Co-toxicity of ATP and Menadione:

Effects on Intracellular Calcium Regulation in Hepatocytes

By

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

The effects of non-lethal oxidative stress on cytosolic calcium regulation in hepatocytes are inadequately understood. It was hypothesized that non-lethal oxidative stress and calcium mobilizing agonists are co-toxic and that non-lethal oxidative injury would result in impaired responsiveness to receptor mediated-calcium mobilization. **Methods:** Oxidative injury was induced in a dose-dependent manner in cultured hepatocytes using the prooxidant chemical, menadione. A lethal (100μM) and a non-lethal (10μM) dose of menadione were defined. Co-toxicity of the calcium-mobilizing purinergic receptor agonist, ATP, and the effects of menadione on ATP-related calcium signaling were determined by incubating hepatocytes for 2 hours with menadione and then exposing hepatocytes to normally non-toxic concentrations of ATP. A propidium iodide exclusion assay was used to assess for cell necrosis. Nuclear stains with Hoechst 33258 and a fluorescein-based TUNEL assay were used to assess apoptosis. Cytosolic free calcium was evaluated using Fura-2-AM. **Results:** Data were expressed as 1) % cell necrosis ± standard error and 2) % baseline $[Ca^{++}]_i$ ± standard error.

Effect of menadione and ATP on cell viability (% necrosis)

	Ca^{++}-containing media		Ca^{++}-free media	
	no ATP	100μM ATP	no ATP	100μM ATP
Control	4.3% ± 1%	4.8% ± 1%	15.5% ± 2%	15.5% ± 2%
10 μM menadione	6.7% ± 2%	21.1% ± 2%*	16.0 ± 3%	15.0% ± 2%
100 μM menadione	10.7% ± 5%	37.0% ± 5%*	22.0% ± 3%	19.0% ± 2%

(*$p < 0.05$ vs. no ATP)

Menadione alone or in combination with ATP did not result in apoptosis. Early

calcium changes, ($[Ca^{++}]_{i\ max}$) were measured at 1-3 minutes post ATP exposure. Late calcium changes ($[Ca^{++}]_{i\ max}$) were measured after 15 minutes of ATP exposure.

Effect of menadione and ATP on $[Ca^{++}]_i$ signaling

	Early $[Ca^{++}]_i$ 100μM ATP	Late $[Ca^{++}]_i$ 100μM ATP
Control	550% ± 26%	313% ± 29%
10 μM menadione	270% ± 12%*	274% ± 26%
100 μM menadione	219% ± 16%*	711% ± 68%*

(*$p < 0.05$ vs. controls)

Conclusions: Menadione and ATP are co-toxic through a calcium - dependent mechanism. Lethal and non-lethal oxidative injury leads to the disruption of normal receptor-mediated calcium signaling in hepatocytes. These data suggest that in complex systems, such as the intact liver, the combination of otherwise non-lethal toxins and signaling agents can cause lethal hepatocyte injury and altered regulation of intracellular calcium.

GENERAL INTRODUCTION

The ultimate effect of any form of cell injury is cell death. Cell death can occur via two different pathways. First, cells can undergo necrotic cell death that is characterized by disorganized cell lysis and loss of intracellular components. Secondly, cells can die via apoptosis that is characterized by organized compartmentalization of intracellular components followed by cell lysis. Both necrosis and apoptosis play important roles in pathophysiology (Popper, 1986). Non-lethal injury is, however, also very important in long-term pathophysiologic processes such as aging and tumorigenesis (Searle, 1982). To modify the processes involved in such long-term physiologic and pathophysiologic processes, it is important to study the effects of both lethal and non-lethal insults.

One way in which cells can be injured is through oxidative stress. Oxidative stress occurs in all systems that employ aerobic metabolism. On one hand, aerobic metabolism is beneficial as there is an increased yield of ATP per unit substrate used. On the other hand, oxygen toxicity can be a significant problem (Fridovich, 1983). Oxidative damage can affect DNA, proteins, lipids and carbohydrates that can lead to the disruption of critical enzyme and organelle function subsequently leading to cell death (Rosser 1995).

One very important way in which lethal oxidative injury has been

shown to affect cell function is through the perturbation of intracellular calcium regulation. Intracellular calcium concentration regulates the secretion of enzymes, cell growth, cell to cell communication and a host of other events (Berridge, 1993, Rosser, 1995). Intracellular calcium concentrations are regulated through several positive and negative feedback systems (Berridge, 1993). Strict regulation of intracellular calcium concentration is important as high intracellular calcium levels interfere with normal physiologic processes and can lead to the death of the cell (Rosser, 1995). Lethal levels of reactive oxygen species (ROS) have been shown to disrupt calcium regulation preceding cell blebbing and death (Jewell, 1982). Increased calcium levels associated with oxidative stress activate degradative hydrolases which have been shown to cause cell death (Rosser, 1993).

While disruption of calcium regulation is clearly associated with lethal oxidative stress (Jewell, 1982), little information is available concerning calcium regulation during non-lethal oxidative stress. It has been hypothesized that non-lethal oxidative injury will be associated with a loss of intracellular calcium regulation capability. This might lead to increases in $[Ca^{++}]_i$ or to disturbances in calcium signaling responses. However, the effect of oxidative stress on calcium signaling in hepatocytes is not yet completely understood. In addition to effects on $[Ca^{++}]_i$, non-lethal oxidative stress may cause a normal response to a calcium-mobilizing hormone to become lethal by disrupting other homeostatic mechanisms (e.g. inhibition of Ca^{++} ATPases, activation of proteases, increased protein degradation (Herman, 1990)). As

such, calcium signaling agents (i.e. ATP, phenylephrine, vasopressin etc.) may be toxic to cells exposed to non-lethal levels of oxidative stress. As a result of the loss of calcium regulation induced by oxidative stress, normally non-toxic calcium-mobilizing hormones could result in lethal cell injury.

The main goal of this thesis is to evaluate the hypothesis that non-lethal levels of oxidative stress in combination with normally non-toxic calcium mobilizing agents would result in toxic injury to hepatocytes. An additional goal is to evaluate the effect of non-lethal oxidative stress on receptor-mediated calcium mobilization.

BACKGROUND AND LITERATURE REVIEW

CELL INJURY

Injury to a cell is defined as a perturbation or stimulus that is beyond the range of normal perturbations or stimulation of the cell (Trump, 1992). Following injury, the cell may adapt by developing a new steady state and therefore survive for a long period of time, or the cell may not have the capability to overcome the injury. Lethal cell injury occurs when the cell cannot adapt to the injury and dies. Non-lethal cell injury occurs when the cell develops an altered steady state and survives. The importance of non-lethal

cell injury is often overlooked because it is not as clearly definable as lethal cell injury.

CELL DEATH

Accepting that there may be a large degree of overlap between different types of cell death, most authorities agree that there are two basic types of cell death; necrosis and apoptosis (Rosser, 1995 and Patel, 1995). It has been shown that severe levels of oxidative damage cause cellular necrosis (Bellomo, 1985, Rosser, 1995). Although studies are limited in hepatocytes, oxidative stress may induce apoptosis in some cell models (Patel, 1995). For the purposes of this project, it was important to understand and investigate the possibility of both types of cell death in our model studying the effects of oxidative stress and calcium signaling on hepatocytes.

NECROSIS

Necrosis has been defined as injury resulting in the loss of plasma membrane integrity and vital metabolic functions (e.g. ATP synthesis) (Rosser, 1995). It is usually a consequence of pathological injury as opposed to physiologic processes. Morphologically, necrosis is characterized by intracellular organelle swelling, pyknotic nuclear changes, and formation of plasma membrane blebs that exclude organelles (Rosser, 1995). Non-specific DNA fragmentation also occurs. Cell swelling and eventually cell lysis occurs leading to the loss of electrochemical gradients and leakage of

cellular enzymes (e.g. lactate dehydrogenase (LDH)). Cells that have undergone necrosis are unable to exclude low molecular weight nuclear dyes such as trypan blue and propidium iodide. Stroke, myocardial infarction and hepatitis are all pathologic processes accompanied by large levels of necrotic cell death.

ASSESSING CELL NECROSIS

Necrotic cell death occurs when cell membrane integrity is lost therefore allowing the cell to become "leaky" (Kodamanti, 1991). When membrane integrity is lost, intracellular macromolecules are released from the cell and extracellular macromolecules are allowed into the cell. Measuring intracellular macromolecule release or extracellular molecule entry into dying cells are the classic methods for assessing necrotic cell death (Rosser, 1995). Assessment of cell death in cultured hepatocytes for this project involved measuring the release of the intracellular macromolecule, lactate dehydrogenase (LDH), or the exclusion of the low molecular weight DNA binding fluorophore, propidium iodide (PI).

LDH release -- LDH is a convenient and effective marker for cell necrosis and is widely used because of the stability of the enzyme activity in the incubation or culture medium (Kodavanti, 1991). The LDH assay is commonly used as a measure of hepatocyte viability (Rosser, 1995).

The rationale for use of the LDH assay is that as cells die, the membrane breaks down and allows the leakage of the cytosolic

macromolecule, LDH, into the incubation medium. By removing part of the incubation medium and measuring the LDH content one can determine how much LDH is lost from the cells and therefore use it as a measure of cell viability. By exposing cells to agents that completely disrupt cell membranes (e.g. digitonin) at the end of the experiment, the total releasable LDH can be determined. LDH release may then be expressed as a percent of total releasable LDH.

PI exclusion– PI is a polar, low molecular weight, DNA binding fluorophore that is impermeable to living cells. When a cell dies, membrane integrity is lost and PI enters the cell binding to DNA. Upon binding to DNA, PI becomes fluorescent. When cells are then exposed to a wavelength of light equal to 540 nm, the nuclei of dead cells, with DNA bound to PI, will emit light at a wavelength of 635 nm. This light can be detected as a red, or "rhodamine"-based nuclear fluorescence in a cell suspension or in individual hepatocytes using digitized fluorescent microscopy.

APOPTOSIS

Apoptosis is another type of cell death. Apoptosis is quite different from necrosis as it is highly regulated. Wyllie et. al. proposed in 1980 that apoptosis occurred as a result of a gene directing programmed cell death (Alison, 1994). It is for this reason that apoptosis is often considered a physiologic process even when occurring in response to a pathologic stimuli (Doherty, 1993, Patel, 1995).

Apoptosis is characterized morphologically by several distinct events. The first obvious change is the cleavage of the DNA by endonucleases, acting initially at points of interaction with the nuclear scaffold and then at exposed sites between nucleosomes (Doherty, 1993). The process of apoptosis continues with cell shrinkage, nuclear fragmentation, cytoplasmic budding, and the formation of membrane blebs containing organelles (Patel, 1995). Cellular contents are not emptied and DNA cleavage is nonrandom between nucleosomes. In apoptosis, membrane integrity is initially maintained and organelles remain functioning.

Apoptosis, although it is a form of cell death, is very important and, in fact, is vital to the proper functioning of several physiologic processes. Apoptotic cell death is a required part of embryonic development, maintenance of the immune system, and turnover of the intestinal epithelium (Trump, 1992). Cytotoxic T lymphocytes bind to virally infected cells and within a few minutes, apoptosis occurs (Vaux 1993). The action of the T-lymphocytes, through the activation of apoptosis, protects from the further infection due to the virus. Immunologically-mediated apoptosis has been suggested as an important contributor to liver damage in chronic hepatitis C and autoimmune hepatitis along with other types of liver disease (Patel, 1995). This illustrates the potential importance of apoptosis in addition to necrosis in clinically important liver disease.

