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Title: MITOCHONDRIAL GLUTATHIONE AND THE EFFECT OF PERTURBED CALCIUM HOMEOSTASIS ON RAT HEPATOCYTE THIOLS

Abstract approved: Redacted for privacy
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Recent studies have demonstrated that perturbations of intracellular thiol and calcium homeostasis may be important events in the early development of cell injury by toxic chemicals.

Incubation of isolated rat hepatocytes in a calcium free medium, severely depleted intracellular Ca^{2+} levels and resulted in the loss of both cytosolic and mitochondrial glutathione (GSH), which preceded cell injury. Elevation of endogeneous α-tocopherol levels, by supplementing the hepatocyte suspension with vitamin E-succinate, inhibited the loss of GSH and reversed cell injury. Increased levels of GSH in the presence of vitamin E-succinate were induced by an apparent α-tocopherol-mediated effect on GSH biosynthesis, indicating a close relationship between these two important cellular antioxidant systems.

Perturbation of intracellular calcium homeostasis in hepatocytes by the administration of A23187, a calcium ionophore, in the presence of different concentrations of extracellular Ca^{2+}, revealed a striking correlation between the loss of mitochondrial GSH

and cell injury. The loss of mitochondrial GSH was preceded by a loss of cytosolic GSH, whereas protein thiol levels were not significantly affected until after the depletion of non-protein thiols. Lipid peroxidation did not have a clear association with cell injury, induced by A23187 in the presence of extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, antioxidants prevented A23187-induced cell injury and loss of mitochondrial GSH and thus dissociated the mobilization of intracellular Ca^{2+} from the expression of toxicity. In the presence of extracellular Ca^{2+}, cell injury as well as the loss of mitochondrial GSH were only partially prevented by antioxidant treatment. These results demonstrate that the level of mitochondrial GSH is critical for cell survival during calcium ionophore-induced perturbation of cellular calcium homeostasis.

Incubation of isolated mitochondria with t-butylhydroperoxide revealed that oxidized glutathione is not transported out of hepatocyte mitochondria, as a response to oxidative stress. These results indicate greater susceptibility of protein thiols in mitochondria to oxidation than in the rest of the cell, and may explain the protective role of mitochondrial GSH in cell injury.

Mitochondrial Glutathione and the Effect of Perturbed Calcium Homeostasis on Rat Hepatocyte Thiols

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ABBREVIATIONS:

BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea

BSA, bovine serum albumin

BSO, buthionine sulfoximine

CS, citrate synthase

DPPD, diphenyl-p-phenylene diamine

EDTA, ethylene diamine tetraactetic acid

EGTA, ethylene glycol bis(Ã-aminoethyl ether)N,N'-tetraacetic acid

Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

GSH, glutathione

GSSG, oxidized glutathione

HPLC, high performance liquid chromatography

LDH, lactate dehydrogenase

Me_2SO, dimethyl sulfoxide

PCA, perchloric acid

protein-SH, protein-thiols

Pr-SSG, glutathione-protein mixed disulfides

RCR, respiratory control ratio

RR, ruthenium red

t-BuOOH, tertiary-butyl hydroperoxide

TBA, thiobarbituric acid

vit E-succinate, α-tocopherol-succinate

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MITOCHONDRIAL GLUTATHIONE AND THE EFFECT OF PERTURBED CALCIUM HOMEOSTASIS ON RAT HEPATOCYTE THIOLS

I. INTRODUCTION.

The elucidation of the cellular processes involved in cell death are of vital importance to biology and medicine. In addition to a basic understanding about a normal phase in the life of every organism, this could help in the prevention and treatment of a wide range of human diseases. The death of a cell can be an irreversible response to injury which is defined as any perturbation that alters the normal homeostasis in the cell.

The focus of this thesis is on the role of thiols in cell injury, with special attention to mitochondrial glutathione. GSH is involved in a number of important cell functions and has been shown to play a critical role in cellular defense against a variety of chemical agents. To investigate the role of thiols in toxicity on a broader basis, cell injury was induced without the use of exogeneous toxicants, but by affecting intracellular Ca^{2+} homeostasis, which is believed to be involved in the early stages of cell death. Cell injury was achieved by manipulating the intra- and extracellular levels of Ca^{2+} and by the use of a calcium ionophore.

Chapter II briefly discusses pertinent background information and current ideas on: the model systems used in this study; the role

of GSH in cell injury; mitochondrial GSH; and calcium homeostasis and the role of Ca^{2+} in cell injury.

Chapter III describes the changes induced in intracellular glutathione pools during the experimental manipulation of the extracellular Ca^{2+} concentration and endogeneous vitamin E levels in isolated hepatocytes. This chapter summarizes my contribution to two journal articles: Fariss, M. W., Olafsdottir, K., and Reed, D. J. (1984) Biochem. Biophys. Res. Commun. 121, 102-110; and Pascoe, G. A., Fariss, M. W., Olafsdottir, K., and Reed, D. J. (1987) Eur. J. Biochem. in press. This chapter includes data on intracellular K^+ levels in hepatocytes determined by Dr. Gary A Pascoe, and is greatfully acknowledged.

Chapter IV describes the use the calcium ionophore, A23187, as a tool to investigate the effects of altered calcium homeostasis in isolated hepatocytes on cellular thiols and demonstrates a remarkable correlation between the depletion of mitochondrial GSH and cell injury. This chapter has been submitted for publication to Archives of Biochemistry and Biophysics.

Finally, in chapter V the inability of isolated mitochondria to export oxidized glutathione (GSSG), in response to oxidative stress, is demonstrated by using the model compound t-BuOOH as an oxidant. This chapter has been submitted for publication to Biochimica Biophysica Acta.

II. BACKGROUND PERSPECTIVES.

Model Systems.

1. Isolated Hepatocytes.

Suspensions of freshly isolated hepatocytes are being increasingly used in a wide range of biochemical and pharmacological investigations, including studies into the pathways of cell injury.

Isolated hepatocytes provide a very useful cellular system which is an intermediate between tissue homogenates and the intact animal. Hepatocytes also possess certain advantages over other cell-based *in vitro* systems such as perfused liver and liver slices, which can be cumbersome because of the difficulty in obtaining repeated tissue samples and subfractionation of the tissue cannot be obtained rapidly. Advantages of hepatocytes over the whole animal include the control or absence of many factors that cannot be defined *in vivo*, such as neuronal and hormonal influences. Others are: the ease of controlling the composition of the incubation medium; the number of samples that can be collected; and control of the level of agent at the target site.

Excellent viability of freshly isolated rat hepatocytes can be maintained for 4-6 h at 37°C. A high level of organization and most of the biochemical properties of the *in vivo* system is maintained. For example, the rate of drug metabolism is comparable with that in the intact liver (1). This makes it possible to study the interrelationship between various experimental manipulations and

normal cellular metabolism. A suspension of cells from a single liver can be divided into several flasks and can therefore be examined under a variety of different conditions. In addition, the time course of metabolic changes can be readily followed and cells can be rapidly separated into several subcellular fractions. By using electron microscopy, morphological alterations in the cells can be correlated with biochemical changes.

A special advantage of the isolated hepatocyte system for the study of cell injury is the possibility of easily and rapidly separating live cells from dead cells by a centrifugation filtration technique (2), allowing for the cellular events responsible for cell injury to be separated from the events that result from cell death, a very important consideration for the work presented in this thesis. This method also provides a rapid and convenient means for both separating cells from the medium and instantly stopping intracellular processes (Fig. II.1).

Disadvantages of the isolated cell system include: the relatively short viability period; possible damage to membranes and perturbation of their ionic composition due to the isolation procedure; normal contact and communication between cells is lost; and the spatial heterogeneity of the cells is not the same as in the whole organ. These factors have the potential of producing artifacts that may lead to misinterpretation of the data.

2. The Calcium Ionophore, A23187.

The antibiotic, A23187, is a lipid soluble monocarboxylic acid (M.W. 523), that specifically binds divalent but not monovalent cations at neutral pH (Fig.II.2). The A23187-Me^{2+} complex appears to be a neutral, 2:1 species with an order of affinity for various divalent cations of $Mn^{2+}>>Ca^{2+}\simeq Mg^{2+}>Sr^{2+}>Ba^{2+}$ (3). The lipophilic nature of A23187 allows it to enter cells and act as a freely mobile carrier to equilibrate divalent cation concentrations across cellular membranes. A23187 has been widely used as an investigative tool to study the role of divalent cations, principally Ca^{2+}, in various biological systems (4-7) and it appears that all of the effects of A23187 on intact cells can be accounted for by its ionophoretic actions.

At physiological concentrations of extracellular Ca^{2+} Reed and Lardy (4) observed that A23187 induced uptake and accumulation of Ca^{2+} in exchange for proton release from erythrocytes and found that A23187 inhibited mitochondrial ATPase by releasing endogenous Mg^{2+} while uncoupling oxidative phosphorylation by inducing a cyclic energy- dissipating flux of Ca^{2+} (4). At low extracellular Ca^{2+} concentrations, the ionophore induced efflux of intracellular Ca^{2+} from isolated rat hepatocytes (5). In a more careful study with guinea pig hepatocytes, Burgess et al. (8) saw only a brief period of Ca^{2+} accumulation in cells incubated with 1.8 mM Ca^{2+}, followed by a long period of Ca^{2+} extrusion, during which the cellular Ca^{2+} content fell below the pre-ionophore level. A third phase of Ca^{2+} reaccumulation was evident 10-30 min after ionophore administration.

These changes were attributed to the extrusion of intracellular Ca^{2+} and the high activity of the plasma membrane ATPase and reaccumulation when ATP levels decreased. Chen et al. (9) reported a similar net efflux of Ca^{2+} from rat hepatocytes incubated for short periods in medium containing either 20 μM or 1 mM Ca^{2+}. From these experiments it is evident that A23187 induces marked perturbation of intracellular Ca^{2+} homeostasis, and was utilized for that purpose in experiments described in chapter IV.

A23187-induced cell injury is dependent on ionophore and extracellular Ca^{2+} concentrations, with no toxicity observed in the absence of Ca^{2+} after short incubations (6,10,11). A 60% decrease in ATP levels was shown to precede cell injury in cultured hepatocytes exposed to the ionophore (12), which was believed to result from an energy consuming cyclic uptake and release of Ca^{2+} from mitochondria. However, the role of ATP depletion in cell injury was disputed by the fact that concomitant depletion of ATP by ethionine did not potentiate A23187-induced toxicity and 2,4-dinitrophenol depleted ATP much more extensively at non-cytotoxic concentrations (12). Since the ionophore has been shown to cause the activation of Ca^{2+} dependent processes in various cells (13,14), A23187 probably induces a transient elevation in cytosolic free Ca^{2+} levels, which may explain its toxicity (see last topic of this chapter).

Glutathione and its Protective Role in Cell Injury.

Glutathione (L-γ-glutamyl-L-cysteine-L-glycine; Fig. II.3a) is an unusual tripeptide present in mM concentration in most cells. GSH contains a thiol functional group and a γ-glutamyl residue, that makes it resistant to the hydrolytic action of proteases and aminopeptidases, and protects the thiol group from rapid oxidation (15,16). The glycine residue of GSH protects it from the action of γ-glutamyl-cyclotransferase (16). These qualities make GSH uniquely capable of participating in a number of important cellular processes.

The cellular pool of GSH is the result of a dynamic equilibrium between GSH synthesis and turnover. GSH turnover is rapid in hepatocytes, with a halflife of approximately 2 h (17) and occurs mainly through efflux out of the cell into the plasma, where it serves as a major transport mode for cysteine. Although GSH can be taken up by epithelial cells of the intestines, kidneys, and lungs (18-20), no evidence for the transport of GSH into liver cells exists. GSH in hepatocytes must therefore be resynthesized by two ATP-requiring steps in the cytoplasm, first by γ-glutamylcysteine synthetase, inhibited by buthionine sulfoximine (21,22) and by GSH synthetase (Fig. II.3b). The availability of cysteine (0.2-0.5 mM in liver) is the rate limiting factor of GSH biosynthesis (23). Breakdown of GSH occurs extracellularly by γ-glutamyl transpeptidase, mainly at the luminal surface of the renal brush border membrane (24).

In mammalian cells, GSH exists in a few interchanging forms. Under normal steady-state conditions, the reduced parent form prevails with 0.1-1% present as the disulfide (GSSG) (25), or as a mixed disulfide (26) with both protein or nonprotein sulfhydryls. During oxidative stress the disulfide levels can increase markedly via GSH peroxidase, present in both cytosol and mitochondria (27). Mixed disulfides are formed in a reaction catalized by thiol transferase (28):

$$\text{Protein-SH} + \text{GSSG} \longrightarrow \text{Protein-SSG} + \text{GSH}$$

with a ratio of 1:1 GSSG/Pr-SSG found in rat hepatocytes treated with paraquat, t-butylhydroperoxide or nitrofurantoin (29). GSSG is reduced back to GSH via GSSG reductase, drawing reducing equivalents from NADPH. Under conditions of severe oxidative stress the coenzyme can become limiting and GSSG is rapidly exported out of the cell (30) to prevent deleterious effects on cellular integrity and metabolic processes.

Thiol esters of GSH constitute another form of cellular GSH. Although their occurrence is not well documented, the identification of at least 3 distinct GSH thiol esterases in human livers suggests functional significance (31).

GSH has been proposed to play a critical role in cellular defense against electrophilic metabolites and reactive oxygen intermediates (32-34). Support for this hypothesis comes from studies with various toxic chemicals that depleted GSH by different mechanisms (35,36). Severe reduction in GSH levels by fasting (35,37), buthionine sulfoximine (36,38), or diethylmaleate (39,40),

prior to the administration of these drugs further potentiated their toxicity. The role of GSH in the detoxification of reactive metabolites was demonstrated by studies on the metabolisms of bromobenzene and acetaminophen. Direct correlation was shown between hepatic GSH levels and the severity of liver necrosis, and the degree of covalent binding of bromobenzene to macromolecules in vivo (41,42). Similar evidence relating GSH concentration with covalent binding and loss of cell viability was obtained in vitro with isolated rat liver hepatocytes (43). Incubation of isolated hepatocytes with acetaminophen resulted in depletion of GSH and concomitant formation of a glutathione-acetaminophen conjugate. GSH depletion was followed by a rapid loss of cell viability, which was abolished by supplementing the medium with precursors of GSH biosynthesis (44).

The metabolisms of organic hydroperoxides and H_2O_2 by GSH peroxidase are important in protecting cells from oxidative stress. Since GSH and pyridine nucleotide oxidation in mitochondria has been proposed to increase the permeability of the inner membrane to Ca^{2+} (45,46), oxidative stress or severe GSH depletion may affect the redox status enough to alter intracellular Ca^{2+} homeostasis, which is believed to be an early step in cell injury (see the last topic of this chapter). Certain proteins are highly sensitive to changes in the cellular thiol status, including the Ca^{2+} dependent ATP-ases (47), as illustrated by experiments showing that t-butyl hydroperoxide-induced inhibition of plasma membrane Ca^{2+} sequestration was prevented by physiological concentrations of GSH

and 1 mM dithiothreitol (48). The Ca^{2+} ATPases serve as membrane bound Ca^{2+} pumps to maintain low levels of cytoplasmic free Ca^{2+}.

Mitochondrial Glutathione.

Glutathione exists in two separate pools in hepatocytes, 85-90% in the cytoplasm and 10-15% in the mitochondria (17,49,50). Early observations with isolated hepatocytes (17) and with rats *in vivo* (51), indicated that the two pools were metabolically separate with different halflifes, approximately 2 h for cytoplasmic GSH and approximately 30 h for mitochondrial GSH. Evidence suggesting that the two pools were not free to equilibrate included studies with the GSH depleting agent, diethylmaleate, which depleted cytoplasmic GSH in hepatocytes without affecting mitochondrial GSH (17), and the biphasic disappearance of GSH in mice *in vivo* (52,53), after administration of buthionine sulfoximine, an inhibitor of GSH biosynthesis. However, all efforts to identify biosynthesis of GSH inside the mitochondria, including our own (Chapter V), have failed (53). The origin of mitochondrial GSH is thus presently unknown but is assumed to be derived from the cytosol and transported into the mitochondria.

A role for mitochondrial GSH in cytotoxicity was first proposed by Meredith and Reed in 1982 (17). They showed that the onset of cell death correlated with the depletion of mitochondrial GSH, whereas cytosolic GSH could be depleted without affecting cell viability and later that despite a complete depletion of cytosolic

GSH by adriamycin in conjunction with BCNU, LDH leakage increased markedly only after the mitochondrial pool was depleted to less than 10% of the initial value (54). Previously, several reports (40,55-58) had demonstrated that cytotoxicity, as measured by lipid peroxidation, liver necrosis and loss of cellular enzymes *in vivo* and *in vitro*, occurred only if the intracellular concentration of GSH fell below 10-15% of the initial value, which is the amount found in the mitochondria. The important role of mitochondrial GSH in maintaining mitochondrial integrity was demonstrated by showing a correlation between the GSH/GSSG ratio, rather than pyridine nucleotide ratios, and the permeability of isolated mitochondria to Ca^{2+} (59). It was proposed that the GSH/GSSG ratio, by controlling the reduction state of critical sulfhydryl groups, regulates lysophospholipid acyltransferase activity and, therefore, the ability of mitochondria the remain impermeable upon activation of the intramitochondrial Ca^{2+} requiring phospholipase A_2 (59). Botti *et al*. (60) showed that morphological lesions in the mitochondria correlated with the loss of mitochondrial GSH, although these were not correlated with hepatotoxicity. Others have demonstrated a crucial role of sulfhydryl groups in the maintenance of the mitochondrial inner membrane structure (61-63), where GSH may play a role in maintaining the intramitochondrial sulfhydryls in a reduced state (59,63). In contrast, Ku and Billings (64) found no correlation between formaldehyde toxicity and depletion of mitochondrial GSH. Loss of cellular protein thiols appeared more

critical for cell survival. However, formaldehyde is known to react with protein functional groups.

Mitochondria are important stores of intracellular Ca^{2+} (65) and may help regulate the cytoplasmic free Ca^{2+} level (66,67). Alterations in mitochondrial redox status that would change the membrane Ca^{2+} permeability could be important in cell injury, since perturbation of cellular Ca^{2+} homeostasis and increased levels of cytoplasmic free Ca^{2+} are believed to trigger cell injury (see the last topic of this chapter).

