind to the iron atom of the heme moiety associated with soluble guanyl cyclase, thereby activating this enzyme and raising intracellular levels of cyclic guanosine 3',5'-monophosphate (cGMP) [11, 16, 35]. It is believed that enhanced intracellular levels of cGMP regulate blood vessel tone and blood flow by inhibiting the smooth muscle contraction and platelet aggregation. Vascular smooth muscle cells (VSMC) have both constitutive and inducible HO activity and when

exposed to hypoxia, enhanced transcription and translation of HO-1 have been observed in these cells. Enhanced HO-1 expression in hypoxic VSMC leads to drastically increased amounts of CO resulting in elevated levels of cGMP and consequently relaxing the blood vessels [3, 11, 26].

Significantly induced mRNA expression encoding HO-1 in various organs including hearts was observed when rats were exposed to elevated ambient temperature (42°C) for 20 min [6, 18]. Maximum induction was observed 1 h after hyperthermia and reached a value 20–40 times as high as in normothermic rats. Raju and Maines, further verified that hyperthermia-induced expression of HO-1 was a result of its enhanced gene transcription and not due to decreased RNA degradation [18]. Furthermore, the increase in HO-1 mRNA transcription was accompanied by an increase in binding of nuclear factor(s) to the heat shock element in the promotor region of HO-1 gene, as well as with increased levels of HO-1 protein in the heart [18, 36].

Katayose *et al.* have examined the expression of HO-1 in rats subjected to hypoxia or pulmonary artery banding, both of which produce severe right ventricular pressure overload [33]. They found that at 3 days after hypoxia HO-1 mRNA levels were elevated by 4-fold, whereas HSP70 mRNA levels remained unchanged. Pulmonary artery banding markedly and immediately (within 6 h) increased HO-1 mRNA levels in both ventricles indicating its important role in cardioprotection against oxidative stress, since bile pigments, the products of heme catabolism are shown to act as radical scavengers [1, 33].

A period of reversible cardiac dysfunction can occur after brief episodes of coronary occlusion and ischemia, a phenomenon that is called 'stunning'. Among the cellular

organelles whose altered function might account for contractile dysfunction are the sarcoplasmic reticulum, the contractile filaments and the sarcolemma [23–27, 37–39]. Northern blot analysis revealed that the steady state mRNA levels of HO-1 were significantly enhanced by repetitive coronary artery occlusion and reperfusion, i.e. in the stunned myocardium as compared to the control [7]. The time course of induction of the HSP32 gene showed maximal mRNA levels after two cycles of 10 min ischemia and 30 min of reperfusion. Thereafter, expression in the stunned myocardial tissue declined but remained slightly elevated as compared to control. These results indicate that the HO-1 gene responded towards ischemia, but significant myocardial accumulation of mRNA occurred only during the reperfusion phase.

Earlier we and others have reported that myocardial ischemia followed by reperfusion resulted in enhanced expression of heat shock proteins, HSP70 and HSP27 [25, 27, 37–41]. Similar to other heat shock proteins, human HO-1 gene contains a heat shock element (HSE) in its promotor region [20, 32, 36, 39]. Binding of a transcription factor to a HSE sequence in the promotor region of HO-1 gene is regarded as the responsible cellular event for heat shock response. It is likely that also the porcine HO-1 gene contains the HSE region that responds to the ischemic episode, whereby, binding to heat shock factor eventually activates HO-1 mRNA transcription. Differential regulation of HO-1 and vascular endothelial growth factor by heat shock as well as by other stimuli has been reported recently indicating a synergistic regulatory mechanism of these two genes implicated in angiogenesis and cytoprotection [42].

As it has also been shown that gene transfer of human HO-1 into coronary artery endothelial cells promotes angiogenesis again pointing to its implication in the important event of blood vessel growth [43].

Additionally, the heme oxygenase system may function as a defense pathway of the tissue against oxidative stress as bilirubin, produced via this system may act as a physiological antioxidant. It is plausible that the HO system plays an important role during cardiac adaptation to ischemia and reperfusion and participates in the cellular defense mechanisms by which the myocardium regains its function after the ischemic episode. The HO system has been implicated in endogenous CO generation. Though, we did not measure the production of CO, it is presumed that the induced expression of HO-1 may generate enhanced levels of CO during the reperfusion phase, which in turn may modulate the myocardial vascular tone. These results suggest that the myocardial adaptive response to ischemia involves the induction of HO-1, which may be indicative for the existence of a pathophysiologically important defense mechanism by which generation of biologically active products of heme metabolism is accelerated.

HO-1 protein was localized in cardiomyocytes and in the

interstitium: Immunoreactive HO-1 was also found in the perivascular region (mainly fibroblasts) around blood vessels. The presence of HO-1 in cardiomyocytes indicates that these cells possess an endogenous protective mechanism by which bile pigments are produced that act as radical scavengers, particularly during the reperfusion phase after ischemia. The perivascular existence of HO-1 may be attributed to its role in regulating the vascular tone by generating carbon monoxide as a result of haem metabolism. Therefore, it is conceivable that HO-1 plays an important role in improving cardiac function after ischemia by dilating coronary arteries with carbon monoxide via endothelium independent mechanism. As stated earlier, HO-1 gene induction in hearts during the reperfusion phase after ischemia and in the OH⁻ generating system of perfused hearts may be one of the protective mechanisms against oxidative stress.

Acknowledgements

The financial support from NATO, Brussels, Belgium in the form of a Collaborative Research grant (No. CRG. 940256) is gratefully acknowledged.

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Heterogeneity of human red blood cell membrane: Co-existence of heavy and light membranes

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Abstract

The exact chemical composition of the red blood cell (RBC) membrane may vary depending on the methods used to isolate the membrane. We provide evidence here that RBC membrane can be fractionated by differential centrifugation and/or density gradient centrifugation into two distinct types, designated as 'heavy membrane' (HM) and 'light membrane' (LM). The amount of LM is twice that of HM on a per cell basis. HM and LM differ in some biochemical and electrophoretic properties. The total activities of Na⁺, K⁺- and Ca²⁺-ATPases, superoxide dismutase, glutathione peroxidase, catalase and glucose-6-phosphate and 6-phosphogluconate dehydrogenase are significantly higher in LM than HM on a per cell basis. While there is no significant difference in the specific activity of other enzymes between the two membranes, the specific activity of Ca²⁺-ATPase is significantly higher in HM, whereas Na⁺, K⁺-ATPase activity is higher in LM. There is a remarkable difference in the distribution of major ghost polypeptides between these two membranes. Component I of spectrin, component III and a protein with mol. wt. of 165 KDa are present in smaller amounts, whereas component II of spectrin and proteins with mol. wt. of 145, 84 and 76 KDa, respectively, are present in higher amounts in HM than LM. Some proteins such as band 4.1, 48 and 46 KDa are present only in LM, whereas some proteins with mol. wt. of 96, 78 and 43 KDa, respectively are present only in HM. It has been confirmed that these two membranes are not representatives of either (a) right side-out vs. inside out vesicles, or (b) open vs. sealed membranes. Thus HM and LM are two distinct membrane fractions. It is suggested that some part of the membrane is denser than other parts, and during hemolysis of RBCs, the rbc membrane is spliced resulting in two populations, dense and light. (*Mol Cell Biochem* **196**: 141–149, 1999)

Key words: antioxidant enzymes, ATPases, spectrin, cell membrane

Introduction

The erythrocyte membrane has long served as a convenient model system for the study of chemical and physical properties of cell membrane due to its relative simplicity. However, the chemical composition of the red blood cell (RBC) membrane may vary depending on the methods used to isolate the membrane [1–3]. The most commonly used method of erythrocyte ghost preparation is to hemolyze RBC in hypotonic solutions to remove hemoglobin. Even though this technique is widely used, certain important variables, namely pH and ionic strength of the hemolyzing solutions, appear partially responsible for conflicting reports on the composition and function of RBC membrane [3].

Several investigators have encountered problems in preparing a hemoglobin-free ghosts [4–6]. On centrifugation of the hemolyzed RBC, they observed two distinct pellets,

namely a reddish pink translucent loose ghost pellet at the 'top' and an opaque, tightly packed ghost pellet at the bottom. However, these investigators did not determine whether the difference in the physical nature of these pellets is due to differences in their chemical composition. In the present study, we provide evidence that human RBC ghosts are heterogeneous in nature and these two membrane fractions differ both in protein composition and biochemical properties.

Materials and methods

Isolation of red blood cells

Venous blood was collected from healthy volunteers into heparinized tube and processed immediately. RBCs were isolated by centrifugation at 1000 x g for 15 min. The

supernatant and buffy coat were discarded by aspiration and the cells were washed three times with three volumes of isotonic phosphate saline buffer, pH 7.4, and resuspended. The suspension was filtered through a column containing alpha-cellulose and 50 microns dry microcrystalline cellulose (1: 1, Sigma) to ensure that no contaminating leukocytes (rich in lysosomal proteases) and platelets are present [7].

Preparation of erythrocyte ghosts

The RBC suspension was centrifuged and erythrocyte ghosts were prepared by modifying the methods of Dodge *et al.* [3], Steck and Kant [6] and Burton *et al.* [8]. In brief, the RBC's were lysed with 30 volumes of 10 mM phosphate buffer containing 12 mM NaCl, 1 mM EGTA, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and aprotinin (0.1 µg/ml), pH 7.4, at 0–4°C. EGTA was used to chelate calcium since the presence of calcium during the lysis step guarantees the activation of the phosphoinositide-specific phospholipase C and calcium-dependent transglutaminases which could impact the density of the resulting stroma. Furthermore, PMSF and aprotinin were used as protease inhibitors to protect the membrane protein from proteolytic degradation during stroma preparation. The suspension was centrifuged at $20,000 \times g$ for 30 min and the resulting pellet was suspended in 1.25 mM phosphate buffer containing 1 mM EDTA, pH 7.4.

Isolation of red blood cell membranes

The RBC membranes were isolated from the suspension by using either differential centrifugation and/or 35–50% linear sucrose density gradient.

For the differential centrifugation, the supernatant was centrifuged at $10,000 \times g$ for 15 min in a refrigerated Sorvall RC-5 centrifuge using a SS-34 rotor, to obtain a tightly packed pellet, designated as 'heavy membrane' (HM). The supernatant containing a loosely bound pellet was further centrifuged at $100,000 \times g$ for 30 min in a Beckman L8-M ultracentrifuge using a 70 Ti rotor to obtain a tightly packed pellet, designated as 'light membrane' (LM). Both pellets were washed two times with hemolyzing buffer and suspended in a known volume of 0.1 M Tris-HCl/1 mM EDTA (pH 7.4).

For the linear sucrose density gradient, the supernatant was centrifuged at $30,000 \times g$ for 30 min in a refrigerated Sorvall RC-5 centrifuge. Then the entire pellet was suspended in hemolyzing buffer. About 6.0 ml of 35–50% (w/v in 0.1 M Tris-HCl/1 mM EDTA, pH 7.4) linear sucrose gradient was made with a Buchler Polystatic Pump (Buchler Instruments), and 3.0 ml of the membrane suspension (15–20 mg protein) was layered on the top of the gradient and centrifuged at

$50,000 \times g$ for 60 min in a Beckman L8-M ultracentrifuge using a SW-40 rotor [9], to yield two distinct bands. Each band was carefully removed with a Pasteur pipette, and diluted with hemolyzing buffer. The diluted sample was centrifuged at $20,000 \times g$ for 15 min., and the resulting pellet was suspended in a known volume of 0.1 M Tris-HCl/1 mM EDTA (pH 7.4). All operations were carried out at 4°C.

Biochemical assays

RBCs were suspended in 10 mM Tris-HCl/1 mM EDTA buffer, pH 7.4 and stored at –20°C. Aliquot of the suspension of RBC, light and heavy membranes were used for the determination of hemoglobin [10] and protein [11]. Superoxide dismutase (SOD) assay was done as described by Hyland *et al.* [12] and glutathione peroxidase (GSH-PX) activity was measured by the method of Flohe and Gunzler [13]. Catalase activity was estimated as described earlier [14]. The activity of glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGAD) was measured by the method of Beutler [15]. Na⁺, K⁺-ATPase activity was assayed as described by Robinson [16] and Ca²⁺-ATPase activity was assayed by the method of Nandi *et al.* [17].

SDS-PAGE electrophoresis of rbc membrane proteins

SDS-PAGE electrophoresis was performed using the discontinuous buffer system of Laemmli [18] in 0.15 × 7 × 8 -cm 7.5 or 11% isotropic separating gels and 4.5% stacking gels. Electrophoresis was carried out in a Hoeffer Model SE 250 mini vertical slab gel apparatus. Electrophoresis was performed with constant current at 20 mA/ gel. The running time under these conditions was about 50 min.

Suspensions of erythrocyte ghost protein at 0.5–2.0 mg/ml were prepared for electrophoresis by adding the following (to the stated final concentrations): 1% SDS, 5% glycerol, 0.5% beta-mercaptoethanol, 30 mM tris-HCl and 10 µg/ml bromophenol blue (tracking dye). The clear sample solutions were incubated at 100°C for 2–3 min. Molecular weight markers were also incubated in the same medium for 3 min. The silver-staining was performed according to the method of Oakley *et al.* [19] using an ICN rapid-Ag-staining kit. Each silver-stained gel was counter stained with 100 ml of 0.1% (wt/vol) coomassie brilliant blue R-250 (CB)/25% isopropyl alcohol (vol/vol)/ 10% acetic acid overnight and destained with the same solution except CB.

The quantity and molecular weight of the specific proteins (spectrin, band 3, band 4.1 and actin, etc.) were determined by using both internal and external standards in a LKB 2222-010 ultrosan XL equipped with 2400 gel scan software package. Data were treated statistically using the student's *t*-test.

Preparation and separation of inside-out and right side-out vesicles

This was done to determine that the dense and light fractions are not simply representatives of right side-out vs. inside-out vesicles. There are no glycoproteins on the internal side of the erythrocyte membrane; therefore inside-out vesicles do not interact with the lectin, concanavalin A, while right-side-out vesicles do interact [20]. Therefore, a concanavalin A cellulose affinity matrix column was used to separate easily inside-out vesicles for both heavy and light membranes. Concanavalin A was coupled to alpha-cellulose as described by Kondo *et al.* [20]. Vesicles were prepared from both heavy and light membranes at 4°C as described by Steck and Kant [6]. The pelleted ghosts were suspended in 40 vols. of ice-cold 0.5 mM Tris-HCl containing 1 mM EGTA, 0.2 mM DTT, 0.1 mM PMSF and aprotinin (0.1 µg/ml), pH 8.0 for 25 min, washed twice with the same buffer and centrifuged at $28,000 \times g$ for 30 min. The pellet was resuspended in same buffer and then vesiculated by passing through a 27 gauze needle five times. This produced a mixture of right-side-out and inside-out vesicles.

10 ml of vesicles (1.33 mg protein/ml) were added to 10 ml of a 20% (v/v) suspension of concanavalin A- alpha cellulose. After 10 minutes at room temperature, the solution was poured onto a coarse sintered-glass funnel and washed with 50 ml of buffer. The filtrate containing the inside-out vesicles which were not bound to the concanavalin A-cellulose was concentrated by centrifugation at $26,000 \times g$ for 20 min. The purity of the inside-out vesicles in the pellet was established by measurement of glyceraldehyde phosphate dehydrogenase (GPD), a marker of the erythrocyte inner surface and acetylcholine-esterase (ACE), a marker of the erythrocyte outer surface [21]. The right side-out vesicles which were bound to the affinity matrix were eluted by washing with 200 ml of 0.1 M alpha-methylmannoside, and centrifuged at $28,000 \times g$ for 20 min [20]. The purity of the right side-out vesicles in the pellet was established by determination of the relative distribution of GPD and ACE.

Separation of erythrocytes by density gradient

In order to find out whether existence of two species of membranes are due to difference in age of the erythrocytes, we separated erythrocytes into varying ages based on stractan density gradient [22]. Erythrocytes were suspended to about 30% hematocrit in buffered saline containing glucose and potassium [22]. Varying aged erythrocytes were separated by layering on the top of discontinuous density gradients of stractan (Sigma). Four layers (7 ml each) of stractan with densities 1.043, 1.053, 1.081 and 1.111 g/ml were placed upon a dense cushion of 1.165 g/ml to prevent packing of the

cells against the bottom of the centrifuge tube. On the top, 7 ml of 30% cell suspension were layered. The gradient was centrifuged at 4°C in a Sorvall RC-5B centrifuge at 11,000 rpm for 30 min using HB-4 swinging rotor. Four erythrocyte fractions were isolated with a Pasteur pipette: F-1 (lightest), F-2, F-3 and F-4 (densest). Erythrocyte fractions were washed with the buffer several times and centrifuged at 2000 rpm for 10 min in a refrigerated centrifuge to remove stractan. Mean cell volume, and mean hemoglobin concentration of the washed erythrocyte fractions were measured with an electronic counter. Heavy and light membranes were isolated from each cell fraction.

Results

By following the conventional methods for preparing hemoglobin-free ghosts [3, 6, 8], we obtained two pellets (dense and light) after centrifugation of the hemolyzed RBC. Figure 1 illustrates the separation of the erythrocyte membrane fraction by 30–50% linear sucrose density gradients. The upper band is designated as LM and the lower band is designated as HM. Both membranes are also separable by differential centrifugation (see Materials and methods). This indicates that RBC membranes are heterogeneous in nature and they differ in density.

The amount of LM is twice that of HM on a per cell basis (Table 1). There is no significant difference in the amount of

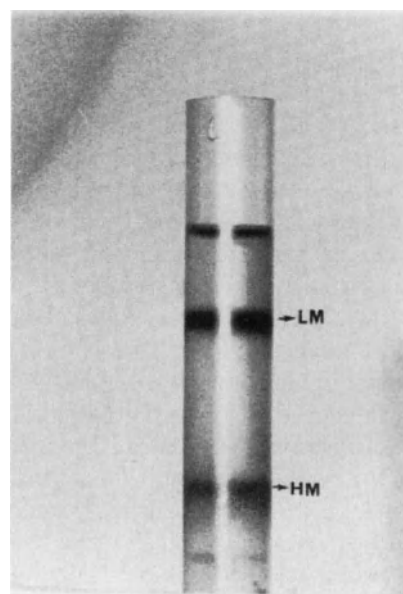


Fig. 1. Fractionation of human RBC membrane on 35–50% linear sucrose gradient. The separation was done by centrifugation at 25,000 rpm. HM represents heavy membrane; LM represents light membrane.

Table 1. Comparison of the specific activity of some important enzymes and calcium content between heavy and light membranes of human red blood cells

Parameters	Heavy	Light
Hemoglobin (ng)	0.12 ± 0.02	0.21 ± 0.03 ^b
Na ⁺ K ⁺ - ATPase (nmol P _i /hr)	75.4 ± 7.2	118.8 ± 8.3 ^a
Ca ²⁺ - ATPase (nmol P _i /hr)	172.1 ± 11.7	108.1 ± 7.4 ^d
SOD (units)	3.3 ± 0.9	4.9 ± 0.8
GSH-PX (μmol NADPH oxidized/min)	0.1 ± 0.0	0.1 ± 0.0
Catalase (μmol H ₂ O ₂ decomposed/min)	3.4 ± 0.3	3.2 ± 0.5
G-6-P dehydrogenase (μmol NADP reduced/min)	7.6 ± 2.0	4.0 ± 0.3
6-PGA dehydrogenase (μmol NADP reduced/min)	3.0 ± 0.6	2.8 ± 0.9
Calcium (fmol × 10 ⁻⁹)	14.1 ± 1.3	14.3 ± 3.9

Results are expressed as mean ± S.E. of 10 samples on the basis of per mg protein. There was no significant difference in the specific activity of SOD, GSH-PX, catalase and NADPH generating enzymes between LM and HM. However, there was a significant difference in the specific activities of Na⁺, K⁺ - and Ca²⁺ ATPases between LM and HM (for a, $p = < 0.01$ and for d, $p = < 0.001$). While the specific activity of Na⁺, K⁺ -ATPase was higher in LM than HM, the specific activity of Ca²⁺-ATPase was higher in HM than LM. Both HM and LM contain trace amount of hemoglobin even though LM contains slightly higher amount than HM (for b, $p = < 0.02$). HM and LM contain 0.39 pg and 0.90 pg protein on per cell basis, respectively.

calcium and specific activity of SOD, GSH-PX, catalase and both NADPH generating enzymes between HM and LM. However, there is a significant difference in the specific activity of both membrane bound ATPases between HM and LM. For example, while the specific activity of Na⁺, K⁺-ATPase is higher in LM than HM, the specific activity of Ca²⁺-ATPase is 1.6 fold higher in HM than LM. This indicates that HM cannot be an aggregated fraction of RBC membrane due to the hypotonic shock. Furthermore, since hemoglobin content is negligible in HM, this cannot be an unhemolyzed or partially hemolyzed RBC. Therefore, LM and HM are two distinct membrane fractions differing in density and biochemical properties.