ASSESSING CELLULAR APOPTOSIS

Non-random DNA cleavage is a unique characteristic of apoptosis. Nuclear fragmentation in cells undergoing apoptosis is uniform and therefore one of the most reliable signs of the occurrence of apoptosis (Searle, 1982). Apoptotic cells have nuclei that are systematically broken into several small circular apoptotic bodies which are detectable under a microscope. Because of the unique nature of the DNA breaks and nuclear fragmentation, assays to assess apoptosis in individual cells generally analyze the breakdown of DNA and the morphologic changes in the cell nucleus.

Assessment of DNA strand breaks with the TUNEL technique - One of the major types of DNA breaks seen with apoptosis is single stranded nick in the DNA. The TUNEL technique (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) uses the enzyme, TdT, to label the 3`-OH termini of the single stranded nicks with fluorescein. Once labeled with fluorescein, fluorescent techniques may be used to determine whether or not apoptosis has occurred.

Assessment of nuclear morphology using Hoescht 33258- Hoescht 33258 is a DNA binding fluorophore which is membrane-permeable to both living and dying cells. When Hoescht 33258 enters a cell, it binds to DNA and fluoresces making the visual analysis of the morphology of the nuclei possible. If the cell is not undergoing apoptosis, the Hoescht stained nucleus is large, intact and circular. If the cell is undergoing apoptosis, the nucleus appears as a cluster of several small circular fragments, as an irregular,

globular shaped structure, or as a small shrunken, dense collection of nuclear material (Patel, 1995).

OXIDATIVE STRESS

Reactive oxygen species (ROS) have been implicated in ischemia/reperfusion, drug, and alcohol induced liver injury (Granger, 1985, Rosen, 1983, Kato, 1990). In fact, ROS are a problem in all systems using aerobic metabolism as molecular oxygen can be converted to ROS. Oxidative stress is, therefore, a potential problem for all mammals. To handle the inevitable presence of ROS, all mammals have complex systems for handling ROS.

ROS are oxidizing agents or "prooxidants" which abstract electrons from other molecules (Rosser, 1995). Reducing agents or "antioxidants" are compounds that donate electrons to oxidizing agents thereby detoxifying them (Rosser, 1995). Oxidative stress occurs when the capacity of the prooxidants outweighs the antioxidant capabilities (Sies, 1985). Oxidative stress occurs when there are excessive amounts of prooxidants or a deficiency of antioxidants.

Menadione

Several chemicals have been identified that promote oxidative stress. One such chemical is menadione (Bellomo, 1985). Menadione is a quinone

which, as it is metabolized, causes oxidative damage by consuming cellular thiols such as glutathione. Glutathione is a very important antioxidant which, when significantly depleted, will lead to conditions of oxidative stress.

The metabolism of menadione, along with any quinone, can follow one of two pathways. First, menadione may undergo a two electron reduction. Two electron reduction of menadione requires the enzyme DT-diaphorase (Bellomo, 1985) as seen in Figure 2. Second, menadione may under go a one electron reduction. The one electron reduction reaction requires a flavoenzyme (e.g. NADPH-cytochrome P-450 reductase, NADH-cytochrome b reductase and NADH-buviquinone oxidoreductase) (Bellomo, 1985). Following the one electron reduction, $O_2^{-\cdot}$ may be formed. If a small amount of $O_2^{-\cdot}$ is formed, it is metabolized by superoxide dismutase (SOD) (Figures 1 and 2) to form Hydrogen peroxide (H_2O_2). H_2O_2 then oxidizes glutathione thus depleting intracellular stores of GSH leading to conditions of oxidative stress. If a large amount of $O_2^{-\cdot}$ is formed, detoxification mechanisms are overwhelmed and oxidative stress occurs.

Prooxidants

Table 1 lists the oxidizing species which are implicated most in oxidative stress (Rosser, 1995). In most systems, $O_2^{-\cdot}$ is the initial ROS generated (figure 1). $O_2^{-\cdot}$ is a natural and inevitable product of aerobic metabolism. $O_2^{-\cdot}$ is generated as a result of healthy physiological activities in mitochondria, microsomes, and peroxisomes. In fact, 1-2% of all molecular

oxygen processed, is converted directly to $O_2^{-\cdot}$ (Kass, 1993). $O_2^{-\cdot}$ is normally converted to hydrogen peroxide by SOD before it can interact with cellular constituents. Hydrogen peroxide can be converted subsequently to several other species, which may or may not have prooxidant capacities. The generation of prooxidants is depicted in Figure 1.

There are several sources of prooxidants in all cells. Some of them are due to pathologic processes, while others are due to physiologic processes. In several pathologic states, mitochondria produce abnormally high levels of $O_2^{-\cdot}$ in association with disturbances in the electron transport chain (Dawson, 1993). The disturbance of the electron-transport chain eventually leads to the depletion of intracellular ATP production. Subsequent disruption of cellular metabolic processes lead to cell death by necrosis. $O_2^{-\cdot}$ and H_2O_2 are also produced due to metabolic processes involving P450 complex enzymes found in microsomes (Morehouse, 1984). Intermediates of the P450 enzymes produced during drug, alcohol or xenobiotic metabolism may also deplete antioxidants. Neutrophils represent a potential exogenous source of prooxidants. One of the functions of neutrophils is killing of bacteria and microorganisms. In addition to protease and other hydrolase release, neutrophils kill infected cells through the formation of $O_2^{-\cdot}$ and the toxic hypochlorous anion (Weiss, 1989). Surrounding tissue and cells may be injured unintentionally by these neutrophil toxins. Finally, cytosolic oxidases (e.g. xanthine oxidase, cyclooxygenase and lipoxygenase) are active during the production of prostaglandins and leukotrienes and may be activated

under conditions of ischemia reperfusion (Rosser, 1995). All of these oxidases can produce $O_2^{-\cdot}$ and singlet oxygen leading to oxidative stress.

Antioxidants

Antioxidants are reducing agents that donate electrons to detoxify prooxidants (Table 1). Some antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase and transferase, act enzymatically (figure 1) while other antioxidants act as free radical scavengers.

The major step in enzymatic detoxification of many prooxidants, involves the activity of SOD. SOD is a family of metalloenzymes that catalyze the conversion of $O_2^{-\cdot}$ to H_2O_2. H_2O_2 can then be detoxified by glutathione peroxidase or catalase to water. Glutathione (GSH) is a key intracellular antioxidant which provides sulfhydral groups which react with oxidative species directly or through the enzyme glutathione-S-transferase (GST). GSH also serves as a substrate for glutathione peroxidase in the detoxification of H_2O_2 (figure 1 and 2). Depletion of GSH can lead to oxidative stress and is often used as a measure of the severity of oxidative stress (Rosser, 1995).

The combination of SOD and glutathione peroxidase / transferase or catalase will detoxify two major prooxidants. In addition to these enzymes that work to deplete prooxidants, cells contain compounds that act as free radical scavengers. Such compounds include ascorbic acid (vitamin C), uric acid, taurine, beta-carotene, and alpha- tocopherol (vitamin E). These free radical scavengers also protect cells from prooxidants and prevent lipid

peroxidation that develops during a variety of forms of oxidative stress (Rosser, 1995).

Oxidative stress: Consequences

When cells are unable to protect themselves from oxidative damage several different outcomes are possible. Non-lethal oxidative stress may occur. This form of injury has been associated with changes in gene expression and transcription, membrane receptor turnover, and membrane protein expression (Houglum, 1991). Oxidation of proteins increases with age and the ability to metabolize these proteins directly correlates with life expectancy in higher vertebrates.

With increasing generation of prooxidants or depletion of antioxidants, severe oxidative stress occurs and results in cell death. While apoptosis has been seen in some models of cell injury, necrosis predominates in severe oxidative stress occurring in hepatocytes (Bellomo, 1985, Rosser, 1995). Oxidative stress may induce necrosis via oxidation of critical cellular proteins, DNA and lipids although the mechanism may differ with both type and severity of injury (Rosser, 1995). Regardless the net effect of severe oxidative stress is hepatocyte death with the subsequent clinical manifestations of acute liver failure.

CALCIUM

Normal calcium physiology

Ionic calcium has a number of important functions in both unicellular and multicellular organisms. The four main biological roles of calcium are structural, electrical, activity as a cofactor for extracellular enzymes and proteins, and its activity as an intracellular regulator. Structurally, calcium is a very important component of skeletal systems of vertebrates and is also a required element of cellular cytoskeleton. Electrically, calcium carries a significant amount of current during an action potential in neurons. Extracellular degradative enzymes such as trypsin, alpha-amylase, DNAase I, and enzymes involved in blood clotting all require calcium to function properly. Intracellularly, changes in calcium concentrations regulate several very important functions as depicted in Table 2.

Intracellular calcium changes regulate the secretion of enzymes, cell growth, cell to cell communication and several other activities vital to healthy, living cells. In addition to the importance of intracellular calcium concentrations in the regulation of normal cellular events, cytosolic calcium has also been implicated in the mechanisms of cell death, cell injury and cell aging (Trump, 1992). Cell aging and cell death are very important events in several physiological and pathological processes.

Receptor-mediated intracellular calcium mobilization

In resting healthy hepatocytes, intracellular calcium concentration is in the range of 100 nM – 200 nM (Smith, 1985). Intracellular calcium regulation by purines is depicted in Figure 3. In Figure 3, an agonist (ATP), acts on a purinergic receptor eliciting the activation of a G-protein. The activated G-protein then activates phopholipase C (PLC) causing the cleavage of membrane bound phosphatidyl-inositol-diphosphate (PIP_2) (Berridge, 1993). The products of PIP_2 cleavage are inositol-triphosphate (IP_3) and diacylglycerol (DAG). Diacylglycerol remains membrane-bound, activating protein kinase C (PKC) while IP_3 diffuses through the cytosol and stimulates IP_3 receptors on the endoplasmic reticulum (ER) causing the release of intracellular calcium stores.

IP_3-induced calcium increase occurs within a few seconds of the purinergic receptor activation (Berridge, 1993). A slower activation of a membrane bound calcium channel also occurs as a result of the activation of the purinergic receptor (Berridge, 1993). The activation of the membrane bound calcium channel allows for extracellular calcium to diffuse into the cell. Increased intracellular calcium causes calcium-induced calcium release (CICR) from the ER (positive feedback) and a further increase in intracellular calcium. The negative feedback mechanisms used to decrease intracellular calcium concentrations, involve the active mitochondrial sequestration of calcium, the binding of calcium by the Ca^{++}-binding molecules and active transport of calcium out of the cell. These negative feedback mechanisms

work to decrease the intracellular calcium concentration.

Role of calcium in oxidative stress

Strict regulation of intracellular calcium concentration is important because overly high calcium levels interfere with normal physiologic processes and can even kill the cell. Calcium regulation is often disrupted during oxidative stress (Rosser, 1995). It has been shown that with lethal levels of oxidative damage, the plasma membrane Ca^{++}-translocase is inhibited leading to an inability to regulate increasing intracellular calcium concentrations (Di Monte, 1984). Activation of Ca^{++}- dependent degradative hydrolases (proteases, phospholipases, etc.) may be responsible for eventual cell lysis that characterizes hepatocyte necrosis (Bellomo, 1985). Although a few studies have examined how cell injury affects Ca^{++} signaling, no studies have tested the effects of low level oxidative stress on cell signaling.