Mitochondrial GSH could also be important in protecting cells against the harmful effects of altered Ca^{2+} homeostasis. Ca^{2+} cycling in and out of the mitochondria would increase respiration due to increased demand for energy. Inside the mitochondria, GSH would be the first line of defense against toxic oxygen species that seem to be unavoidable byproducts of aerobic respiration (68). Chapters III and IV explore this possibility.

Calcium Homeostasis and the Role of Ca^{2+} in Cell Injury.

Calcium plays a central role in the regulation of a wide range of cellular functions (69), acting primarily as a messenger of intracellular signals through a series of binding proteins, including calmodulin, troponin C, and parvalbumins (70).

Cytosolic free Ca^{2+} concentration in hepatocytes (10^{-7} M) is maintained at levels 3-4 orders of magnitude lower than in the extracellular medium (10^{-3} M). This concentration gradient is

maintained by active uptake of Ca^{2+} into the intracellular compartments and by active extrusion of the ion through the plasma membrane (Fig. II.4) (71). Isolated rat liver mitochondria are able to accumulate Ca^{2+} and have been shown to act as buffers of extramitochondrial Ca^{2+} (72,73). However, the results of measurements of <u>in situ</u> mitochondrial calcium contents range widely (1-40 nmol/mg mitochondrial protein (74)) and the role of mitochondria in buffering cytosolic Ca^{2+} remains controversial (66,67,75). The plasma membrane high affinity Ca^{2+} translocase, although important for the long-term maintenance of total cellular calcium levels, was discounted as an important regulator of cytosolic Ca^{2+} in hepatocytes by experiments showing that the steady-state free Ca^{2+} concentration maintained by suspensions of hepatocytes was in the 0.1-0.2 μM range when the plasma membrane was made permeable by digitonin treatment (76). However, in the same study, microsomes were shown to affect mitochondrial Ca^{2+} cycling and alter the set point for free Ca^{2+} homeostasis (76). These investigators conclude that the endoplasmic reticulum appears to be a high affinity, low capacity organelle, whereas the mitochondria have a very large capacity for accumulating Ca^{2+} but do so only at elevated free Ca^{2+} concentrations (10^{-6} M) (77). The relative contribution of the endoplasmic reticulum and the mitochondria to the regulation of cytosolic free Ca^{2+} remains controversial.

Ca^{2+} movement into and out of mitochondria occur via different transport routes. Ca^{2+} uptake is driven by the transmembrane potential generated across the mitochondrial inner membrane during

coupled respiration (78,79). Release of Ca^{2+} from mitochondria can be induced by factors which cause collapse of the transmembrane potential or by selective inhibitors of the uptake route like ruthenium red (80). The release mechanism has been suggested to involve H^+/Ca^{2+} and/or Na^+/Ca^{2+} exchange (81,82) and to be regulated by the redox state of intramitochondrial pyridine nucleotides (46), which can be affected by a number of toxic chemicals, mostly through the oxidation of GSH.

The role of calcium in cell injury has been the subject of much controversy. Initial reports on the pathology of cell death described the accumulation of calcium in necrotic tissue (83). This work stimulated the initiation of later studies with in vitro cell systems in which the calcium content of the extracellular medium could be manipulated. Several reports showed that cell death caused by a variety of different chemicals was dependent on extracellular Ca^{2+} (10,84). These investigators proposed a two step mechanism for toxic cell death. The first step was the disruption of the integrity of the plasma membrane by widely differing mechanisms followed by a "final common pathway": the influx of extracellular calcium across the damaged plasma membrane (84). In contrast, other studies demonstrated that toxicity of several agents was increased in calcium free cell incubations (85-87). These investigators concluded that the absence of extracellular Ca^{2+} predisposes cells to toxic injury and that the entry of extracellular Ca^{2+} occurs after cell death. Recent developments suggest that chemically induced cell injury results from changes in intracellular Ca^{2+} homeostasis and that

increased levels of cytosolic Ca^{2+} precede the onset of cell death. These include studies with the redox active compounds; menadione, t-butylhydroperoxide, and an acetaminophen metabolite, N-acetyl-p-benzoquinone imine, demonstrating that thiol oxidation resulted in impaired function of Ca^{2+}-ATPases leading to a sustained increase in cytosolic Ca^{2+} that preceded cytotoxicity in isolated hepatocytes (88-90). N-Methylformamide was shown to impair the function of the mitochondrial Ca^{2+} pump (91) and CCl_4 treatment of cultured hepatocytes resulted in a sustained increase (100-fold) in cytosolic free Ca^{2+}, preceding the release of cytoplasmic enzymes (92). The CCl_4-induced increase in cytosolic Ca^{2+} did not require extracellular Ca^{2+} and a parallel decrease in Ca^{2+} sequestration by the endoplasmic reticulum was observed (92).

An increase in cytosolic free Ca^{2+} may have a number of potentially adverse consequences on the cell. Phospholipases may be activated directly by Ca^{2+} in various membranes including the plasma, mitochondrial, and endoplasmic reticulum membranes (93). Lysosomal phospholipases do not appear to be involved and lysosomal enzyme release was shown to occur after cell death (94). Changes in membrane phospholipids may result in alterations in membrane permeability, affecting Ca^{2+} transport to further enhance the destructive effect of Ca^{2+}. Stimulation of phosholipid degradation occurs in several different forms of cell injury (95). In particular, Smith et al. (96) demonstrated that the mitochondrial phospholipid cardiolipin, decreased early following acute ischaemic injury in the rat kidney, and inhibition of phospholipid hydrolysis

by chlorpromazine was shown to prevent ischaemic cell death (97). However, Shier and Dubourdieu (98) have found that the phospholipid breakdown induced by A23187 in cultured fibroblasts was complete before toxicity occurred and Nicotera et al. (14) found that while chlorpromazine and dibucaine inhibited phospholipid hydrolysis, these agents did not prevent cystamine toxicity.

An early indication of cell injury is the changed morphology of the cell. Increased cytosolic Ca^{2+} causes actin filaments to be fragmented into short oligomers and actin bundles are disrupted, permitting the cell to undergo a shape change (93). These cytoskeletal changes are induced by Ca^{2+} or Ca^{2+}-calmodulin binding to various proteins associated with the microtubules and the microfilaments (70). Many toxic agents have been shown to cause the formation of small blebs on the surface of isolated hepatocytes which precede cell death (99). These blebs may pinch off the cell, but their content is unknown. A similar phenomenon, termed autophagic vacuoles, occurs within the cell. These contain cytoplasmic components and are eventually fused with lysosomes and digested. These cytoskeletal changes may also alter the permeability of cellular membranes.

The role of Ca^{2+} in the control of proteolysis is well recognized and activation of a nonlysosomal proteolytic system has recently been proposed as the mode by which increased cytosolic Ca^{2+} triggers cell disruption (14). These proteases show specificities for microtubule and myofibril associated proteins but their physiological functions are still unclear (70). Ca^{2+} may also

activate transglutaminases to cause cross-linking of carboxyl groups of glutamic acid and ϵ-amino groups of lysine, resulting in high molecular weight protein aggregates, although relatively high Ca^{2+} concentrations (100 μM) are needed to activate the transglutaminases (70).

It is evident that calcium plays an important role in the pathway of cell death, although its place in the sequence of events still awaits further research. The interrelationship between calcium and thiol homeostases during cell injury is the major topic presented in this thesis.

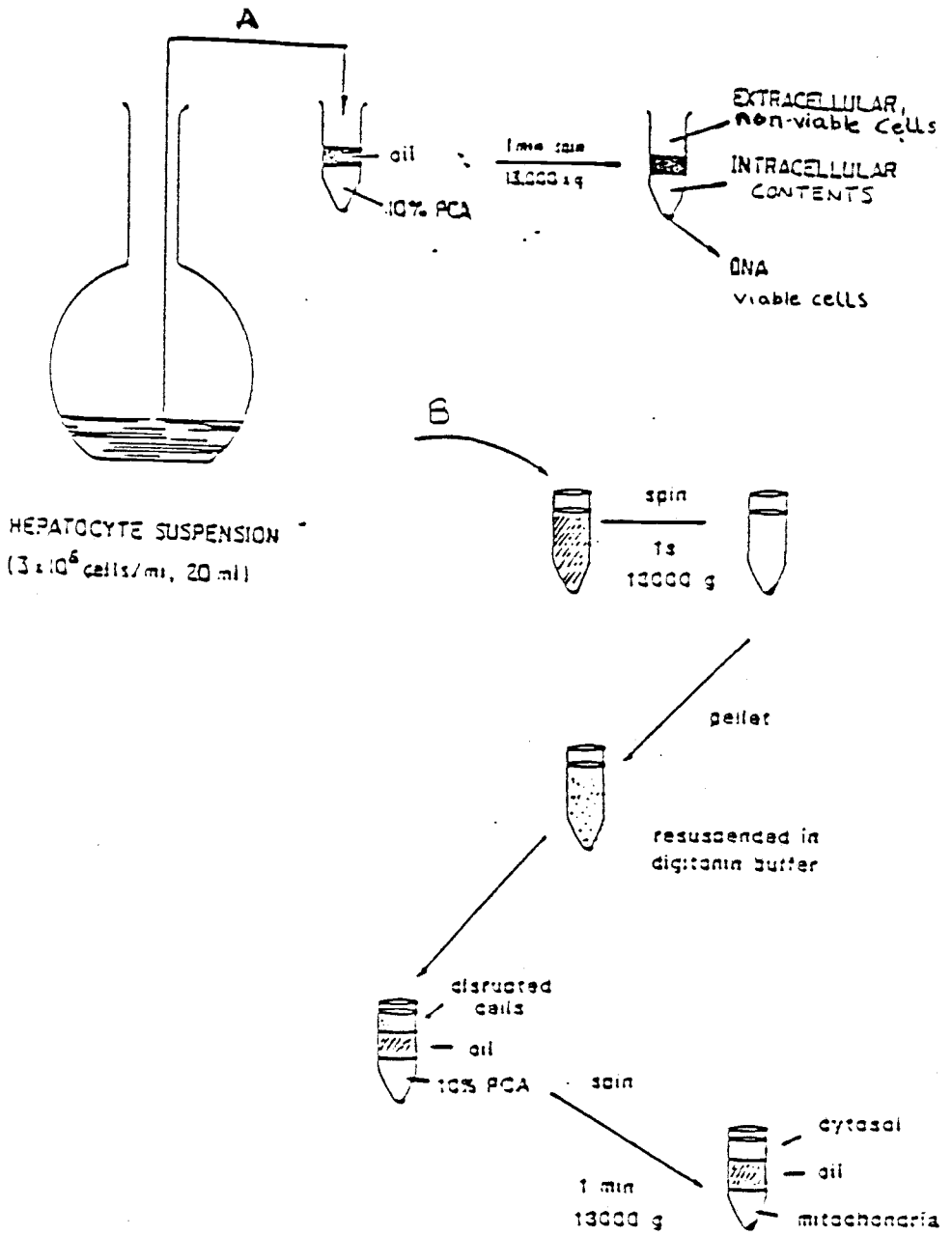
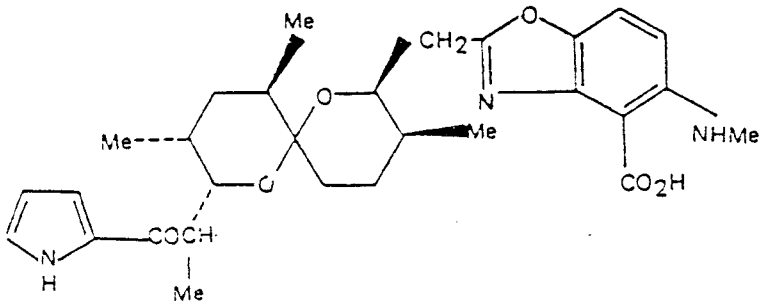


Figure II.1

Centrifugation filtration technique for rapid washing and (A) separation of viable from nonviable cells and (B) separation of mitochondria from cytosol. Digitonin disruption of hepatocytes is described in (17).



Free Acid

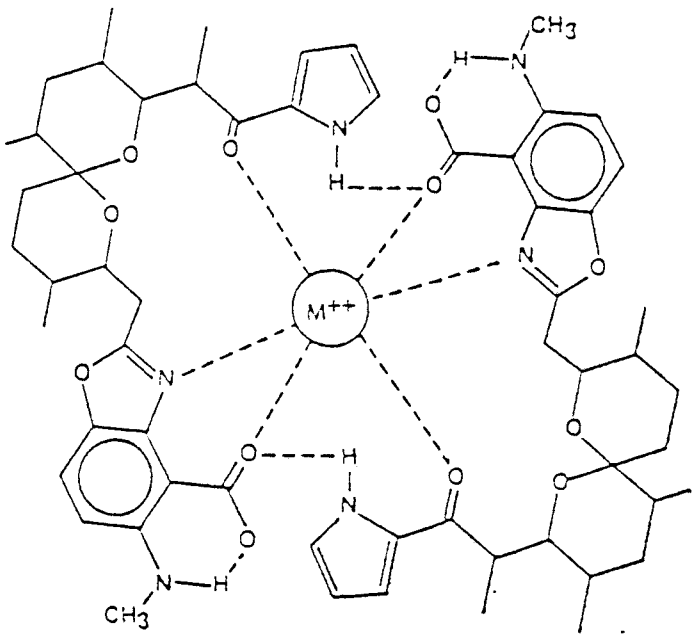
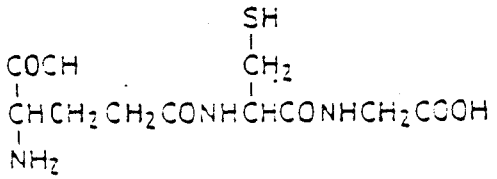


Figure II.2

Structure of the calcium ionophore, A23187, and the A23187-metal complex.

GLUTATHIONE (GSH) :



γ - GLUTAMYL - CYSTEINYL - GLYCINE

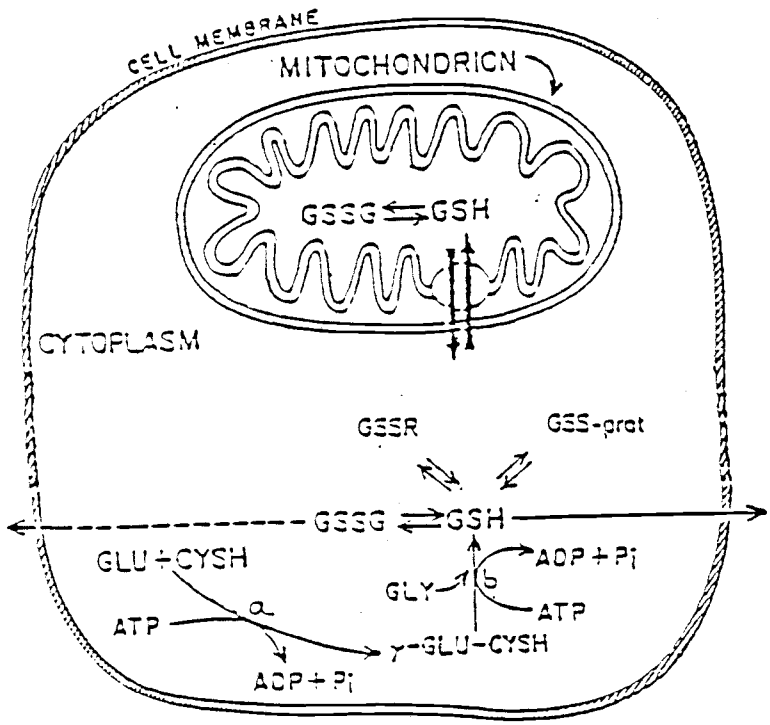


Figure II.3

Structure of glutathione (upper panel) and glutathione homeostasis (lower panel) in the hepatocyte. (a) γ-glutamylcysteine synthetase, (b) glutathione synthetase. Adapted from (50).

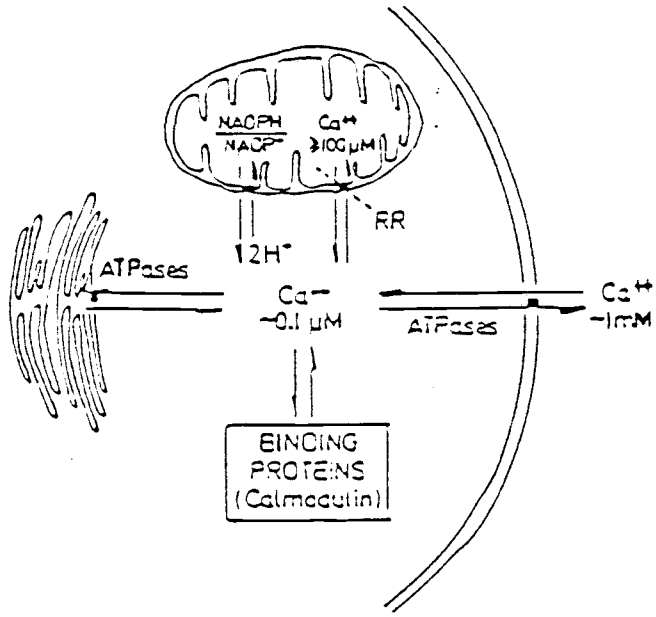


Figure II.4

Calcium homeostasis in the hepatocyte. Adapted from (71).

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III. EFFECT OF EXTRACELLULAR Ca^{2+} AND VITAMIN E ON GLUTATHIONE LEVELS IN ISOLATED HEPATOCYTES

Abstract:

Increased chemical-induced cell injury has been demonstrated in several different cell systems, when Ca^{2+} is left out of the incubation medium.

The incubation of isolated rat hepatocytes in a calcium-free medium, but in the absence of toxic chemicals, resulted in a pronounced loss of cell Ca^{2+}, partial depletion of mitochondrial and cytosolic glutathione (GSH), as compared with hepatocytes incubated with Ca^{2+}. Furthermore, a markedly increased efflux of both reduced and oxidized (GSSG) glutathione was seen from the calcium-depleted cells. These events were followed by a loss of cell K^+, which is an indicator of reversible cell injury, although cell viability was not affected.

Addition of α-tocopheryl-succinate (vit E-succinate) to the Ca^{2+}-depleted cells prevented the loss of K^+ and cytosolic GSH, and partially prevented the loss of mitochondrial glutathione. However, the increased efflux of GSH and GSSG was not prevented, so that higher total levels of glutathione equivalents (intra- plus extracellular) were found in the presence of vitamin E than in its absence. Administration of buthionine sulfoximine (BSO), an inhibitor of GSH biosynthesis, eliminated the GSH sparing effect of

vit E-succinate, indicating that vit E-succinate had prevented the loss of intracellular GSH through stimulation of GSH biosynthesis.