For further characterization, the polypeptide content of HM and LM was analyzed by electrophoresis in 1% sodium dodecylsulfate. The same amount of protein was used for both membranes. Figures 2 and 3 compare the gel patterns of both membrane proteins stained with CB and CB / Ag, respectively. The gel patterns indicate that these membrane fractions differ remarkably in the abundance of major polypeptides. Both LM and HM contain 22 detectable bands, but 16 are CB positive in LM, whereas only 10 of them are CB positive in HM. Several bands are visible in both membranes after Ag-stain followed by CB stain. There is also an indication that HM contains more Ag-positive proteins, i.e., sialoglycoproteins, than LM. Furthermore, some bands, such as band 4.1 and

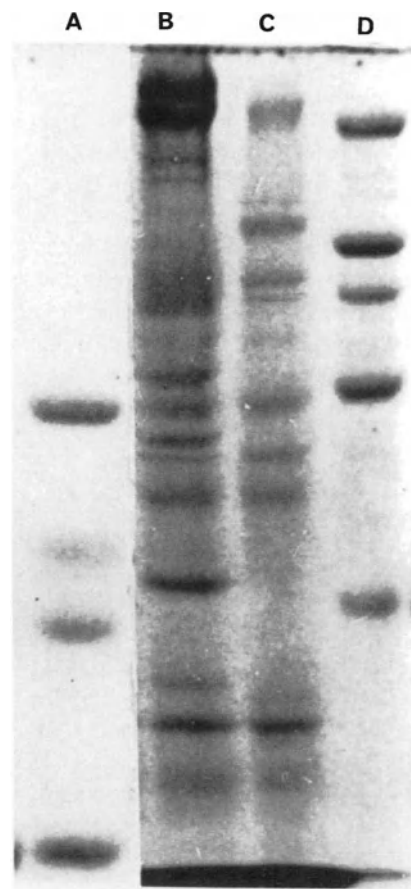


Fig. 2. Electrophorogram of heavy and light membranes of human RBC in 7.5% SDS-PAGE Laemmli gel, stained with coomassie blue (Sigma). Lane A is low mol. wt. protein standards (bovine serum albumin, ovalbumin, pepsin and β -lactoglobulin) from Sigma Chemical Company, St. Louis, MO, USA; lane B is light membrane; lane C is heavy membrane and lane D is high mol. wt. protein standards (myosin, β -galactosidase, phosphorylase b, bovine serum albumin and ovalbumin) from Bio-Rad, Richmond, CA, USA.

proteins with mol. wt. of 48 and 46 KDa, respectively, are only present in LM. Similarly some bands (proteins with mol. wt. of 96, 78 and 43 KDa) are only present in HM and are missing in LM. The gel patterns of LM are similar to those previously reported for ghost membrane [23, 24]. The ultrascan of both membranes also reveals that HM does not contain band 4.1 and contains a very little amount of component I and actin which are predominant as well as important components of ghost membrane (Fig. 4). Currently, we are identifying the major bands by Western blot analysis.

Table 2 shows the percent distribution and approximate molecular weight of major CB stained proteins among HM and LM. HM does not contain several CB stained proteins (250, 165, 155, 88, 78, 65, 45, 41 and 35 KDa). But these proteins are present in LM and have previously been reported

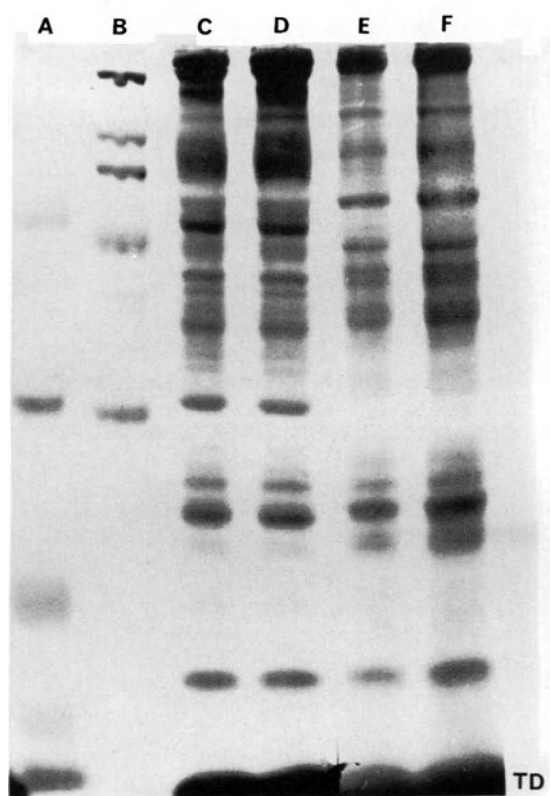


Fig. 3. Electrophorogram of heavy and light membranes of human RBC in 11% SDS-PAGE Laemmli gels, stained with both Ag (ICN Biomedicals, Costa Mesa, CA, USA) and coomassie blue. Lane A is low mol. wt. protein standards (bovine serum albumin, ovalbumin, trypsin, β -lactoglobulin and lysozyme); lane B is high mol. wt. protein standards (myosin, β -galactosidase, phosphorylase b, bovine serum albumin and ovalbumin); Lanes C and D represent light membranes and lanes E and P represent heavy membranes. TD represents the tracking dye bromophenol blue.

by several investigators in ghost membranes [23–26]. HM contains three additional bands (mol. wt. 135, 95, 32 KDa) which are absent in LM. Furthermore, component III (mol. wt. 100 KDa) is present in a significantly lower amount in HM than in LM ($p < 0.001$) whereas proteins with mol. wt. of 70 and 61 KDa, respectively, are present in significantly higher amounts in HM than in LM ($p < 0.001$). Component I cannot be detected in HM by CB stain.

Table 3 shows the percent distribution and approximate molecular weight of major proteins among HM and LM which are positive for double-stain. The double staining picked up some additional bands which were not visible by CB staining only. For example component I, and proteins with mol. wt. of 165, 155 and 84 KDa, respectively, are visible after double staining. Also, band 4.1, which is one of the important cytoskeletal proteins of RBC membrane, is absent in HM, but present in LM. Furthermore, in comparison to LM,

Table 2. Percent distribution of coomassie blue positive proteins among heavy and light membrane of human red blood cells

Band	Tentative identity	Approximate Molecular Wt.	Relative Abundance	
			Heavy	Light
1	Component I	250,000	–	15.9 $\pm$ 0.5
2	Component II	225,000	17.4 $\pm$ 0.6	18.9 $\pm$ 0.4
3		165,000	–	6.2 $\pm$ 0.2
4		155,000	–	4.3 $\pm$ 0.7
5		135,000	15.0 $\pm$ 0.3	–
6	Component III	100,000	9.7 $\pm$ 0.4	15.7 $\pm$ 0.6 ^d
7		95,000	5.6 $\pm$ 0.1	–
8	Band 4.1	88,000	–	2.3 $\pm$ 0.2
9		78,000	–	6.4 $\pm$ 0.2
10		70,000	10.9 $\pm$ 0.5 ^d	4.3 $\pm$ 0.2
11		65,000	–	4.5 $\pm$ 0.3
12		61,000	10.0 $\pm$ 0.4 ^d	2.2 $\pm$ 0.3
13		53,000	5.9 $\pm$ 0.1	5.3 $\pm$ 0.3
14		45,000	–	1.7 $\pm$ 0.2
15	Actin	41,000	–	6.9 $\pm$ 0.2
16		35,000	–	1.5 $\pm$ 0.1
17		32,000	5.7 $\pm$ 0.2	–
18		30,000	12.0 $\pm$ 0.2 ^d	2.3 $\pm$ 0.3
19		22,000	7.9 $\pm$ 0.2 ^d	1.5 $\pm$ 0.2

Approximate molecular weight of each protein band was measured by calibration of the gels with mixtures of molecular weight markers and by using both internal and external standards in a LKB 2222010 ultrosan XL. Some of the major polypeptides was tentatively identified by comparing with the reported values of major ghost polypeptides. Values are mean $\pm$ S.E. of 10 samples. For d, $p < 0.001$.

HM also contains a significantly smaller amount ($p < 0.001$) of two other proteins which are very essential for RBC function, such as component I and actin. In addition, some proteins are present in significantly higher amounts in HM than in LM. For example, component II of spectrin (mol. wt. 230 KDa) and proteins with mol. wt. of 145, 84 and 76 KDa, respectively, are present in a significantly higher amount in HM than in LM ($p < 0.001$).

Discussion

The presence of dense and light membrane fractions has been observed by others [4, 5]; however, they have attributed this occurrence to the fact that erythrocyte membrane proteins are prone to either aggregation or proteolytic degradation [24, 27]. In the present study we filtered the red blood cells through cellulose to remove contaminating leukocytes and platelets [7]. The complete blood count analysis and light microscopic examination ensured that no contaminating leukocytes were present. Therefore, it is unlikely that proteases present in the leukocytes are responsible for the generation of two species of membranes. Furthermore, the

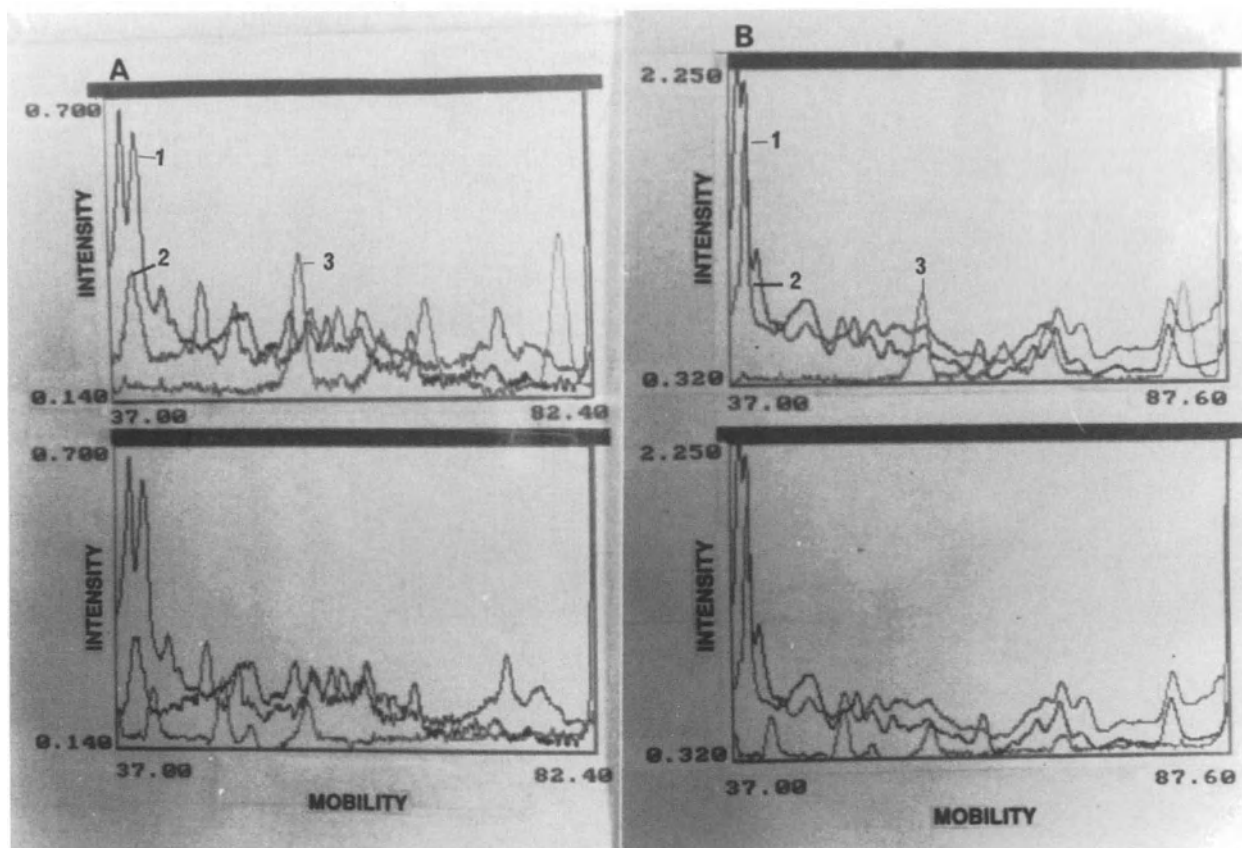


Fig. 4. Scanning of SDS polyacrylamide electrophoresis gels on a LKB 2222-010 UltraScan XL Laser Densitometer. 1 represents light membrane; 2 represents heavy membrane; 3 represents either low mol. wt. protein standards (top) or high mol. wt. protein standards (bottom). Panel A represents 7.5% gel stained with CB and panel B represents 11 % gel stained with both AG/CB.

presence of EGTA as a calcium chelator during the lysis step guaranteed the inactivation of both phosphoinositide-specific phospholipase C and calcium-dependent transglutaminases which otherwise could impact the density of the resulting stroma.

We have recently reported that physical stress to the subjects from whom blood is obtained causes an increase in the amount of heavy membrane and this effect is more pronounced in individuals with sickle cell trait RBC than normal RBC [28]. This indicates that the dense fraction must contain some important component of RBC membrane which is affected by exercise. Since we added PMSF and aprotinin as protease inhibitors during the lysis step, it is unlikely that the presence of proteases in the red cell stroma are responsible for the observed differential effect of exercise on the relative distribution of light and heavy membranes among normal and sickle cell trait RBC.

Gel profiles and percent distribution of major proteins among HM and LM also indicate that HM and LM are two distinct membrane fractions. We still do not know, however, whether they originate from the same cell population or from

different cell populations. The shape and deformability of RBC are properties of the structural organization of membrane proteins and lipids. Ultrastructural studies indicate that spectrin and actin are the major components of the cytoskeletal network in RBC; bands 4.1 and 4.2 are the minor components [29]. Several reports have described the organization of erythrocyte membrane with regard to their asymmetry and their interaction with other membrane components [30, 31]. It is known that cross-linking between spectrin component I and actin is essential for the regulation of cell shape and deformability of RBC [32, 33]. Since HM and LM differ in spectrin, actin and component III content, we speculate that HM and LM are functionally different.

We have observed higher levels of sialoglycoproteins in HM than LM. Component III (band 3, a 100,000 mol. wt. protein) and component I of spectrin were absent in HM but present in LM. Also, Na^+ , K^+ -ATPase activity was found to be lower in HM than LM. A schematic model of the arrangement of some of the erythrocyte membrane polypeptides as proposed by Juliano [34] is shown in Fig. 5. This model indicates that sialoglycoproteins are located on outside,

Table 3. Percent distribution of major coomassie blue and silver stained positive proteins among heavy and light membranes of human red blood cells

Band	Identity (Tentative)	Mol. Wt. Approximate	Relative Abundance	
			Heavy	Light
1	Component I	250,000	1.2 ± 0.0	17.4 ± 1.2^d
2	Component II	230,000	18.2 ± 1.2^d	10.0 ± 1.2^a
3		165,000	1.6 ± 0.2	6.2 ± 1.0
4		155,000	1.2 ± 0.7^d	1.9 ± 0.5
5		145,000	12.1 ± 1.5	2.1 ± 0.7
6	Component III	100,000	7.2 ± 1.1	12.8 ± 1.4^c
7		96,000	3.5 ± 0.2	—
8	Band 4.1	88,000	—	4.0 ± 0.3
9		84,000	9.8 ± 2.1^c	3.4 ± 0.8
10		80,000	1.4 ± 0.1	5.7 ± 0.9
11		78,000	2.2 ± 0.3	—
12		76,000	7.3 ± 1.1^b	3.3 ± 0.9
13		70,000	5.4 ± 0.7	4.3 ± 0.4
14		66,000	5.3 ± 0.4	2.3 ± 0.2
15		60,000	3.5 ± 0.7	1.1 ± 0.3
16		56,000	4.0 ± 0.1	3.4 ± 0.2
17		50,000	1.5 ± 0.0	0.4 ± 0.0
18		48,000	—	0.3 ± 0.1
19		46,000	—	0.9 ± 0.1
20		43,000	0.9 ± 0.1	—
21	Actin	41,000	0.8 ± 0.1	6.4 ± 1.1^d
22		31,000	1.4 ± 0.1	2.0 ± 0.1
23		27,000	3.0 ± 0.9	4.4 ± 1.0
24		24,000	4.6 ± 0.9	6.6 ± 1.0
25		22,000	2.1 ± 0.7	0.9 ± 0.1

Approximate molecular weight of each protein band was measured by calibration of the gels with mixtures of molecular weight markers and by using both internal and external standards in a LKB 2222-010 ultrosan XL. Some of the major polypeptides were tentatively identified by comparing with the reported values of major ghost polypeptides [20]. Values are mean $\pm$ S.E. of 10 samples. Component I, component III, actin and a protein with mol. wt. of 165K are present in significantly higher amounts in light membrane than in heavy membrane (for c, $p < 0.01$ and for d, $p < 0.001$). Component II, and proteins with mol. wt. 145, 84 and 76K, respectively, are present in significantly higher amounts in heavy membrane than in light membrane (for b, $p < 0.02$, for c, $p < 0.01$ and for d, $p < 0.001$).

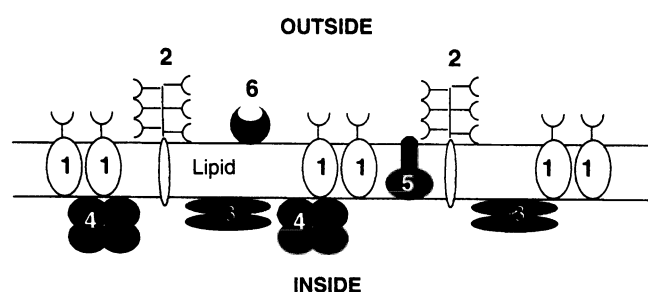


Fig. 5. A model of erythrocyte membrane. 1. 100,000 mol. wt. protein. 2. Sialoglycoprotein. 3. Spectrin. 4. Glyceraldehyde-3-phosphate dehydrogenase. 5. (Na^+-K^+) -ATPase. 6. Acetylcholinesterase. Only some of the membrane polypeptides are shown.