HYPOTHESIS

Based on the understanding of receptor-mediated calcium release and the effects of oxidative stress on calcium regulation and cell viability, I developed the hypothesis that non-lethal levels of oxidative stress, in combination with normally non-toxic calcium mobilizing agents, could result in toxic injury to hepatocytes.

OBJECTIVES

In order to test this hypothesis, my specific objectives are:

- to determine if lethal oxidative injury, as a result of menadione treatment, causes hepatocyte necrosis or apoptosis;
- to determine the concentrations of menadione which cause lethal oxidative injury and those which cause non-lethal oxidative injury;
- to evaluate $[Ca^{++}]_i$ regulation under basal and ATP-stimulated states in cells treated with lethal and non-lethal levels of menadione;
- to determine if the difference in calcium regulation in response to ATP is associated with a difference in cell mortality; that is, to determine if extracellular ATP and menadione are "co-toxic".

MATERIALS AND METHODS

Materials– Collagenase; collagen; HEPES; trypsin inhibitor; pentobarbital; Pyruvate; NADH; Digitonin; Propidium Iodide; Digitized Video Fluorescent Microscopy system; Hoechst 33258; paraformaldehyde; PBS; In Situ Cell Death Detection Kit, Fluorescein (TUNEL); Diethylmaleate; CMAC; Fura-2-AM; menadione; ATP; Thapsigargin.

Hepatocyte Isolation and Culture– After anesthesia with pentobarbital hepatocytes were isolated from male Sprague-Dawley rats (250-300 g) as previously described (Alpini1994). Briefly, a midline

abdominal incision was preformed. The portal vein was cannulated and perfused with calcium free Hanks Balanced Salt Solution. To avoid excessive pressure build up in the liver, the inferior vena cava was cut. After perfusion with the calcium free salt solution, the liver was perfused with calcium containing Hanks Balanced Salt Solution, collagenase and trypsin inhibitor for approximately 10-15 minutes. The liver was then removed from the abdominal cavity and placed in a petri dish. Mechanical dissociation and differential centrifugation were used to obtain hepatocytes, which were cultured in Waymouth's media supplemented with 10% fetal calf serum and 100 nM insulin. Hepatocytes were used for experimentation within the first 8 hours of culture thus minimizing dedifferentiation and loss of agonist receptor sensitivity (unpublished observations, Fisher 1996).

Fluorescence Microscopy– Cultured hepatocytes were incubated on a heated microscope stage at 37°C exposed to ambient air in a non-perfusing buffer system. After loading hepatocytes with the fluorophore of interest, intracellular fluorescence was quantitated using a digitized videofluorescence microscopy system (DVFM) consisting of a Leitz DM-IRB inverted fluorescence microscope, an 8 bit digital camera (Optikon Corporation), Axon Imaging software (Axon Instruments) and a Pentium-based computer system.

Assessment of cell death– Two types of cell death were assessed.

Necrosis

Viability assessment by measurement of LDH release - Each culture dish was

treated with a variable concentration of the agent of interest. At 2 hour intervals for a total of 6 hours, a 20 µl sample of the incubation buffer was collected and immediately frozen at -20°C. At the end of the experiment, each dish was treated with 300 mM digitonin and a 20 µl sample was taken to determine total releasable LDH. LDH activity in each sample was determined and was expressed as a % of total releasable LDH.

The LDH assay involves measuring the reactants of the following reaction with lactate dehydrogenase as the enzyme.

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

In order for the reaction to take place, the following were placed in a quartz cuvette: 17 µl of incubation media (sample)

450 µl of assay buffer

200 µM final concentration of NADH

800 µM final concentration of sodium pyruvate

The cuvette was then placed in a spectrophotometer for the measurement of NADH depletion by UV absorbance at 340 nm. NADH depletion was measured for 2 minutes. The magnitude of NADH depletion is proportional to the amount of LDH in the sample.

To calculate cellular viability following the LDH assay some assumptions are required. 0% of cells exposed to digitonin are assumed to be alive with 100% LDH being released. 100% of control cells are assumed to be alive with 0% release of LDH. With these two assumptions, cell viability

can be determined by the following equation.

$$1 - \frac{\text{LDH released}}{\text{Total releasable LDH}} = \%\ \text{cells viable}$$

Viability assessment by measurement of PI Exclusion-- Hepatocytes were cultured on a slide in incubation medium containing 1 μM PI. Experimental manipulation was carried out, the slide was placed in the viewing dish and mounted on the scope. Cells were exposed to light with a wavelength of 540 nm. Nuclei stained with PI, emitted light at a wavelength of 635 nm and were visibly red. At least 1000 cells were assessed for PI staining. Because of the potential for observer bias, the investigator was blind to the experimental manipulation. The number of PI positive nuclei was determined as a percentage of total nuclei (dead cells / total cells).

Apoptosis

Assessment of apoptosis using Hoechst 33258 staining and assessment of nuclear morphology-- After experimental manipulation, cultured hepatocytes were incubated for 10 - 20 minutes at 37°C with 15 μM Hoechst 33258. Following incubation, nuclear morphology was assessed on a fluorescence microscope with an excitation wavelength of 352 nm and an emission wavelength of 461 nm. Cells were considered apoptotic if they exhibited 1) fragmented nuclei, 2) grossly irregular nuclei, globular in appearance or 3) severely shrunken, intensely stained nuclei (Patel, 1995). Because of the potential for observer bias, the investigator was blind to the experimental manipulation.

TUNEL assay -- Cultured hepatocytes were exposed to the experimental manipulation and were fix air dried with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 minutes at room temperature. Slides were rinsed with PBS and incubated in permeabilisation solution (0.1% Trition X-100, 0.1% sodium citrate) for 2 minutes on ice. Cells were then rinsed twice with PBS. 50 µl of a commercially available fluorescein-based TUNEL reaction mixture was then placed on the slide and incubated in a humidified chamber for 60 minutes at 37°C in the dark. Cells were then rinsed three times with PBS and analyzed. Because of the potential for observer bias, the investigator was blind to the experimental manipulation.

Measurement of cellular GSH – After experimental manipulation, hepatocytes were incubated with 40 µM CMAC at 37°C. After precisely 20 minutes the reaction was quenched with 200 µl of 50% trichloroacetic acid. The samples were analyzed on a Sequoia fluorometer (excitation wavelength-360 nm, emission wavelength-430 nm) and fluorescence values recorded.

To determine the [GSH] in each sample as a percentage of controls, some assumptions were made. Cellular GSH concentration was depleted to 5% of controls by treatment for 30 minutes with diethyl-maleate (DEM) (Rosser, 1993). Control cells were assumed to contain 100% GSH possible. With these two assumptions, GSH concentration of the samples could be calculated using the equation of a straight line, $y = mx + b$, where y is %GSH

in the sample, m is the slope of the line, x is the measured fluorescence of the sample and b is the y intercept (extrapolated).

Measurement of intracellular calcium concentration ($[Ca^{++}]_i$) -- Because intracellular calcium is so important to several cellular mechanisms, its measurement and study are necessary. Measurement of $[Ca^{++}]_i$ was achieved using fura-2-AM. Fura-2-AM is a very sensitive fluorescent probe useful for measuring intracellular calcium concentrations. Cells exposed to extracellular concentrations of fura-2-AM will take up the probe. Once inside the cell, fura-2-AM is exposed to cellular esterases which remove the ester moiety and render the probe trapped inside the cell. Fura-2 displays a Ca^{++}-dependent fluorescence excited at 340 nm and a Ca^{++}-independent fluorescence excited at 380 nm (Lemasters, 1986). The ratio of the fluorescence values at these two wavelengths can then be used to quantitatively determine cytosolic free calcium.

When cells are exposed to excitation wavelengths of 340 nm and 380 nm with an emission wavelength of 510 nm, the concentration of intracellular calcium can be determined using the following equation.

$$[Ca^{++}]_i = Kd\ ((R - R_{min}) / (R_{max} - R_{min}))\ (F_{min} / F_{max})$$

[Grynkeiwicz, 1985]

R is the measure of actual fluorescence measured at 340 nm and 380 nm, respectively. R_{min} is the value of R at zero calcium. R_{max} is the value of R at saturating calcium concentration. F_{min} / F_{max} is the ratio of fluorescence

measured at zero calcium and saturating calcium for 380 nm excitation respectively. Kd is a constant of 224 (Grynkeiwicz, 1985). Calibration of digitized video fluorescent microscopic equipment was carried out daily to determine R_{min}, R_{max}, and F_{min} / F_{max}.

Prior to experimental manipulation, cells were incubated for 30 minutes at 37°C with 5 μM fura-2-AM. Cells were then placed on the fluorescence microscope stage and exposed to the experimental manipulation of interest. $[Ca^{++}]_i$ was determined every 30 seconds for 45 minutes. Fluorescent ratio values were recorded and intracellular calcium concentrations were determined as described above.

Statistical Analysis-- All data are expressed as means $\pm$ standard error of the mean from a minimum of five separate experiments employing hepatocytes from different animals. Statistical significance was considered at the $p < 0.05$ level. Differences between more than two groups were analyzed using a one-way ANOVA with a Tukey-Kramer correction. Differences between two groups were analyzed using an unpaired t-test. Qualitative data were analyzed using a Chi-squared test with a Yates correction factor. The software package JMP In (SAS institute Inc.) was employed for all statistical analysis.

EXPERIMENTAL DESIGN AND RESULTS

Does menadione induced oxidative stress cause hepatocyte death by necrosis or apoptosis?

Hepatocytes were exposed to a range of concentrations of menadione and were evaluated over the subsequent 6 hours for necrosis and apoptosis. Apoptosis due to menadione treatment in hepatocytes was not significantly different from controls (Control 0.4% $\pm$ 0.02%, 10μM menadione 0.5% $\pm$ 0.02%, 100μM menadione 0.5 % $\pm$ 0.01 at 6 hours). In contrast, menadione produced dose-dependent hepatocyte necrosis (Figure 4). The severity of injury paralleled glutathione depletion measured over the initial 2 hours of menadione exposure (Figure 5). Based on these results, it was concluded that, menadione induces cell death through necrosis but not apoptosis and is associated with progressive oxidative stress manifested by progressive intracellular glutathione depletion.

What concentration of menadione results in lethal versus non-lethal oxidative injury?

In order to analyze the differences between the effects of lethal and non-lethal oxidative stress, definitions of lethal and non-lethal injury must be established. In order to clearly separate the two types of injury, definitions were arbitrarily chosen so as to minimize any potential overlap between the two types of injury.

Lethal oxidative stress-- Lethal oxidative injury was defined to occur when GSH levels were depleted to < 25% of controls within two hours of menadione exposure and when, after 6 hours of treatment with menadione, < 30% of cells were viable.

Non-lethal oxidative stress-- Non-lethal oxidative injury was defined to occur when GSH levels were depleted no more than 50% of control levels within 2 hours of menadione exposure and when, after 6 hours of treatment with menadione, > 80% of cells were viable.

As seen in Figure 4, 100 μM menadione resulted in < 30% cell viability after 6 hours while 10 μM menadione resulted in 90% viability after 6 hours. Figure 5 shows that 100 μM menadione depletes GSH to a level < 25% of controls while 10 μM menadione depletes GSH to a level approximately 50% of controls.