It is concluded that incubation of hepatocytes in a Ca^{2+}-free medium, predisposes the cells to chemical-induced injury due to compromised thiol pools in the cells. Elevated levels of cellular α-tocopherol prevent the thiol loss by affecting GSH synthesis.

Introduction:

The cellular expression of a toxic insult depends on the status of the cells endogenous protective systems, particularly the glutathione redox system (1). Glutathione is the most abundant non-protein thiol in the hepatocyte cytoplasm, and plays major roles in the detoxification of chemicals, by conjugation via GSH-S-transferases or as a reductant (2-4). During oxidative stress both GSH, and the membrane bound antioxidant, α-tocopherol, become susceptible to oxidation and hence depletion (5). Deficiencies of either antioxidant increase the susceptibility of the cell to injury (3,6-8).

Recent reports have begun to establish relationships between the redox systems of GSH and vitamin E (9-14) and direct regeneration of α-tocopherol via the GSH redox cycle has been reported in a cell free system (12). Yoshida et al. (13) reported that vitamin E deficiency in chick liver resulted in increased levels (3-fold) of GSH and GSH synthesis over controls, but Costagliola et al. (14) reported that high doses of vitamin E in humans resulted in

significantly higher levels of GSH in red blood cells. The effect of vitamin E on GSH synthesis and turnover is therefore not clear.

Over the years a variety of evidence has indicated that extracellular Ca^{2+} plays a critical role in the initiation of toxic cell death (15-17). Schanne et al. (16) demonstrated that a number of chemicals were cytotoxic only in the presence of high concentrations of extracellular Ca^{2+}. However, several other investigators have shown that the absence of extracellular Ca^{2+} potentiates the hepatotoxicity of a variety of compounds whose proposed mechanism of toxicity are quite different. Smith et al. (18) reported that carbon tetrachloride, bromobenzene, and ethylmethyl sulfonate, were far more toxic to isolated hepatocytes in the absence of extracellular Ca^{2+} than in its presence. Acosta and Sorensen (19) reported that the toxicity of $CdCl_2$ to cultured hepatocytes was accelerated in a Ca^{2+} free medium. Studies in this laboratory (3) had also demonstrated an increased rate of cell death in isolated hepatocytes incubated in a Ca^{2+} free medium and exposed to a combination of the chemotherapeutic drugs, adriamycin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). These studies further revealed that the increased rate of cell death was accompanied by an accelerated loss of intracellular GSH as compared with treated cells incubated with Ca^{2+}. These results suggested that the incubation of hepatocytes in Ca^{2+} free medium increased the susceptibility of cells to chemical-induced death by altering cellular protective systems.

To test this hypothesis, we examined the effect of incubating isolated rat hepatocytes in medium devoid of extracellular Ca^{2+} on

cellular thiol pools and cell injury, but without toxic chemicals. We also investigated the effect of elevated cellular α-tocopherol levels on these parameters to establish whether a relationship exists between these antioxidant systems.

This study revealed that cellular thiol pools were severely compromised when hepatocytes were incubated in the absence of extracellular Ca^{2+}, leading to extensive loss of cellular Ca^{2+} and reversible cell injury. Elevated levels of cellular α-tocopherol prevented cell injury and mostly inhibited changes in thiol status, apparently by affecting GSH biosynthesis.

Materials and Methods:

Isolation and Incubation of hepatocytes:

Parenchymal liver cells were prepared as previously described (20,21) from male Sprague-Dawley rats (175-225 g) with access to standard rat chow ad-libitum. It is important to note that this diet is considered sufficient in vitamin E content. Freshly isolated hepatocytes were suspended in 125 ml culture flasks (2×10^6 cells/ml) in Fischer's medium (20 ml) modified as described (22), and supplemented with 0 or 3.5 mM $CaCl_2$, 10 mM Hepes and 0.2 mM cystine immediately before use. Fetal calf serum was omitted from the incubation medium. α-Tocopheryl-succinate (vit E-succinate, Sigma Chemical Co, St. Louis, MO), 25 μM, was added to the incubation medium in 20 μl dimethyl sulfoxide (Me_2SO) 15 min prior to the 0 h. Equivalent volume of 25 μM succinic acid (disodium salt, Sigma) in

50% Me_2SO was added to control flasks with no effect on the parameters measured. BSO (Sigma) was added in saline to a final concentration of 0.5 mM at 0 h where indicated. Each flask was slowly rotated at 37°C under a constant flow of water-saturated 95% air, 5% CO_2, and aliquots were removed hourly for analyses.

Isolation of mitochondria and preparation of cell pellets and extracts:

Mitochondria were isolated from incubated hepatocytes at each time point as described by Meredith and Reed (23). In brief, cells were disrupted in a 0.8 mg/ml digitonin buffer for 30 s and then immediately spun through a dibutyl phthalate oil layer into 10% perchloric acid, thus forcing the intact mitochondria through the oil leaving the cytoplasmic contents in the layer above the oil.

Similarly, viable hepatocytes were separated from nonviable cells and medium by centrifugation through dibutyl phthalate into 10% perchloric acid to release the intracellular contents for analysis (22).

Biochemical analyses:

Cell viability was monitored by measuring lactate dehydrogenase (LDH) activity in the incubation medium with a Beckman TR analyzer (24). Cell viability is expressed as the % of the total cellular LDH activity that remains associated with the cells. Reversible cell injury was monitored by determining the total intracellular $[K^+]$ with a flame photometer (25), assayed in the acid layer after centrifugation of viable cells through the oil layer.

The number of viable cells and mitochondria isolated through the dibutyl phthalate oil layer were quantitated by analyzing the perchloric acid-precipitate for DNA content (26). Nuclei are not affected by the digitonin treatment, and are centrifuged through the oil layer along with the mitochondria, allowing for quantitation of nuclear DNA and therefore of viable cells used for mitochondrial analysis.

Acid-soluble glutathione pools were analyzed by the HPLC method of Reed et al. (27) with several modifications. Intracellular glutathione was determined in the acid layer, which had been supplemented with a known amount of γ-glutamyl glutamic acid (Vega Biochemicals, Tucson, AZ). Each sample was treated with the iron chelator, 1,10-phenanthroline, and iodoacetic acid, to final concentrations of 1 and 5 mM respectively, and a base-solution ($KOH/KHCO_3$, 3.0 M/2.4 M) was added to reach pH 8-9, judged by including the pH indicator m-cresol-purple in the assay system. After 1 h incubation in the dark, 1% fluorodinitrobenzene in ethanol (v/v) was added to the reaction mixture. This sample was stored in the dark for 24 h at room temperature and then analyzed by HPLC with γ-glutamyl glutamic acid as an internal standard. Oxidized glutathione in control cells was 1.4 ± 0.3% of the total glutathione.

The efflux of glutathione from hepatocytes was determined as described (21), by taking advantage of the reaction of extracellular GSH with cystine, which was added to the medium and is not taken up by isolated hepatocytes (28). This reaction allows for the distinction between GSH and GSSG efflux, since most of the

extracellular GSH bound in the form of cysteinylglutathione is not available for autooxidation.

GSH-protein mixed disulfides (Pr-SSG) were analyzed on the acid-precipitated cell pellet (29).

Determination of intracellular [Ca^{2+}] was by atomic absorption spectrophotometry, performed in the acid layer after centrifugation of whole cells through dibutyl phthalate, as described (22). [Ca^{2+}] in the "calcium-free" Fischer's medium was <80 μM.

Statistics:

Values are expressed as sample means ± standard error (SEM). Student's "t" test was used to determine differences between sample means.

Results:

Incubation of isolated rat hepatocytes in a Ca^{2+} free medium resulted in an extensive loss of cellular Ca^{2+}, as compared with cells incubated with 3.5 mM extracellular Ca^{2+} (Table III.1). The drop in cellular calcium levels was already complete at 0 h and did not change appreciably over the 5 h incubation period. Since these cells contain only about 10% of the whole cell calcium content of control cells, they are considered Ca^{2+} depleted.

The depletion of cellular Ca^{2+} resulted in an enhanced loss of intracellular K^{+}, which is an indicator of reversible cell damage (30). The increased loss of K^{+} started at the third hour and reached a loss 20% greater than that of controls at 5 h. However, LDH

leakage, an indicator of irreversible cell damage (30), was not affected (Table III.1).

Another consequence of eliminating Ca^{2+} from the medium was a reduction in the levels of cytosolic and mitochondrial GSH. The levels of both GSH pools fell steadily, to approximately 50% of the initial concentration over a 5 h period (Fig. III.1). In contrast, no loss of GSH was seen from hepatocytes incubated in the presence of Ca^{2+} over the same period (Fig. III.1). It should be noted that the loss of thiols occurred prior to the loss of intracellular K^{+}.

A comparison of the total intracellular GSH concentration in hepatocytes incubated with or without Ca^{2+} for 5 h reveals a difference of approximately 14.5 nmol/10^6 cells (Fig. III.1). Since GSH is not broken down within hepatocytes, the possible explanations for the loss of intracellular GSH from hepatocytes incubated without Ca^{2+} include: an accelerated efflux of GSH; an enhanced formation and efflux of GSSG; a depressed rate of GSH biosynthesis; or increased formation of GSH mixed disulfides. As seen in Table III.2, GSH efflux was indeed accelerated from the Ca^{2+} depleted cells. Within a 5 h period, hepatocytes incubated without Ca^{2+} released approximately 8.8 nmol/10^6 cells more GSH than cells incubated with Ca^{2+}. Similarly, the formation and release of GSSG from the hepatocytes was enhanced in the absence of Ca^{2+}. The efflux of GSSG was approximately 2.2 nmol(GSH equivalents)/10^6 cells/5 h greater than in hepatocytes incubated with Ca^{2+} (Fig. III.2). The efflux of GSH + GSSG thus accounts for a major part of the missing intracellular GSH, or 11.0 nmoles out of 14.5 nmoles. No increase in the levels of Pr-

SSG were detected in the Ca^{2+} depleted cells (0.2 ± 0.1 nmol/10^6 cells at 0 and 5 h in both systems), so the missing 3.5 nmoles of GSH may have been lost as undetected acid-soluble mixed disulfides, or the GSH biosynthesis was depressed by depletion of intracellular calcium.

Addition of vit E-succinate (25 μM) to a suspension of hepatocytes in a calcium free medium, which had previously been shown to result in a marked elevation of the cellular α-tocopherol content (31), did not affect the loss of intracellular Ca^{2+}, but the loss of intracellular K^+ was completely reversed (Table III.1). This demonstrates that cell injury was not due to the loss of cellular Ca^{2+} per se, but rather to a consequence of the Ca^{2+} depletion that was preventable by high levels of intracellular α-tocopherol.

The addition of vit E-succinate to the calcium depleted cells also mostly reversed the loss of intracellular GSH (Fig. III.1). Surprisingly, vit E-succinate administration had no effect on the accelerated efflux of GSH and only a minimal inhibitory effect on the enhanced oxidation of GSH to GSSG (Table III.2). Similarly, supplementation with vit E-succinate did not affect the formation of Pr-SSG. Thus, the maintenance of intracellular GSH content and protection against injury in calcium-depleted cells with elevated α-tocopherol content was due neither to an α-tocopherol-mediated prevention of GSH oxidation to GSSG or Pr-SSG, nor to an inhibition of GSH efflux from the cell. Other possible explanations could be α-tocopherol-mediated increase in GSH biosynthesis or prevention of GSH loss to undetected pools, normally occurring in control cells.

To test the first possibility, the effect of vit E-succinate on GSH pools was monitored during the inhibition of glutathione biosynthesis. Incubation of both calcium-depleted and calcium-supplemented cells with BSO (0.5 mM), an inhibitor of the rate limiting step of GSH biosynthesis (32), resulted in a continuous loss of intracellular GSH to 2.2 ± 0.1 nmol/10^6 cells in calcium-depleted cells and to 5.7 ± 0.6 in calcium adequate cells (Fig. III.2). The addition of vit E-succinate did not inhibit the increased loss of GSH in the calcium-depleted cells treated with BSO, indicating that α-tocopherol enhanced the formation of intracellular GSH in non-BSO-treated cells by affecting biosynthesis of GSH.

The net changes in hepatocyte glutathione contents, intra- plus extracellular, over the 5 h incubation period are depicted in Table III.3. As previously mentioned, the total change in the levels of glutathione in calcium-depleted cells was 3.5 nmoles lower than in calcium-supplemented cells, or the apparent GSH synthesis rate was 1.64 nmol/10^6 cells/h as compared to 2.38 nmol/10^6 cells/h in controls. The addition of vit E-succinate to the calcium-depleted cells increased the rate of glutathione formation by 117% from 1.63 to 3.54 nmol/10^6 cells/h, a rate significantly greater (46%) than the rate of GSH formation in control cells. This stimulatory effect of vit E-succinate on glutathione formation was not observed in cells incubated with 3.5 mM Ca^{2+}.

Discussion:

The presence of extracellular Ca^{2+} appears to be required for maintenance of normal metabolic functions and protective systems in isolated rat hepatocytes. The absence of extracellular Ca^{2+} stimulates the release of calcium from intracellular storage and eventually out of the cell. Our working hypothesis, based on recent observations on chemically induced injury (33,34), is that the calcium, which is released abnormally, abruptly increases the usually low cytosolic free Ca^{2+} levels, triggering cell injury through the activation of various Ca^{2+} dependent processes.

Although cell death as determined by the leakage of cytosolic enzymes was not a consequence of the Ca^{2+} depletion described in this study, there was an associated cell injury as evidenced by the leakage of intracellular K^{+}, one of the more sensitive indicators of cell injury, since it precedes loss of LDH from the cell and the ability to exclude trypan blue (30). The events described herein can therefore be considered to reflect early changes in cellular defense systems during cell injury associated with altered intracellular calcium homeostasis.

Previous studies have shown that chemically induced cell death is accelerated when hepatocytes are incubated without extracellular Ca^{2+} (18-20). One possible explanation for this enhanced toxicity is our finding that the absence of extracellular Ca^{2+} results in diminished levels of intracellular GSH. Lower levels of intracellular GSH would reduce the capacity of the GSH protective

system and the proposed threshold level, necessary for cell survival (35), would be reached earlier.

Meredith and Reed (23,36) demonstrated that the onset of cell death by ethacrynic acid and adriamycin/BCNU, correlated better with the depletion of mitochondrial GSH than with that of cytosolic GSH. These investigators suggested that chemically induced cell death occurs in hepatocytes only after the GSH pool in mitochondria has been severely reduced. In view of this hypothesis, the diminished levels of mitochondrial GSH observed in hepatocytes incubated without calcium may also explain the increased susceptability of these cells to chemically induced cell death. However, since both pools of GSH are depleted at the same rate in our system, their hypothesis cannot be verified.

One major function of intracellular GSH is to maintain membrane protein thiol groups in the reduced form (37). Depletion of protein thiols affects many cell functions including membrane permeability and various enzymatic activities (38-40). Recent studies suggest that the impairment of Ca^{2+} sequestration, due to thiol oxidation in the Ca^{2+}-ATPases, is the crucial event leading to increased cytosolic free Ca^{2+} and hepatocellular injury (38). Consequently, hepatocytes incubated without extracellular Ca^{2+} and thus with lower levels of GSH, might be more susceptible to the toxic effects of compounds which form oxygen free radicals or electrophilic metabolites.

It has been suggested that the rate of GSH efflux is simply a reflection of the intracellular GSH content (41,42). This hypothesis is not supported by the present study in which hepatocytes with the

lowest intracellular GSH concentrations showed the highest GSH efflux rates. Increased rates of formation and release of GSSG from liver have been observed under conditions of oxidative stress (43-45) and are believed to reflect the intracellular activity of GSH peroxidase. Consequently, the observed increase in GSSG formation suggests that a Ca^{2+} free environment causes an "oxidative stress" in hepatocytes. Accelerated efflux of GSH, however, has not been associated with oxidative stress, so its significance is unknown, but possibly functions to maintain the extracellular thiol status that may be altered in the absence of Ca^{2+}.

Vit E-succinate supplementation of the Ca^{2+}-free medium prevented cell injury in the calcium depleted hepatocytes without affecting total intracellular Ca^{2+} levels, demonstrating that the maintenance of total intracellular calcium levels does not explain the protection afforded isolated hepatocytes incubated with extracellular Ca^{2+}. In addition to preventing cell injury, elevated levels of intracellular α-tocopherol mostly reversed the loss of GSH, supporting the contention that diminished levels of intracellular GSH were responsible for the cell injury observed in Ca^{2+} depleted cells. This GSH-sparing effect of vit E-succinate appeared to be mediated through an enhancement of GSH biosynthesis, since GSH levels were not increased by vit E-succinate when GSH biosynthesis was inhibited. Since GSSG formation was only partially and non-significantly inhibited by vit E-succinate, our data do not support the hypothesis that α-tocopherol directly replaced GSH as an antioxidant. Although a direct cytosolic action of α-tocopherol on the enzymatic

biosynthesis of GSH appears unlikely, this has been suggested as the mechanism of the observed increase in GSH content of human erythrocytes loaded with α-tocopherol (14). In partial support of this hypothesis, GSH formation and activity of γ-glutamylcysteine synthetase have been shown to be slightly, albeit non-significantly, diminished in vitamin E-deficient isolated rat hepatocytes after 5 h incubation (8). The nature of the α-tocopherol-mediated stimulation of GSH biosynthesis remains to determined.

We have shown that incubation of isolated hepatocytes in the absence of extracellular Ca^{2+} results in a loss of cytosolic and mitochondrial GSH which may explain the increased chemical toxicity seen under these incubation conditions. The GSH loss and resulting cell injury can be reversed by increased levels of cellular α-tocopherol, mediated through increased synthesis of GSH, indicating a close relationship between the two antioxidant systems.

Table III.1:

Intracellular levels of Ca^{2+}, K^{+}, and LDH in isolated hepatocytes incubated with or without extracellular Ca^{2+} and vit E-succinate*.