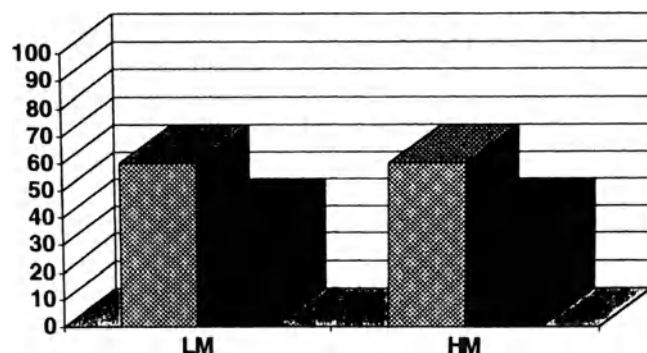


Fig. 6. Observed distribution profile of right side-out and inside-out vesicles prepared from light and heavy membranes. ▨ represents inside-out vesicles and ■ represents right side-out vesicles.

spectrin is located on inside, and both band 3 (100,000 mol. wt. protein) and Na^+ , K^+ -ATPase are integral proteins, therefore, it may be argued that HM represents right-side out whereas LM represents inside-out vesicles. However, we have been able to prepare right side-out and inside-out vesicles in equal distribution from both heavy and light membranes (Fig. 6). This was confirmed by the measurement of the activities of GPD and ACE of both inside-out and right side-out vesicles isolated from each membrane. Almost all of the GPD activity and only 10–15% of the ACE activity was confined to the inside-out vesicles from both membranes (data not shown). However, if HM and LM were representatives of right side-out and inside-out vesicles, we would have anticipated a difference in the distribution of GPD and ACE activities between the inside-out and right side-out vesicles (Fig. 7). Therefore, it is unlikely that the dense and light fractions are simply the representations of right side-out and inside-out vesicles. On similar ground, it is unlikely that the heterogeneity in rbc membranes is due to differences in

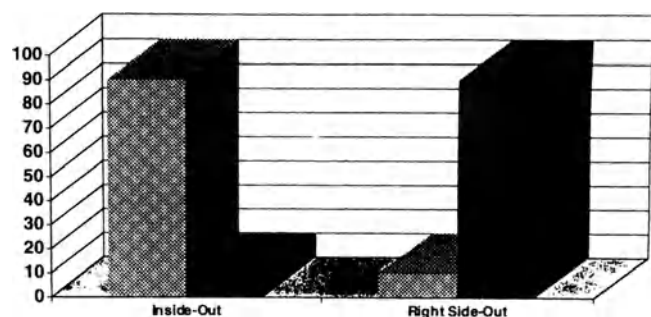


Fig. 7. Anticipated profile assuming that heavy membrane and light membrane are representatives of right side-out and inside-out vesicles, respectively. ▨ represents glyceraldehyde-3-phosphate dehydrogenase and ■ represents acetylcholinesterase.

sealing. If one of the membranes is open and the other is sealed, we would have observed a marked difference in the binding of these two membranes to the Con A/cellulose column.

It is well known that different subpopulations exist among erythrocytes [35]. Also, it is known that erythrocyte membrane undergoes change in lipid and protein content, enzyme activity, ion permeabilities, size and deformability during its aging process [22, 36–38].

Thus, it may be argued that HM derives from heavy cells, i.e., aged cells and LM from light, i.e., young cells. However, we have isolated four populations of erythrocytes (Fig. 8) by discontinuous stractan gradients [22] and each fraction yielded two distinct species of 'ghosts' (heavy and light). Furthermore, we have recently reported that tread mill exercise causes an increase in the amount of HM and a corresponding decrease in the amount of LM [28] and also a loss of deformability in the RBC of sickle cell trait individuals [39]. This suggests that HM and LM must originate from the same cell populations, since short term exposure to exercise would not yield different cell populations. It is however possible that HM is the site of the cell membrane most affected by aging processes or other external factors such as exercise. Currently, we are carrying out studies on the relative distribution of proteins in HM and LM of RBCs of different

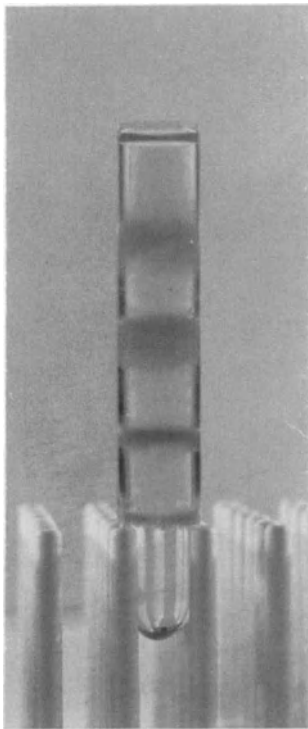


Fig. 8. Fractionation of varying aged erythrocytes on the discontinuous stractan density gradient.

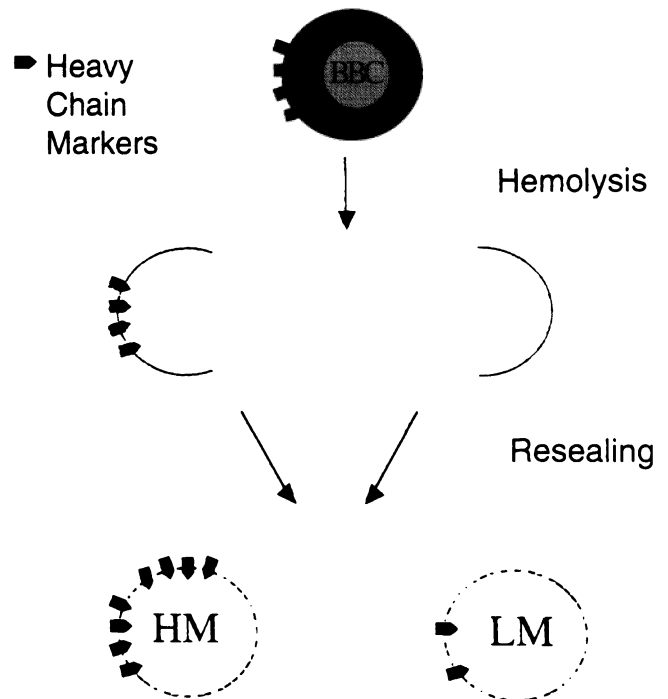


Fig. 9. Proposed mechanism of heterogeneity in rbc membrane. HM heavy membrane and LM = light membrane.

densities (ages). It has been reported by Piomelli *et al.* [40] that density of RBCs increase with aging process.

The results from this study indicate that some part of the rbc membrane is denser than other parts. It is possible that during the process of hemolysis of RBCs, the membrane is spliced which results in the formation of two populations of membranes, HM and LM (Fig. 9). These membranes may or may not be sealed depending on the nature of buffer.

Acknowledgement

We thank Ms. Karen Childs, Cynthia Weaver and Pam Patterson for collecting the blood specimens. We thank Dr. M. A. South for her encouragement in this work, Brenda Green-Jarvis for graphics, and Dr. Maria de Fatima Lima for her help in scanning gels. This work was supported by Grants from Army (DAMD 17-87-7027), National Institutes of Health (NIH-GM08198), and National Science Foundation (HRD 9450476).

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Schisandrin B protects against myocardial ischemia-reperfusion injury by enhancing myocardial glutathione antioxidant status

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Abstract

The effects of Schisandrin B (Sch B) and dimethyl-4, 4'-dimethoxy-5,6,5',6'-dimethylene-dioxy-biphenyl-2,2'-bicarboxylate (DDB) treatment on myocardial ischemia-reperfusion (IR) injury in isolated perfused rat hearts were examined under both *in vitro* and *ex vivo* conditions. *In vitro* administration of liposome-entrapped Sch B or DDB during reperfusion did not protect against myocardial IR injury, whereas ascorbic acid or Trolox supplemented perfusate produced protective effect, as evidenced by the significant decrease in the extent of lactate dehydrogenase leakage as well as an improvement in contractile force recovery. Myocardial protection afforded by N-acetyl-L-cysteine supplemented perfusate was not accompanied by the enhancement in contractile force recovery. In *ex vivo* experiment, pretreatment of Sch B (0.6/1.2 mmol/kg/day $\times$ 3) protected against IR-induced myocardial damage in a dose-dependent manner. The myocardial protection was associated with an enhancement in myocardial glutathione antioxidant status, as indicated by significant reductions in both the extent of IR-induced reduced glutathione depletion and inhibition of Se-glutathione peroxidase and glutathione reductase activities. In contrast, the inability of DDB pretreatment to enhance myocardial glutathione antioxidant status resulted in a failure in preventing IR injury. The ensemble of results suggests that the myocardial protection afforded by Sch B pretreatment, which was unlikely due to free radical scavenging action, may be mainly mediated by the enhancement of myocardial glutathione antioxidant status, particularly under oxidative stress conditions. (Mol Cell Biochem **196**: 151–156, 1999)

Key words: Schisandrin B, dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene-dioxybiphenyl-2,2'-bicarboxylate, myocardial ischemia-reperfusion, glutathione, glutathione reductase, glutathione peroxidase

Abbreviations: CCl₄ – carbon tetrachloride; DDB – dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene-dioxy-biphenyl-2,2'-bicarboxylate; FS – *Fructus Schisandrae*; GPX – Se-glutathione peroxidase; GRD – glutathione reductase; GSH – reduced glutathione; IR – ischemia-reperfusion; LDH – lactate dehydrogenase; NAC – N-acetyl-L-cysteine; Sch B – Schisandrin B; TRX – Trolox; VC – ascorbic acid

Introduction

Fructus Schisandrae (FS), the fruit of *Schisandra chinensis* (Turcz.) Baill., has been used as tonic and astringent in traditional Chinese medicine for centuries [1]. Schisandrin B (Sch B, see Fig. 1a), a dibenzo[a,c]cyclooctadiene derivative isolated from FS, has early been shown to protect against carbon tetrachloride (CCl₄)-induced hepatotoxicity by

enhancing the hepatic glutathione antioxidant system [2]. Dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene-dioxy-biphenyl-2,2'-bicarboxylate (DDB, Fig. 1b), an intermediate compound derived from the synthesis of Schisandrin C (another dibenzo[a,c]cyclooctadiene derivative from FS), has been shown to possess antioxidant activity [3] and protect against CCl₄-induced hepatocellular damage [4]. Preliminary studies in our laboratory have demonstrated that Sch B

pretreatment could also produce cardioprotective effect on ischemia-reperfusion (IR) injury [5]. However, the biochemical mechanism involved in the cardioprotective action of Sch B is still unclear. It would also be of interest to investigate whether the two structurally related compounds, namely Sch B and DDB, produce similar pharmacological action in the myocardium. In the present study, we examined the effect of Sch B or DDB treatment on myocardial IR injury in isolated Langendorff-perfused rat hearts under both *in vitro* and *ex vivo* experimental conditions. The effects of known antioxidants such as Trolox (TRX), ascorbic acid (VC) and N-acetyl-L-cysteine (NAC) were also investigated for comparison under *in vitro* conditions. In *ex vivo* experiments, the effect of Sch B or DDB pretreatment on tissue glutathione antioxidant status was examined in control and IR-challenged hearts in order to explore the biochemical mechanism involved in the cardioprotection.

Materials and methods

Chemicals and herbal material

VC and TRX were purchased from Aldrich Chemical Co. (Buchs, Switzerland). NAC, phosphatidylcholine and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DDB was purchased from Guangzhou Xun Xing Pharmaceutical Company (Guangzhou, P.R. China). All other chemicals were of analytical grade. Solvents used for high-performance liquid chromatography were of HPLC grade. FS was imported from mainland China. It was authenticated and supplied by a commercial dealer (Lee Hoong Kee Ltd.) in Hong Kong. Sch B was purified from the petroleum ether extract of FS as described in Ip *et al.* [6].

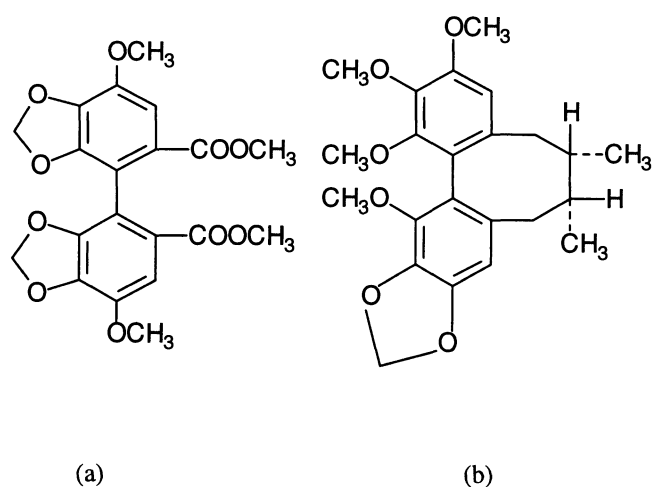


Fig. 1. Chemical structures of (a) Sch B and (b) DDB.

Preparation of liposome-entrapped Sch B/DDB

Since both Sch B and DDB have very low water solubility, liposome entrapped drug was prepared for perfusion in *in vitro* experiments. Liposomes were prepared by a method modified from Van Rooijen *et al.* [7]. Phosphatidylcholine (37.5 mg) and cholesterol (5.5 mg) were dissolved in 20 ml methanol/chloroform mixture (1: 1, v/v) containing in a 500 ml round-bottom flask and dried under reduced pressure at 37°C by rotary evaporation to form a thin film on the interior wall of the flask. After the addition of 10 ml chloroform containing either 0.096 g Sch B or 0.1 g DDB, the chloroform was removed by rotary evaporation at 37°C again. Then the thin film was dispersed in 10 ml modified Krebs-Henseleit perfusion buffer by gentle rotation for 10 min at room temperature (22°C). The preparation was kept at room temperature for 2 h, sonicated in a waterbath for 3 min and kept at room temperature again for 2 h. Finally, 390 ml perfusion buffer was added to make a 400 ml of monophasic perfusate containing the drug at 600 µM. Empty liposomes, which were prepared by the same procedure except omitting the drug, were used for control experiments.

Animal pretreatment

Male adult Sprague-Dawley rats (~ 8 weeks old; 260–290 g) were maintained on a 12-h dark/light cycle at about 22°C and allowed food and water *ad libitum*. Animals were randomly divided into groups. In the pretreatment groups with IR, rats (n = 5 in each group) were treated intragastrically with Sch B or DDB at increasing daily doses of 0.6/1.2 mmol/kg or 1.2/2.4 mmol/kg, respectively, for 3 days. In the pretreatment groups without IR, rats (n = 3 in each group) were treated intragastrically with Sch B or DDB at a daily dose of 1.2 mmol/kg, respectively, for 3 days. The two drugs were suspended/dissolved in olive oil. Control animals (n = 8) were given the olive oil only. Twenty-four hours after the last dosing, control or drug pretreated rats were anesthetized with diethylether. The heart was excised and subjected to IR challenge as described below.

Isolated-perfused rat heart

The heart was excised quickly and immediately immersed in ice-cold and heparinized (50 unit/ml) saline. The aorta was cannulated and then transferred to a warm and moistured chamber of the perfusion apparatus. The heart was retrogradely perfused according to the Langendorff method as described in [8]. The apex of the heart was attached via a metal hook to an unextendable cotton thread which was connected to a force displacement transducer (Grass FT03), and the

isometric contractions of the heart were recorded on a polygraph (Grass Model 7-8 P).

Myocardial IR

After an initial 30-min of perfusion for equilibration, the isolated heart was subjected to a 10-min period of 'no-flow' normothermic global ischemia followed by a 15-min reperfusion. For testing the *in vitro* effect of the drug on myocardial IR injury, perfusate containing liposome-entrapped Sch B or DDB, VC, TRX or NAC at a concentration of 300 or 600 μM was used for perfusing hearts prepared from untreated animals ($n = 5$ in each group) during the reperfusion period. In the IR control group, isolated hearts were either perfused with buffer ($n = 6$) or empty liposome-containing buffer ($n = 6$) during the reperfusion period. Since there was no detectable difference in terms of myocardial IR injury between the two groups, the data were pooled together (i.e. $n = 12$). The non-ischemic control (i.e. non-IR) hearts were perfused for 25 min after equilibration, without subjecting to 10 min of ischemia. Coronary effluent was collected in 1-min fraction at increasing time intervals during the course of equilibration and reperfusion. The fraction was immediately put on ice until assay. Myocardial IR injury was assessed by measuring the extent of lactate dehydrogenase (LDH) leakage during the reperfusion period. LDH activity of in all fractions of coronary effluent was assayed as described in [8]. The extent of LDH leakage was estimated by computing the area under the curve (AUC) of the graph plotting the percent of LDH activity (with respect to the mean preischemic value measured during the equilibration period) against the reperfusion time (1–15 min) as described in [8].

Biochemical analysis

Myocardial tissue samples were rinsed with ice-cold homogenizing buffer (50 mM Tris(hydroxymethyl) amino-methane, 0.1 mM EDTA, pH 7.6) after the IR experiment. Tissue homogenate was prepared by homogenizing 0.8 g of myocardial tissue in 8 ml ice-cold homogenizing buffer with two 10-sec bursts of a tissue disintegrator (Ika Ultra turax T25) at 135,000 rpm. Myocardial cytosolic fraction was prepared by diluting 1 ml tissue homogenate with 3 ml homogenizing buffer and centrifuging at 40,000 g for 30 min at 4°C. Reduced glutathione (GSH) level was measured by an HPLC method modified from Reed *et al.* [9] as described in [10]. Activities of glutathione reductase (GRD) and S-glutathione peroxidase (GPX) were measured as described in [11].

Statistical analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple range test to detect the inter-group difference. Significant difference was determined when $p < 0.05$.

Results and discussion

As shown in Fig. 2a, a 10-min period of ischemia followed by 15-min reperfusion caused an increase in LDH leakage in isolated perfused rat hearts. The increased extent of LDH leakage was associated with an incomplete (48%) recovery of contractile force at 15 min after reperfusion (Fig. 2b). Isolated hearts subjecting to ischemic challenge received perfusate supplemented with VC or TRX at 300 μM showed protection against IR-induced myocardial damage, with the extent of LDH leakage being decreased by 65 and 56%, respectively. The reduction in the extent of LDH leakage was paralleled by a small but significant improvement in contractile force recovery in these hearts (Fig. 2b). When doubling the concentration to 600 μM , NAC containing perfusate only produced a smaller degree of protection (19%) against IR injury (Fig. 2a). However, the myocardial protection of NAC was not accompanied by the enhancement in contractile force recovery (Fig. 2b). While perfusing hearts with empty liposomes did not affect the extent of IR injury (data not shown), administration of Sch B or DDB liposomes containing perfusate (600 μM) during reperfusion did not protect against myocardial IR injury. Reactive oxidant species have been shown to be involved in the development of myocardial IR injury [12]. This is supported by the cardioprotection afforded by VC and TRX supplemented perfusate (Figs 2a and 2b), which is also consistent with the finding that both VC and TRX could reduce the extent of myocardial infarction in an open chested canine model of IR injury [13]. Electron spin resonance studies showed that reperfusion of isolated hearts following a brief period of ischemia was associated with a burst of free radical formation [14–16]. The myocardial protection afforded by VC and TRX perfusion probably involves scavenging of free radicals arising from the reperfusion process [17–18]. The observation that NAC, a glutathione enhancing agent, produced only a small degree of protection against myocardial IR injury suggests that the free radical scavenging activity of NAC may be lower than those of VC and TRX under the present experimental conditions. The inability of liposome-entrapped Sch B and DDB to prevent myocardial IR injury indicates the lack of free radical scavenging activities in these preparations. It has been reported that liposome-entrapped lipid-soluble antioxidants, such as butylated hydroxytoluene, in the perfusate was able to protect against myocardial IR injury when applying during the post-ischemic period in isolated-perfused

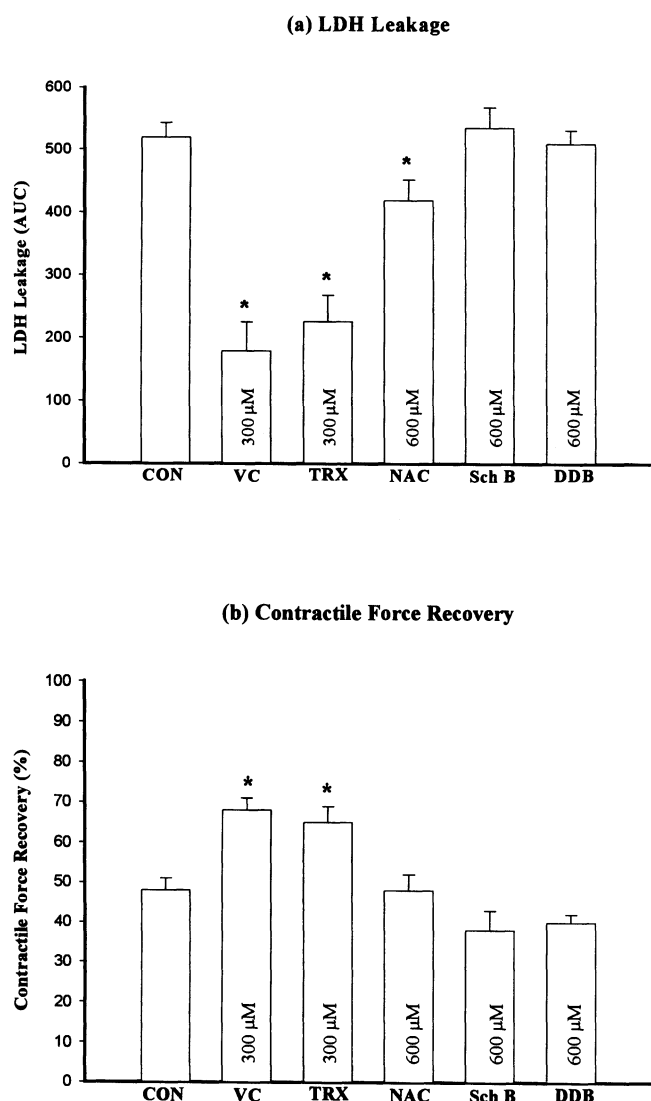


Fig. 2. Effect of Schisandrin B and DDB perfusion on myocardial ischemia-reperfusion injury in isolated Langendorff-perfused rat hearts. Isolated hearts prepared from unpretreated animals were subjected to ischemia-reperfusion (IR) injury as described in Materials and methods. Perfusate containing ascorbic acid (VC, $n = 5$), Trolox (TRX, $n = 5$) or N-acetyl-L-cysteine (NAC, $n = 5$) or liposome-entrapped Schisandrin B (Sch B, $n = 5$) or dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-bicarboxylate (DDB, $n = 5$) at the indicated dose was used for reperfusion. Control (CON, $n = 12$) hearts received perfusion with buffer or empty liposome-containing buffer. Each bar represents the mean $\pm$ S.E.M. (a) LDH leakage was obtained by subtracting the value measured from isolated perfused hearts without subjecting to IR (640 ± 13.3 , $n = 5$) from that of the respective IR hearts; (b) contractile force recovery was estimated by comparing the contractile force at 15 min of reperfusion with that measured at the end of the equilibrium period. *Significantly different from the CON group.

rat hearts [19]. The failure of Sch B and DDB in myocardial protection is unlikely due to the unavailability of the drug caused by liposome entrapment.