Using the above criteria, it was defined that 100 μM menadione induces lethal oxidative stress while 10 μM menadione induces non-lethal levels of oxidative stress. These concentrations of menadione were used to assess the effect of lethal and non-lethal oxidative stress on ATP-induced calcium signaling.

What effect does lethal and non-lethal oxidative stress have on early basal $[Ca^{++}]_i$ values?

Neither lethal nor non-lethal oxidative stress concentrations affect basal levels of calcium over the first 2 hours of observation when compared

to control cells (Table 3). Thus menadione does not appear to modulate baseline $[Ca^{++}]_i$ over a period of 2 hours under these experimental conditions.

What effect does lethal and non-lethal menadione exposure have on ATP-generated calcium responses?

Most hepatocytes in both the lethal and non-lethal groups were viable after 2 hours. This 2 hour time point was chosen to assess responses to calcium-mobilizing agonists in these viable but "oxidatively stressed" hepatocytes. $[Ca^{++}]_i$ was determined every minute for 45 minutes in hepatocytes exposed to ATP after this 2 hours of treatment with menadione. Control hepatocytes not treated with menadione show an early, dose-dependent, IP_3-related calcium spike (figure 6). Late calcium concentrations were lower than the early peak calcium levels (figure 7). In contrast, early ATP induced calcium spikes in cells exposed to both lethal and non-lethal oxidative stress are significantly less than those of control cells (figure 6). Late $[Ca^{++}]_i$ in cells exposed to non-lethal levels of oxidative stress are not significantly different from control cells (figure 7). However, late $[Ca^{++}]_i$ in cells exposed to lethal levels of oxidative stress are significantly higher than controls and the increases of $[Ca^{++}]_i$ are generally followed by leakage of fura-2-AM from cells, suggesting cell membrane lysis and cell death.

These data indicate that early calcium spikes are significantly less in cells exposed to lethal and non-lethal oxidative stress, while late calcium spikes are significantly higher only in the cells exposed to lethal oxidative

stress.

Are effects on calcium responses due to depletion of intracellular calcium stores?

Severe oxidative stress has been shown to deplete intracellular calcium stores (Bellomo, 1985). If this occurred in this study, mobilization of calcium from internal stores might be decreased in cells undergoing oxidative stress, thus explaining the decrease in the initial IP_3-related $[Ca^{++}]_i$ increase observed in hepatocytes treated with menadione. To determine the integrity of intracellular calcium stores, thapsigargin was employed. Thapsigargin is a cell permeable compound which inhibits the calcium pump found on the endoplasmic reticulum and causes the discharge of intracellular calcium (Thomas and Hanley, 1994). Hepatocytes loaded with fura-2-AM were placed on the microscope stage and treated with 200 nM thapsigargin. Calcium concentrations were calculated every 30 seconds for the subsequent 20 minutes. Control cells treated with 200 nM thapsigargin release calcium with maximum spikes of 400% of baseline values (Figure 8). Viable hepatocytes were exposed to 200 nM thapsigargin after 2 hours of lethal oxidative stress. Peak $[Ca^{++}]_i$ after thapsigargin exposure was similar to control cells (Figure 8). Thus, intracellular calcium store depletion does not explain the differences observed in ATP-induced $[Ca^{++}]_i$ changes during non-lethal and lethal oxidative stress.

Are the changes in $[Ca^{++}]_i$ regulation after ATP exposure in menadione treated cells associated with increased cell mortality?

Because increases in cytosolic $[Ca^{++}]_i$ may exacerbate cell injury during oxidative stress, we assessed whether menadione and ATP in combination were more toxic than either of the compounds applied alone. Control cells, cells exposed to non-lethal oxidative stress and cells exposed to lethal oxidative stress were analyzed for necrotic cell death with and without ATP exposure. Figure 9 shows that the combination of menadione (at both 10 μM and 100 μM) and any concentration of ATP is more toxic than either menadione or ATP alone. The combined toxicity shows a dose-response relationship. Thus, in addition to resulting in dysregulation of $[Ca^{++}]_i$ response to ATP, non-lethal and lethal oxidative stress induced by menadione sensitizes hepatocytes to otherwise non-toxic concentrations of calcium mobilizing receptor agonist ATP.

Is the co-toxicity of menadione and ATP dependent on extracellular calcium influx?

Influx of extracellular calcium is commonly associated with cell injury (Rosser, 1995). If extracellular calcium influx was responsible for the co-toxicity of menadione and ATP, exclusion of extracellular calcium should decrease the toxicity induced by ATP in menadione treated hepatocytes. When menadione treated hepatocytes were exposed to ATP in a medium free of extracellular calcium the increase in cell death was no longer

observed (figure 10). Thus, the observed exacerbation of menadione toxicity by ATP is dependent on extracellular calcium influx.

DISCUSSION

The goal of this thesis was to investigate the effects of oxidative stress on calcium regulation and viability in hepatocytes. Firstly, I wanted to determine if toxic concentrations of menadione induced apoptosis or necrosis in hepatocytes. The data show that oxidative stress induced by menadione results in hepatocyte necrosis but not hepatocyte apoptosis.

Secondly, I was interested in determining if the regulation of intracellular calcium was disrupted in hepatocytes exposed to lethal and non-lethal oxidative stress. In order to study this question I developed a definition of lethal and non-lethal oxidative stress in isolated hepatocytes. Our data showed that $[Ca^{++}]_i$ responses to calcium signaling agents (ATP) were abnormal in cells exposed to different levels of oxidative stress.

Thirdly, I wanted to determine if this dysregulation of calcium responses during non-lethal and lethal oxidative stress was associated with a sensitization to otherwise non-toxic concentrations of the calcium mobilizing receptor agonist, ATP (i.e. a co-toxicity of oxidative stress and ATP). The data confirmed that oxidative stress sensitizes hepatocytes to ATP and demonstrated co-toxicity of ATP and the prooxidant, menadione.

Fourthly, I was interested in the mechanism behind the observed co-

toxicity. The data suggest that the exacerbation of non-lethal and lethal oxidative injury by ATP is dependent on extracellular calcium influx.

The ultimate result of oxidative stress is cell death via necrosis or apoptosis. It has been shown that hepatocytes undergoing oxidative stress due to the effects of alcohol die via apoptosis at low alcohol concentrations and via necrosis at high alcohol concentrations (Higuchi, 1996). Through review of the literature, it is apparent that oxidative stress in general, can cause hepatocyte necrosis or apoptosis.

Menadione-induced oxidative stress has been shown to cause apoptosis at low concentrations and necrosis at high concentrations in cell types such as kidney cells (Chiou, 1997), osteoblasts (Sun, 1997), and pancreatic acinar cells (Sata, 1997). It has been shown that menadione causes necrosis in hepatocytes but there is no clear evidence that shows menadione causes apoptosis (Denda, 1991). The data presented in this thesis also shows that toxic concentrations of menadione lead to hepatocyte necrosis rather than hepatocyte apoptosis.

Menadione was found to cause oxidative stress at a concentration of 10 μM, after 6 hours of exposure as indicated by the decrease in intracellular glutathione. At 10 μM, however, menadione did not cause a significant level of necrosis. When treated with 10 μM menadione, hepatocytes were capable of reaching a level of steady state which did not result in significant death. 10 μM menadione was determined to be the best model of non-lethal oxidative stress because it was the concentration that resulted in the lowest level of

mortality, while at the same time depleted GSH to a significant level. Menadione, after 6 hours of exposure, was found to cause lethal hepatocyte injury at a concentration of 100 μM menadione.

One of the early effects of cell injury is an alteration in calcium homeostasis and regulation (Nicotera, 1992). Xenobiotics causing oxidative stress can interfere with signal transduction at different levels through a loss of normal calcium responses (Bellomo, 1987). It has also been shown that cell injury due to heavy metal contamination can interfere with intracellular calcium transport and with the proper function of calcium channels and pumps (Hughes, 1989).

My study demonstrates that calcium mobilization and regulation in hepatocytes undergoing oxidative stress was significantly altered as compared to controls. Although 2 hours of treatment with an eventually toxic concentration of menadione (100 μM) and with a non-toxic concentration of menadione (10 μM) did not cause a significant change in basal calcium concentrations, early calcium peak concentrations reached in response to treatment with extracellular ATP were significantly different when compared to controls. Both non-lethal and lethal concentrations of menadione (10 μM and 100 μM) caused a significantly lower early or IP_3-related calcium peak.

As previously discussed, calcium stored in the endoplasmic reticulum is mobilized by IP_3 within 1 to 3 minutes following ATP exposure. Calcium concentration in cellular compartments (e.g. mitochondria) may be depleted by millimolar concentrations of menadione (Bellomo, 1985). I

considered that 10 μM and 100 μM menadione might be depleting calcium pools in the endoplasmic reticulum. Treatment of hepatocytes with thapsigargin, which inhibits the calcium pump on the endoplasmic reticular membrane and causes calcium extrusion from the endoplasmic reticulum showed that these stores of calcium were intact following menadione treatment. The explanation for the lower IP_3-related calcium peak is therefore due to some other mechanism disturbance than simply a decrease in endoplasmic reticular stores of calcium. Future studies would need to focus on the upstream pathway of receptor binding, IP_3 and DAG generation and IP_3 binding to the endoplasmic reticulum in order to determine the mechanism responsible for this observation (figure 3).

When analyzing the effects of menadione on calcium concentration after 15 minutes of ATP exposure, I observed that intracellular calcium concentration of hepatocytes exposed to 100μM menadione is significantly higher than controls (Figure 7). We also observed that both lethal and non-lethal concentrations of menadione combined with ATP were more toxic than either agent given alone (Figure 9). It has been shown, that in response to lethal injury, calcium levels rise beyond normal levels (Jewell, 1982). It has also been shown that increased cytosolic calcium concentration then leads to the activation of calcium-dependent degradative hydrolases including proteases (calpains), phospholipases (phospholipase-A_2), and endonucleases (Nicotera, 1992, Glende, 1986, McConkey, 1990). Activation of these hydrolases eventually results in cell necrosis (Rosser, 1995). My

data indicate that late calcium levels rise well above those of control cells when treated with ATP after exposure to 100 μM menadione. Perhaps it is the activation of calpains, phospholipases and endonucleases induced by the increased $[Ca^{++}]_i$ which leads to the increased cell death observed. Indeed, exclusion of extracellular calcium decreased toxicity of ATP in hepatocytes exposed to 100 μM menadione confirming a calcium dependent mechanism for the observed co-toxicity. However, this study was not designed to evaluate the downstream intracellular mechanisms for the observed calcium-dependence of toxicity.