	Ca^{2+} (nmol/10^6 cells)	K^{+} (%initial content)	LDH
3.5 mM Ca^{2+}	16.4 ± 0.8*	85 ± 4*	76.6 ± 0.6
0 mM Ca^{2+}	2.14 ± 0.21	67 ± 3	74.4 ± 0.8
0 mM Ca^{2+} + vit E-succ.	2.21 ± 0.30	84 ± 3	78.8 ± 0.7

*Hepatocytes were incubated for 5 h in the presence of 0 mM or 3.5 mM extracellular Ca^{2+}, or 0 mM Ca^{2+} and 25 μM vit E-succinate. Ca^{2+}, K^{+}, and LDH levels were determined as described under materials and methods. Values are mean ± SEM, n=4-10. *$p < 0.05$ vs 0 mM Ca^{2+}.

Table III.2:

Efflux of GSH and GSSG from hepatocytes incubated with or without 3.5 mM Ca^{2+} and vit E-succinate*.

	GSH	GSSG
	(nmol GSH equiv./10^6 cells)	
3.5 mM Ca^{2+}	8.15 ± 0.82*	1.14 ± 0.38*
0 mM Ca^{2+}	15.8 ± 1.08	3.28 ± 0.80
0 mM Ca^{2+} + vit E-succinate	16.2 ± 1.48	2.63 ± 0.88

*Hepatocytes were treated as in Table III.1. Glutathione levels were assayed in the medium as described under materials and methods. Values are mean ± SEM, n=7. *$p < 0.05$ vs other treatments.

Table III.3:

Effect of vit E-succinate on net changes in the levels of total glutathione in hepatocytes incubated with or without 3.5 mM Ca^{2+}*.

	Intracellular GSH	extracellular GSH	GSSG	Total GSH formed/5 h	GSH /h
	(nmol GSH equiv./10^6 cells)				
- Ca^{2+}	-10.7	+15.8	+3.28	+8.4	1.63 ±0.4*
+ Ca^{2+}	+2.60	+8.15	+1.15	+11.9	2.38 ±0.1
- Ca^{2+} + vit E	-1.22	+16.3	+2.63	+17.7	3.54 ±0.1*
+ Ca^{2+} + vit E	+2.53	+8.10	+0.79	+11.4	2.28 ±0.1

*Hepatocytes were incubated as described in Table III.1. 0 h Glutathione was subtracted from 5 h glutathione. Values are mean ± SEM, n=7. *$p < 0.05$ vs all other treatments.

Figure III.1 Effect of extracellular Ca^{2+} and vit E-succinate on the levels of (A) cytosolic and (B) mitochondrial GSH in isolated hepatocytes. Hepatocytes were incubated in the presence of 0 mM (□), or 3.5 mM extracellular Ca^{2+} (■), or 0 mM Ca^{2+} and 25 μM vit E-succinate (▲), and GSH levels were determined as described under materials and methods. Values are mean ± SEM, n=7-10. *$p < 0.05$ vs other treatments.

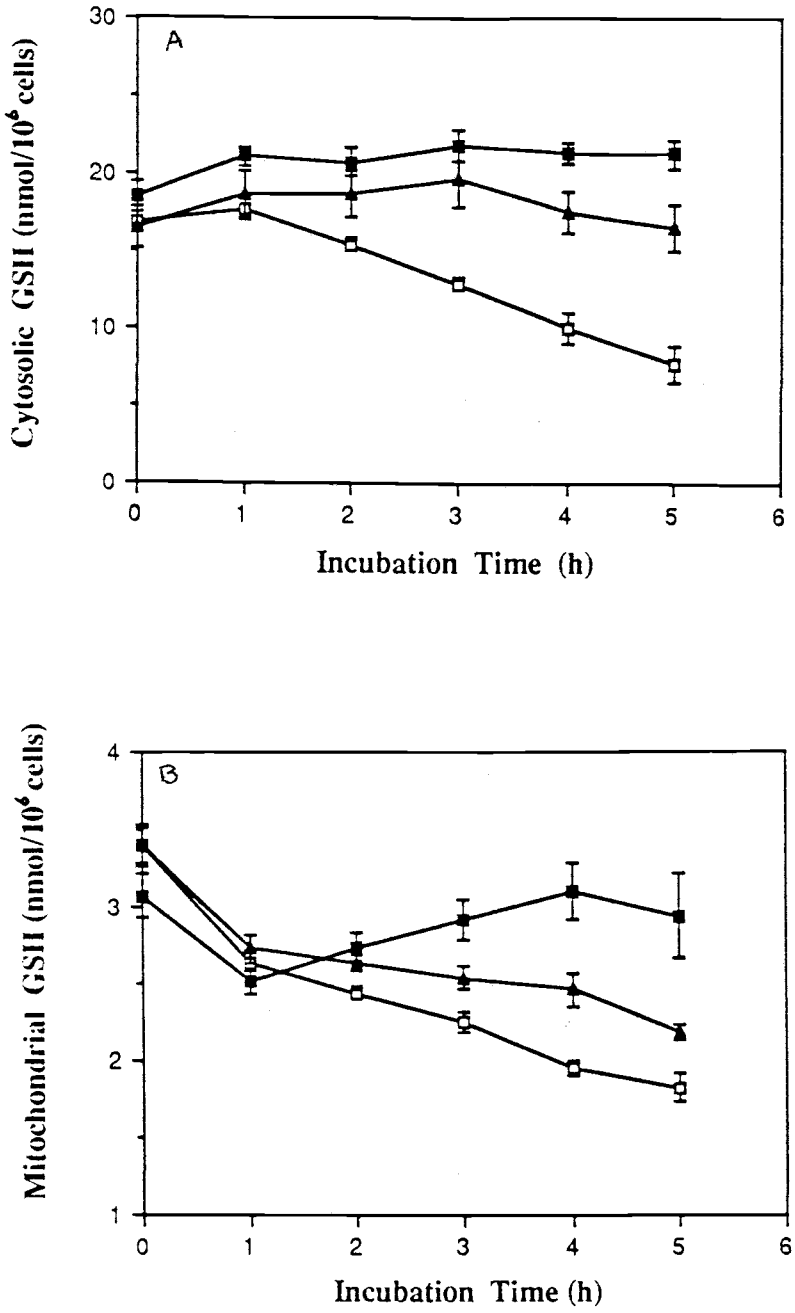
A
Cytosolic GSH (nmol/10⁶ cells)
30
20
10
0
0
1
2
3
4
5
6
Incubation Time (h)
B
Mitochondrial GSH (nmol/10⁶ cells)
4
3
2
1
0
1
2
3
4
5
6
Incubation Time (h)

Figure III.1

Figure III.2 Effect of BSO on the levels of intracellular GSH in isolated hepatocytes incubated with or without extracellular Ca^{2+} and vit E succinate. Hepatocytes were incubated in the presence of 0 mM (□), or 3.5 mM extracellular Ca^{2+} (■), or 0 mM Ca^{2+} and 25 μM vit E-succinate (▲) and GSH levels were determined as described under materials and methods. Values are mean ± SEM, n=5. *$p < 0.05$ vs other treatments.

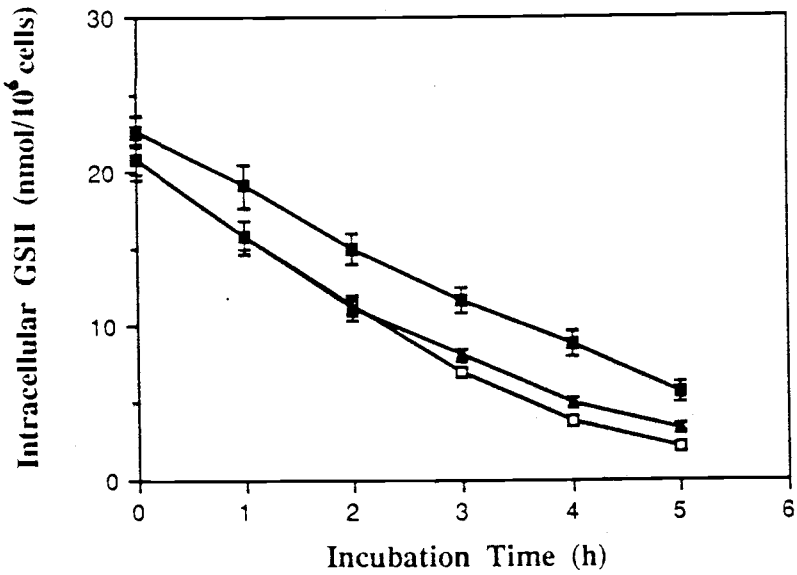
Intracellular GSH (nmol/10⁶ cells)
30
20
10
0
0
1
2
3
4
5
6
Incubation Time (h)

Figure III.2

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IV. MITOCHONDRIAL GLUTATHIONE AS A CRITICAL DETERMINANT IN CALCIUM IONOPHORE-INDUCED INJURY TO ISOLATED HEPATOCYTES

Abstract:

In this study the calcium ionophore, A23187, was used to determine the effects of disrupted Ca^{2+} homeostasis on cellular thiols and lipid peroxidation. Isolated rat hepatocytes were incubated with different concentrations of extracellular Ca^{2+} and A23187, to induce accumulation or loss of cellular Ca^{2+}. These treatments resulted in loss of mitochondrial and cytosolic glutathione (GSH), loss of protein-thiols, extensive lipid peroxidation and cell injury. This injury was dependent on the concentrations of ionophore and extracellular Ca^{2+}. An excellent correlation was found between cell injury and the loss of mitochondrial GSH, while the loss of cytosolic glutathione preceded both these events. The time course of protein-thiol loss appeared secondary to the loss of non-protein thiols while lipid peroxidation did not correlate with toxicity. In the absence of extracellular Ca^{2+}, the antioxidants α-tocopherol and diphenyl-p-phenylene-diamine both totally prevented A23187-induced cell injury and loss of mitochondrial GSH and thus dissociated the mobilization of intracellular Ca^{2+} from the expression of toxicity. In the presence of extracellular Ca^{2+}, cell injury as well as the loss of mitochondrial GSH were only partially prevented by antioxidant treatment. The mitochondrial calcium channel blocker, ruthenium red, protected hepatocytes from A23187-induced injury only in the absence

of extracellular Ca^{2+}. Leupeptin, an inhibitor of Ca^{2+}-activated proteases, and dibucaine, a phospholipase inhibitor, did not affect cytotoxicity. Our results demonstrate that the level of mitochondrial GSH is critical for cell survival during ionophore-induced perturbation of cellular Ca^{2+} homeostasis.

Introduction:

Glutathione, a thiol tripeptide that functions as an intracellular reductant, has been shown to play an important protective role in cell injury caused by a variety of chemicals (1-3). GSH exists in two separate pools in the cell, 85-90% in the cytoplasm and 10-15% in the mitochondria (4-6).

A protective role for mitochondrial GSH in cytotoxicity was first proposed by Meredith and Reed in 1982 (6). They showed that the onset of cell injury in isolated rat hepatocytes by ethacrynic acid correlated with the depletion of mitochondrial GSH, whereas the cytosolic pool could be depleted without affecting cell viability. Previously, several reports (7-11) had demonstrated that cytotoxicity, as measured by lipid peroxidation, liver necrosis and loss of intracellular enzymes *in vivo* and *in vitro*, occurred only if the intracellular concentration of GSH fell below 10-15% of the initial value, which is the same level found in mitochondria.

Mitochondria perform functions that are essential for cellular integrity, most of which depend on intact mitochondrial membranes. It has been estimated that approximately 2% of hepatic mitochondrial O_2 consumption generates H_2O_2 (12). Therefore, mitochondrial GSH

could play a critical role in the defense against endogenous membrane peroxidation and subsequent damage by reducing H_2O_2 via GSH peroxidase.

Mitochondrial GSH may also be important in regulating inner membrane permeability by maintaining intramitochondrial sulfhydryl groups in the reduced state (13,14). Certain proteins are highly sensitive to changes in the cellular thiol status, including the Ca^{2+} dependent ATP-ases (15) which serve as membrane bound Ca^{2+} pumps to maintain low levels of cytoplasmic Ca^{2+}. Interference of Ca^{2+} homeostasis and increased levels of cytoplasmic free Ca^{2+} are believed to trigger cell injury (16) that, if not reversed, will eventually lead to cell death. Ca^{2+} and thiol homeostases in the mitochondria are believed to be closely linked either directly (14) or through the pyridine nucleotides (17); an imbalance in one could affect the status of the other, and diminish cell viability.

The lipid soluble ionophore, A23187, has been widely used as an investigative tool to study the role of divalent cations, principally Ca^{2+}, in various biological systems (18-21). At physiological concentrations of extracellular Ca^{2+}, Reed and Lardy (18) observed that A23187 induced uptake and accumulation of Ca^{2+} in exchange for proton release from erythrocytes, and found that A23187 inhibited mitochondrial ATPase by releasing endogenous Mg^{2+}, while uncoupling oxidative phosphorylation by inducing an energy dissipating cyclic flux of Ca^{2+} (18). At low extracellular Ca^{2+} concentrations the ionophore induced the efflux of intracellular Ca^{2+} from isolated rat

hepatocytes (19). This ability of A23187 to move Ca^{2+} in and out of cells renders it especially useful in toxicological studies.

In this report we examined some of the biochemical consequences of altered Ca^{2+} homeostasis in isolated hepatocytes, induced by controlling the Ca^{2+} concentration in the extracellular medium in the presence of A23187. We demonstrate that the expression of A23187-induced toxicity strongly correlated with mitochondrial GSH depletion, which was preceded by the loss of cytosolic GSH, while the loss of protein-SH appeared to occur after the loss of non-protein thiols. Lipid peroxidation did not have a clear association with cell injury cused by A23187 and high extracellular Ca^{2+} concentrations.

Materials and Methods:

Isolation and Incubation of hepatocytes:

Parenchymal liver cells were prepared as previously described (22,23) from male Sprague-Dawley rats with access to standard rat chow ad-libitum. Freshly isolated hepatocytes were suspended in 125 ml culture flasks (2×10^6 cells/ml) in Fischer's medium (20 ml) modified as described (24), and supplemented with 10 mM Hepes. Fetal calf serum was omitted from the incubation medium. A23187 (2, 5, 20 μM from 4.8 mM stock solution), α-tocopheryl-succinate (vit E-succinate, 25, 200 μM from 25, and 50 mM stock solutions) (Sigma Chemical Co, St. Louis, MO) and diphenyl-p-phenylene-diamine (DPPD, 25 μM from 25 mM stock solution) (Aldrich Chemical Co,), were added

to the incubation medium in dimethyl sulfoxide (Me_2SO). Ethacrynic acid (100 μM) and dibucaine (100 μM) (Sigma) were added in ethanol, methionine (0.67 mM) and ruthenium red (RR, 25 μM) (Sigma) in H_2O, whereas leupeptin (0.2 mg/ml) and EGTA (in 2.5 mM excess of Ca^{2+}) (Sigma) were added directly to the incubation medium. Equivalent volumes of the vehicles were added to control flasks with no effect on the parameters measured. All agents, except the ionophore, were administered to the hepatocyte suspensions 15 min prior to the 0 h. A23187 was added immediately following the 0 h sampling. Each flask was slowly rotated at 37°C under a constant flow of water-saturated 95% O_2, 5% CO_2, and aliquots were removed hourly for analyses.

Isolation of mitochondria and preparation of cell pellets and extracts.

Mitochondria were isolated from incubated hepatocytes at each time point as described by Meredith and Reed (6). In brief, cells were disrupted in a 0.8 mg/ml digitonin buffer for 30 s and then immediately spun through a dibutyl phthalate oil layer into 10% perchloric acid, thus forcing the intact mitochondria through the oil leaving the cytoplasmic contents in the layer above the oil.

Similarly, viable hepatocytes were separated from nonviable cells and medium by centrifugation through dibutyl phthalate into 10% perchloric acid to release the intracellular contents for analysis (24).

Biochemical analyses:

Cell viability was monitored by measuring lactate dehydrogenase (LDH) activity in the incubation medium with a Beckman TR analyzer (25).

The number of viable cells and mitochondria isolated through the dibutyl phthalate oil layer were quantitated by analyzing the perchloric acid-precipitate for DNA content (26). Nuclei are not affected by the digitonin treatment, and are centrifuged through the oil layer along with the mitochondria, allowing for quantitation of nuclear DNA and therefore of viable cells used for mitochondrial analysis.

Acid-soluble glutathione pools were analyzed by the HPLC method of Reed et al. (27) with modifications as described (28). Oxidized glutathione in control cells was 1.4 ± 0.3% of the total glutathione. Protein thiols were analyzed in the acid-precipitated whole cell pellet with Ellman's reagent (29), with detection at 440 nm instead of 412 nm to avoid absorbance interference by A23187.

Lipid peroxidation was determined by measuring the amount of thiobarbituric acid (TBA)-reactive substances released into 0.25 ml of the cell incubation medium (24,30).

Determination of intracellular [Ca^{2+}] was done in the acid layer after centrifugation of whole cells through dibutyl phthalate, by atomic absorption spectrophotometry as described (24).

Statistics:

Values are expressed as sample means ± standard error (SEM). Student's "t" test was used to determine differences between sample means.

Results:

Effect of A23187 on cell viability and mitochondrial GSH:

This laboratory had previously proposed an important role for mitochondrial GSH in the expression of toxicity by several agents (6,31). Since Ca^{2+} appears to play a central role in cell injury, we investigated the relationship between mitochondrial GSH and perturbed Ca^{2+} homeostasis induced by A23187. As seen in table IV.1, the levels of total Ca^{2+} in isolated hepatocytes were markedly affected by calcium ionophore treatment; accumulation or loss of intracellular Ca^{2+} could be readily induced by controlling the A23187 and calcium concentration of the extracellular medium. Since an exchangeable pool of intracellular Ca^{2+} has been located in the mitochondria (32,33) and the accumulation of Ca^{2+} in hepatocytes (2.5 fold) was shown to be sequestered mainly by the mitochondria (34), this suggested that a major part of the intracellular Ca^{2+} flux induced by A23187 may have occurred at the mitochondria.

Exposure of the hepatocytes to A23187 (2, 5, 20 μM) also involved drastic changes in cell viability and thiol status. In the presence of 3.5 mM extracellular Ca^{2+}, ionophore treatment resulted in a dose-dependent loss of cell viability over a 5 h period (Fig.