In order to investigate whether Sch B or DDB pretreatment can produce a direct effect on the myocardium, isolated perfused rat hearts prepared from drug pretreated animals were subjected to IR injury. As shown in Fig. 3a, isolated hearts prepared from Sch B-pretreated rats showed a dose-dependent protection against IR-induced tissue damage, with the extent of LDH leakage being reduced by 36 and 60% at daily doses of 0.6 and 1.2 mmol/kg, respectively. Sch B pretreated hearts (1.2 mmol/kg) also showed a better recovery of contractile force following IR challenge (Fig. 3b). DDB pretreated hearts did not protect against myocardial IR injury, even though the daily dosage was double that of Sch B.

The antioxidant mechanism involved in the cardioprotective action of Sch B pretreatment was further investigated by examining the effect on myocardial glutathione antioxidant status. As shown in Figs 4a, 4b and 4c, the IR-induced myocardial injury was associated with an impairment in myocardial glutathione antioxidant status, as evidenced by a depletion in tissue GSH (44%) as well as decreases in GPX (30%) and GRD (18%) activities in ischemic-reperfused hearts, when compared with those of the non-IR control. While Sch B pretreatment at a daily dose of 1.2 mmol/kg did not enhance the myocardial glutathione antioxidant status in hearts without subjecting to IR, it protected against the IR-induced impairment in myocardial glutathione antioxidant status, as indicated by increases in GSH level (64%), GPX (25%) and GRD (24%) activities in ischemic-reperfused hearts when compared with those of the unpretreated IR control. As regards the myocardial level of GPX and GRD proteins, it is unlikely that Sch B pretreatment could induce the synthesis of these enzyme proteins because the enzyme activities of the Sch B-pretreated and non-IR hearts were not significantly changed (Figs 4b and 4c). DDB pretreatment, at the same dosage as Sch B, did not produce any detectable effect on myocardial glutathione antioxidant status in control and IR-treated hearts when compared with the respective controls (Figs 4a, 4b and 4c). The maintenance of myocardial glutathione antioxidant status is important in protecting against free radical-induced tissue damage [20–21]. The glutathione antioxidant system, comprising mainly GSH, GPX and GRD, serves as an effective cellular defense against oxidative stress. In essence, the GRD-catalyzed regeneration of GSH from its oxidized form can sustain the GSH-dependent free radical scavenging activity or GPX activity in decomposing hydrogen peroxide or other organic hydroperoxides [22]. It has been reported that GSH-depleted rat hearts were found to be more susceptible to IR injury [23]. The IR-induced impairment in myocardial glutathione antioxidant status, as shown in the present study (Figs 4a, 4b and 4c), may be caused by the free radical-induced GSH depletion and enzyme inactivation. Sch B pretreatment, which presumably sustained the functioning of myocardial glutathione antioxidant system (Table 3), could prevent tissue

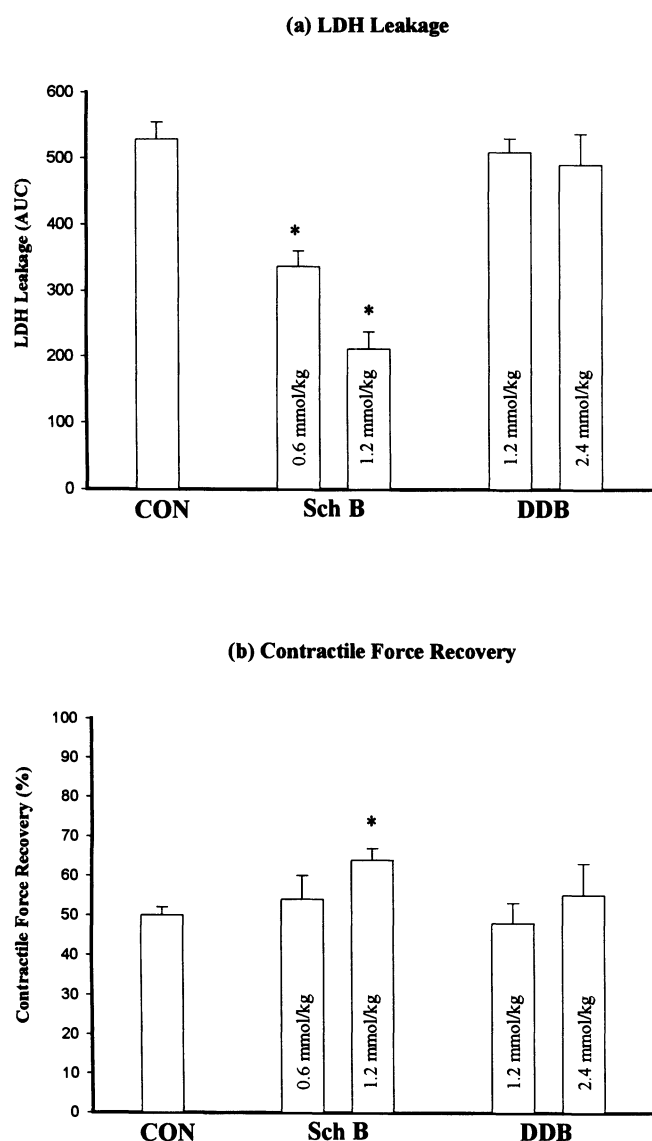


Fig. 3. Effect of Schisandrin B and DDB pretreatment on myocardial ischemia-reperfusion injury in isolated Langendorff-perfused rat hearts. Isolated hearts prepared from animals pretreated orally with Sch B or DDB at the indicated dose for 3 days were subjected to IR injury as described in Materials and methods. CON animals were given olive oil only. Each bar represents the mean $\pm$ S.E.M., with $n = 8$ for CON and $n = 5$ for the drug-pretreated group. *Significantly different from the CON group.

damage caused by IR challenge. In this connection, GSH supplemented cardioplegic solution was found to be able to prevent the post-ischemic cardiac dysfunction and contractility in canine hearts [24]. To the contrary, reperfusion the previously ischemic myocardium with liposome-entrapped Sch B supplemented perfusate did not protect against IR injury or prevent the IR-induced decreases in GPX and GRD activities (data not shown). The inability of DDB pretreatment to enhance myocardial glutathione antioxidant status also resulted in the failure in preventing IR injury.

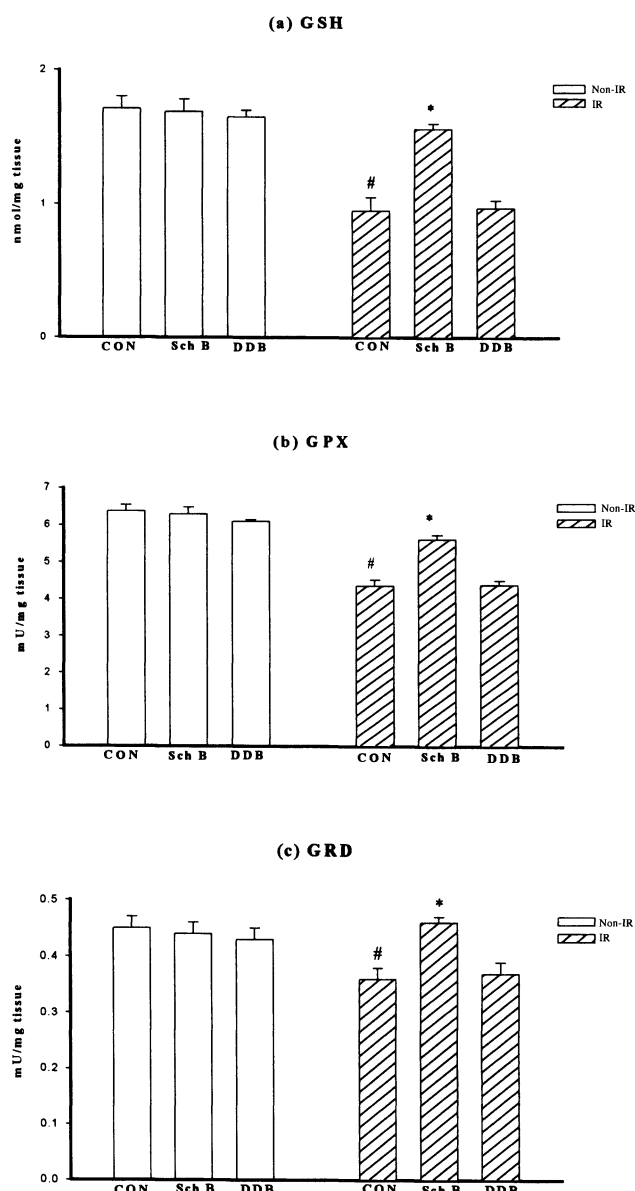


Fig. 4. Effect of Schisandrin B/DDB pretreatment on myocardial glutathione antioxidant status in control or ischemic-reperfused rat hearts. Animals were treated orally with Sch B or DDB at a daily dose of 1.2 mmol/kg for 3 days as described in Fig. 3. CON animals were given olive oil only. After the IR experiment, the myocardial tissue samples were subjected to biochemical analyses as described in Materials and methods. Myocardial (a) reduced glutathione (GSH); (b) Se-glutathione peroxidase (GPX) and (c) glutathione reductase (GRD) activities were measured. Each bar represents the mean $\pm$ S.E.M., $n = 5$ for all groups except the non-IR Sch B and DDB groups of which $n = 3$. #Significantly different from the non-IR CON group. *Significantly different from the IR CON group.

In conclusion, the failure of Sch B to prevent myocardial IR injury under *in vitro* conditions suggests that the myocardial protection afforded by Sch B pretreatment was unlikely due to the free radical scavenging action. The cardioprotective action of Sch B may be attributed to the beneficial effect on

myocardial glutathione antioxidant status. This postulation is supported by the observation that the failure of DDB pretreatment in protecting against myocardial IR injury was accompanied by the absence of any effect on myocardial glutathione antioxidant status. The ensemble of results suggests that the cardioprotective action of Sch B is likely mediated by the enhancement of myocardial glutathione antioxidant status, particularly under oxidative stress conditions.

Acknowledgement

This work was supported in part by a research fund from Lee Kum Kee Group (Hong Kong).

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Scavenging of superoxide anion radical by chaparral

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Abstract

Chaparral is considered to act as an antioxidant. However, the inhibitory effects of chaparral on specific radical species are not well understood. Using electron paramagnetic resonance (EPR) spectroscopy in combination with spin trapping techniques, we have found that chaparral scavenges superoxide anion radical ($O_2^{\cdot-}$) in a dose-dependent manner. 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a spin trapping agent and the reaction of xanthine and xanthine oxidase as a source of $O_2^{\cdot-}$. The kinetic parameters, IC_{50} and V_{max} , for chaparral scavenging of $O_2^{\cdot-}$ were found to be 0.899 $\mu\text{g/mL}$ and 8.4 ng/mL/sec , respectively. The rate constant for chaparral scavenging $O_2^{\cdot-}$ was found to be $1.22 \times 10^6 \text{ g}^{-1}\text{s}^{-1}$. Our studies suggest that the antioxidant properties of chaparral may involve a direct scavenging effect of the primary oxygen radical, $O_2^{\cdot-}$. (Mol Cell Biochem **196**: 157–161, 1999)

Key words: chaparral, EPR, spin trapping, superoxide anion radical

Introduction

Personal health care practices that may include the use of dietary supplements are becoming increasingly common in the United States. Health aid products marketed as dietary supplements include botanicals, vitamins, and minerals. Chaparral is a herbal preparation which is made by grinding leaves of an evergreen desert shrub known as the creosote bush or 'greasewood'. The leaves can be brewed for tea or made into capsules or tablets. Chaparral has been recommended as a free radical scavenger to retard aging, maintain proper skin conditions, and protect against various other health disorders [1, 2]. However, its direct effects on reactive oxygen species (ROS) are not well understood. To address whether chaparral can act as a direct scavenger of free radicals, we have investigated the effects of chaparral on ROS by spin trapping in combination with electron paramagnetic resonance (EPR) techniques. If chaparral scavenges free radicals, the characteristic EPR signals of trapped free radical species generated in the given reaction

systems should decrease with increasing amount of added chaparral. In this study, we report new experimental evidence that chaparral is capable of scavenging the superoxide anion radical ($O_2^{\cdot-}$).

Materials and methods

Xanthine, xanthine oxidase, superoxide dismutase (SOD, from human erythrocytes), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 3-cyano-proxyl free radical, and dimethyl sulfate-oxide (DMSO) were purchased from Sigma Chemical Company.

Chaparral was purchased from Turtle Island Herbs, Inc., Soline Star Route, Boulder, CO 80302, USA. The herb is ethically wild harvested. This preparation is a crude extract of fresh chaparral leaf (*Larrea Glutinosa*) with 60% ethanol (food grain alcohol) and water. One mL of this preparation was evaporated under pure nitrogen and yielded a 125 mg dark brown residue. The residue was dissolved in DMSO to

obtain a stock solution. Before EPR experiment, we have tested whether chaparral inhibits xanthine oxidase activity by monitoring the uric acid formation at 290 nm. Chaparral (up to 10 $\mu\text{g/mL}$) did not exhibit significant inhibition on the rate of uric acid formation, indicating that chaparral does not inhibit xanthine oxidase.

The typical spin-trapping reaction mixture consisted of DMPO (70 mM, equivalent to 7.9×10^{-3} g/mL), xanthine (20 μM) and xanthine oxidase (0.02 unit/mL) in air-saturated sodium/potassium phosphate buffered DMSO (7 M) solution, pH 7.4, in the absence or presence of chaparral extract. The reaction was initiated by adding xanthine oxidase stock solution which was prepared in 10 mM sodium/potassium phosphate buffered solution, pH 7.4.

Individual samples were placed in the EPR cavity using an EPR aqueous flat cell ($60 \times 8.5 \times 0.25$ mm, inner diameter) for the measurements of trapped radicals at a given incubation time at room temperature. The commercial 3-cyano-proxyl free radical was used as the nitroxide standard for determining relative concentrations of spin adduct of DMPO with $\text{O}_2^{\cdot-}$. The recordings of EPR spectra were performed with a Varian 109 EPR spectrometer. Unless otherwise stated, the EPR parameters were set at 100 KHz, X-band; microwave frequency, 9.73 GHz; microwave power, 20 mW; modulation amplitude, 0.5 G; time constant, 0.25 sec; scan time, 960 sec; and receiver gain, 5×10^4 .

Results

EPR spectra of superoxide radical spin adduct

The reaction between xanthine and xanthine oxidase was used as a source of $\text{O}_2^{\cdot-}$ [3, 4]. Figure 1 shows the EPR spectra of DMPO- $\text{O}_2^{\cdot-}$ obtained in the reaction mixtures of xanthine (20 μM) and xanthine oxidase (0.02 units/mL) containing DMPO (70 mM) in the absence and presence of indicated amount of chaparral. The EPR spectrum in Fig. 1a was obtained in a control reaction mixture in the absence of chaparral. This spectrum exhibits $3 \times 2 \times 2$ splitting lines, which typically results from the interactions of an uncoupled electron with a primary nitrogen atom along with the secondary β - and γ -protons. The hyperfine splitting constants of the signal ($a_N = 13.4$ G, $^b a_H = 10.3$ G, and $^c a_H = 1.3$ G) are consistent with previously reported values for DMPO- $\text{O}_2^{\cdot-}$ [4–7] indicating that the signal results from trapped $\text{O}_2^{\cdot-}$. Chaparral was found to significantly inhibit the formation of DMPO- $\text{O}_2^{\cdot-}$. As shown in Fig. 1b–d, chaparral, at concentrations as low as 0.925 $\mu\text{g/mL}$, inhibited ~55% of the EPR signal intensity (Fig. 1c), and at 1.98 $\mu\text{g/mL}$, inhibited EPR signal intensity ~75% of control (Fig. 1d). From these data, it is evident that chaparral is an effective scavenger for $\text{O}_2^{\cdot-}$.

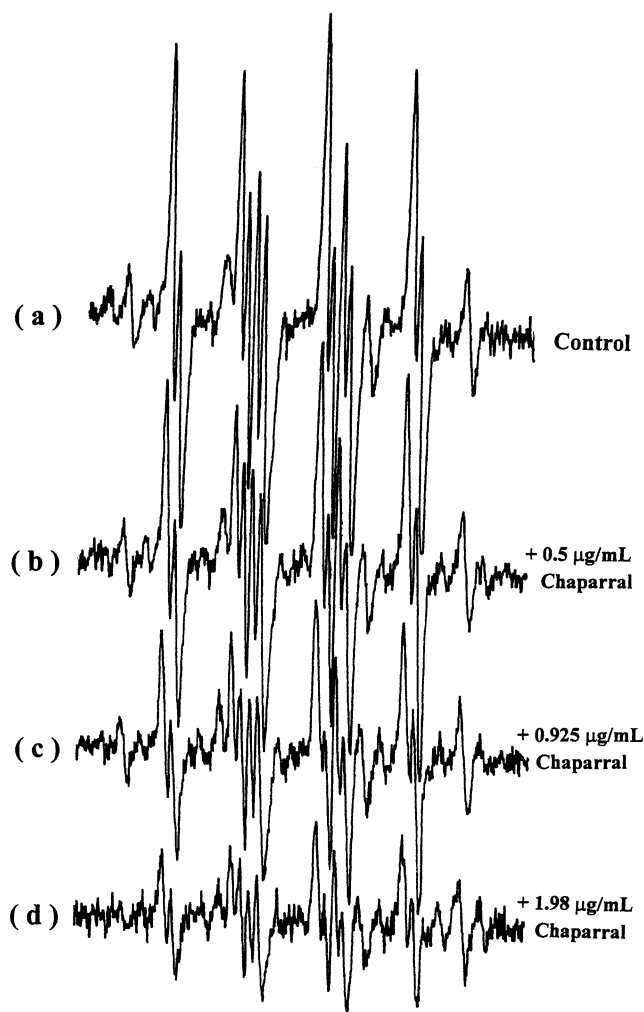


Fig. 1. EPR spectra of spin adduct of superoxide anion radical observed during the reaction of xanthine (20 μM) and xanthine oxidase (0.02 units/mL) containing 70 mM DMPO in air-saturated phosphate buffered 7 M DMSO solution in the presence of: (a) 0; (b) 0.5 $\mu\text{g/mL}$; (c) 0.925 $\mu\text{g/mL}$; and (d) 1.98 $\mu\text{g/mL}$, chaparral. EPR spectroscopy settings were the same as described under Materials and methods.