In contrast to hepatocytes exposed to 100 μM menadione and ATP, those exposed to 10 μM menadione and ATP had a much lower intracellular calcium concentration after 15 minutes of exposure to ATP (Figure 8). In fact, the calcium concentrations were not significantly different from those of control cells. Because of the lower level of intracellular calcium concentration, it may be expected that cell death associated with 10 μM menadione and ATP would not be significantly different from control cells and ATP. As seen in Figure 9, this was not the case. Non-lethal oxidative stress in conjunction with treatment with ATP caused more cell death than with either non-lethal oxidative stress or ATP alone. Because this co-toxicity occurs without an increase in intracellular calcium, these data suggest that the toxicity is not due to an increase in intracellular calcium concentration. However, exclusion of extracellular calcium clearly decreases the toxic effect of ATP in these hepatocytes exposed to non-lethal oxidative stress. It may

be that the changes in $[Ca^{++}]_i$ in response to ATP in these cells are delayed and would be detected by $[Ca^{++}]_i$ measurements longer after exposure to ATP. Alternatively, the effect of the increased $[Ca^{++}]_i$ associated with ATP exposure in cells undergoing non-lethal oxidative stress may damage critical cellular systems. In this case, despite the restoration of $[Ca^{++}]_i$ over time to a level similar to controls, the cascade of cell death may continue through the subsequent activation of non-calcium dependent pathways (Rosser, 1995). Thus, exclusion of extracellular calcium would protect against this initial calcium-dependent injury and prevent subsequent non-calcium dependent hepatocyte necrosis. This issue remains unclear but does not contradict the concept that otherwise non-toxic levels of ATP can convert non-lethal oxidative stress to lethal hepatocyte injury through calcium dependent mechanisms.

SUMMARY

Based on the data presented, menadione leads to GSH depletion and eventually to necrotic cell death in hepatocytes. Non-lethal oxidative stress decreases the hepatocyte's ability to mobilize intracellular calcium in response to ATP when compared to controls. Lethal oxidative stress decreases the hepatocyte's ability to mobilize intracellular calcium in response to ATP when compared to controls. In addition, lethal oxidative stress results in a late increase in cytosolic calcium levels after ATP

exposure. Thus, oxidative stress disrupts normal responses to receptor-mediated calcium mobilization in hepatocytes. Both non-lethal and lethal oxidative stress sensitized hepatocytes to toxic effects of ATP. This sensitization required the presence of extracellular calcium indicating that menadione and ATP are co-toxic through a calcium-dependent mechanism.

Table 1. Common prooxidants and antioxidants

Prooxidants	Antioxidants
superoxide anion ($O_2^{\cdot-}$)	superoxide dismutase (SOD)
hydrogen peroxide (H_2O_2)	glutathione peroxidase + glutathione transferase
hydroxyl radical ($OH^{\cdot}$)	glutathione catalase
hyperchlorous acid (OHCl)	ascorbic acid
chloramine	uric acid
singlet oxygen	taurine
peroxyradicals	beta-carotene
	alpha-tocopherol

Table 2. Summary of the Four Biological roles of Calcium

I Structural	***Form of Calcium***	***Example***
	Calcium phosphate	Bone, teeth
	Calcium phospholipid	Membrane fluidity and integrity
	Calcium proteinate	Cell adhesion, gap junctions, connexions
	Calcium nucleate	Chromatin structure
II Electrical	***Form of Calcium***	***Example***
	Ionic	current carried during action potential
III As a cofactor for proteins	***Protein requiring calcium***	***Source of protein***
	alpha-amylase	saliva
	trypsin	pancreas and duodenum
	DNAase I	pancreas
	Transglutaminase	serum
	blood clotting factors	serum
	antibody-antigen complex	serum
IV As an intracellular regulator	***Phenomenon regulated***	***Example***
	Cell movement	Muscle contraction
	Secretion	Neurotransmitter, endocrine and exocrine releases
	Cell division	Egg fertilization
	Membrane permeability	Substrate uptake, cation permeability in action potentials, cell to cell communication

Table 3 Basal Calcium Concentration in Controls and Menadione treated Hepatocytes

Menadione Exposure	*$[Ca^{++}]_i$ nM*
none	200 ± 20
10 μM	216 ± 14
100 μM	194 ± 26

Hepatocytes were incubated in KRH buffer with 2 mM $CaCl_2$ for 2 hours with the designated concentration of menadione. $[Ca^{++}]_i$ was measured after this exposure period using Fura-2-AM and digitized video fluorescence microscopy.

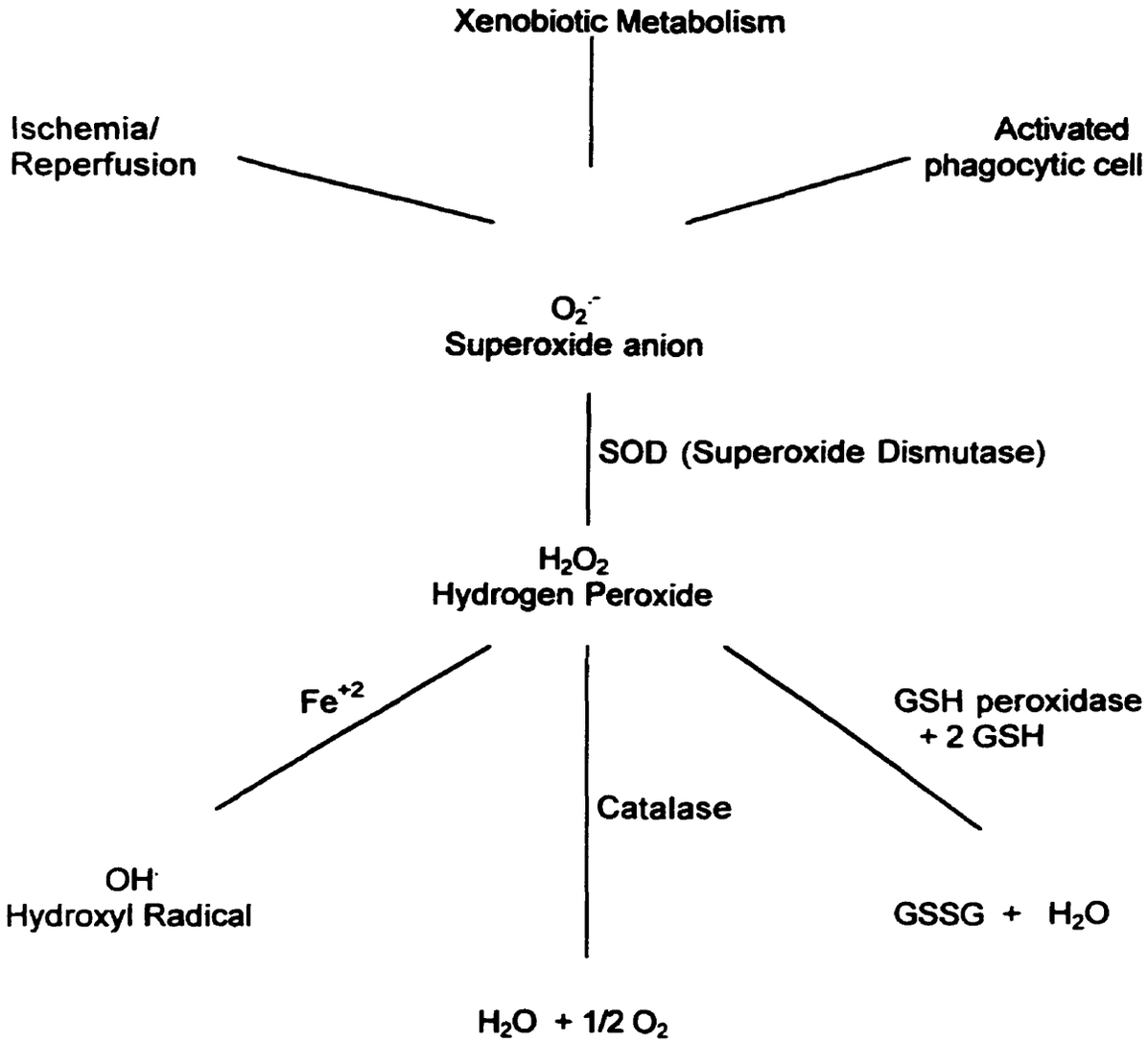

Figure 1 Generation of prooxidants and enzymatic detoxification of hydrogen peroxide and the superoxide anion.

$O_2^{\cdot-}$ is usually the first prooxidant formed but is quickly metabolized. It is enzymatically converted to H_2O_2 which can be detoxified to water or converted to other prooxidants.

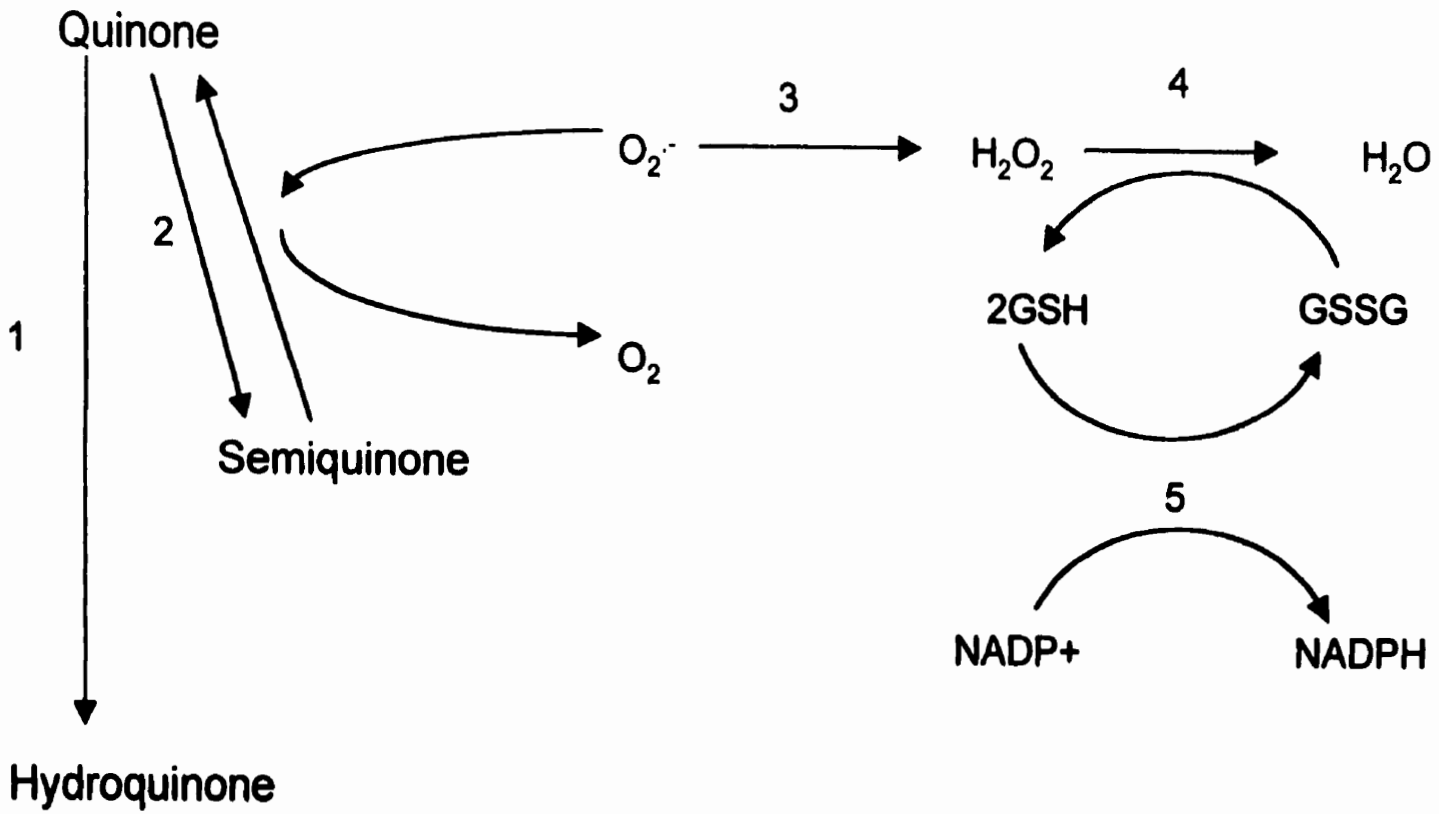

Figure 2 Metabolism of redox active quinones (i.e. menadione) by isolated rat hepatocytes. Numbers refer to different enzyme catalyzed steps: 1. DT-diaphorase; 2. Flavoenzyme; 3. Superoxide dismutase; 4. GSH peroxidase; 5. GSH reductase.