IV.1A). With 20 μM A23187, a sharp increase in LDH leakage was observed at 1 h which gradually increased to almost total cell death at 5 h. With the control cells (0 μM A23187), LDH leakage remained less than 20% at 5 h. Similarly, A23187 induced a dose-dependent loss of mitochondrial GSH in the presence of 3.5 mM extracellular Ca^{2+} (Fig. IV.1B). Again with 20 μM A23187, a sharp decrease in the levels of mitochondrial GSH occurred early so that less than 50% of the initial GSH level remained at 1 h. Comparing panels A and B of Fig. IV.1 reveals a striking correlation between LDH leakage and depletion of mitochondrial GSH.

To further demonstrate the relationship between mitochondrial GSH and A23187-induced cell injury, LDH leakage and mitochondrial GSH levels were monitored during the experimental manipulation of extracellular Ca^{2+} concentrations during exposure of hepatocytes to 20 μM A23187. Sequentially lowering the levels of extracellular Ca^{2+} (3.5, 1, 0 mM) had the same effect as reducing the ionophore concentration, i.e. the toxicity of 20 μM A23187 was reduced when the extracellular Ca^{2+} concentration was lowered (Fig. IV.2A). Lowering the levels of extracellular Ca^{2+} also reduced the loss of mitochondrial GSH induced by 20 μM A23187 (Fig. IV.2B). However, a much greater loss of mitochondrial GSH in proportion to the extent of LDH leakage was seen in the absence than in the presence of extracellular Ca^{2+}, which might indicate a different mechanism of action of cell injury when Ca^{2+} is lost from the cell rather than accumulated by the cell. Linear regression analysis of cell

viability vs levels of mitochondrial GSH gave a correlation coefficient of r=0.90.

A23187 treatment also resulted in the loss of cytosolic GSH from hepatocytes (Fig. IV.3). This loss, however, preceded both the loss of mitochondrial GSH and the onset of cell injury (Fig. IV.2). This was particularly apparent in the absence of extracellular Ca^{2+} where 30% of the initial level of cytosolic GSH was lost after 1 h (Fig. IV.3), whereas mitochondrial GSH levels were unaffected until 2 h, and were followed by increased LDH leakage at 3 h (Fig. IV.2).

To further test our hypothesis, hepatocyte GSH pools were experimentally increased or decreased prior to A23187-administration. First, the hepatocytes were preincubated with the GSH precursor, methionine, for 2 hours, followed by A23187 administration. This treatment initially raised mitochondrial GSH by 2 nmol, and reduced the A23187-induced loss of cell viability and loss of mitochondrial GSH (Fig. IV.4A). Similarly, a 50% depletion of mitochondrial GSH by 30 min incubation with ethacrynic acid prior to A23187 administration greatly potentiated the toxicity of the ionophore (Fig. IV.4B).

Prevention of A23187-induced cell injury:

In attempts to ascertain the mechanism of A23187-induced GSH loss and toxicity, we investigated the effects of several agents on changes in cell viability and mitochondrial GSH levels induced by A23187.

Two antioxidants, α-tocopherol and DPPD, were found to reduce A23187-induced cell injury, essentially in the same manner. It should be noted, however, that in order to reach the same

intracellular concentration of α-tocopherol in all incubations, 200 μM vit E-succinate was used when Ca^{2+} was present in the medium, and 25 μM vit E-succinate was used in the absence of extracellular Ca^{2+}, since the Ca^{2+} status of the cells has been found to affect the hydrolysis of the vit E-ester (35). As seen in Fig. IV.5, the antioxidant-dependent protection was highly dependent on the Ca^{2+} concentration of the medium. In the absence of extracellular Ca^{2+}, A23187-induced cell injury was eliminated by both agents (from 40.8% without antioxidants to 19.5% LDH leakage at 5 h with DPPD, Figs. IV.2 and IV.5). At 1 mM extracellular Ca^{2+}, cell injury was substantially reduced (from 44.3% to 29.1%), whereas with 3.5 mM Ca^{2+} the antioxidants afforded protection only during the last two hours. In the same manner, DPPD (and vit E-succinate, data not shown) prevented A23187-induced loss of mitochondrial GSH (Fig. IV.5B). In the absence of extracellular Ca^{2+} almost no loss of mitochondrial GSH was seen over 5 h, whereas in the presence of extracellular Ca^{2+} the antioxidants delayed the loss of mitochondrial GSH, which correlated with delayed loss of cell viability.

The calcium chelator, EGTA, in 2.5 mM excess of extracellular Ca^{2+}, prevented both the early sharp rise in cell injury and the loss of mitochondrial GSH caused by 20 μM A23187 and Ca^{2+}. The extent of cell injury and loss of mitochondrial GSH resembled that observed in the absence of extracellular Ca^{2+} (data not shown). Furthermore, in the presence of EGTA, cell injury and loss of mitochondrial GSH were slightly less than that observed in the absence of extracellular Ca^{2+}; LDH leakage was 31.2 ± 2.3% versus 40.8 ± 3.9% after 5 h (Table

IV.2). To test whether this small but significant protection might be due to EGTA prevention of Ca^{2+} cycling across mitochondrial membranes, the hepatocytes were treated with A23187 and the mitochondrial calcium channel blocker, RR. As seen in table IV.2, RR markedly increased ionophore toxicity in the presence of 3.5 mM extracellular Ca^{2+} but only slightly with 1 mM Ca^{2+} when the calcium gradient across the cell was smaller. However, in the absence of extracellular Ca^{2+}, RR partially prevented A23187-induced injury, supporting our hypothesis that Ca^{2+} cycling was at least partially responsible for cell injury when the hepatocytes were incubated in the absence of extracellular Ca^{2+}.

Finally, dibucaine, a phospholipase inhibitor and leupeptin, an inhibitor of Ca^{2+}-activated nonlysosomal proteases (36), had no protective effect on cell injury (Table IV.2) nor loss of mitochondrial GSH induced by 20 μM A23187 in the presence of 1 mM Ca^{2+} (data not shown).

Effect of A23187 on protein thiols and lipid peroxidation:

Loss of cellular protein thiols and stimulation of lipid peroxidation are both commonly associated with chemical-induced cell injury. These parameters were monitored during A23187-induced cytotoxicity to investigate the effects of perturbed Ca^{2+} homeostasis on membrane components. Protein-SH levels were decreased by A23187 treatment (Fig. IV.6), although little correlation with cell injury was found. For example, although very little difference was seen in protein-SH levels between hepatocytes incubated with 0 mM or 1 mM Ca^{2+}, with or without antioxidants, these treatments resulted in very

different patterns of LDH leakage, as seen in Fig. IV.5A. With 3.5 mM Ca^{2+}, the steady loss of protein-SH, which was preventable by DPPD for 1 h, was in contrast with the early sharp drop in cell viability that was not preventable by DPPD (Fig. IV.5A). By comparing Figs. IV.5 and IV.6 it is apparent that the protein-SH levels in ionophore treated cells decreased later than the mitochondrial GSH levels, and after the onset of cell injury.

Unusually high levels of TBA-reactive materials, a measure of lipid peroxidation, were found in the medium of hepatocytes incubated with A23187. Ionophore treatment resulted in a dose-dependent stimulation of lipid peroxidation (data not shown) in agreement with the dose-dependent extent of cell injury (Fig. IV.1A). However, 20 μM A23187 was found to induce greater levels of TBA-reactive materials with decreasing levels of extracellular Ca^{2+} (Fig. IV.7), in sharp contrast to its effects on cell injury, whereby the least amount of lipid peroxidation was observed with 3.5 mM Ca^{2+} with which LDH leakage was greatest (Fig. IV.2B). This does not preclude the possibility that lipid peroxidation contributed to the cell injury in the absence of extracellular Ca^{2+}, when both lipid peroxidation and cell injury were totally reversed by antioxidants. To test this possibility, the relationship between A23187-induced cell injury and lipid peroxidation in the absence of extracellular Ca^{2+} was investigated. As seen in Table IV.3, the toxicity of A23187 was not dose-dependent in the absence of extracellular Ca^{2+}; all three doses induced the same loss of cell viability. However, again lipid peroxidation was dependent on the ionophore dose. These data

indicate that lipid peroxidation can be dissociated from a role in A23187-induced cytotoxicity. In further support of this, although both vit E-succinate and DPPD also totally eliminated lipid peroxidation in the presence of Ca^{2+}, they reduced LDH leakage only by 6-20% (Fig. IV.5 and Table IV.2).

Discussion:

In the present study, ionophore-induced perturbation of cellular Ca^{2+} homeostasis was utilized to demonstrate the critical role of mitochondrial GSH in cell injury. Numerous evidence suggest that increased levels of cytoplasmic free Ca^{2+} precede the onset of cell death (16). However, whether this increase results from the influx of Ca^{2+} from the environment (37,38) or from the release of stored intracellular Ca^{2+} to the cytosol (39,40) has been extensively debated. Both influx of extracellular Ca^{2+} induced by A23187, as well as the disturbance of intracellular Ca^{2+} homeostasis induced by A23187 in the absence of extracellular Ca^{2+}, caused marked decreases in the levels of mitochondrial GSH that were in excellent correlation with the loss of cell viability. Ionophore-induced cell injury and loss of mitochondrial GSH were reduced by lowering the dose of A23187 or by lowering the Ca^{2+} concentration in the medium. Furthermore, A23187-induced toxicity was alleviated by increasing the initial GSH content of the mitochondria by preincubation of the hepatocytes with methionine, and was enhanced dramatically by depleting mitochondrial GSH by 50% prior to ionophore administration.

Further evidence for the importance of mitochondrial GSH in cell injury is provided by the observations that agents which afforded protection against the loss of mitochondrial GSH also reduced cell injury. The antioxidants DPPD and vit E-succinate reduced the loss of mitochondrial GSH during ionophore treatment and inhibited the loss of cell viability. Since ionophore-induced Ca^{2+} loss or accumulation in the hepatocytes was not affected by the presence of vit E or DPPD (data not shown), we conclude that the antioxidants did not prevent the incorporation of A23187 into cellular membranes. We have recently hypothesized that vit E-succinate prevents both chemical-induced and non-chemical-induced injury to hepatocytes via the maintenance of non-protein (41) and protein thiols (42-44). The ability of DPPD to similarly prevent losses of protein-SH groups and mitochondrial GSH, concomitant with maintenance of cell viability, suggests that the thiol-sparing action is not specific to vit E, but is related to its antioxidant properties. Whether antioxidants such as vit E or DPPD function to preserve thiol levels specifically in the mitochondrion as a protective mechanism against chemical-induced toxicity remains to be investigated.

Particularly striking in this study was the effect of the antioxidants on the loss of mitochondrial GSH and cell viability in the absence of extracellular Ca^{2+}. Under these conditions, an ionophore-induced loss of 80% of mitochondrial GSH was completely prevented by the presence of DPPD or vit E-succinate, which correlated well with their total prevention of cell injury. Thus,

the ionophore-induced toxicity in the absence of extracellular Ca^{2+} was not the direct result of the loss of cellular Ca^{2+}, but was related to the consequences of alterations in intracellular Ca^{2+} homeostasis that were preventable by an increased antioxidant level in the cell. Our evidence thus appears to dissociate the mobilization of stored intracellular Ca^{2+} from the expression of toxicity in these cells, although we are unable to say whether a significant increase in cytosolic free Ca^{2+} accompanied the ionophore treatment.

On the other hand, the lack of total prevention by the antioxidants of A23187-induced toxicity in the presence of Ca^{2+} probably indicates a different mechanism of toxicity associated with the influx of extracellular Ca^{2+}. Further support for this contention was lent by the differential action of RR on A23187-induced toxicity. In the absence of Ca^{2+}, RR partially prevented cell injury, whereas it potentiated toxicity in the presence of Ca^{2+}. This indicates that Ca^{2+} cycling and resulting ATP depletion played a role in cell injury only when the flux of Ca^{2+} was directed out of the cell. In the presence of Ca^{2+}, increased toxicity might be due to increased levels of cytoplasmic free Ca^{2+}, when re-uptake of Ca^{2+} into the mitochondria was inhibited by RR.

Recently, various investigations have reported that protein thiols, more so than non-protein thiols, are critical for the maintenance of cell viability during toxic chemical insult (45-47). In fact, we have similarly shown that, in the near total absence of intracellular GSH, cell viability ultimately depended on the

maintenance of protein thiol levels (42). In the present study, the findings that LDH leakage from hepatocytes exposed to A23187 showed a better correlation with mitochondrial GSH loss than with cellular protein thiol loss may indicate that cytotoxicity due specifically to altered Ca^{2+} homeostasis is primarily related to loss of mitochondrial function. Furthermore, this suggests that intracellular Ca^{2+} homeostasis and the mitochondrial thiol redox system are intimately linked in maintaining cell viability.

It has also been proposed that following the loss of membrane protein thiol groups and subsequent increase in cytoplasmic Ca^{2+}, activation of phospholipases (48) or nonlysosomal proteases (36) by Ca^{2+} triggers the final cell membrane damage and ultimate loss of cell viability. The failure of dibucaine and leupeptin to prevent Ca^{2+}-induced toxicity in our system does not support these hypotheses, although the use of other known inhibitors of these enzymes would be necessary to exclude these processes as a final pathway of toxicity.

The role of lipid peroxidation in cytotoxicity is still unclear (49). A23187-treatment induced very high levels of TBA-reactants in hepatocytes, 4-6 times that of non-ionophore-treated cells. It is unclear why lipid peroxidation accompanies ionophore treatment, since A23187 itself is not an oxidant, but it probably relates to the type of oxidative injury we have reported to be present in Ca^{2+}-depleted cells (28,41). However, no apparent relationship existed between the extent of lipid peroxidation and A23187-induced cell injury.

Many studies have indicated a role for thiol groups in the retention and transport of Ca^{2+} from mitochondria (14,17,50) and our findings herein suggest that these two processes and mitochondrial GSH are interrelated and closely regulated. The link between Ca^{2+} flux and mitochondrial GSH is not obvious, but may lie in changes in the distribution of various ions and therefore loss of regulation within the mitochondria. The importance of Ca^{2+} cycling, ATP levels, and membrane potential in relation to the loss of mitochondrial GSH are currently under investigation in our laboratory.

Table IV.1

Dose-response of A23187 on the levels of Ca^{2+} in isolated hepatocytes incubated in the presence or absence of 3.5 mM extracellular Ca^{2+}*.

A23187 (μM)	0	2	5	20
- Ca^{2+}	2.06 ±.21	1.55 ±.13	1.24 ±.26	1.07 ±.18
+ Ca^{2+}	11.0 ±0.4	15.8 ±2.4	19.7 ±2.8	26.5 ±4.5

*Hepatocytes, 2 x 10^6 cells/ml, were incubated in a modified Fischer's medium with 10 mM Hepes, 0.2 mM cystine and with or without 3.5 mM $CaCl_2$ at 37°C under an atm. of 95% O_2, 5% CO_2, for 5 h. The calcium ionophore, A23187, was added in Me_2SO after the 0 h time point. Whole cell Ca^{2+} (nmol/10^6 cells) was determined in viable hepatocytes after 1 h incubation, as described under materials and methods. Values are mean ± SEM, n=4.

Table IV.2

Effect of several agents on LDH leakage (% total) from hepatocytes incubated for 5 h with 20 μM A23187 and different concentrations of extracellular Ca^{2+}*.

Agent	none	vit E	EGTA	RR	dibu.	leup.
0 mM Ca^{2+}	40.8 ±3.9	20.7* ±2.0	31.2* ±2.3	33.1* ±2.4		
1 mM Ca^{2+}	44.3 ±2.5	29.5* ±5.5	30.8* ±1.5	49.1 ±4.0	54.7 ±3.1	56.2 ±5.1
3.5 mM Ca^{2+}	72.0 ±6.0	66.1 ±3.0	30.7* ±3.1	100*		

*Hepatocytes were treated as in Fig. IV.2. [Vit E] = 25 μM without Ca^{2+} or 200 μM with Ca^{2+} in the medium. EGTA was added to the medium in 2.5 mM excess of Ca^{2+}. [RR] = 25 μM, [dibucaine] = 100 μM, and [leupeptin] = 0.2 mg/ml. Values are mean ± SEM, n=3-8, except for dibu. and leup. mean ± range, n =2. * $p < 0.05$ vs "none".

Table IV.3

Dose-response of A23187 on LDH leakage and lipid peroxidation in hepatocytes incubated for 5 h in the absence of extracellular Ca2+.*

A23187 (μM)	0	2	5	20
LDH activity (% total)	25.6* ±0.8	38.5 ±2.4	44.3 ±5.1	40.8 ±0.9
TBA reactants (nmol/10^6 cells)	3.18* ±.13	5.34* ±.20	8.58* ±.84	10.95* ±.16

*Hepatocytes were treated as in Table IV.1, except without $CaCl_2$ in the medium. Lipid peroxidation was determined by measuring the amount of TBA-reactive substances in 0.25 ml of the cell incubation medium as described under materials and methods. Values are mean ± SEM, n=4-9. * $p < 0.05$ vs all other treatments.

Figure IV.1 Dose-response of A23187 on (A) LDH leakage and (B) mitochondrial GSH levels of isolated hepatocytes incubated in the presence of 3.5 mM extracellular Ca^{2+}. Hepatocytes were incubated as described in table IV.1 but with 3.5 mM $CaCl_2$ in the medium. The calcium ionophore, A23187, 0 μM (○), 2 μM (□), 5 μM (●), 20 μM (■), was added in Me_2SO after the 0 h time point. LDH activity in the medium is indicated as the % of the total LDH activity in the cells. Mitochondrial GSH was determined as described under materials and methods. Values are mean ± SEM, (A) n=3-8, (B) n=5-8. * $p < 0.05$ vs all doses, ** p, 0.1 vs 2 μM A23187.