Effect of chaparral on EPR signal intensity

Chaparral inhibition of DMPO- $\text{O}_2^{\cdot-}$ EPR signal intensity is depicted in Fig. 2. The maximum inhibition can be obtained by plotting $[1-I/I_0]^{-1}$ against $[\text{Chaparral}]^{-1}$. $[1-I/I_0]$ is the fraction of inhibition of EPR signal intensity of DMPO- $\text{O}_2^{\cdot-}$ and I/I_0 is the ratio of the EPR signal intensity in the presence and absence of chaparral. The intercept on the Y-axis represents a reciprocal value of the fraction of inhibition. If this value is equal to 1, it indicates 100% inhibition. If the value is larger than 1, for example, 2, it represents 50% inhibition. When $[1-I/I_0]^{-1}$ was plotted against $[\text{Chaparral}]^{-1}$, a straight line with a correlation coefficient of 0.981 was obtained (Fig. 2, inset) and the intercept value extrapolated

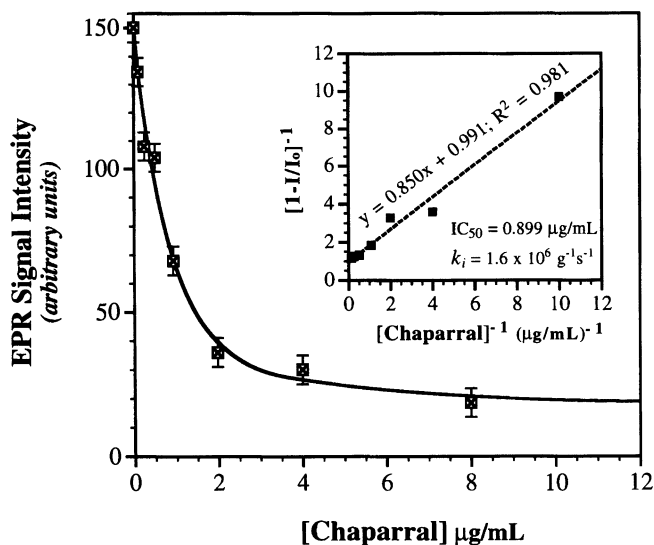


Fig. 2. Graph of chaparral inhibition of DMPO- $O_2^{\cdot-}$ EPR signal intensity. The reaction mixture was same as in Fig. 1 in the absence and presence of indicated concentration of chaparral. Inset, data obtained are presented on reciprocal coordinates, that is, $(1-I/I_0)-1$ against $[Chaparral]^{-1}$, where $(1-I/I_0)$ is the fraction of inhibition of DMPO- $O_2^{\cdot-}$ signal and $1/I_0$ is the ratio of the rates of EPR signal intensity of DMPO- $O_2^{\cdot-}$ in the presence and absence of chaparral. EPR spectroscopy settings were the same as described under Materials and methods.

on the Y-axis was found to be 0.991. Therefore, the maximum inhibition can be considered to be 100%. The ratio of slope to intercept provides the IC_{50} value, the concentration of chaparral required to cause a 50% inhibition of EPR signal intensity, and this ratio was found to be 0.899 $\mu\text{g/mL}$, which is consistent with our estimated value of chaparral concentration that caused 50% inhibition of EPR signal intensity. No inhibition of EPR signal was observed in the reaction mixture incubated under identical conditions in the absence of chaparral, indicating that the inhibition of EPR signal of DMPO- $O_2^{\cdot-}$ is entirely chaparral dependent.

In order to quantitatively confirm the inhibition of DMPO- $O_2^{\cdot-}$ formation by chaparral in this system, we used the methods reported by Buettner *et al.* [8]. If we assume that the velocity of chaparral inhibition against the formation of spin adduct is equal to the rate of formation of spin adduct when the EPR signal intensity of DMPO- $O_2^{\cdot-}$ was suppressed by 50%, then we can obtain the following equation:

$$k_i = k_{app} \cdot [DMPO]/[CH] \quad [1]$$

where k_i is the apparent rate constant of chaparral inhibition of DMPO- $O_2^{\cdot-}$ formation, and k_{app} is a pH-dependent apparent rate constant of DMPO trapping $O_2^{\cdot-}$ and $\cdot\text{OOH}$. Using the IC_{50} value of chaparral described above, 0.899 $\mu\text{g/mL}$, and k_{app} of 26.5 $\text{M}^{-1}\text{s}^{-1}$ (identical to $1.83 \times 10^2 \text{ g}^{-1}\text{s}^{-1}$), the calculated value for DMPO trapping $O_2^{\cdot-}$ and $\cdot\text{OOH}$ at pH

7.4, we obtained a rate constant of $1.63 \times 10^6 \text{ g}^{-1}\text{s}^{-1}$ for chaparral inhibition of DMPO- $O_2^{\cdot-}$ formation in this DMSO-aqueous solution (pH 7.4).

Effect of chaparral on the rate of superoxide radical elimination

Since chaparral does not inhibit xanthine oxidase activity (data not shown), there exists a competition between DMPO and chaparral for available $O_2^{\cdot-}$. Therefore, the kinetic parameters of chaparral, as the competitor in the test system, can be obtained. In a typical reaction, xanthine and xanthine oxidase generates $O_2^{\cdot-}$, which in turn either spontaneously dismutates ($2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$ or $2HOO\cdot \rightarrow H_2O_2 + O_2$ or $O_2^{\cdot-} + \cdot\text{OOH} + H^+ \rightarrow H_2O_2 + O_2$) or react with DMPO to form DMPO- $O_2^{\cdot-}$ and DMPO- OOH spin adducts. Chaparral inhibits the spin adduct formation by scavenging $O_2^{\cdot-}$. Since the maximum concentration of DMPO- $O_2^{\cdot-}$ generated was determined to be $\sim 15 \mu\text{M}$, and DMPO concentration in the reaction system is three order of magnitude larger than $O_2^{\cdot-}$ concentration, we assume that all $O_2^{\cdot-}$ can be trapped and DMPO- $O_2^{\cdot-}$ formation reaches a maximum velocity (V_{max}). Therefore, DMPO- $O_2^{\cdot-}$ can represent the relative concentration of $O_2^{\cdot-}$. Since one DMPO molecule can trap one $O_2^{\cdot-}$, the concentration of reduced DMPO- $O_2^{\cdot-}$ spin adduct should be identical to the concentration of $O_2^{\cdot-}$ eliminated by chaparral. If reduced EPR signal intensity is directly proportional to the concentration of chaparral, a first-order kinetic plot should yield a straight line. Under steady state-conditions, the decrease in concentration of $O_2^{\cdot-}$ could be expressed as follows:

$$dV = -d[O_2^{\cdot-}]/dt = -d[DMPO-O_2^{\cdot-}]/dt = k_s[CH] \cdot [O_2^{\cdot-}] \quad [2]$$

where dV is the velocity of scavenging of $O_2^{\cdot-}$ by chaparral and k_s is the rate constant of chaparral scavenging of $O_2^{\cdot-}$. Under a given condition, the values of dV can be obtained at different concentrations of chaparral. Figure 3 depicts the velocity of chaparral scavenging of $O_2^{\cdot-}$ as a function of chaparral concentration. The hyperbolic nature of the curve in Fig. 3 indicates that chaparral scavenged $O_2^{\cdot-}$ to completion. The Lineweaver-Burk plot (Fig. 3, inset) demonstrated linearity with a correlation coefficient of 0.982. The reciprocal of the intercept value of the plot, V_{max} the maximum rate of chaparral scavenging $O_2^{\cdot-}$ in this system, was found to be 8.4 ng/mL/sec . The ratio of slope to intercept from Fig. 3 provides K_d/k_s , the P value [9, 10], which is the concentration of chaparral at one-half the $O_2^{\cdot-}$ to be scavenged. This P value was found to be 0.899 $\mu\text{g/mL}$, which is similar to the IC_{50} value obtained in Fig. 2. Using a $K_d = 1.1 \text{ s}^{-1}$, the value reported previously for the spontaneous decay of $O_2^{\cdot-}$ [11]. The k_i value was calculated to be $1.22 \times 10^6 \text{ g}^{-1}\text{s}^{-1}$ which is

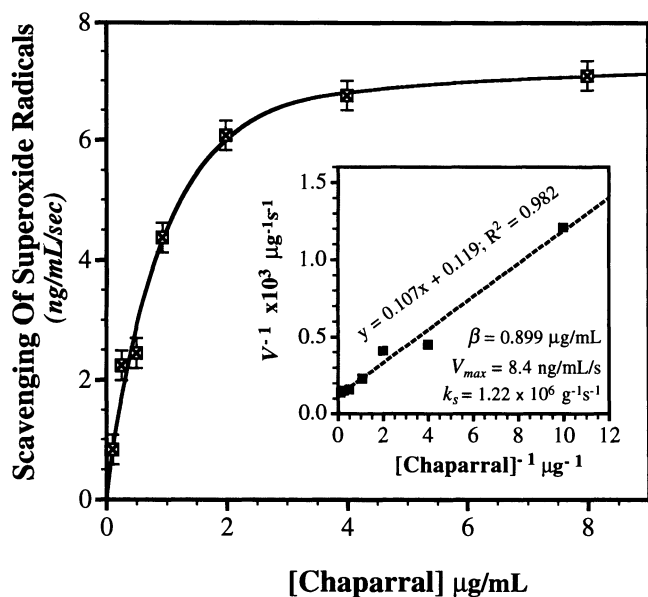


Fig. 3. The rate of $O_2^{\cdot-}$ scavenging as a function of chaparral concentration during the reaction of xanthine (20 μ M) and xanthine oxidase (0.02 units/mL) in air-saturated phosphate buffered 7 M DMSO aqueous solution in the presence of 70 mM DMPO. Inset, the inverse plot of the rate of DMPO- $O_2^{\cdot-}$ reduction versus chaparral concentration. EPR spectroscopy settings were the same as described under Materials and methods.

close to the k_i value of $1.6 \times 10^6 \text{ g}^{-1}\text{s}^{-1}$ for chaparral inhibition of DMPO- $O_2^{\cdot-}$ formation obtained by the method described above. It is clearly evident that chaparral inhibits DMPO- $O_2^{\cdot-}$ formation by scavenging $O_2^{\cdot-}$. These data strongly support the hypothesis that chaparral is an efficient scavenger for $O_2^{\cdot-}$.

Discussion

Our results demonstrate that chaparral is able to directly scavenge $O_2^{\cdot-}$. The kinetic data further demonstrated that the scavenging of $O_2^{\cdot-}$ by chaparral is effective and is dependent upon its concentration. Although chaparral has been considered to act as an antioxidant [1, 2], to date, very little direct evidence was available to support this claim. Our EPR studies demonstrate that chaparral is indeed an effective scavenger for $O_2^{\cdot-}$. This was confirmed by comparing the rates of DMPO- $O_2^{\cdot-}$ formation in the absence and presence of chaparral. Thus, the rate of DMPO- $O_2^{\cdot-}$ formation decreased with increased chaparral concentration (Figs 1 and 2). Additional support for this conclusion came from the EPR kinetic study, in which the Lineweaver-Burk plots resulted in a linear relationship with a correlation coefficient of 0.982 (Fig. 3 inset), indicating that there is a stoichiometric relationship between chaparral

concentration and scavenging of $O_2^{\cdot-}$. The rate constant, k_c , for chaparral inhibition of DMPO- $O_2^{\cdot-}$ formation was found to be $1.6 \times 10^6 \text{ g}^{-1}\text{s}^{-1}$, which is identical to the rate constant k_i for chaparral scavenging $O_2^{\cdot-}$. The maximum velocity (V_{max}) of $O_2^{\cdot-}$ scavenged by chaparral was found to be 8.4 ng/mL/sec. The IC_{50} value, the concentration of chaparral required to cause 50% inhibition of EPR signal intensity, was found to be 0.899 μ g/mL, which is equal to the β value, the concentration of chaparral required to scavenge half the $O_2^{\cdot-}$. These data demonstrate that chaparral is a powerful $O_2^{\cdot-}$ scavenger, capable of virtually eliminating $O_2^{\cdot-}$.

It is well known that $O_2^{\cdot-}$ is a primary reactive oxygen radical species that, by itself, is not very active. However, it can dismutate to form H_2O_2 [12–15]. In the presence of transition metal ions, such as Fe^{2+} and Cr^{5+} , H_2O_2 will be converted to $\cdot OH$ by the Fenton-type or Fenton-like reaction. $\cdot OH$ is a secondary product of $O_2^{\cdot-}$ dismutation and has been shown to be a biologically toxic oxygen radical species, which can not be detoxified by enzymatic mechanisms [16–18]. Although many antioxidants have been shown to protect against oxidative stress, most of them can react only with secondary reactive oxygen species, for example, $\cdot OH$, rather than $O_2^{\cdot-}$ [17]. Therefore, the protective mechanism of these agents is virtually a competitive process. In contrast to $\cdot OH$, $O_2^{\cdot-}$ is a much less toxic primary oxygen radical species. It is evident that by eliminating $O_2^{\cdot-}$, and thereby, blocking production of more damaging ROS, chaparral may provide more effective protection against oxidative damage to biomolecules.

Acknowledgements

This work was supported by US Army Center for Health Research and National Institute for Occupational Safety and Health funding to Colorado State University.

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Role of Fe(III) in Fe(II)citrate-mediated peroxidation of mitochondrial membrane lipids

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Abstract

In this report we study the effect of Fe(III) on lipid peroxidation induced by Fe(II)citrate in mitochondrial membranes, as assessed by the production of thiobarbituric acid-reactive substances and antimycin A-insensitive oxygen uptake. The presence of Fe(III) stimulates initiation of lipid peroxidation when low citrate:Fe(II) ratios are used ($\leq 4:1$). For a citrate:total iron ratio of 1:1 the maximal stimulation of lipid peroxidation by Fe(III) was observed when the Fe(II):Fe(III) ratio was in the range of 1:1 to 1:2. The lag phase that accompanies oxygen uptake was greatly diminished by increasing concentrations of Fe(III) when the citrate:total iron ratio was 1:1, but not when this ratio was higher. It is concluded that the increase of lipid peroxidation by Fe(III) is observed only when low citrate:Fe(II) ratios were used. Similar results were obtained using ATP as a ligand of iron. Monitoring the rate of spontaneous Fe(II) oxidation by measuring oxygen uptake in buffered medium, in the absence of mitochondria, Fe(III)-stimulated oxygen consumption was observed only when a low citrate:Fe(II) ratio was used. This result suggests that Fe(III) may facilitate the initiation and/or propagation of lipid peroxidation by increasing the rate of Fe(II)citrate-generated reactive oxygen species. (Mol Cell Biochem **196**: 163–168, 1999)

Key words: Fe(II)citrate, free radicals, iron, lipid peroxidation, mitochondria, reactive oxygen species

Abbreviations: EGTA – Ethyleneglycolbis (β -aminoethyl-ether)- N,N,N',N' -tetraacetic acid; HEPES – 2-hydroxyethyl-1-piperazineethanesulfonic acid; MES – morpholinoethanesulfonic acid; MOPS – morpholinopropanesulfonic acid; TBARS – thiobarbituric acid-reactive substances

Introduction

It is recognized that lipid peroxidation plays an important role in cell injury and death under oxidative stress conditions (for reviews, see refs. [1, 2]). Peroxidative destruction of internal cell membranes, such as the endo(sarco)plasmic reticulum and mitochondria, is linked to the disruption of both Ca^{2+} homeostasis and cellular energetics [2–7]. In order to induce lipid peroxidation in biomembranes, several oxidizing agents have been tested, including the hydroxyl

radical in ionizing radiation studies [8], triplet acetone [9, 10], perhydroxyl radical [11], the peroxynitrite anion [12], and certain iron complexes [5, 6, 13–18].

It is proposed that iron complexes of low molecular weight are involved in important mechanisms of cell injury, such as post-ischemic oxidative damage and liver injuries associated with iron-overload diseases (for reviews, see refs. [19, 20]). We have previously reported that micromolar amounts of Fe(II)citrate [5, 6] or Fe(II)ATP [18] induce lipid peroxidation of mitochondrial membranes and alterations in

membrane proteins. Although it has been studied extensively, the mechanism by which iron complexes of low molecular weight initiate lipid peroxidation is still controversial. The hydroxyl radical ($\text{HO}\cdot$), an important radical in cellular oxidative stress, is often dismissed as an initiator of lipid peroxidation because $\text{HO}\cdot$ scavengers do not usually inhibit peroxidation promoted by iron complexes [21–23]. A possible $\text{HO}\cdot$ formation in specific hydrophobic sites, involving iron bound to membranes, is controversial [23]. Minotti and Aust [14, 22] have proposed that a ferrous-ferri-dioxygen ($\text{Fe(II)-Fe(III)-O}_2$) complex is the species that initiates lipid peroxidation. Others favor the participation of the perferryl (Fe(II)-O_2 or $\text{Fe(III)-O}_2\cdot$) [15, 24, 25] or ferryl ion (FeO^{2+} or FeOH^{3+}) (for review, see ref. [23]).

In the present study, we investigated the influence of Fe(III) on Fe(II) -citrate-mediated lipid peroxidation of mitochondrial membranes, measured as antimycin A-insensitive oxygen uptake and formation of thiobarbituric acid-reactive substances (TBARS). Our results show that Fe(III) stimulates both spontaneous Fe(II) oxidation and Fe(II) -citrate-induced lipid peroxidation only when low citrate: Fe(II) ratios are used.

Materials and methods

Reagents and solutions

Ferrous ions ($\text{Fe(NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) solutions were prepared in Milli-Q water and used immediately. Fe(III) -citrate solutions were prepared daily by dissolving FeCl_3 (4 mM final concentration) in 4 mM citric acid and adjusting the pH to 7.2 by the addition of KOH. The citrate present in the Fe(III) solution was taken into account in calculating the total citrate content mentioned in figure legends. Antimycin A, citrate, EGTA, HEPES, rotenone and thiobarbituric acid were obtained from Sigma Chemical Co (St. Louis, MO, USA).

Isolation of rat liver mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight. The homogenate was prepared in 250 mM sucrose, 1.0 mM EGTA and 5.0 mM HEPES buffer, pH 7.2. The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80–100 mg/ml. These mitochondria contained 8–10 nmol/mg endogenous calcium as determined by atomic absorption spectroscopy. The respiratory control (respiratory rate of state 3/state 4) of mitochondrial preparations were more than 4.0, measured with succinate (2 mM) as substrate.

Standard incubation procedure

The experiments were carried out at 30°C in a reaction medium containing 130 mM KCl, 10 mM HEPES buffer pH 7.2, 5.0 μM rotenone, 0.5 μM antimycin A and 10 μM Ca^{2+} as determined by atomic absorption spectroscopy. Other additions are indicated in the figure legends.

Oxygen uptake measurements

Oxygen uptake was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a glass chamber equipped with magnetic stirring. The lag phase of the oxygen uptake burst was defined as the time required to reach the maximal velocity of oxygen consumption after Fe(II)/Fe(III) addition (see example in Fig. 1).

Thiobarbituric acid-reactive substances (TBARS)

TBARS production by mitochondria was measured according to Buege and Aust [26]. Briefly, 0.4 ml samples were taken after 10 min incubation in the conditions described above and mixed with 0.4 ml of 1% thiobarbituric acid in 0.05 N NaOH, 0.2 ml of 20 % H_3PO_4 and 40 μl of 10 N NaOH. The

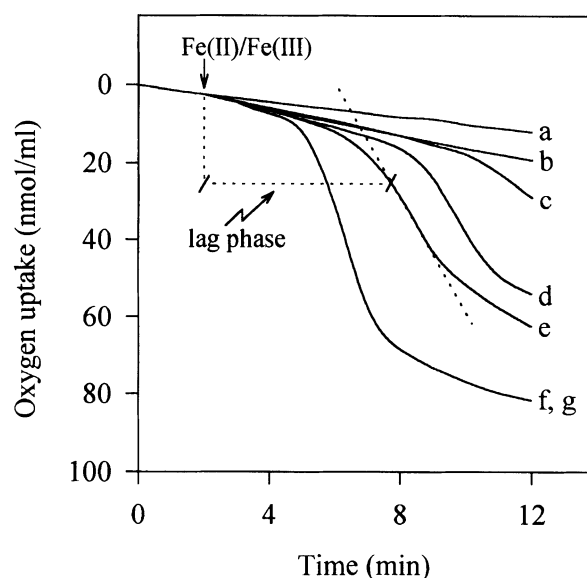


Fig. 1. Effect of Fe(III) on oxygen uptake due to lipid peroxidation induced by Fe(II) -citrate: Dependence on citrate:iron ratio. Rat liver mitochondria (RLM – 1 mg protein/ml) were incubated in standard reaction medium containing 0.05 mM (b, c), 0.1 mM (d, e) or 2 mM (a, f, g) citrate. At the arrow, 25 μM Fe(II) was added to all experiments (except in line a) and 25 μM Fe(III) was added to experiments represented by lines a, c, e and g. Data presented are representative of 3 experiments reproducible within 10%.

mixture was heated at 90–100°C for 15 min in the presence of 1 mM butylated hydroxytoluene. After cooling, 1.5 ml butanol was added to the solution. The mixture was shaken and centrifuged at 3000 rpm during 5 min. The optical density of the organic layer was determined at 535 nm. The molar extinction coefficient used to calculate TBARS concentrations was $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ [26].