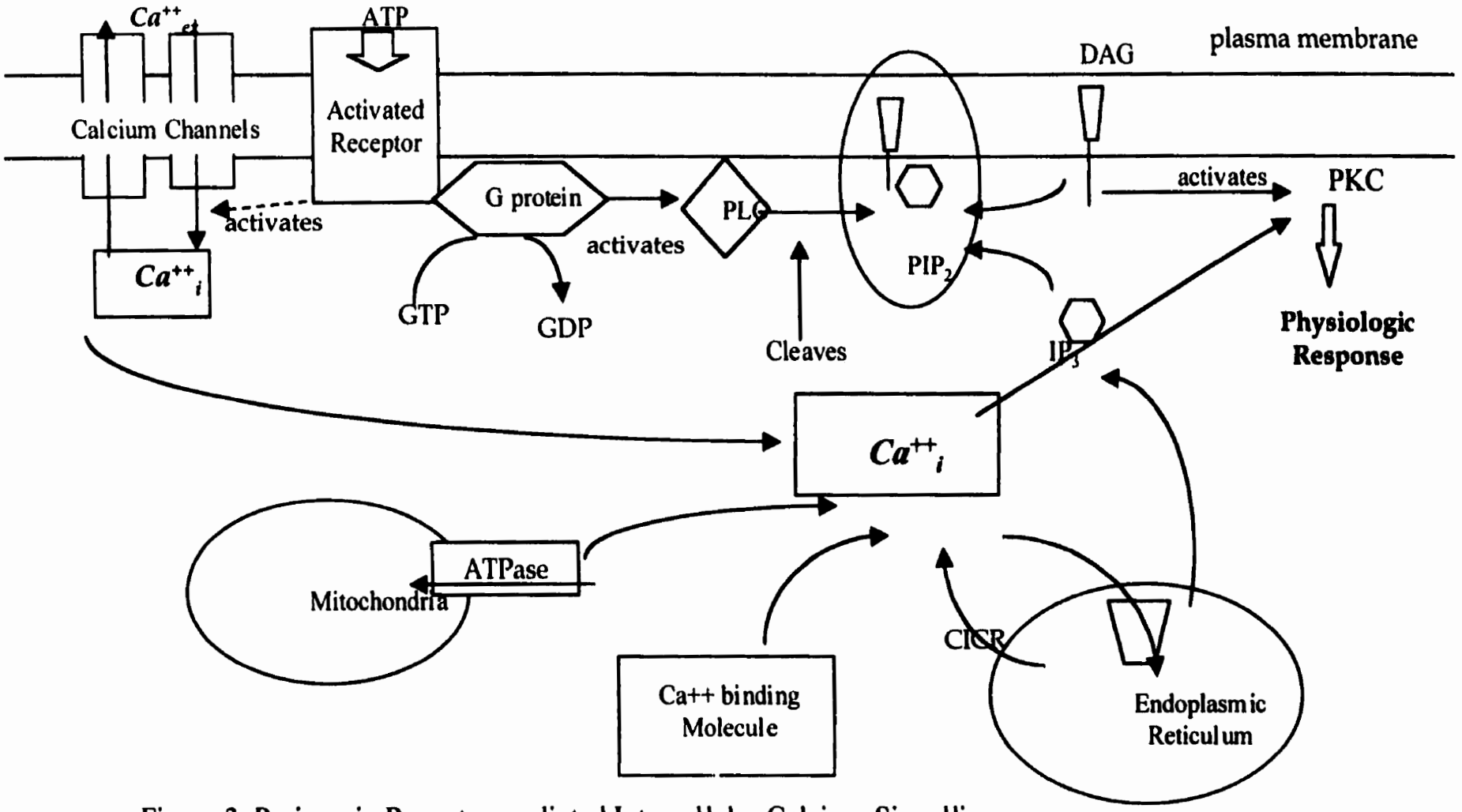

Figure 3 Purinergic Receptor mediated Intracellular Calcium Signalling
Established pathways in hepatocytes (solid lines). Pathways documented in other cell lines (dotted lines).

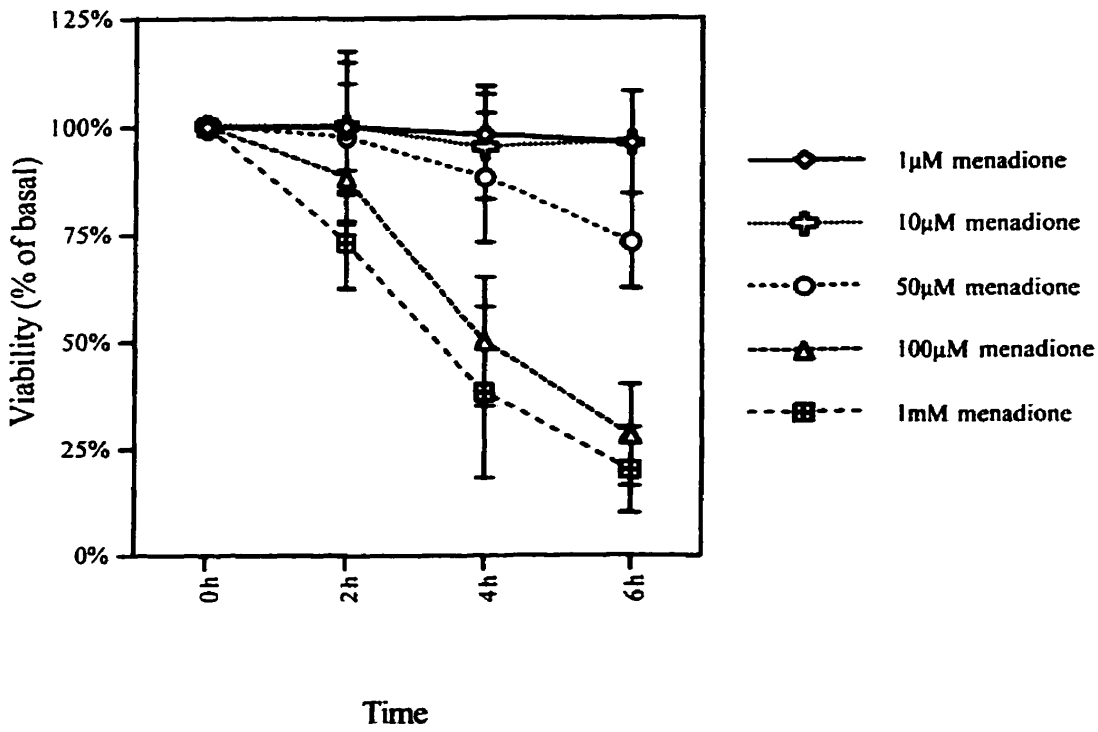

Figure 4. Hepatocyte viability with menadione treatment

Hepatocytes were treated for 6 hours with varying concentrations of menadione. At 0, 2, 4 and 6 hours, viability was determined using the LDH assay (see methods). Menadione induces a time a dose-dependent hepatocyte necrosis. A similar pattern of injury was seen in cell evaluated with the propidium iodide assay (see results).

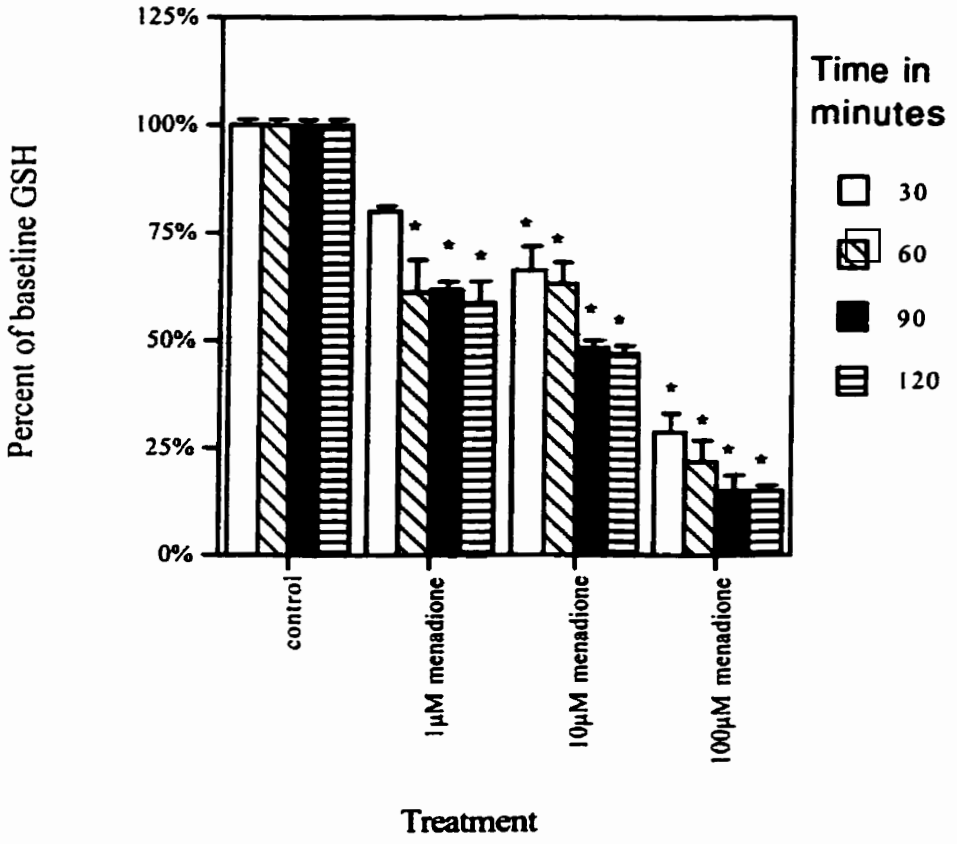

Figure 5. Cellular glutathione depletion with menadione treatment Hepatocytes were incubated with varying concentrations of menadione and GSH content was determined at 0, 30, 60, 90 and 120 minutes (see methods). Menadione induces a dose and time dependent depletion of hepatocyte glutathione.