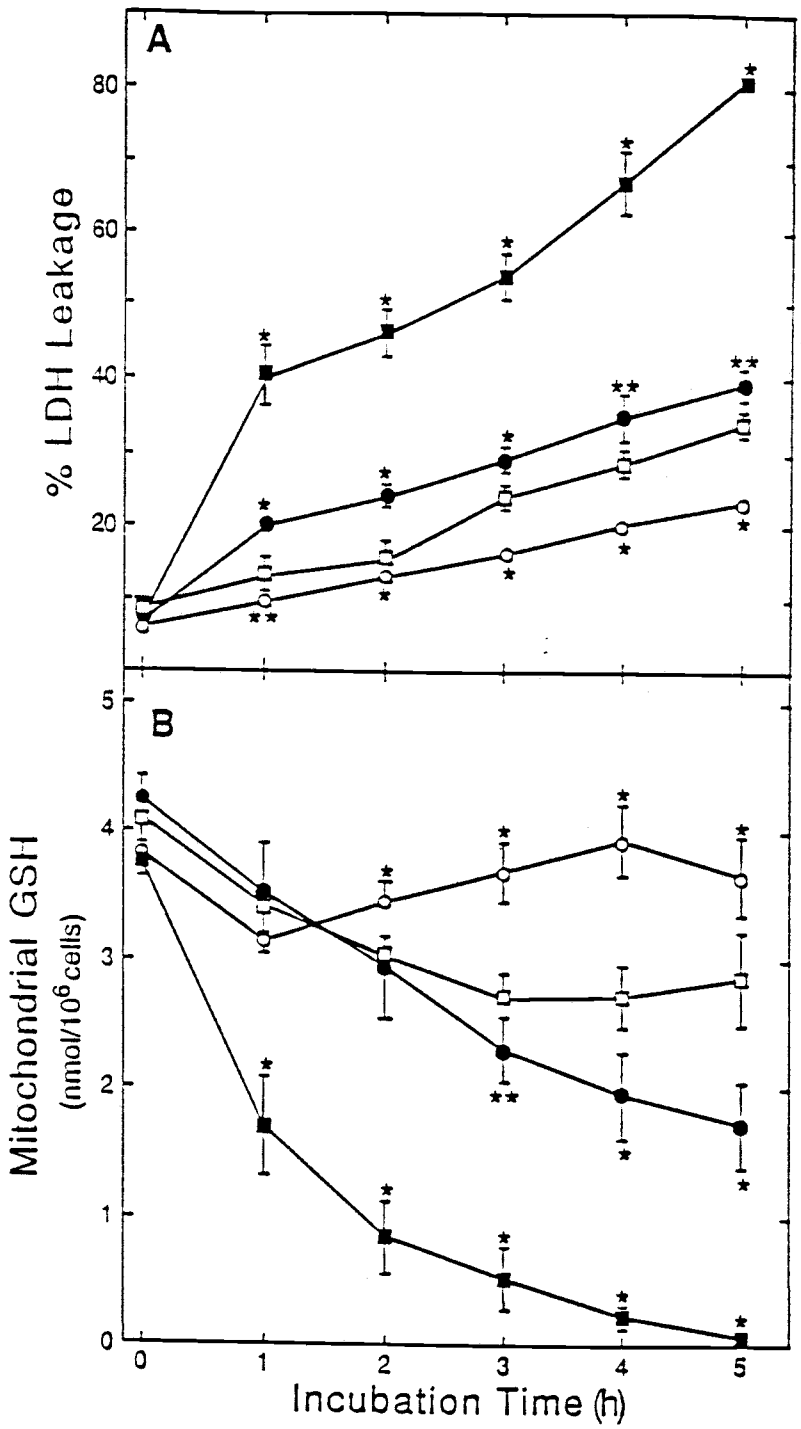
A
B
% LDH Leakage
Mitochondrial GSH
(nmol/10^6cells)
Incubation Time (h)
0
20
40
60
80
0
1
2
3
4
5

Figure IV.1

Figure IV.2 Effect of different extracellular Ca^{2+} concentrations on (A) LDH leakage and (B) mitochondrial GSH levels in A23187-treated hepatocytes. Hepatocytes were treated as in Fig. IV.1 except with 0 mM (○), 1 mM (□) or 3.5 mM extracellular Ca^{2+} (△). 20 μM A23187 in Me_2SO was added at 0 h. Values are mean ± SEM, n=3-8. * $p< 0.05$ vs all treatments, ** $p< 0.05$ vs 3.5 mM Ca^{2+}.

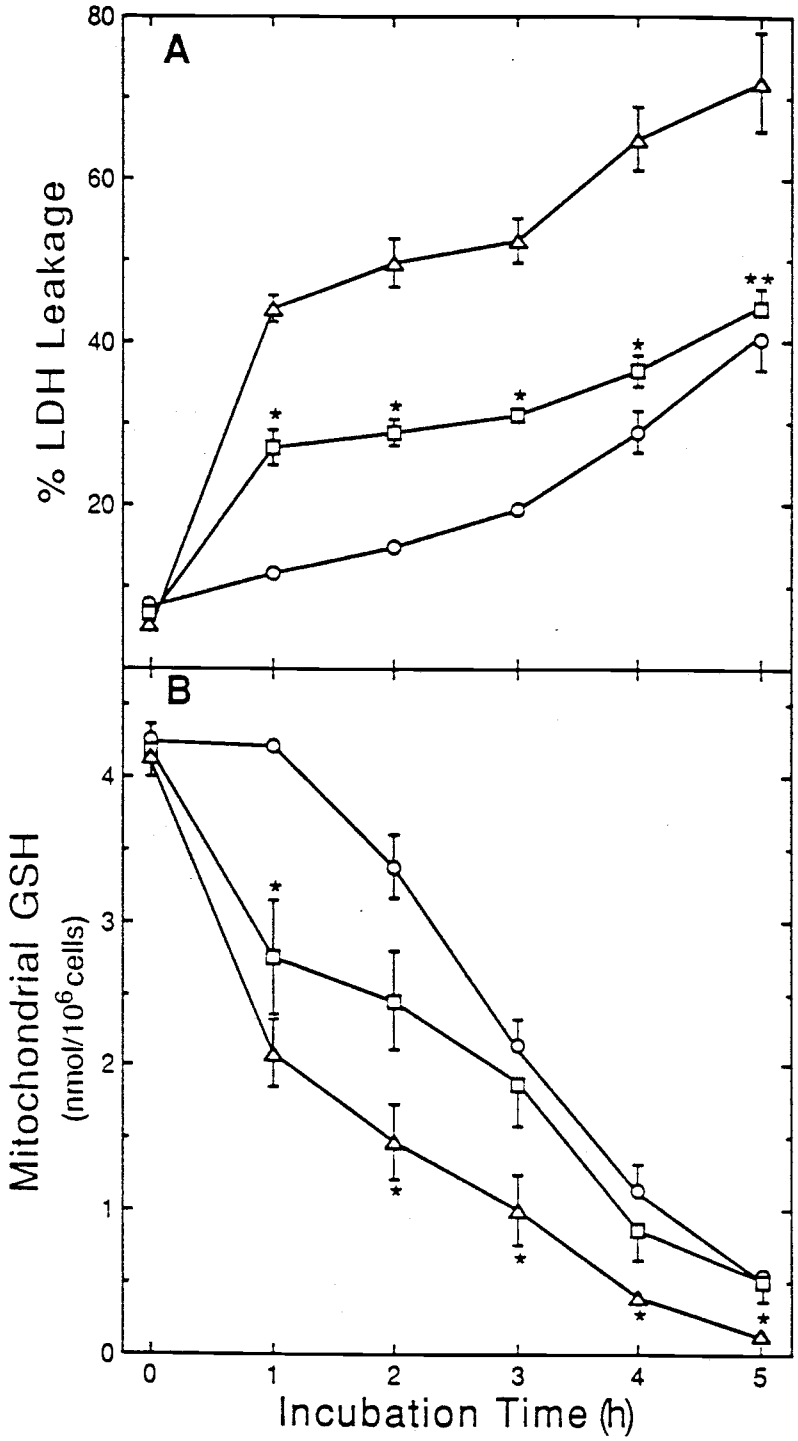
A
B
% LDH Leakage
Mitochondrial GSH
(nmol/10^6 cells)
Incubation Time (h)
0
20
40
60
80
1
2
3
4
5
*
**

Figure IV.2

Figure IV.3 Effect of different extracellular Ca^{2+} concentrations on the levels of cytoplasmic GSH in A23187-treated hepatocytes. Hepatocytes were treated as in Fig. IV.2 with 0 mM (o), 1 mM (□), 3.5 mM (△) extracellular Ca^{2+}. Values are mean ± SEM, n=3-8. * $p < 0.05$ vs all treatments.

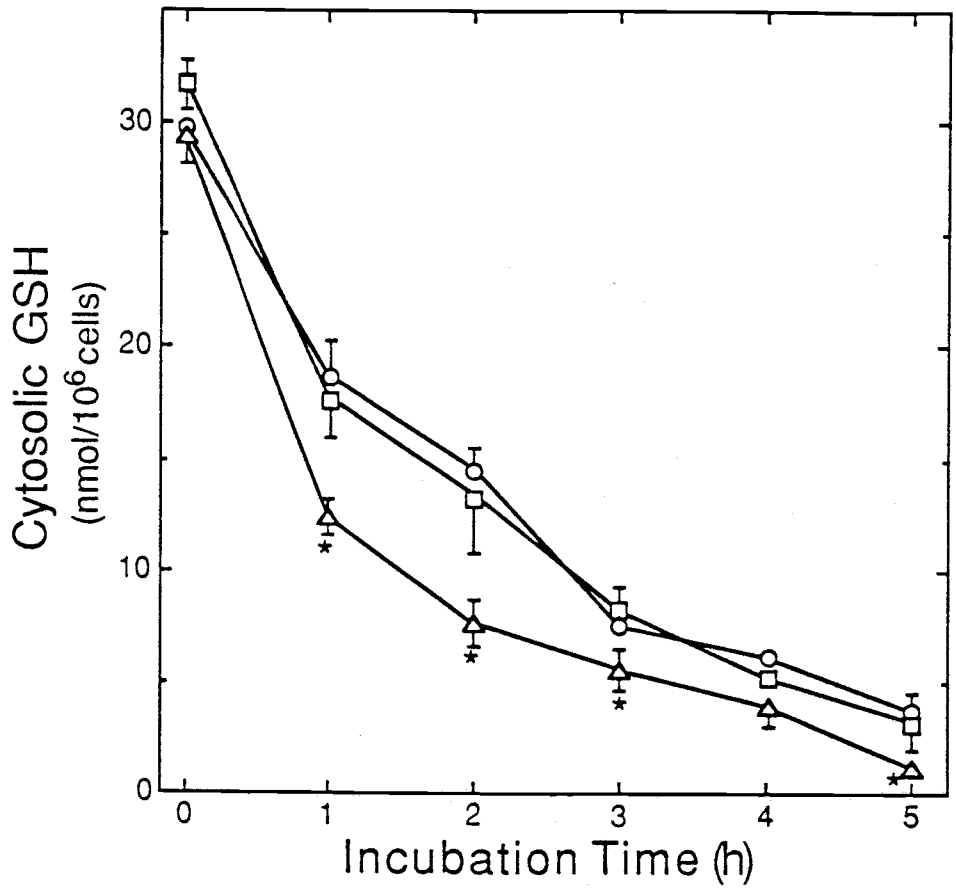
Cytosolic GSH
(nmol/10^6cells)
30
20
10
0
0
1
2
3
4
5
Incubation Time (h)
*
*
*
*

Figure IV.3

Figure IV.4 Effect of manipulations of the initial GSH levels in isolated hepatocytes on the LDH leakage (upper panels) and loss of mitochondrial GSH (lower panels) induced by A23187. A: Hepatocytes were incubated in modified Fischer's medium supplemented with 10 mM HEPES, 1 mM $CaCl_2$ and 0 mM (o) or 0.67 mM methionine (●). After 2 h incubation the cells were resuspended in fresh medium without methionine and 20 μM A23187 was added at time 0 h. B: Hepatocytes were incubated as in Table IV.1 except with 1 mM $CaCl_2$ and 0 μM (□) or 100 μM ethacrynic acid (■). After 30 min incubation, the cells were resuspended in fresh medium without ethacrynic acid and 20 μM A23187 was added at time 0 h. One experiment representative of three.

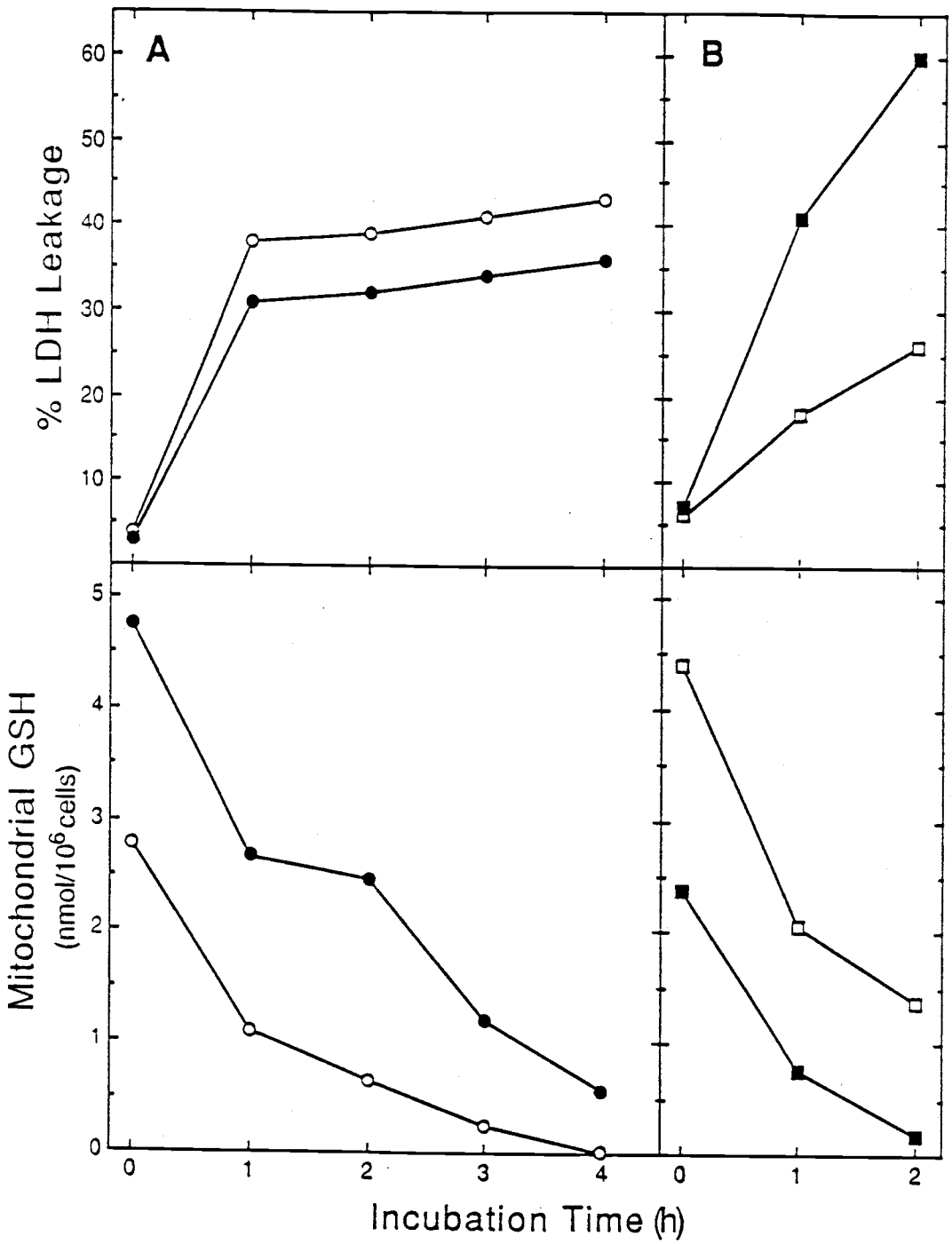
A
B
% LDH Leakage
Mitochondrial GSH
(nmol/10^6 cells)
Incubation Time (h)
0
10
20
30
40
50
60
1
2
3
4
5

Figure IV.4

Figure IV.5 Effect of DPPD[#] on (A) LDH leakage and (B) mitochondrial GSH levels in A23187-treated hepatocytes. Hepatocytes were treated as in Fig. IV.2 with 0 mM (●), 1 mM (■), 3.5 mM extracellular Ca^{2+} (▲), and with 25 μM DPPD. 20 μM A23187 in Me_2SO was added at 0 h. Values are mean ± SEM, n=4-8. * $p < 0.05$ vs - DPPD (Fig. IV.2).
[#]Results with vit E were essentially identical, see Table IV.2.

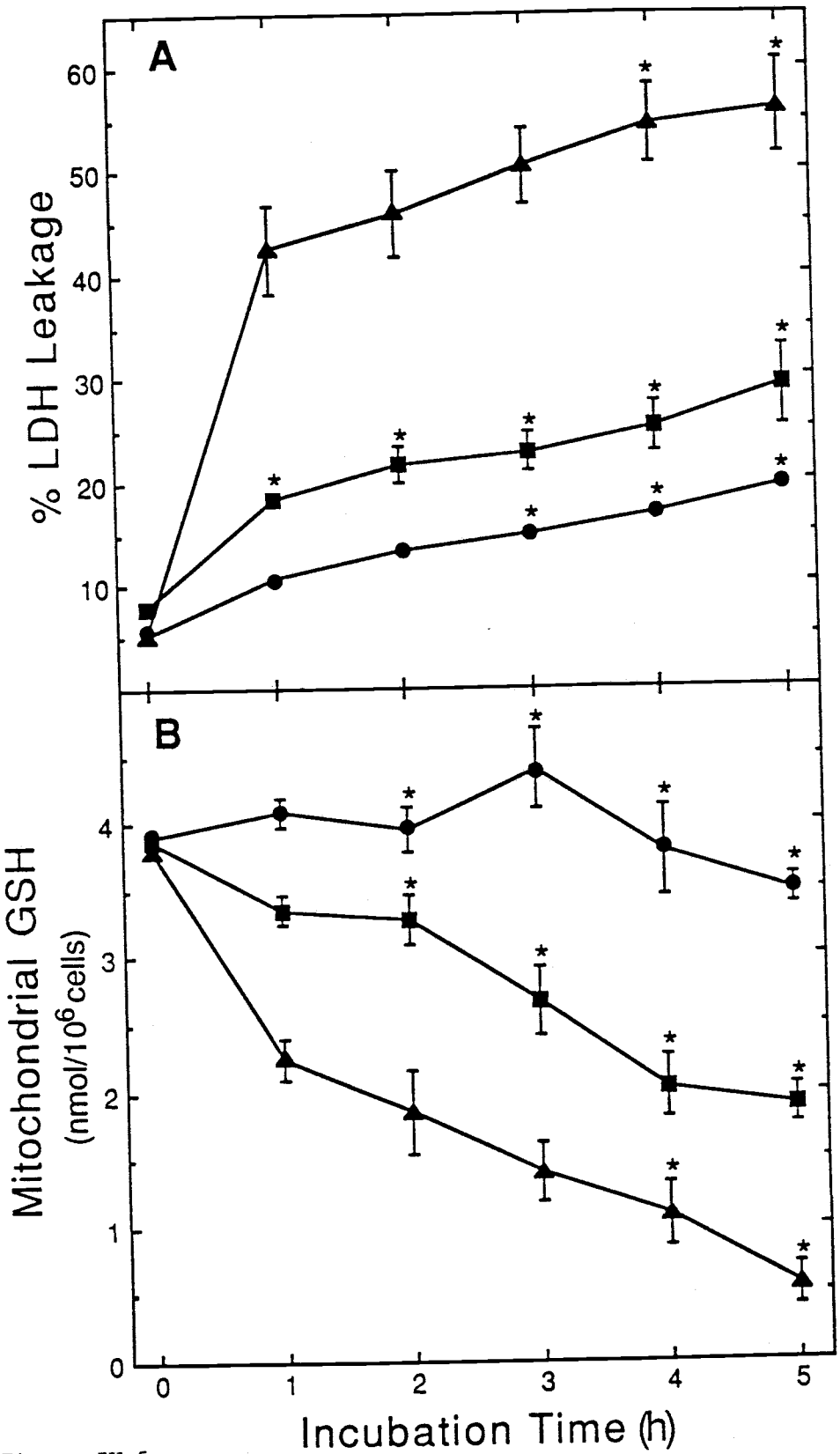
A
% LDH Leakage
60
50
40
30
20
10
B
Mitochondrial GSH
(nmol/10^6 cells)
4
3
2
1
0
0
1
2
3
4
5
Incubation Time (h)

Figure IV.5

Figure IV.6 Effects of different extracellular Ca^{2+} concentrations and DPPD on protein thiol levels in A23187-treated hepatocytes. Hepatocytes were treated as in Fig. IV.2, with 0 mM (○,●), 1 mM (□,■), 3.5 mM extracellular Ca^{2+} (△,▲) and with (filled symbols) or without (open symbols) 25 μM DPPD. Protein-SH levels at 0 h were 147.4 ± 2.6 nmol/10^6 cells and were determined as described under materials and methods. Values are mean ± SEM, n=5-8. * $p < 0.05$ vs + DPPD.