Results

Figure 1 shows that addition of 25 μM Fe(II) to a mitochondrial suspension in the presence of a high concentration of citrate (2 mM) caused a transitory burst of antimycin A-insensitive oxygen uptake (line f). Previous data from our group [18] showed that this oxygen burst is inhibited by butylated hydroxytoluene, demonstrating that it is due to lipid peroxidation of the mitochondrial membrane. Co-addition of 25 μM Fe(III) and 25 μM Fe(II), in the presence of 2 mM citrate (line g), induced the same oxygen uptake burst profile obtained in line f. When lower concentrations of citrate were employed (0.05 mM, lines b–c and 0.1 mM, lines d–e), the rate of iron-dependent lipid peroxidation decreased. Lipid peroxidation mediated by iron has been reported to be dependent on the citrate concentration [14]. Under these conditions of low citrate concentrations, the oxygen uptake burst obtained in the presence of Fe(II) plus Fe(III) (lines c and e) was significantly greater than in the presence of Fe(II) alone (lines b and d). Moreover, the lag phase that accompanies the oxygen uptake burst was

reduced from 7 min in the presence of 0.1 mM citrate plus Fe(II) (line d) to 5.5 min by Fe(III) addition (line e). These results indicate that, at low citrate levels, Fe(III) stimulates Fe(II)-induced lipid peroxidation. In the experimental conditions of Fig. 1, spontaneous Fe(II) oxidation is responsible for up to 5 nmols of O_2 uptake (result not shown). The appropriated corrections due to spontaneous Fe(II) oxidation do not result in significant alterations in the data presented in Fig. 1. A detailed study of spontaneous Fe(II) oxidation will be presented in Fig. 5.

Minotti and Aust [14] obtained peroxidation of microsomal phospholipid liposomes by Fe(II)citrate with optimum activity occurring at a Fe(II):Fe(III) ratio of 1:1. The effect of different Fe(II):Fe(III) ratios on mitochondrial lipid peroxidation was assessed by measuring total oxygen uptake (Fig. 2A) or TBARS formation (Fig. 2B). In the presence of a citrate:total iron ratio of 1:1 (●) (citrate = 0.05 mM; iron being either Fe(II), Fe(III) or a mixture of Fe(II) plus Fe(III)) maximal stimulation of lipid peroxidation occurred when the Fe(II):Fe(III) ratio was in the range of 1:1 to 1:2. In the presence of a high citrate concentration (2 mM) (■), this optimal range of Fe(II):Fe(III) ratio was not observed, rather lipid peroxidation diminished in proportion to the decrease in the initial Fe(II) concentration.

Figure 3 shows the influence of 25 μM Fe(III) on total oxygen uptake (panel A) and on the lag phase that accompanies the oxygen uptake burst (panel B) when different citrate:Fe(II) ratios (from 2:1–32:1, with Fe(II) at 25 μM) were used. As expected, an increase in citrate concentration induced an increase in total oxygen uptake (panel A), both in the

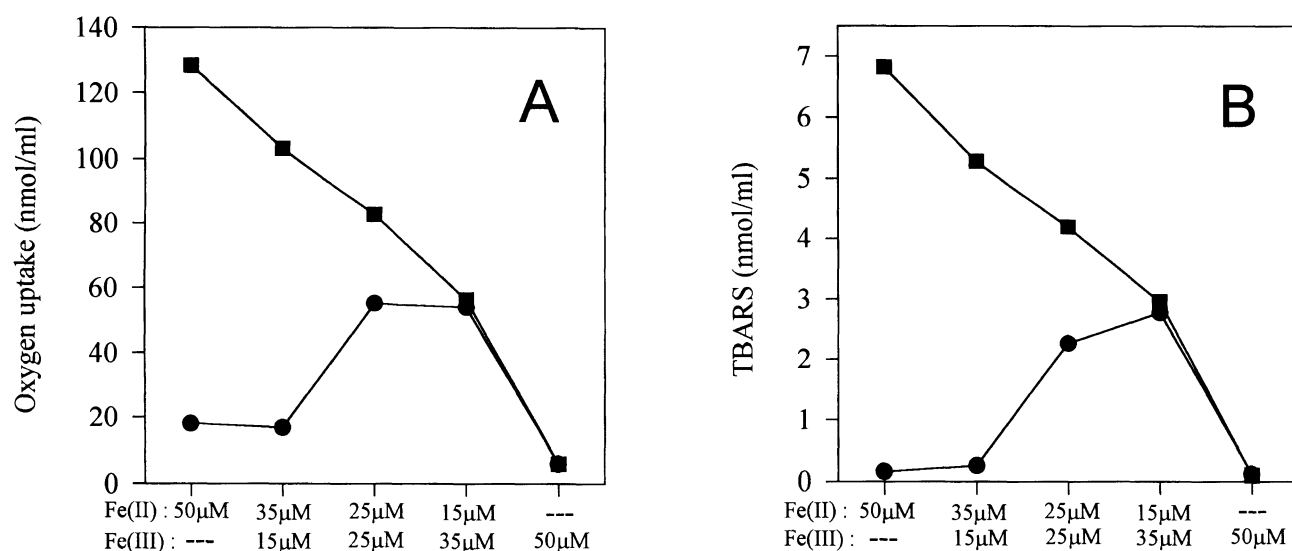


Fig. 2. Effect of the Fe(II):Fe(III) ratio on oxygen uptake (A) or TBARS formation (B) due to lipid peroxidation induced by Fe(II)citrate: Dependence on citrate concentration. RLM (1 mg protein/ml) were incubated in standard reaction medium containing 0.05 mM (●) or 2 mM citrate (■). Fe(II), Fe(III) or both were added at 2 min to obtain the concentrations indicated on the abscissa. Total oxygen uptake and TBARS formation were determined 12 min after Fe(II)/Fe(III) addition. Data are presented as means of 3 experiments reproducible within 10%.

presence (■) and in the absence (●) of Fe(III). However, only at low citrate:Fe(II) ratios (2:1 and 4:1, which corresponds to 50 and 100 μ M citrate, respectively) Fe(III) significantly stimulated the total oxygen uptake (Fig. 3A). At low citrate concentrations, Fe(III) (■) also shortens significantly the lag phase that accompanies the oxygen uptake burst induced by Fe(II)citrate (●) (Fig. 3B). These results strongly suggest that Fe(III) stimulates the Fe(II)-mediated lipid peroxidation reaction mainly in its initial phase. At high citrate levels, Fe(III) lacks the ability to stimulate lipid peroxidation of the mitochondrial membrane.

Figure 4 shows that when citrate and Fe(III) concentrations were increased together (■) (so that the ratio between citrate and total Fe(II) plus Fe(III) remained at 1:1, with a fixed concentration of Fe(II)) the lag phase was reduced more rapidly than in the absence of Fe(III) (●). Stimulation of Fe(II)citrate-mediated lipid peroxidation by Fe(III) did not occur at high citrate levels (2 mM, ▲). In the presence of 5 μ M Fe(II) and 2 mM citrate (result not shown) a lag phase of 6–7 min preceding the oxygen consumption was observed. In this situation, the addition of Fe(III) (5–50 μ M) did not alter whatsoever the lag period.

The initial rate of Fe(II) spontaneous oxidation was measured by monitoring the oxygen consumption in buffered medium in the absence of mitochondria (Fig. 5). A Fe(II) concentration of 200 μ M was used in order to obtain more significant results. The presence of Fe(III) (40–200 μ M) stimulates the initial oxygen consumption rate when a low

citrate:Fe(II) ratio (0.4 mM citrate) (■) was used. In the presence of 8 mM citrate (●), the presence of Fe(III) did not increase oxygen consumption.

Discussion

It has been reported that Fe(III) is able to stimulate Fe(II)-dependent lipid peroxidation [14, 15, 22, 24, 27]. This phenomenon has been linked to different hypothetical mechanisms for initiation of lipid peroxidation: direct interaction of Fe(III) with membrane lipids [27], increased concentration of iron-derived radicals [15] or formation of a Fe(II)-Fe(III)-O₂ complex [14, 22]. The results presented in this paper show that Fe(III) stimulates lipid peroxidation of rat liver mitochondrial membrane only at low citrate:Fe(II) ratios. At low citrate concentrations, Fe(III) induces a significant decrease in the initiation phase of the lipid peroxidation analyzed as the lag period that accompanies the oxygen uptake burst (Figs 1, 3B and 4). Similar results were obtained using ATP as a ligand of iron (results not shown). Although the mechanism by which Fe(III) stimulates lipid peroxidation remains unknown, we propose that Fe(II)citrate complex could have its redox potential decreased by a co-complexation of Fe(III) in the presence of low citrate levels. This would promote increased rates of both Fe(II) oxidation (Fig. 5) and oxyradical production, thus stimulating mitochondrial lipid peroxidation. The presence of high levels

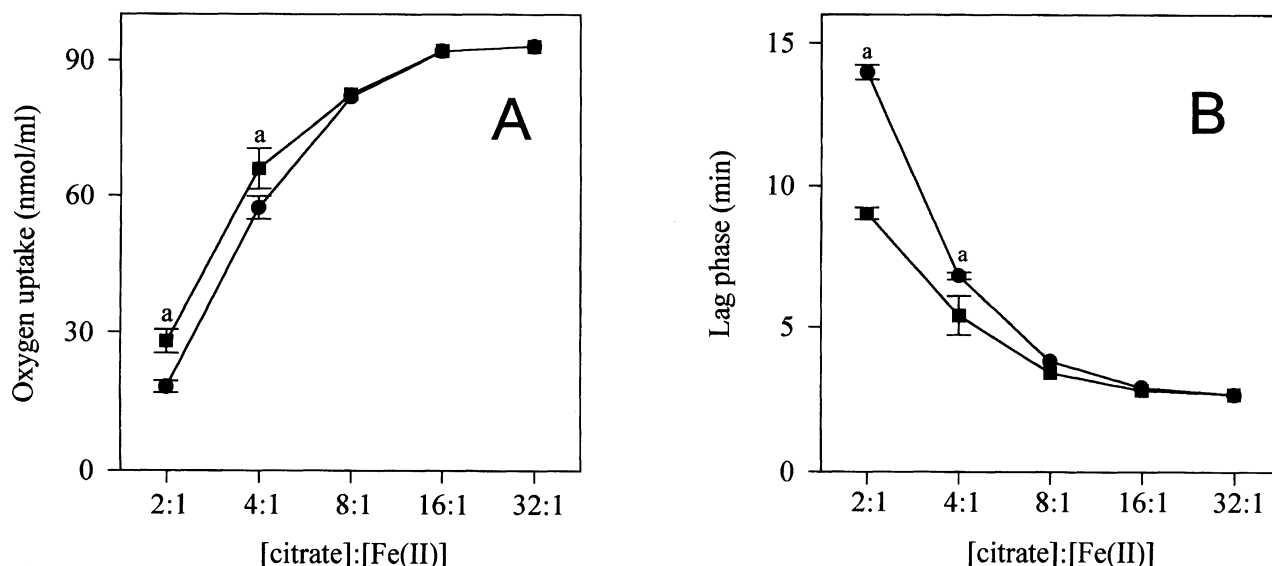


Fig. 3. Stimulation of Fe(II)citrate-induced lipid peroxidation by Fe(III). RLM (1 mg protein/ml) were incubated in standard reaction medium containing concentrations of citrate necessary to obtain the citrate:Fe(II) ratios indicated on the abscissa. Either 25 μ M Fe(II) (●) or 25 μ M Fe(II) plus 25 μ M Fe(III) (■) were added at 2 min. In (A), total oxygen uptake was determined 10 min after Fe(II)/Fe(III) addition. In (B), the lag phase that accompanies oxygen uptake burst was determined as the time required to reach the maximal velocity of oxygen consumption after Fe(II)/Fe(III) addition (as shown in Fig. 1). Data are presented as means of 3 experiments $\pm$ S.D. Note: (a) $p < 0.05$ for unpaired Student's two-tailed t -test.

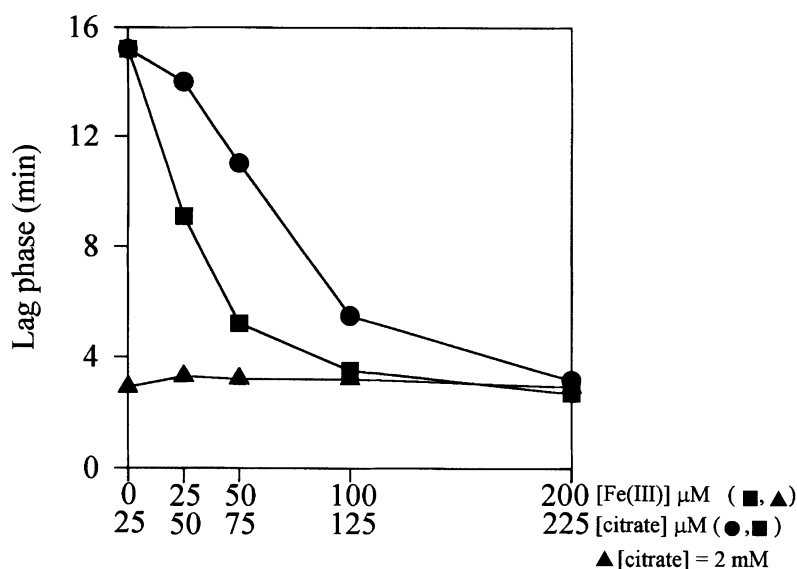


Fig. 4. Effect of Fe(III) on the lag phase that accompanies oxygen uptake due to lipid peroxidation induced by Fe(II)citrate: Dependence on citrate concentration. RLM (1 mg/ml) were incubated in standard reaction medium containing either 2 mM citrate (▲) or concentrations indicated on the abscissa (●, ■). At 2 min, either 25 μM Fe(II) (●) or 25 μM Fe(II) plus the concentrations of Fe(III) indicated on the abscissa (■, ▲) were added. Data are presented as means of 3 experiments reproducible within 10%.

of citrate would drive Fe(III) out of the putative Fe(II)-citrate-Fe(III) complex to form an unreactive Fe(III)citrate complex. Accordingly, Tadolini and Hakin [28] have observed that Fe(III) promotes an increase in deoxyribose degradation,

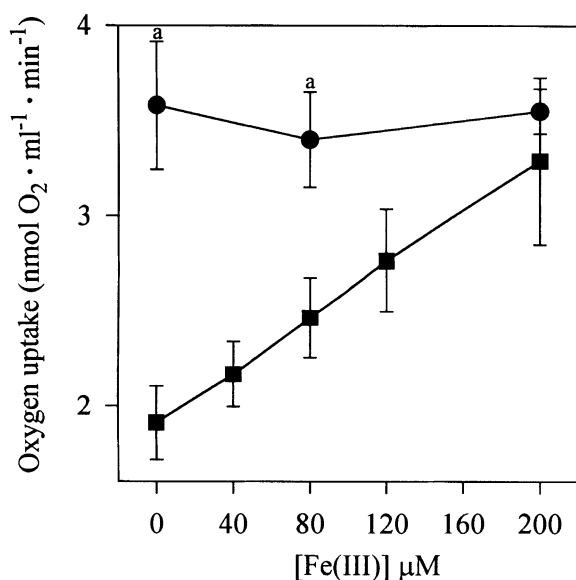


Fig. 5. Effect of Fe(III) on oxygen uptake due to spontaneous Fe(II) oxidation: Dependence on citrate concentration. The experiments were conducted in standard reaction medium (without the addition of RLM) in the presence of 0.4 mM (■) or 8 mM (●) citrate. Oxygen uptake was determined during the first 30 sec after the addition of 200 μM Fe(II). Data are presented as means of 5 experiments $\pm$ S.D. Note: (a) $p < 0.05$ for unpaired Student's two-tailed t -test.

as an index of oxyradical formation, caused by Fe(II) autoxidation in MES or MOPS buffered media (in the absence of citrate). In this last case the buffer could act as a low affinity ligand of iron.

Another interpretation for the increase in lipid peroxidation promoted by Fe(III) has been presented by Minotti and Aust [14, 22], who proposed that the Fe(II)-Fe(III)-O₂ complex initiates lipid peroxidation. This conclusion was supported by the observation that maximal lipid peroxidation occurred when a critical 1:1 ratio of Fe(II):Fe(III) was present. This observation was based only on experiments conducted without organic ligands of iron [22] or in the presence of low citrate concentrations [14]. However, as shown by the present results, the 1:1 ratio of Fe(II):Fe(III) does not produce maximal levels of lipid peroxidation when high citrate concentrations are used.

In conclusion, we propose that: (i) Fe(III) stimulates the autoxidation of Fe(II)citrate by forming a putative co-complex, in the presence of low citrate levels, leading to increased formation of oxyradicals and lipid peroxidation; and (ii) Fe(III) ions could also interact with the mitochondrial phospholipids [27] when low citrate levels are present, somehow facilitating the initiation or propagation process of lipid peroxidation, as proposed for others trivalent metal ions [29]. High levels of citrate would remove Fe(III) from the membranes. Both mechanisms (i) and (ii) could take place at the same time, thus explaining the role of Fe(III) in lipid peroxidation. In any case, the modulation of Fe(II)-mediated lipid peroxidation caused by Fe(III) and citrate is possibly of *in vivo* significance during processes of hepatic oxidative stress in iron-overload conditions.

Acknowledgements

This work was supported by grants from CNPq, FAPESP, FAP-DF, PADCT and PRONEX (Brazil) to A.E.V. and M.H.-L. R.F.C. was supported by a Wellcome Trust Fellowship. We thank Alicia J. Kowaltowski and Martha Sorenson for valuable comments.

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Studies on hepatic oxidative stress and antioxidant defence systems during arteether treatment of *Plasmodium yoelii nigeriensis* infected mice

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Abstract

Reactive Oxygen species play an important role in pathology during malaria infection. The status of hepatic oxidative stress and antioxidant defence indices was studied during *Plasmodium yoelii nigeriensis* (*P. y. nigeriensis*) infection in mice and arteether treatment of *P. y. nigeriensis* infected mice. *P. y. nigeriensis* infection caused a significant increase in hepatic xanthine oxidase, rate of lipid peroxidation, reduced glutathione (GSH) and glutathione reductase with progressive rise in parasitemia. This was accompanied by a significant decrease in hepatic superoxide dismutase (SOD) and catalase with increase in parasitemia. Arteether treatment (10 mg/kg body weight of mice) of infected mice from day 2 of post infection resulted in complete clearance of parasitemia on day 4 of post infection which was accompanied by restoration of all the oxidative stress and antioxidant defence indices to normal levels. (Mol Cell Biochem **196**: 169–173, 1999)

Key words: malaria, reactive oxygen species (ROS), superoxide anion, glutathione, superoxide dismutase, catalase

Introduction

Forty percent of the world's population is at risk of malaria infection and each year over one million individuals (primarily African children) die of this disease [1]. Malaria has traditionally been treated with quinolines such as chloroquine, quinine, mefloquine and primaquines and antifolates like Fansidar (sulfadoxine-pyrimethamine). Unfortunately, most of the strains of *Plasmodium falciparum*, the parasite responsible for fatal malaria infection have become resistant to chloroquine and other commonly used antimalarials [2]. Therefore endoperoxides like artemisinin (qinghaosu) and its derivatives like arteether form a promising class of antimalarials.