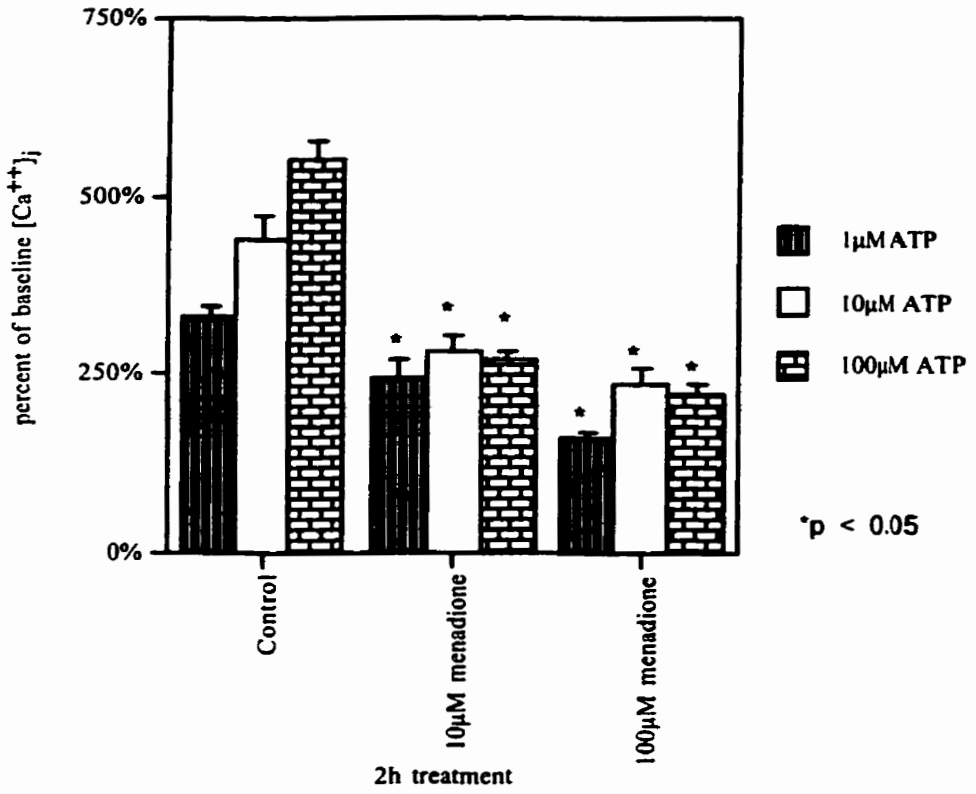

Figure 6 Early intracellular calcium peak in hepatocytes undergoing non-lethal and lethal oxidative stress following ATP exposure

Cells were treated for 2 hours with menadione, loaded with FURA-2-AM and viewed on the DVFM system stage (see methods). Baseline $[Ca^{++}]_i$ and $[Ca^{++}]_i$ at 1-3 minutes after exposure to ATP was determined. Early $[Ca^{++}]_i$ peak in response to ATP was suppressed in hepatocytes undergoing both lethal and non-lethal oxidative stress.

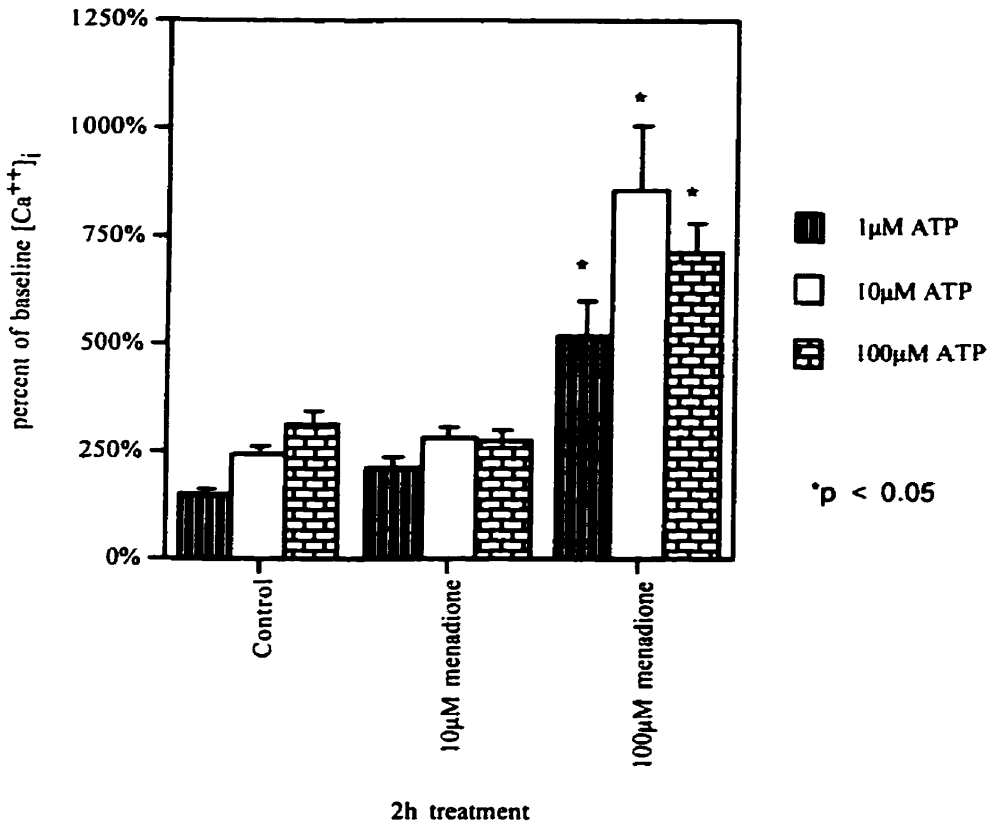

Figure 7. Late intracellular calcium peak in hepatocytes undergoing non-lethal and lethal oxidative stress following ATP exposure

Cells were treated for 2 hours with menadione, loaded with FURA-2-AM and viewed on the DVFM system stage (see methods). Baseline $[Ca^{++}]_i$ and $[Ca^{++}]_i$ 15 minutes after exposure to ATP was determined. Late $[Ca^{++}]_i$ peak in response to ATP was exaggerated in hepatocytes exposed to lethal oxidative stress.

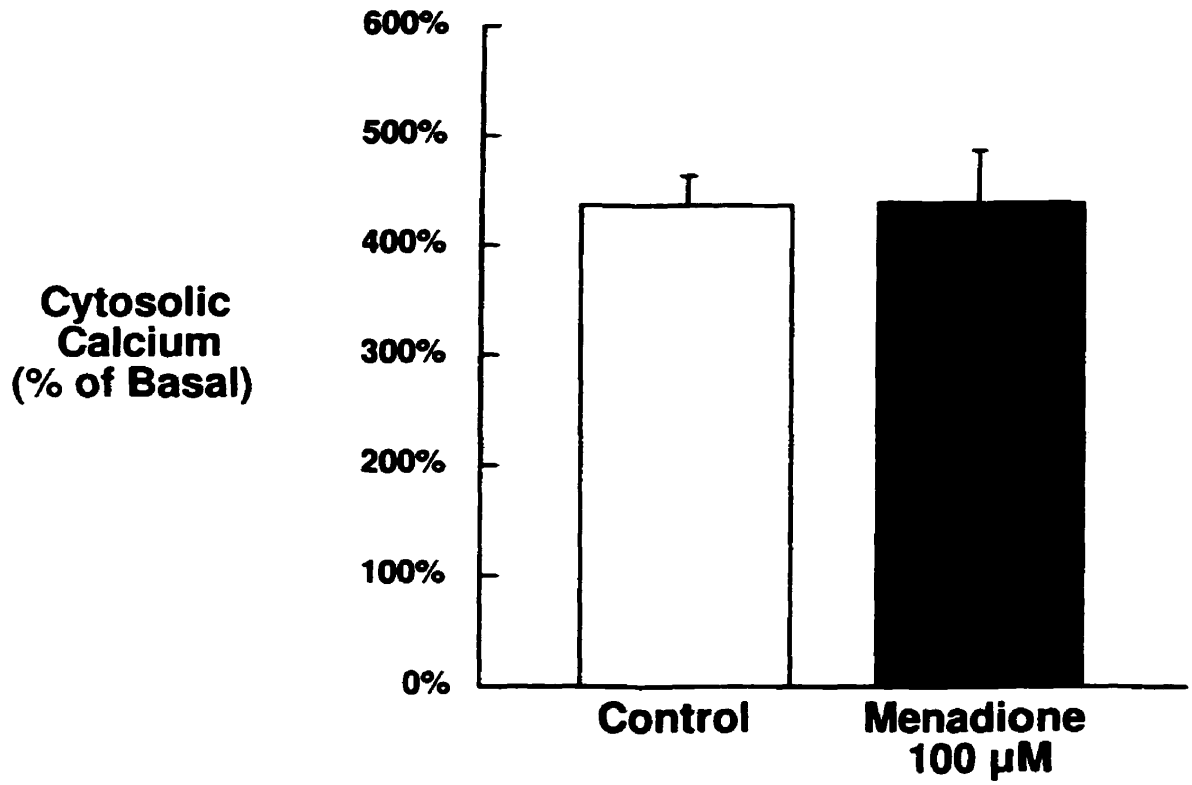

Figure 8. Intracellular calcium stores of control cells versus menadione treated with hepatocytes

After 2 hours of incubation in media with and without menadione, hepatocytes loaded with FURA-2-AM were treated with 200nM thapsigargin and peak $[Ca^{++}]_i$ was determined. No difference in $[Ca^{++}]_i$ response to thapsigargin was observed between control and menadione treated hepatocytes.

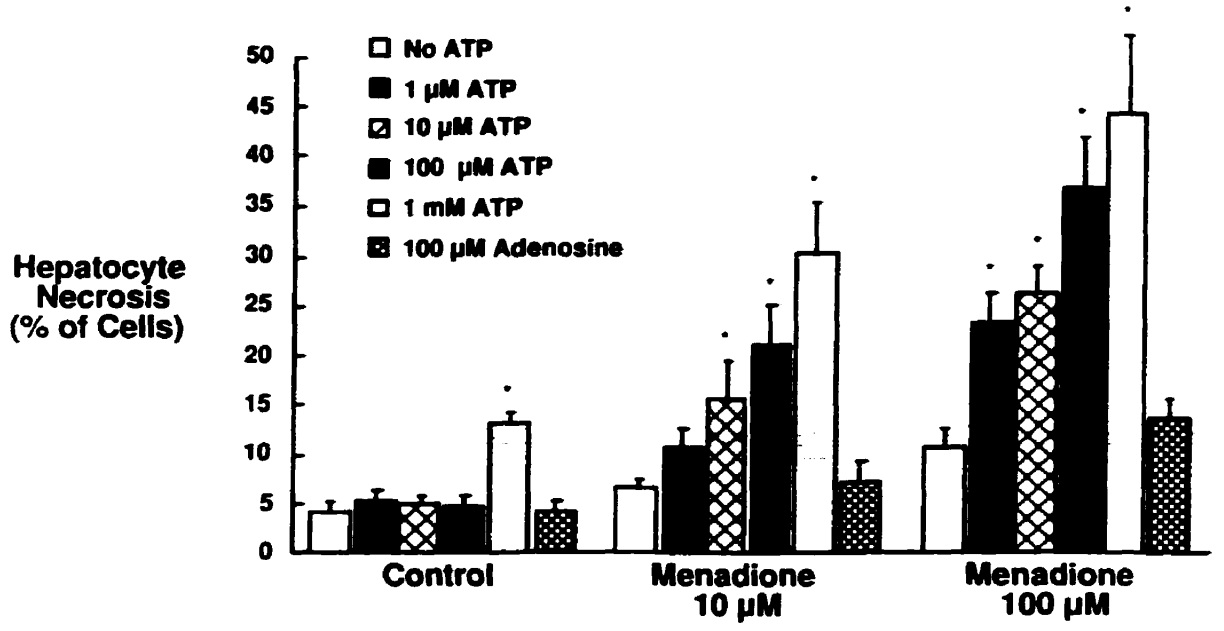

Figure 9 Hepatocyte necrosis associated with menadione and ATP treatment in the presence of extracellular calcium

Cells were treated for 2 hours with menadione followed by treatment with ATP for 30 minutes in a calcium containing (2 mM) KRH buffer. Cells were then loaded with propidium iodine and assessed for cell necrosis using DVFM. ATP accentuated both lethal and non-lethal oxidative cell death in the presence of extracellular calcium.