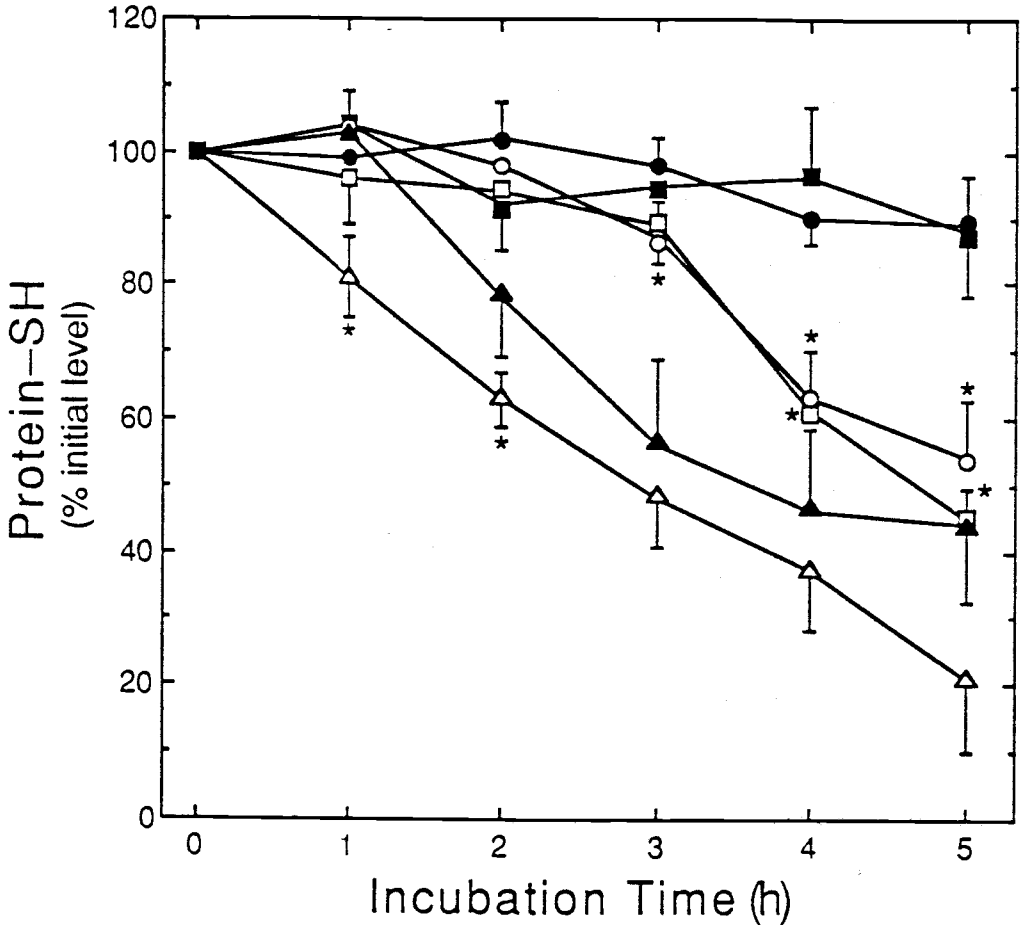
Protein–SH
(% initial level)
120
100
80
60
40
20
0
0
1
2
3
4
5
Incubation Time (h)

Figure IV.6

Figure IV.7 Effect of different extracellular Ca^{2+} concentrations on A23187-induced lipid peroxidation in isolated hepatocytes. Hepatocytes were treated as in Fig. IV.2 with 0 mM (○), 1 mM (□), 3.5 mM extracellular Ca^{2+} (△). Lipid peroxidation was determined by measuring the amount of TBA-reactive substances in 0.25 ml of the cell incubation medium as described under materials and methods. Values are mean ± SEM, n=3-8. * $p < 0.05$ vs all treatments.

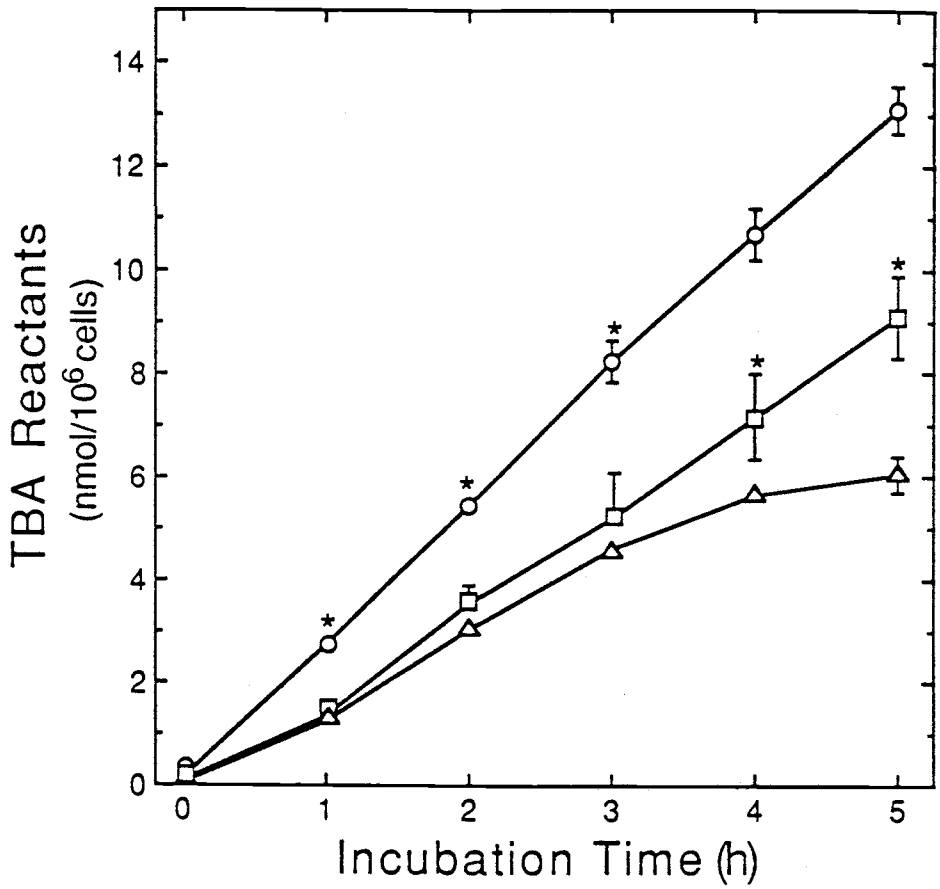
TBA Reactants
(nmol/10^6 cells)
Incubation Time (h)
0
2
4
6
8
10
12
14
0
1
2
3
4
5
*
*
*
*
*

Figure IV.7

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V. RETENTION OF OXIDIZED GLUTATHIONE BY ISOLATED RAT LIVER MITOCHONDRIA DURING HYDROPEROXIDE TREATMENT

Abstract:

The addition of tertiary-butyl hydroperoxide (t-BuOOH) to isolated mitochondria resulted in oxidation of approximately 80% of the mitochondrial reduced glutathione (GSH) independent of the dose of t-BuOOH (1-5 mM). Although t-BuOOH administration affected the respiratory control ratio (RCR), the mitochondria remained coupled and loss of the matrix enzyme, citrate synthase, was not increased over the control and was less than 3% over 60 min. A slow loss of GSH out of the coupled non-treated mitochondria was not increased by t-BuOOH treatment, in fact, a dose-dependent drop of GSH levels occurred in the medium. However, no GSSG was found outside the mitochondria indicating the necessary involvement of enzymes in the t-BuOOH-induced conversion of GSH to GSSG. The absence of GSSG in the medium also suggests that, unlike the plasma membrane, the mitochondrial membranes do not have the ability to export GSSG, as a response to oxidative stress.

Concomitant with the oxidation of GSH inside the mitochondria was the formation of GSH-protein mixed disulfides (Pr-SSG), with approximately 1% of the mitochondrial protein thiols involved. However, depending on the dose of t-BuOOH, the levels of GSH recovered very quickly to control levels, via the reduction of oxidized GSH (GSSG) and a slower reduction of Pr-SSG.

Our results demonstrate the inability of mitochondria to export GSSG during oxidative stress and may explain the protective role of mitochondrial GSH in cytotoxicity.

Introduction:

Endogeneous oxidative stress is an unavoidable consequence of aerobic metabolism, which in eucaryotes occurs mostly in the mitochondria. Reduction of oxygen in the respiratory chain is often incomplete and involves the formation of toxic oxygen intermediates. It has been estimated that 2% of mitochondrial O_2 consumption generates H_2O_2 (1). H_2O_2 can lead to the formation of the very reactive hydroxyl radical and if unchecked will result in the formation of lipid hydroperoxides that can damage mitochondrial membranes and function.

Since mitochondria have no catalase (2), they rely solely on GSH peroxidase to detoxify hydroperoxides (1). GSH peroxidase utilizes the reducing equivalents of GSH, the most abundant cellular non-protein thiol, with 10-15% located in the mitochondria (3-5). Mitochondrial GSH has been shown to be a critical target in cell injury induced by ethacrynic acid (5) and the calcium ionophore, A23187 (Olafsdottir, K., and Reed, D. J., unpublished results). Reduction of endogeneous hydroperoxides, via GSH peroxidase, may be the key function of mitochondrial GSH. This reaction results in the formation of oxidized GSH (GSSG), which is reduced back to GSH via

GSSG reductase, also present in the mitochondria (6), with the consumption of reducing equivalents from NADPH.

GSH and pyridine nucleotide oxidation in mitochondria have been shown to increase the permeability of the inner membrane to Ca^{2+} (7,8) through the oxidation of protein thiol groups (9). This suggests that oxidative stress or severe GSH depletion may affect the redox status in the mitochondria enough to alter intracellular Ca^{2+} homeostasis, which is believed to be an early step in cell injury (10,11). During severe oxidative stress, high levels of GSSG could have deleterious effects on cell integrity and metabolic processes. Several investigators have shown that GSSG is actively released from cells undergoing an oxidative challenge (12-15). Eklöw et al. (15) demonstrated a rapid and extensive release of GSSG, that totally accounted for the loss of intracellular GSH, from hepatocytes treated with t-BuOOH, with only a transient increase in GSSG seen inside the cells before efflux. The GSSG efflux was preceded by a marked decrease in the cellular NADPH/$NADP^+$ redox level, supporting an earlier contention (16) that the GSSG efflux pathway is a relieve valve for the cell to avoid highly oxidative states. No evidence exists for the operation of this pathway in the mitochondria, although it has been assumed to exist (17).

Experiments by ourselfes (Olafsdottir, K., and Reed, D. J., unpublished results) and others (17) have not provided any evidence for biosynthesis of GSH inside mitochondria. On the other hand, uptake of GSH into hepatocyte mitochondria could not be demonstrated experimentally in our laboratory, and similar experiments by others

using isolated mitochondria have been negative (3). The origin of mitochondrial GSH is thus presently unknown.

In the present study, we attempted to characterize further some properties of the mitochondrial pool of glutathione by inducing oxidative stress in isolated mitochondria. We demonstrate the presence of a dynamic GSH system inside mitochondria, capable of detoxifying low doses of organic peroxides, but without the release of GSSG into the medium. This may indicate a greater susceptibility to protein thiol oxidation inside the mitochondria than in the rest of the cell.

Materials and Methods:

All chemicals were reagent grade or better. t-BuOOH was purchased as a 95% solution in ethanol from Sigma (St. Louis, MO, USA).

Preparation of mitochondria:

Liver mitochondria were isolated from 250-300g, fed, male Sprague-Dawley rats, by the method of Schnaitman and Greenwalt (18) and washed 2 x in the isolation medium, which contained 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mg/ml BSA (defatted), pH 7.4, and stored on ice until used. The yield of mitochondria was 25-35%. Cytosolic contamination was assessed by determining the lactate dehydrogenase (LDH) activity (19) in the mitochondrial suspension and was determined to be negligible after two washes.

Incubations:

Mitochondria were resuspended at 5 mg/ml, in a medium containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris/Cl, 5 mM succinate, 1m M EDTA, pH 7.4, and with 0.01% BSA (defatted), except in experiments with BCNU. The mitochondrial suspensions, 5 ml in 25 ml erlenmeyer flasks, were slowly rotated in a gyratory shaker at 30°C. Non-treated mitochondria remained well coupled (RCR > 2) for up to 210 min under these conditions. t-BuOOH (1-5 mM) or 100% ethanol (vehicle) were added immediately after the 0 min time point. At each time point 0.5-1 ml of the mitochondrial suspension were layered on top of a dibutylphthalate oil layer and spun for 1 min at 13000xg, into 10% perchloric acid (PCA), thereby rapidly washing the mitochondria free of the media and releasing the intramitochondrial content into the acid.

Biochemical analysis:

Acid-soluble glutathione pools were analyzed by the HPLC method of Reed et al. (20) with modifications as described (21). Oxidized glutathione in non-treated mitochondria was 2.1 ± 0.2% of the total glutathione. GSH-protein mixed disulfides were analyzed in the acid-precipitated mitochondrial pellet by the method of Livesey and Reed (22). Mitochondria were washed twice in 0.5 ml 100% ethanol, by sonication. The pellets were resuspended in 0.5 ml of 50 mM N-morpholinopropane sulfonic acid buffer, pH 8.0, containing 25 mM 1,4-dithiothreitol and incubated with shaking for 60 min at 37°C. The sample was then deproteinized with 70% PCA, frozen in liquid

nitrogen, thawed and the supernatant was used for the determination of GSH.

Protein-thiol levels were determined on a different acid-precipitated mitochondrial pellet with Ellman's reagent according to Di Monte et al. (23).

Protein determinations were performed on the same PCA pellets by the method of Peterson (24).

The activity of citrate synthase (CS) in the media was determined according to Srere (25) and expressed as the % of the total CS activity of the mitochondrial suspension.

The respiratory control ratio was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, USA) in a 3 ml thermostatized chamber equipped with magnetic stirring. The RCR was determined at 25°C according to Estabrook (26) using succinate as substrate and 1 mg mitochondrial protein.

Results:

Isolated rat liver mitochondria could be maintained functional with a RCR > 2 for 210 min at 30°C, if incubated in a carefully chosen medium and slowly shaken with a circular motion. The most critical ingredient for maintenance of RCR appeared to be a metal ion chelator. Despite the apparent impermeability of the mitochondrial membranes, a steady loss of GSH out of the non-treated mitochondria was observed. The GSH loss was most rapid at first, with

approximately 0.06 nmol/mg/min lost the first 10 min and approximately 0.02 nmol/mg/min after that. Most of the GSH was recovered in the media and no formation of GSSG or Pr-SSG was detected. The apparent efflux of GSH from mitochondria could not be prevented by the addition of: rotenone (50 μM); α-tocopherol (25 μM); α-tocopheryl-succinate (25 μM); desferoximine (0.1 mM); cysteine (0.1 mM); glycine (1 mM); glutamic acid (1 mM); glutathione (2 mM); nor ATP (2 mM), to the incubation medium. However, lowering the incubation temperature to 24°C, slowed down the rate of GSH loss and when the mitochondria were kept on ice, no GSH was lost out of the organelles (data not shown).

Exposure of isolated rat liver mitochondria to t-BuOOH (0, 1, 2, 5 mM) resulted in a rapid loss of about 80% of the initial mitochondrial GSH within 10 min incubation at 30°C (Fig. V.1A). The loss of GSH was not dose-dependent. With 1 and 2 mM t-BuOOH, the GSH levels returned to control levels at 20 and 30 min respectively, whereas with 5 mM t-BuOOH, GSH levels did not recover during the 60 min incubation, although GSH was never totally depleted. Concomitantly, increased levels of GSSG and Pr-SSG were found inside the mitochondria (Fig. V.1B,C). With all three doses of t-BuOOH, the initial rate of disulfide formation appeared the same, with about the same number of Pr-SSG as GSSG being formed. Recovery of GSSG to control levels coincided with the recovery of GSH, whereas Pr-SSG returned to control levels at a slower rate.