Liver is one of the most important organs to be affected during malaria infection and free radicals have been implicated to play an important role in the liver pathology [3]. During *P. berghei* infection there is an increase in hepatic xanthine oxidase and lipid peroxidation which indicates an increased

generation of oxidative stress in the liver [4]. Cells are protected from free radical damage by antioxidant defence system viz., reduced glutathione (GSH) and glutathione reductase (GR). Glutathione reductase causes reduction of oxidised glutathione to reduced glutathione. GSH serves as a sulphhydryl buffer which protects the –SH groups of proteins from the damaging effects of reactive oxygen species (ROS) [5]. The other antioxidant system is represented by superoxide dismutase (SOD) and catalase. The enzyme superoxide dismutase catalyzes dismutation of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) while catalase causes dissipation of H_2O_2 to water and oxygen. Previous studies have shown an increase in the glutathione system and a decrease in SOD and catalase during *P. berghei* infection in mice [4].

The endoperoxides are a promising class of antimalarial drugs which meet the challenges posed by drug-resistant parasites and rapid progression of malarial illness [2]. Artemisinin is a sesquiterpene lactone. Dihydroartemisinin is the reduced lactol derivative of artemisinin and arteether

is the β -ethyl ether of dihydroartemisinin. Artemisinin and its derivatives are toxic to malaria parasites in nanomolar concentrations whereas micromolar concentrations are required for toxicity to mammalian cells [6]. The present study was undertaken to study the effect of arteether treatment on hepatic oxidative stress and antioxidant defence indices in *Plasmodium yoelii nigeriensis* (*P. y. nigeriensis*) infected mice.

Materials and methods

Animals

Colony bred albino mice (Swiss strain) weighing about 20–25 g maintained in an air conditioned room (temperature $25 \pm 1^\circ\text{C}$) free from infection were used. The animals were divided into 4 groups each consisting of four animals. They were Group 1. Normal mice which served as control Group 2. *P. y. nigeriensis* infected mice, Group 3. Normal mice treated with arteether and Group 4. *P. y. nigeriensis* infected mice treated with arteether.

Parasite

The strain of *P. y. nigeriensis* used in this study was obtained from Prof. P.C.C. Garnham (Imperial College, Field Station, Silwood Park, Berkshire, UK). Mice in groups 2 and 4 were inoculated with 10^6 infected erythrocytes.

Monitoring of parasitaemia

Blood smears were prepared from tail blood vein of infected mice, fixed in methanol and stained in giemsa (prepared by mixing 20 ml of giemsa and 80 ml of 0.05 M phosphate buffer, pH 7.2) for 30 min. The slides were washed in tap water, dried and examined in at least fifty fields of microscope to determine the percentage of infected erythrocytes.

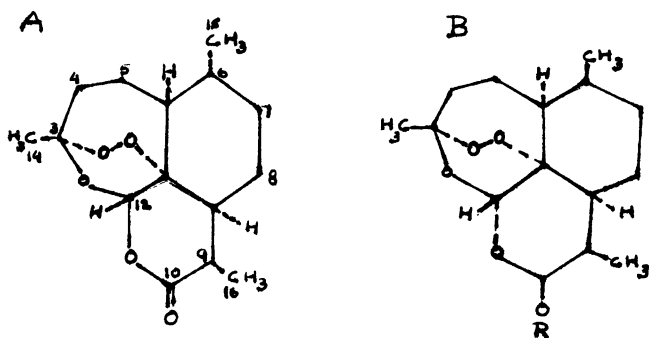


Fig. 1. (A) Artemisinin; (B) Lactol derivatives including dihydroartemisinin (R = H), arteether (R- CH_2CH_3).

Chemicals

All the chemicals used in the study were of analytical grade and purchased from Sigma Chemical Company, St. Louis, USA.

Methods

The mice were killed by cervical dislocation. The livers were perfused with saline, removed of adhering materials and washed with chilled buffered KCl (150 mM KCl in 10 mM NaH_2PO_4 - Na_2HPO_4 , pH 7.4) and homogenized using Potter-Elvehjem homogenizer to prepare 10% homogenate (wt/vol). The homogenate was successively centrifuged at 600, 8000, 15000 and 10000 $\times$ g to obtain various subcellular fractions. The rate of lipid peroxidation (LPO) was measured by the method of Ultey *et al.* [7]. Catalase was assayed as described by Abie [8] and reduced glutathione (GSH) level was monitored by the method of Beutler *et al.* [9]. All the above parameters were analysed in the whole homogenate. Glutathione reductase was assayed in 600 $\times$ g supernatant by the method of Racker [10]. Cu-Zn superoxide dismutase (SOD) was partially purified in 15000 $\times$ g supernatant [11] and assayed according to Nishikimi *et al.* [12]. Xanthine oxidase was assayed in 100,000 $\times$ g supernatant by the method of Chander *et al.* [13]. Protein was estimated according to the method of Lowry *et al.* [14].

Analysis were carried out using Shimadzu double beam spectrophotometer UV-190 for time course reaction and Bausch and Lomb spectrophotometer Model-21 for others. Fresh tissue samples were used for investigation. The data were analysed for significance using Student's *t*-test. P values < 0.05 were considered significant.

Results

P. y. nigeriensis infection in mice caused an increase in the activity of hepatic xanthine oxidase which showed an increase of 44, 122 ($p < 0.002$) and 444% ($p < 0.001$) at low, middle and high parasitemia respectively with respect to normal mice (Table 1). The rate of hepatic lipid peroxidation showed an increase of 38 ($p < 0.05$), 99 ($p < 0.001$) and 140% ($p < 0.001$) at low, middle and high parasitemia respectively with respect to normal mice (Table 1). The levels of reduced glutathione increased by 29 ($p < 0.002$), 109 ($p < 0.001$) and 189% ($p < 0.001$) at low, middle and high parasitemia respectively when compared to normal mice. The activity of glutathione reductase increased by 28 ($p < 0.01$), 67 ($p < 0.001$) and 161% ($p < 0.001$) respectively with respect to normal mice (Table 1). With progressive rise in parasitemia hepatic SOD showed a decrease of 5 ($p < 0.001$), 26 ($p < 0.001$)

Table 1. Sequential changes in hepatic oxidative stress and antioxidant defence indices during *Plasmodium yoelii nigeriensis* infection in mice

Sl. Parameters	Normal	Parasitemia		
		Low < 10%	Middle 20–30%	High > 50%
1. Xanthine oxidase ¹	0.09 ± 0.01	0.13 ± 0.03	0.2 ± 0.04**	0.49 ± 0.03***
2. Lipid peroxidation ²	2.45 ± 0.45	3.37 ± 0.62*	4.87 ± 0.25***	5.87 ± 1.25***
3. Reduced glutathione ³	6.57 ± 0.43	8.5 ± 0.7**	13.75 ± 0.64***	19.0 ± 0.91***
4. Glutathione reductase ⁴	3.87 ± 0.47	4.95 ± 0.42**	6.45 ± 0.66***	10.12 ± 0.85***
5. Superoxide dismutase ⁵	150.87 ± 1.03	143.0 ± 2.4***	112.25 ± 2.06***	98.87 ± 0.85***
6. Catalase ⁶	3.75 ± 0.64	3.55 ± 0.64	2.87 ± 0.25**	1.17 ± 0.27***

Values are ± S.D. of 4 mice in each group. *p < 0.05 when compared to normal mice; **p < 0.01 when compared to normal mice; ***p < 0.001 when compared to normal mice. ¹units mg protein⁻¹. OD 1.00 corresponds to one unit; ²nmoles of malonyldialdehyde formed h⁻¹ mg protein⁻¹; ³nmoles of reduced glutathione g fresh tissue⁻¹; ⁴values × 10⁻² umoles of NADPH oxidised min⁻¹ mg protein⁻¹; ⁵Units mg protein⁻¹. One unit is the amount of enzyme which inhibits formazone formation by 50%; ⁶Values × 10³ umoles of H₂O₂ dissipated min⁻¹ mg protein⁻¹.

Table 2. Parasitemia level during arteether treatment of *Plasmodium yoelii nigeriensis* infected mice

Sl. Groups No.	Parasitemia on day (post infection)				
	2	3	4	5	6
1. Normal	Nil	Nil	Nil	Nil	Nil
2. Normal + Arteether*	Nil	Nil	Nil	Nil	Nil
3. Infected	0.5 ± 0.4	2.12 ± 0.047	7.37 ± 0.94	24.75 ± 4.11	49.5 ± 3.31
4. Infected + Arteether	1.25 ± 0.64	0.27 ± 0.26	Nil	Nil	Nil

Values are ± S.D. of 4 mice in each group. *Arteether given from day 2 of post infection (10 mg/kg body wt) by intramuscular route.

Table 3. Status of hepatic oxidative stress and antioxidant defence indices during arteether treatment of normal and *Plasmodium yoelii nigeriensis* infected mice

Sl. Parameters	Normal	Infected	Normal + Arteether	Infected + Arteether
1. Xanthine oxidase ¹	0.085 ± 0.007	0.485 ± 0.007***	0.12 ± 0.05	0.09 ± 0.04
2. Lipid peroxidation ²	2.45 ± 0.45	5.87 ± 1.25***	3.0 ± 0.4	2.1 ± 0.27
3. Reduced glutathione ³	6.57 ± 0.43	19.0 ± 0.91***	6.75 ± 0.28	6.25 ± 0.64
4. Glutathione reductase ⁴	3.87 ± 0.47	10.125 ± 0.85***	4.32 ± 0.42	4.37 ± 0.85
5. Superoxide dismutase ⁵	150.87 ± 1.03	98.87 ± 0.85***	152.5 ± 2.08	150.25 ± 1.25
6. Catalase ⁶	3.75 ± 0.645	1.17 ± 0.27***	3.87 ± 0.62	4.37 ± 0.62

Values are ± S.D. of 4 mice in each group. *Parasitemia 50%; ^bParasitemia-nil. ***p < 0.001 with respect to normal mice. ¹Units mg protein⁻¹. OD 1.00 corresponds to one unit; ²nmoles of malonyldialdehyde formed h⁻¹ mg protein⁻¹; ³nmoles of reduced glutathione g fresh tissue⁻¹; ⁴Values × 10⁻² umoles of NADPH oxidised min⁻¹ mg protein⁻¹; ⁵Units mg protein⁻¹. One unit is the amount of enzyme which inhibits formazone formation by 50%; ⁶Values × 10³ umoles of H₂O₂ dissipated min⁻¹ mg protein⁻¹.

and 34% (p < 0.001) while catalase showed a decrease of 5 (p < 0.5), 23 (p < 0.02) and 69% (p < 0.001) at low, middle and high parasitemia respectively when compared to normal mice (Table 1).

Arteether treatment (10 mg/kg body wt. of mice) from day 2 of post infection resulted in a significant decrease of parasitemia on day 3 followed by complete clearance of parasitemia on day 4 (Table 2). This was accompanied by restoration of all the oxidative stress and antioxidant defence indices to normal levels (Table 3). Arteether treatment of normal mice caused no significant alteration of any oxidative stress and antioxidant defence indices (Table 3).

Discussion

Malarial parasites invading erythrocytes initiate pathological process in various other tissues including the liver. Hepatomegaly is a common sign of malaria infection [15], which is accompanied by the change in the colour of liver from pink to dark brown and finally black. The black colour is attributed to the deposition of malaria pigment, hemozoin in the reticuloendothelial cells [16]. During acute malaria the liver becomes extremely friable and easily torn. Malaria pigment is the end product of the digestion of hemoglobin by the parasite. The pigment is a ferric ion containing

porphyrin conjugated with protein moiety derived from partial proteolysis of globin portion of hemoglobin. Other pathological changes include dilated sinusoids filled with polymorphonuclear cells, swelling and disorganization of mitochondrial cristae, impairment of mitochondrial respiration and phosphorylation [17]. The biochemical changes in the liver during malaria include depletion of liver glycogen, lipid infiltration in the centrilobular region, decrease in the nucleic acid content (DNA and RNA) and an increased activity of acid and alkaline nucleases (DNase and RNase) [18]. Other features which point to liver dysfunction include jaundice, elevated serum transaminases, rapid development of hypoalbuminaemia, prolonged coagulation indices, failure of gluconeogenesis and reduced metabolic clearance of antimalarial drugs [19].

Free radicals are important mediators of tissue injury during malaria infection [3]. In the present study *P. y. nigeriensis* infection resulted in an increased generation of superoxide anion ($O_2^{\cdot-}$) as shown by the increased activity of hepatic xanthine oxidase with the progressive rise in parasitemia. Increase in the levels of hepatic xanthine oxidase occurs during other protozoal infections [20] which suggests that it acts as a non-specific defence mechanism against the parasite. Increased $O_2^{\cdot-}$ production leads to an increase in the rate of hepatic lipid peroxidation which causes impaired membrane functioning, decreased fluidity and inactivation of membrane bound enzymes [21]. The increase in hepatic GSH and glutathione reductase during *P. y. nigeriensis* infection may be an attempt of the host to meet the challenge of increased oxidative stress generated due to the presence of parasite. GSH traps the reactive oxygen species preventing them from reacting with critical cellular constituents like phospholipids, nucleic acids and proteins [22]. The decrease in hepatic SOD and catalase might be due to their inhibition/inactivation of $O_2^{\cdot-}$ [23]. The pathology of malaria is thus commensurate with the excessive production of ROS or decrease in activities of SOD and catalase.

Arteether treatment of *P. y. nigeriensis* infected mice from day 2 of post infection resulted in complete clearance of parasitemia within 48 h. Continued treatment of arteether to infected mice prevented relapse of parasitemia. Artemisinin and its derivatives have been shown to be selectively toxic to malaria parasites [2] due to the enhanced uptake of the drug by the parasites [24]. Artemisinin derivatives have been shown to affect parasites differently from other oxidant drugs. Instead of reacting with oxygen and producing large quantities of ROS, artemisinin itself becomes a free radical in the reaction catalysed by iron [25]. Therefore in the present study arteether treatment of *P. y. nigeriensis* infected mice resulted in complete clearance of blood parasitemia which was associated with the restoration of oxidative stress and antioxidant defence indices to normal levels.

Acknowledgements

The authors are thankful to the Director, CDRI, Lucknow and Head, Division of Biochemistry, for providing the necessary lab facilities. One of us (N.J.S.) is thankful to NIH, USA for providing Senior Research Fellowship. This is CDRI Communication No. 5750.

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Effect of *Salmonella typhimurium* enterotoxin (S-LT) on lipid peroxidation and cell viability levels of isolated rat enterocytes

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Abstract

Reactive oxygen species (ROS) are potent mediators of inflammatory disorders and may be of pathophysiological importance in *S. typhimurium* induced tissue damage. This study was carried out to investigate if ROS play a role in mediating the enterocyte damage during *in vitro* exposure to *Salmonella typhimurium* enterotoxin (S-LT). The ROS generation was detected by measuring the changes in the enterocyte arachidonic acid (AA) metabolism (measured indirectly by estimating the level of enterocyte damage in the absence and presence of the cyclooxygenase inhibitor, indomethacin) and xanthine oxidase activity. The enterocyte damage was estimated by measuring the changes in the level of lipid peroxidation and cell viability. The results obtained showed that the exposure of isolated rat enterocytes to S-LT resulted in an increased XO activity; an increased arachidonic acid metabolism, dose and time dependent increase in the level of lipid peroxidation and decreased cell viability. Lipid peroxidation decreased and cell viability increased in the presence of the antioxidant enzymes superoxide dismutase (SOD) or catalase. Thus the *in vitro* exposure of the enterocytes to S-LT is accompanied by an increased generation of ROS which may induce the lipid peroxidation of the enterocyte membrane thereby leading to a loss of cell viability. (Mol Cell Biochem **196**: 175–181, 1999)

Key words: enterotoxin, lipid peroxidation, *Salmonella typhimurium*, oxygen free radicals

Introduction

S. typhimurium mediated intestinal infection is accompanied by gross structural damage to the mucosa which causes generalized impairment of absorption. Several potential virulence factors of *S. typhimurium* may contribute to intestinal mucosal damage. These factors include epithelial invasion [1], synthesis of an enterotoxin (S-LT) [2] and cytotoxin [3] and induction of an inflammatory response [4]. However the exact bacterial factors responsible for diarrhoea still remain poorly characterized.

It has been established that *S. typhimurium* encodes a cholera like enterotoxin [2]. Previous studies have shown that S-LT may trigger the mucosal arachidonic acid cascade thereby leading to the production of prostaglandins [5, 6].

S-LT treatment has also been seen to impair the mucosal GSH levels [7] which can trigger the conversion of xanthine oxidase (XO) from its dehydrogenase form to its free radical producing form [8]. Increased arachidonic acid metabolism and high levels of enzyme XO may lead to excess mucosal ROS production. The resulting oxidant stress may overwhelm the endogenous defenses that regulate ROS production during normal metabolism, particularly if there are comparatively low levels of endogenous antioxidants as in that *S. typhimurium* infected ileal mucosa [7]. Consequent tissue injury by ROS results from their direct reaction with all kinds of biochemical substances present in the cell. The most important cellular damage caused by ROS is lipid peroxidation wherein these reactive species attack the lipids in the membrane and cause peroxidation resulting in complete destruction of the mem-

brane [9]. Reactive metabolites of oxygen are also believed to contribute to diarrhoea by acting as secretagogues.

In this study we have investigated if ROS play a role in mediating the S-LT induced enterocyte damage. We measured the ROS production and the damage caused to enterocytes during *in vitro* exposure to S-LT. In addition the protective role, if any of superoxide dismutase (SOD) and catalase during S-LT induced damage was also investigated.

Bacterial strain

S. typhimurium strain, 1402/84, obtained from Central Research Institute, Kasauli was used for the study. The strain was identified by the standard biochemical tests and by serotyping. The colony forming units (CFU) of the strain were determined by the spread plate technique.

Preparation of S. typhimurium toxin (S-LT)

For the preparation of S-LT, the bacterial cells harvested by centrifugation from a 6 h culture in Casamino yeast extract (incubated at 37°C with longitudinal shaking., 100 oscillations per min) were extracted with polymyxin B (Sigma) solution [6 g per litre of phosphate buffered saline (PBS; NaCl 8 g/l, KCl 0.2 g/l, Na₂HPO₄ 1.43 g/l, KH₂PO₄ 0.2 g/l, pH 7.3) [10]. The extract was centrifuged at 8000 g for a period of 30 min and the supernatant was concentrated by ultrafiltration (PM-10 Amicon filters). It was then filter sterilized (0.2 µm filters) and stored at 4°C. The toxin moiety present in the polymyxin extract of *S. typhimurium* was purified to homogeneity [11]. The enterotoxigenicity of the purified S-LT was checked by the rabbit ileal loop test [12], rabbit skin permeability test [13] and enzyme linked immunosorbent assay [14]. In all the three tests cholera toxin (CT) was used as the positive control.

Isolation of enterocytes

For the isolation of enterocytes the ileum, isolated from control rats, was flushed with warm O₂ saturated PBS to force out the intestinal contents. The ileum was then cut into half and the enterocytes were isolated by chelation elution method [15]. The cells obtained were sedimented, washed and suspended in 2 mM Tris-HCl buffer (pH 7.2) and purified [16]. Our cell preparations were found to be composed of > 90–95% epithelial cells when viewed under contrast microscope as well as when stained preparations were examined microscopically.

Enterocytes obtained were sonicated and a portion of the cell suspension was centrifuged at 105,000 g for 1 h. The pellet constituted the cell membrane and supernatant was the cytosol.

Exposure of enterocytes to S-LT

Exposure to S-LT was done firstly by incubating enterocytes (10⁶ cells) at 37°C with 10 µl (10 µg) of the toxin for different time periods separately (0, 2, 4, 6, 8, 10 and 12 min) and secondly by incubating enterocytes (10⁶ cells) with different volumes of S-LT separately [0, 1 µl (1 µg), 3 µl (3 µg), 5 µl (5 µg), 7 µl (7 µg), 9 µl (9 µg) and 11 µl (11 µg)] for a period of 10 min. After the exposure the toxin was removed by centrifugation and the cells were suspended in fresh Tris-HCl buffer (2 mM, pH 7.2). The extent of lipid peroxidation was estimated by measuring the MDA levels formed as explained later in this section. (Fig. 1.)