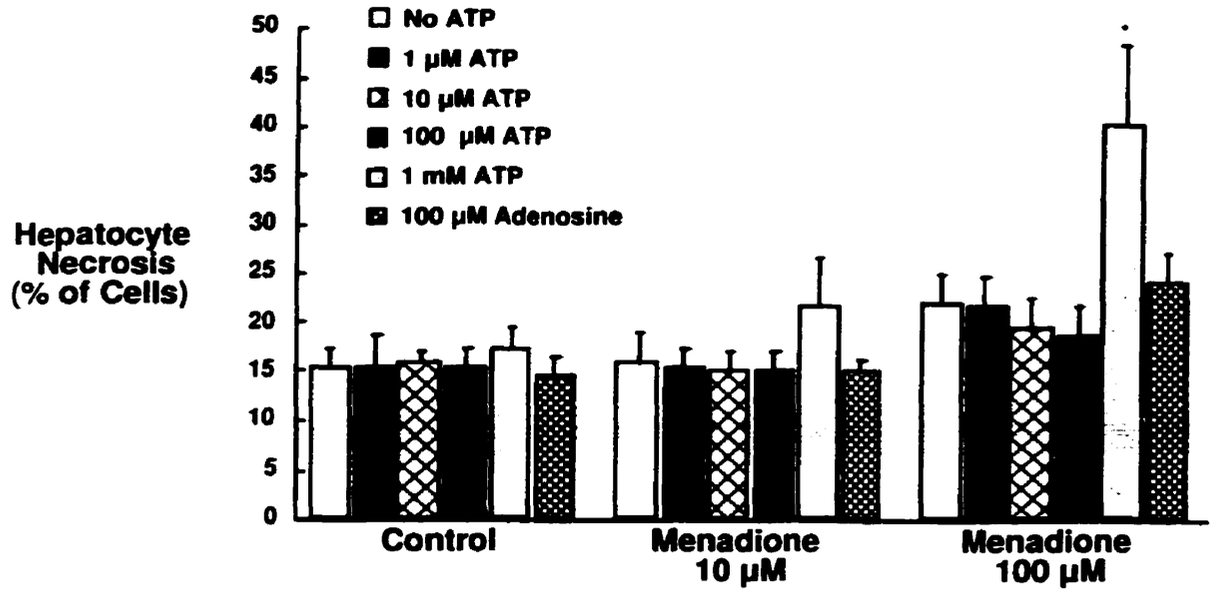

Figure 10. Hepatocyte necrosis associated with menadione and ATP treatment in the absence of extracellular calcium

Cells were treated for 2 hours with menadione followed by treatment with ATP for 30 minutes in a calcium free (0 mM) KRH buffer. Cells were then loaded with propidium iodine and assessed for cell necrosis using a digitized video fluorescent microscopy system. ATP did not accentuate either lethal or non-lethal oxidative cell death in the absence of extracellular calcium.

REFERENCES

Alison MR and Sarraf CE (1994) Liver cell death: patterns and mechanisms. Gut, 35: 577-581.

Alpini G, Phillips JO, Vroman B and LaRusso NF (1994) Recent Advances in the Isolation of Liver Cells. Hepatology, 20(2): 494-510.

Bellomo G and Orrenius S (1985) Altered Thiol and Calcium Homeostasis in Oxidative Hepatocellular Injury. Hepatology, 5(5): 876-882.

Bellomo G, Thor H and Orrenius S (1987) Alteration in Inositol Phosphate Production during Oxidative Stress in Isolated Hepatocytes. The Journal of Biological Chemistry, 262(4) 1530-1534.

Berridge MJ (1993) Inositol triphosphate and calcium signaling. Nature, 361: 315-325.

Campbell AK. Intracellular Calcium its Universal Role as Regulator: *Chapter 1*, Toronto, John Wiley & Sons Limited, pp 15-17, 1983.

Chiou TJ, Zhang J and Ferrans VJ (1997) Cardiac and Renal Toxicity of Menadione in Rat. Toxicology, 124(3): 193-202.

Dawson TL, Gores GJ, Niemenen A, Herman B, Lemasters J (1993) Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. American Journal of Physiology 264: c961-967.

Denda A, Kimie S, Tang Q, Tsugiuchi T, Tsutsumi M, Amanuma T, Murata Y, Nakae D, Maruyama H, Kurokawa Y and Konishi Y (1991) Induction of 8-hydroxydeoxyguanosine but not initiation of carcinogenesis by redox enzyme modulations with or without menadione in rat liver. Carcinogenesis, 12(4): 719-726.

Di Monte D, Bellomo G, Thor H (1984) Menadione-induced cytotoxicity is associated with protein thiol oxidation and alterations in intracellular calcium homeostasis. Arch biochem Biophys 235: 343-350.

Doherty PC (1993) Cell-Mediated Cytotoxicity. Cell, 75: 607-612.

Fridovich I (1983) Superoxide Radical: an endogenous toxicant. Annual Review of Pharmacology and Toxicology, 23: 239-257.

Glende EA and Pushpendran KC (1986) Activation of phospholipase A2 by carbon tetrachloride in isolated rat hepatocytes. Biochemical Pharmacology, 35: 3301-3307.

Granger DN and Kvietys PR (1985) Recent Advances in Measurement of Gastrointestinal Blood Flow. Gastroenterology 88(4): 1073-1076.

Grynkiewicz G, Poenie M and Tsien RY (1985) A New Generation of Ca^{2+} Indicators with Greatly Improved Fluorescence Properties. The Journal of Biological Chemistry, 260(6): 3440-3450.

Herman B, Gores GJ, Nieminen AL, Dawanishi T, Harman A and Lemasters JJ (1990) Calcium and pH in Anoxic and Toxic Injury. Toxicology 21(2): 127-148.

Higuchi H, Kurose I, Kato S, Miura S and Ishii H (1996) Ethanol-induced apoptosis and oxidative stress in hepatocytes. Alcohol-Clin-Exp-Res 20(9): 340A-346A.

Houglum K, Brenner DA, Chojkier M (1991) d-alpha-Tocopherol Inhibits Collagen alpha-1(I) Gene Expression in Cultured Human Fibroblasts. Journal of Clinical Investigation, 87: 2230-2235.

Hughes BP and Barritt GJ (1989) Inhibition of the liver cell receptor-activated calcium inflow system by metal ion inhibitors of voltage-operated calcium channels but not by other inhibitors of calcium inflow. Biochim. Biophys.

Acta 1013: 197-205.

Jewell SA, Bellomo G, Thor H and Orrenius S (1982) Bleb Formation in Hepatocytes During Drug Metabolism is Caused by Disturbances in Thiol and Calcium Ion Homeostasis. Science 217: 1257-1258.

Kass G, Bellomo G, Juedes M, Orrenius S (1993) Toxic effects of calcium on mitochondria. Methods in Toxicology 2: 378-388.

Kato S, Kawase T, Alderman J, Inatomi N and Lieber CS (1990) Role of Xanthine Oxidase in Ethanol-induced Lipid Peroxidation in Rats. Gastroenterology 98(1): 203-210.

Kodavanti PRS and Mehendale HM. Hepatotoxicology: *Biochemical Methods of Studying Hepatotoxicity*, Florida, CRC Press, pp241-325, 1991.

Konstantinov AA, Peskin AV, Popova EY, Khomutov GB and Ruuge EK (1987) Superoxide generation by the respiratory chain of tumor mitochondria. Biochimica et Biophysica Acta 894: 1-10.

Lemasters JL, DiGuiseppi J, Nieminen AL and Herman B (1987) Blebbing, free calcium and mitochondrial membrane potential preceding cell death in hepatocytes. Nature 325: 78-81.

McConkey DJ, Chow SC, Orrenius S and Jondal M (1990) NK cell-induced cytotoxicity is dependent on a calcium increase in the target. FASEB Journal 4: 2661-2664.

Morehouse LA, Thomas CE and Aust SD (1984) Superoxide Generation by NADPH-Cytochoursome P-450 Reductase: The Effect of Iron Chelators and the Role of Superoxide in Microsomal Lipid Peroxidation. Archives of Biochemistry and Biophysics 232(1): 366-377.

Nicotera P, Bellomo G and Orrenius S (1992) Calcium-mediated Mechanisms in Chemically Induced Cell Death. Annual Review of Pharmacology and Toxicology 32: 449-470.

Patel T and Gores G (1995) Apoptosis and Hepatobiliary Disease. Hepatology 21(6): 1725-1741.

Popper H (1986) General Pathology of the Liver: Light Microscopic Aspects Serving Diagnosis and Interpretation. Seminars in Liver Disease 6(3): 175-184.

Rosen GM, Singletary WV, Rauckman EJ and Killenberg PG (1983) Acetaminophen Hepatotoxicity: an alternative mechanism. Biochemical Pharmacology 32(13): 2053-2059.

Rosser B, Powers S, Gores G (1993) Calpain Activity Increases in Hepatocytes Following Addition of ATP. The Journal of Biological Chemistry 268(31): 23593-23600.

Rosser BG and Gores G (1995) Liver Cell Necrosis: Cellular Mechanisms and Clinical Implications. Gastroenterology 108: 252-275.

Sata N, Klonowski-Stumpe H, Han B, Haussinger D and Niederau C (1997) Menadione Induces both Necrosis and Apoptosis in Rat Pancreatic Acinar AR4-2J cells. Free Radical Biology and Medicine 23(6): 844-850.

Searle J, Kerr J and Bishop C (1982) Necrosis and Apoptosis: Distinct Modes of Cell Death with Fundamentally Different Significance. Pathology Annual 17(2): 229-259.

Sies H. Oxidative Stress: *Introductory Remarks*, Florida, Academic Press, pp1-7, 1985.

Smith MT, Evans CG, Thor H and Orrenius S. Oxidative Stress: *Quinone-*

Induce Oxidative Injury to Cells and Tissues, Florida, Academic Press, pp 91-113, 1985.

Sun JS, Tsuang YH, Huang WC, Chen LT, Hang YS and Lu FJ (1997) Menadione-induced Cytotoxicity to Rat Osteoblasts. Cellular and Molecular Life Sciences 53(11-12): 967-976.

Thomas D and Hanley M (1994) Pharmacological Tools for Perturbing Intracellular Calcium Storage. Methods in Cell Biology 40: 65-89.

Trump BF and Berezesky IK (1992) The Role of Cytosolic Calcium in Cell Injury, Necrosis and Apoptosis. Current Opinion in Cell Biology 4: 227-232.

Vaux DL (1993) Toward an Understanding of the Molecular Mechanisms of Physiological Cell Death. Proc. Natl. Acad. Sci. USA 90: 786-789.

Weiss SJ (1989) Tissue Destruction by Neutrophils. The New England Journal of Medicine 320(6): 365-374.

Wyllie AH, Kerr JFR and Currie AR (1980) Cell Death: the significance of apoptosis. Int. Rev. Cytol. 68: 251-306.