The loss of GSH from non-treated mitochondria could mostly be accounted for in the media (Fig. V.1E). However, when the total

intramitochondrial levels of glutathione (GSH + 2xGSSG + Pr-SSG) in t-BuOOH treated mitochondria were computed, it was apparent that about 20% of the control mitochondria glutathione was not accounted for when the mitochondrial redox potential was low (Fig. V.1D). However, as GSH, GSSG, and Pr-SSG levels returned to normal, total levels of glutathione in the treated mitochondria were the same as the control, indicating that the missing glutathione had not been lost out of the mitochondria in the form of GSH or GSSG, but rather had been present in a reversibly formed disulfide within the mitochondria that was not detected by our HPLC method. Further support for the observation that no glutathione was lost from the mitochondria as GSSG was the fact that no GSSG was found in the media. This was despite the fact that GSH in the media decreased in a dose-dependent manner with t-BuOOH treatment (Fig. V.1E), which suggested that a reaction between GSH and t-BuOOH had taken place, that did not involve the formation of GSSG.

Treatment of isolated mitochondria with t-BuOOH also resulted in a slow dose-dependent loss of RCR, although the mitochondria remained coupled throughout the experiment (Fig. V.2). However, this did not result in any significant loss of citrate synthase, a mitochondrial matrix enzyme, and thus indicated functional rather than structural damage to the mitochondria. The level of CS activity in the media after 60 min incubation was: 2.2 ± 0.4%, 3.0 ± 0.4%, 2.4 ± 0.9%, and 2.8 ± 0.2% from mitochondria treated with: 0, 1, 2, and 5 mM t-BuOOH respectively.

In order to activate a potential efflux pathway for GSSG in the mitochondria, attempts were made to increase the levels of intramitochondrial GSSG, by treating the mitochondria, in conjunction with 1 mM t-BuOOH, with the GSSG reductase inhibitor, BCNU (27), or iodoacetamide, an inhibitor of thioltransferase (28), which has been shown to catalyze the oxidation and reduction of protein thiols and protein disulfides (29). Although 75 μM BCNU inhibits hepatocyte GSSG reductase by 90% in 60 min (30), and 150 μM BCNU greatly potentiates the loss of mitochondrial GSH induced by adriamycin in isolated hepatocytes (31), 75, 150, or 300 μM BCNU did not significantly prevent the reduction of GSSG in isolated mitochondria. Only with 300 μM BCNU was any effect observed, the levels of GSSG at 10 min were increased by 50% over treatment with 1 mM t-BuOOH alone (data not shown), reducing the GSH/GSSG ratio to approximately 0.4 from approximately 100 in non-treated mitochondria. The GSSG levels, however, were returned to zero by 20 min although the levels of Pr-SSG were reduced back to normal at a slower rate. As before no GSSG was found in the medium. Addition of 300 μM BCNU 5 min prior to t-BuOOH administration did not change these results. Since the activity of BCNU was confirmed by testing its inactivation of hepatocyte GSSG reductase, it is not clear why BCNU was not more effective in inhibiting mitochondrial GSSG reductase. Possible explanations could be; a very slow uptake of BCNU into the mitochondria; or the protein, shown to mediate BCNU degradation (32) to form 2-chloroethyl isocyanate, the actual inhibitor of GSSG reductase (27), may not be present in the mitochondria.

Administration of 300 μM iodoacetamide in conjunction with 1 mM t-BuOOH failed to prevent the formation of Pr-SSG (data not shown), although 10 μM iodoacetamide inhibits purified rat liver thioltransferase by 90% (28). In fact, the levels of Pr-SSG were greatly increased over treatment with 1 mM t-BuOOH alone, or by 20% at 10 min and by 400% by 30 min, confirming that iodoacetamide was taken up by the mitochondria.

The levels of total protein thiols in the mitochondria were 123 ± 6 nmol/mg mitochondrial protein, and were not significantly changed by the t-BuOOH treatment. Since the observed formation of 1 nmol Pr-SSG/mg mitochondrial protein would account for only 1% oxidation of total protein thiols, this loss would be too small to be detected by our protein thiol assay.

Discussion:

t-BuOOH treatment is accompanied by a decrease in intracellular GSH and NADPH concentrations and a release of GSSG from the perfused rat liver (33) and isolated rat hepatocytes (15). The rate of GSSG release is believed to be proportional to the activity of GSH Peroxidase (12). We have demonstrated herein that GSSG is formed in isolated mitochondria during oxidative stress induced by t-BuOOH-treatment, but despite a drastic decrease in the mitochondrial GSH/GSSG ratio no release of GSSG into the medium was observed. This was true when GSSG levels were quickly reduced back to GSH, as well as during a sustained perturbation of the GSH/GSSG ratio, or when

GSSG levels were greater than 50% of total mitochondrial glutathione for 60 min. The possibility that exported GSSG may have been reduced in the media was discounted by the finding that no GSH equivalents were missing inside the mitochondria after glutathione levels recovered to those of non-treated mitochondria. These results indicate that a pathway for GSSG efflux is absent in hepatocyte mitochondria and that all GSSG formed inside mitochondria must be reduced in situ.

Since the RCR was lowered in a dose-dependent fashion by t-BuOOH treatment, sustained depletion of mitochondrial GSH and high levels of Pr-SSG induced by t-BuOOH, correlated with loss of mitochondrial function. Although this did not result in structural damage to the mitochondria, these results confirm the importance of mitochondrial GSH in maintaining intramitochondrial protein thiol groups in the reduced state, to preserve the integrity of mitochondrial membranes (34). Since mitochondria appear to lack the ability to export GSSG, this further indicates that mitochondria may be more susceptible to protein thiol oxidation than the rest of the cell. This may help explain why the loss of mitochondrial GSH, rather than cytosolic GSH, has been shown to be critical in some types of cell injury (5).

Since GSH was continuously lost at a slow rate from coupled mitochondria, these data further demonstrate an apparent efflux pathway for GSH in rat liver mitochondria. Earlier studies had shown an outward diffusion of GSH from mitochondria, with 25%-56% of the endogeneous level gone by 10 min incubation at 30°C (3). Since

coupled mitochondria are impermeable to protons, and 2 mM GSH in the medium did not affect the rate of GSH release in our system, a simple diffusion down a concentration gradient cannot explain the loss of GSH from isolated mitochondria. The loss of GSH from non-treated mitochondria was approximately 30% of the starting level in 1 h. Since the halflife of mitochondrial GSH in isolated hepatocytes was reported to be approximately 30 h (5), it can be assumed that the rate of GSH efflux observed from isolated mitochondria was induced by the artificial surroundings and does not occur in vivo, although the ability of mitochondria to export GSH has been demonstrated. However, more studies are needed to establish the nature of this GSH transport system.

The formation of 1 nmol Pr-SSG/1 nmol GSSG is consistant with the equilibrium constant for the reaction between GSSG and protein thiols, which is close to 1 (35). Iodoacetamide, a potent inhibitor of thioltransferase, did not inhibit the formation of Pr-SSG in isolated mitochondria, suggesting that the disulfide formation occurred nonenzymatically or via a different enzyme not inhibited by iodoacetamide. It has been suggested that the formation of Pr-SSG in the rat lung is a mechanism for maintaining NADPH levels during oxidative stress (36). In contrast, oxidation of protein thiols has been correlated with increased toxicity of several agents (10,37,38), possibly by affecting membrane permeability, so the physiological significance of this reaction is not fully understood.

It is also interesting to note that a small but significant amount of mitochondrial GSH (approximately 10%) could not be depleted

by raising the concentration of t-BuOOH. These data are in agreement with the results of Jocelyn and Cronshaw (39), who used chlorodinitrobenzene to deplete GSH. These investigators suggested that GSH might be sequestered within the mitochondrial matrix.

In summary, we have demonstrated that isolated mitochondria do not appear to export GSSG as a response to oxidative stress. This raises questions about the consequences of mitochondrial protein thiol oxidation for cell viability.

Figure V.1 Dose-response of t-BuOOH on (A) GSH, (B) GSSG, (C) Pr-SSG, and (D) total glutathione levels in isolated mitochondria and (E) on GSH levels in the incubation medium. Mitochondria were incubated and glutathione was determined as described under materials and methods. 0 mM (○), 1 mM (●), 2 mM (▲), 5 mM (■) t-BuOOH was administered in 100% ethanol immediately after the zero h time point. Values are mean ± SEM,
n=3-5.

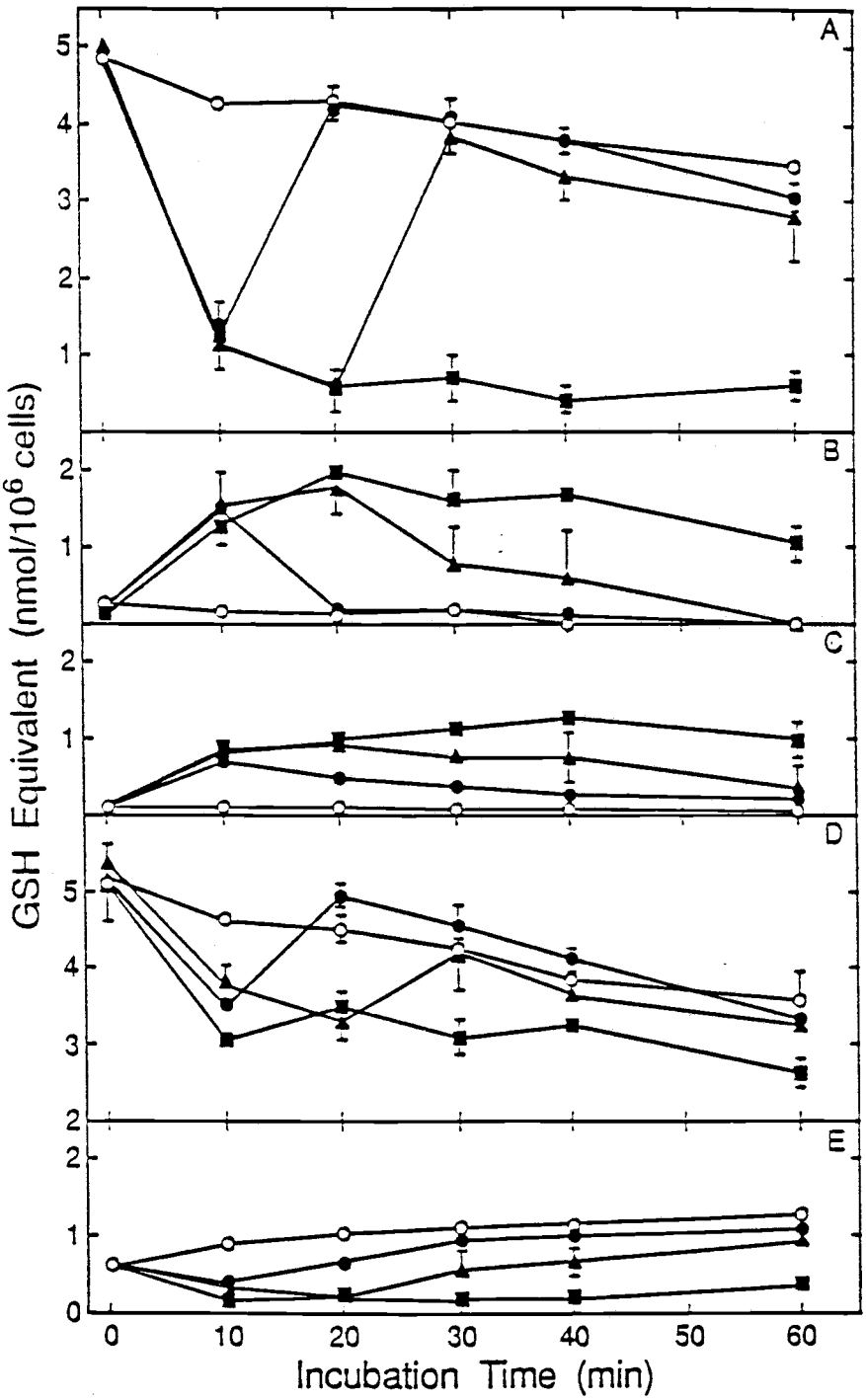
A
B
C
D
E
GSH Equivalent (nmol/10^6 cells)
Incubation Time (min)
0
10
20
30
40
50
60

Figure V.1

Figure V.2 Dose-response of t-BuOOH on RCR of isolated mitochondria. Mitochondria were incubated and RCR was determined as described under materials and methods. 0 mM (○), 1 mM (●), 2 mM (▲), 5 mM (■) t-BuOOH. Values are mean ± SEM, n=3-5, except mean ± range, n=2 for 5 mM t-BuOOH.

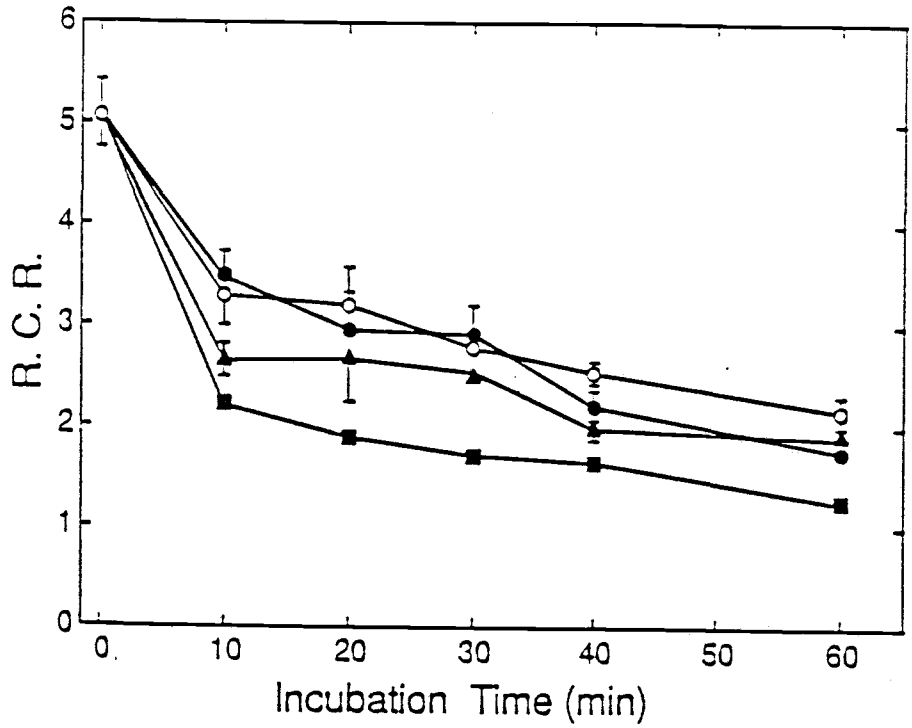

Figure V.2

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VI. CONCLUSION AND COMMENTS:

It is evident that therapeutic interventions designed to manipulate the processes involved in cell death could potentially result in the control and possible prevention of many human diseases. Abnormal calcium regulation appears to be a common thread relating altered physiologic functions or morphology to the disease. Transient increases in cytosolic Ca^{2+} concentrations are a normal part of many essential processes, such as contraction, secretion, endocytosis, transport, cell growth and cell division. However, sustained increased levels of cytosolic free Ca^{2+} can activate deleterious Ca^{2+}-dependent processes, that can interfer with normal cell metabolism and ultimately precipitate death, if not reversed. This perturbation of Ca^{2+} homeostasis appears to be related to impaired sequestration of intracellular Ca^{2+}, which has been shown to be an early event in cell injury in *in vitro* systems. *In vivo*, disturbances of calcium homeostasis are implicated for example in defective bone mineralization, renal failure, hypertension, parturient paresis in ruminants, some forms of muscular dystrophy and seizure disorders.

A close relationship between Ca^{2+} and thiol homeostasis has been demonstrated in this thesis. Both cytosolic and mitochondrial GSH were greatly affected by altered Ca^{2+} homeostasis, leading to oxidation of protein thiol groups and cell injury. In particular, the levels of mitochondrial GSH appeared critical for cell survival, as demonstrated by a close correlation between the loss of

mitochondrial GSH and LDH from hepatocytes treated with the Ca^{2+} ionophore, A23187, in the presence of different concentrations of extracellular Ca^{2+}.

A possible explanation for the protective role of mitochondrial GSH during cell injury was suggested by experiments with hydroperoxide-treated isolated mitochondria. The mitochondria were found not to export GSSG as a response to oxidative stress. Instead, an increase in Pr-SSG was observed inside the mitochondria, correlating with loss of mitochondrial function. Mitochondrial protein thiols may thus be more sensitive to GSH oxidation than other cellular protein thiols, where highly oxidative states and adverse thiol/disulfide redox status can be avoided by the export of GSSG out of the cell.

The relationship between cellular thiol and calcium homeostases needs to be further explored. The link between Ca^{2+} flux and thiol loss is not obvious, but may be related to the apparent oxidative state induced in calcium-depleted cells (chapter III) and in calcium ionophore-treated cells (chapter IV). Incubation of isolated hepatocytes in the absence of extracellular Ca^{2+}, resulted in accelerated efflux of GSH, GSSG and a marked increase in lipid peroxidation (3.2 ± 0.2 nmol TBA reactants/10^6 cells/5 h), exceeding levels found in hepatocytes treated with toxic concentrations of various chemicals in the presence of Ca^{2+}, (data not shown). Administration of the calcium ionophore resulted in even higher levels of lipid peroxidation, especially in the absence of extracellular Ca^{2+}. This unusually high oxidative state was further

reflected by high levels of intracellular GSSG (Olafsdottir, K., and Reed, D. J., unpublished results), that correlated closely with the extent of lipid peroxidation. Experiments demonstrating that the oxidation of glutathione was not an artifact, included the addition of GSH into the acid-layer in which the hepatocytes were disrupted, demonstrating no oxidation of the exogeneously added GSH during extensive oxidation of endogeneous GSH. However, the high levels of GSSG did not involve the formation of Pr-SSG nor correlate with cell injury and, in the absence of extracellular Ca^{2+}, coexisted with normal levels of intracellular NADPH (Olafsdottir, K., and Reed, D. J., unpublished results). It is therefore intriguing to speculate on a possible compartmentation within the cell, induced by Ca^{2+}-mediated changes in the cytoskeleton, that would prevent the action of thioltransferase and GSSG reductase. Aw et al., 1987, proposed that during anoxia, a rapid change occurs in mitochondrial regulation of ATP synthase activity and ionic fluxes. Such a regulation would provide an anoxic steady state in which the cells have reduced functional ability but increased capacity to endure a brief anoxic period. A similar mechanism may exist to endure periods of oxidative states induced by altered Ca^{2+} homeostasis.

More research to determine the underlying cause of Ca^{2+}-induced oxidative stress should be interesting and will undoubtedly clarify the mechanism by which increased cytosolic free Ca^{2+} triggers cell injury.

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