It was observed that exposure of enterocytes (10⁶ cells) to 9 µl (9 µg) of SLT for a period of 10 min induced a maximum increase in the levels of MDA formed. Therefore, throughout the present study, 10⁶ enterocytes were exposed to 9 µl (9 µg) of S-LT for a period of 10 min and various parameters studied.

Neutralization of S-LT by monoclonal antibodies against purified CT

The immunologic cross reactivity between CT and S-LT was used to confirm that the observed enterocyte damage was due to the exposure to enterotoxin only. M_{ab} against CT was kindly provided by Dr G B Nair, National Institute of Cholera and Enteric Diseases, Calcutta. M_{ab} was diluted 1:250, 1:500, 1:750, 1:1000, 1:1250, 1:1500 and 1:1750 with 10 mM PBS in 0.5% BSA. 9 µl of S-LT and 9 µl of M_{ab} of different dilutions were mixed well and incubated at 37°C for 30 min. Then the enterocytes (10⁶ cells) were exposed to the total volume of this M_{ab} treated S-LT as described previously and the level of lipid peroxidation was estimated.

The results obtained showed that anti CT M_{ab} dilutions upto 1:1500 were found to produce same results as those of control confirming that the observed enterocyte damage was due to exposure to S-LT only. (Fig. 2.)

Biochemical estimations xanthine oxidase activity

XO activity of the control and S-LT treated enterocytes (cytosolic fraction) was measured spectrophotometrically following uric acid production at 295 nm [17]. The activity was expressed as nm of Uric acid produced/min/mg/protein.

Peroxidation parameters studied

MDA formation

The measurement of lipid peroxidation in the sonicated enterocytes was done by following the formation of the

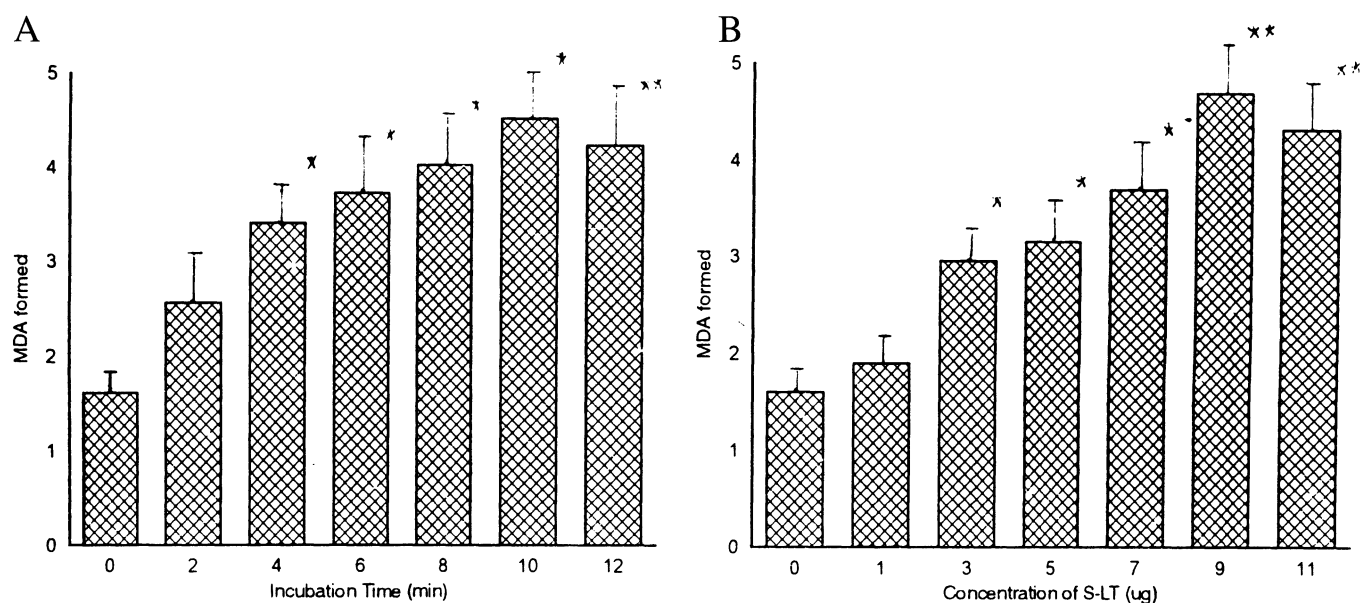


Fig. 1. Lipid peroxidation in isolated rat enterocytes following an exposure to S-LT. Data represents mean $\pm$ S.E.M. of six different experiments. The results are expressed as nm of MDA formed/mg protein. * $p < 0.01$ as compared to control cells. ** $p < 0.001$ as compared to control cells.

chromophore between thiobarbituric acid (TBA) and malondialdehyde [18]. The absorbance of the chromophore was measured at 535 nm in a spectro photometer. The results were expressed as nm of MDA formed/mg protein

Conjugated diene formation

Lipid peroxidation is accompanied by a rearrangement of polyunsaturated fatty acid double bonds, leading to the formation of conjugated dienes. For measuring the formation

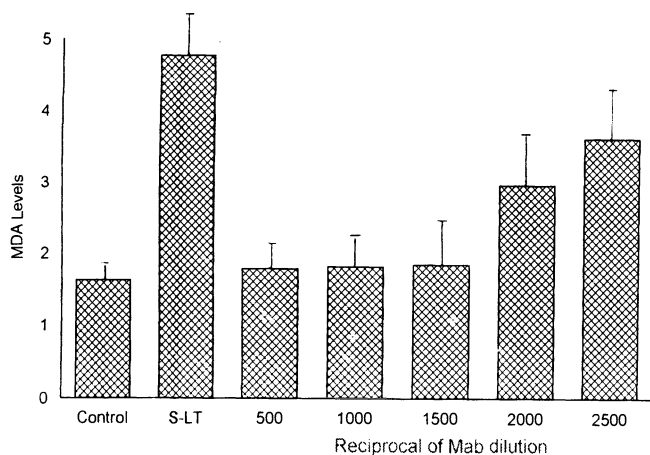


Fig. 2. Neutralization of S-LT by monoclonal antibodies (M_{ab}) raised against purified CT. Values are expressed as mean $\pm$ S.E.M. of six determinations. The results are expressed as nm of MDA formed/mg protein.

of conjugated dienes, the total lipids from the sonicated enterocytes were extracted [19], dissolved in 1 ml heptane and read at 233 nm. The results were expressed as μ moles of conjugated dienes formed/mg protein.

Estimation of cell viability

Lactate dehydrogenase (LDH) release was used to determine cell viability. In brief, the control as well as the S-LT treated cells were suspended in Tris-HCl buffer (2 mM, pH 7.2); centrifuged at 1 000 g for 10 min and the viability ascertained by LDH assay in both supernatant and cell suspension. Total LDH activity of the cell suspension was measured by adding Triton X-100 to a final concentration of 5%, and the percentage of viable cells, was calculated using the total activity. LDH assay was done spectrophotometrically by following the reduction of NADP⁺ at 340 nm [20]. Specific activity was expressed as μ moles of NADP⁺ reduced/min/mg protein.

Exposure of enterocytes to S-LT in the presence of modulators

Enterocytes (10^6 cells) were exposed to 9 μ l of S-LT in the absence and presence of 0.1 mg SOD or 500 I.U. catalase or different concentrations of indomethacin (0–2 mM). After the exposure the toxin and the modulator were removed by centrifugation and the enterocytes were suspended in fresh Tris-HCl buffer (2 mM, pH 7.2). The extent of lipid per-

oxidation was estimated by measuring the MDA levels formed. (Fig. 3.)

Protein estimation

The protein estimation was done using bovine serum albumin as the standard [21].

Statistical analysis

All results were expressed as mean $\pm$ S.E.M. of six different observations. The data was analysed by student's *t*-test (unpaired) *p* value < 0.05 was taken as significant.

Results

Enterotoxicity of purified S-LT

Purified S-LT gave positive results in the rabbit ileal loop test, rabbit skin permeability test and ELISA. Incubation with 1:1500 dilution of M_{ab} completely neutralized the secretory and permeability factor activity of S-LT (Table 1).

ROS generation

There was a significant increase ($p < 0.01$) in the XO activity of the enterocytes exposed to S-LT (48.15 ± 3.76 units/mg protein) as compared to the control cells (30.45 ± 2.16 units/mg protein).

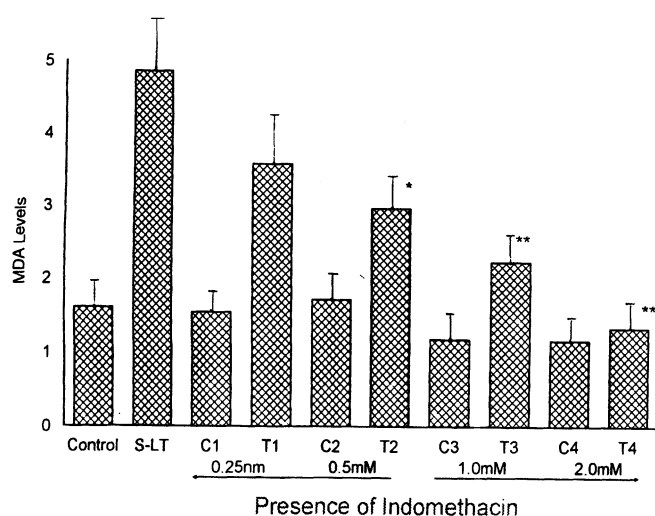


Fig. 3. Effect of indomethacin on S-LT induced lipid peroxidation. Data represents mean $\pm$ S.E.M. of six different observations. The results are expressed as nm MDA formed/mg protein. **p* < 0.05 as compared to S-LT treated cells. ***p* < 0.01 as compared to S-LT treated cells.

Table 1. Enterotoxicity of purified S-LT

Samples	Rabbit ileal loop Response [volume of fluid, (V,ml) /length of the loop (l,cm)]	Permeability factor activity (blueing diameter, mm)	ELISA (O.D. at 495 nm)
S-LT	1.42 $\pm$ 0.04	8 $\pm$ 0.05	0.41
S-LT treated with 1:1500 Mab	ND	ND	0.006
CT	1.85 $\pm$ 0.08	5.89 $\pm$ 0.03	3.20

Values are expressed as mean $\pm$ S.E.M. of six different experiments. ND – (Not detectable).

Damage caused to enterocytes

Table 2 shows the enterocyte damage during exposure to S-LT. There was a significant increase in the extent of lipid peroxidation in the S-LT treated enterocytes as compared to the control cells. A decrease in the percentage of viable cells in the enterocytes treated with S-LT was also seen.

Table 3 shows the protective effect of antioxidants on enterocyte damage during exposure to S-LT. The presence of SOD during the exposure of enterocytes to S-LT blocked the increase in the lipid peroxidation level by 41.23% as compared to its absence. In the presence of catalase the increase in the extent of peroxidation was blocked by 45.77%.

Table 2. Enterocyte damage during exposure to S-LT

Samples	MDA Content (nmol MDA formed/mg protein)	Conjugated dienes formed (μ moles/mg protein)	Viable cells (%)
Control cells	1.61 $\pm$ 0.32	5.16 $\pm$ 1.23	89.32 $\pm$ 5.83
S-LT treated cells	4.85 $\pm$ 0.70**	14.78 $\pm$ 2.89*	50.64 $\pm$ 6.32**

Values are expressed as mean $\pm$ S.E.M. of six different experiments **p* < 0.05 ; ***p* < 0.01 as compared to the control cells as assessed by students unpaired *t*-test.

Table 3. Enterocyte damage during exposure to S-LT in the absence and presence of antioxidants

Samples	MDA Content (nm MDA formed/mg protein)	Viable cells (%)
Control cells	1.61 $\pm$ 0.32	89.32 $\pm$ 5.83
+ SOD	1.50 $\pm$ 0.23	87.93 $\pm$ 6.82
+ Catalase	1.48 $\pm$ 0.26	86.89 $\pm$ 4.23
Cells treated with S-LT	4.85 $\pm$ 0.70	50.64 $\pm$ 6.32
+ SOD	2.85 $\pm$ 0.52*	68.23 $\pm$ 4.01*
+ Catalase	2.63 $\pm$ 0.42*	70.12 $\pm$ 5.09*

Values are expressed as mean $\pm$ S.E.M. of six different experiments **p* < 0.05 as compared to treated cells.

The presence of antioxidants also blocked the decrease in the percentage of viable cells. SOD and catalase blocked the decrease in the percentage of viable cells by 34.73 and 38.46% respectively.

Discussion

The findings of the present study show that S-LT treated enterocytes produce increased levels of ROS, as detected by amplified XO activity and increased AA metabolism. Further these free radicals appear to mediate the enterocyte damage occurring during this exposure.

The enzyme XO is capable of generating $O_2^{\cdot-}$ by converting hypoxanthine or xanthine to uric acid [22]. XO exists in the normal healthy cells predominantly as a NAD^+ reducing xanthine dehydrogenase (XDH) and can be converted to an oxygen radical producing form (XO) in a variety of pathological states either by the proteases released from the inflammatory cells or by the depletion of the thiol groups. During the conversion of XDH to XO there is either the oxidation of the sulfhydryl groups or the proteolysis of a small portion of the enzyme [8].

A previous study has suggested that XO mediated production of ROS contributes to a loss of intestinal barrier function [23]. Further, the XO pathway has been seen to be a major source of ROS in the ischemic reperfusion injury of the gut [22]. In the present study, the XO activity of the S-LT treated enterocytes was found to be significantly higher than the control cells. We have found in a previous study that the administration of S-LT in the ligated rat ileal loop depletes the enterocyte GSH levels [5]. This impairment of GSH might be either due to its oxidation on exposure to ROS [24] or alternatively ROS might impair the activity of γ -glutamylcysteine synthetase [25], an enzyme that catalyses the rate limiting step of GSH synthesis. It appears that, in the present study the exposure of enterocytes to S-LT might deplete the GSH levels which can further trigger the conversion of xanthine oxidase from its dehydrogenase form to its free radical producing form.

Another source of ROS in the enterocyte could be the arachidonic acid metabolism. Arachidonic acid is metabolized via cyclooxygenase and lipoxygenase pathways to yield prostaglandin and leukotrienes respectively. In general, the formation of AA metabolites in living cells requires first the generation of free AA within the cells [26] and this generation which is a rate limiting step for the formation of active metabolites is generally thought to involve phospholipase A2 activation cleaving the esterified AA from various cellular phospholipids. A previous study has shown that H_2O_2 stimulates phospholipase A2 mediated AA release in cultured intestinal epithelial cells [27].

AA and metabolites are of fundamental importance in the gastrointestinal tract. Accordingly, alterations in AA meta-

bolism may play an essential role in the pathophysiology of various gastrointestinal disorders such as inflammatory bowel disease [28]. In the present study the alterations in the AA metabolism were studied indirectly by measuring the extent of enterocyte damage in the absence and presence of the cyclooxygenase inhibitor, indomethacin. It was seen that the presence of indomethacin significantly decreased the extent of lipid peroxidation of enterocytes suggesting that the S-LT treatment involves the generation of ROS by the AA metabolism. There are several potential mechanisms whereby ROS might stimulate the production of prostaglandins. Firstly peroxides are important cofactors for prostaglandin biosynthesis, as demonstrated by the exogenous addition of either H_2O_2 or lipid peroxides to tissues [29]. Secondly H_2O_2 might stimulate the release of free AA from the membrane phospholipids thereby activating the rate limiting step of prostaglandin biosynthesis [27]. In the intestine the prostaglandin biosynthesis could be stimulated by the peroxide mediated activation of inflammatory or mesenchymal cells [29]. Prostaglandin synthesis in the lamina propria of the intestine might initiate changes in the enterocyte electrolyte transport by directly stimulating adenylate cyclase or could activate receptors on the enteric nervous system with resulting release of secretory neurotransmitters.

ROS are detrimental to the integrity of the biological tissues and mediate their injury. The cytotoxic effects of ROS result mainly from the peroxidation polyunsaturated fatty acids. Since polyunsaturated fatty acids are present in high concentrations in the cellular membrane, peroxidation of these membrane constituents lead to the disintegration of cells and irreversible cell and tissue damage. A measure of the lipid peroxidation products such as MDA and conjugated diene is an indication of the extent of peroxidation. In this study, there was a significant increase in the lipid peroxidation level of enterocytes upon exposure to S-LT as compared to the control cells. Thus the exposure to S-LT is accompanied by the oxidative damage of the enterocyte membrane. An increase in the extent of lipid peroxidation of the mucosa has also been observed during the ischemia reperfusion injury [30].

The occurrence of lipid peroxidation in biological membranes has far reaching consequences. It causes impairment of membrane functioning [31], decreased membrane fluidity, inactivation of membrane bound receptors and increased non-specific permeability to ions such as calcium. In a previous study, the lipid peroxidation of the intestinal mucosa led to the luminal secretion of water and electrolytes [32]. In another study, a significant increase in the lipid peroxidation led to the death of the cultured hepatocytes [33].

In the present study also, the increase of lipid peroxidation was accompanied by the loss of cell viability. The relationship between lipid peroxidation and cell viability has been a matter of constant debate. This problem is compounded by the fact that when cells die by any means their lipids may be

peroxidized. Therefore, the mere presence of lipid peroxidation cannot be taken as evidence that this process participated in the mechanism of cell injury. However, if cell death is a consequence of the lipid peroxidation of the cellular membrane, then the addition of an antioxidant would be expected to prevent the cell death as well as the peroxidation of cellular lipids. In the present study, the presence of SOD or catalase during the exposure to S-LT protected the enterocytes from the loss of cell viability and accumulation of MDA. These results imply that cell death may probably be a consequence of lipid peroxidation of enterocyte membrane.

The importance of oxygen-derived metabolites in S-LT mediated enterocyte damage was further studied by measuring the extent of lipid peroxidation and cell viability in the presence of antioxidants. Both $O_2^{\cdot-}$ and H_2O_2 were found to be mediators of damage to enterocytes as determined by a significant decrease in the extent of lipid peroxidation and loss of cell viability in the presence of SOD and catalase respectively.

Administration of exogenous SOD has also proved to be beneficial in the acetic acid induced colitis model and there is preliminary clinical evidence that liposomal encapsulated CuZn SOD may be of benefit in Crohn's disease (CD) and ulcerative colitis (UC) [34, 35]. In another study CuZn SOD has been seen to reduce the lucigenin amplified chemiluminescence by mucosal biopsy specimens isolated from rats with acetic acid induced colitis [36]. In coiticosteroid resistant CD, a combination of SOD and desferrioxamine was found to be effective [37]. Antioxidants have also been successfully used in experimental models of intestinal inflammation. [34, 38].

In contrast to the above mentioned studies some systems have shown increased toxicity with increasing concentrations of SOD suggesting that H_2O_2 or H_2O_2 interaction with metals or other $O_2^{\cdot-}$ derived metabolites is important in providing injury [39, 40]. H_2O_2 is injurious to the rectal mucosa in humans when applied topically [41] and intraperitoneal catalase has been seen to improve inflammation in rat acetic acid induced colitis [34]. An antioxidant effect for catalase has also been demonstrated in both acetic acid induced colitis and UC biopsy specimens [42].

In a previous study, both $O_2^{\cdot-}$ and H_2O_2 , whether created by stimulated neutrophils or by the enzyme XO have been seen to be damaging to the isolated enterocytes [43]. It appears that in the present study the ability of both H_2O_2 and $O_2^{\cdot-}$ to induce toxicity during exposure to S-LT may be due to their capacity to generate other ROS such as hydroxyl radical and singlet oxygen which then initiate a radical chain reaction, causing lipid and organic peroxide formation. H_2O_2 has been seen to induce injury in an intestinal epithelial cell line both directly and by the generation of the hydroxyl radical [44].

Although this work did not attempt to quantitate $O_2^{\cdot-}$ or H_2O_2 or define the exact reactive O_2 species causing enterocyte

injury, it did show the importance of ROS in mediating the enterocyte damage during exposure to S-LT. These ROS may be derived from the enterocyte XO and AA metabolism. ROS may damage the enterocytes by inducing the lipid peroxidation of the enterocyte membrane thereby leading to a loss of cell viability.

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