Reactive Oxygen Species Generated by Depletion of Cellular Glutathione Mediate Cadmium Cytotoxicity

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REACTIVE OXYGEN SPECIES GENERATED BY DEPLETION OF CELLULAR GLUTATHIONE MEDIATE CADMIUM CYTOTOXICITY

by

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Studies were put forth to test whether or not reactive oxygen species, which are influenced by the cellular level of reduced glutathione, mediate the toxicity of cadmium. Cadmium has been a focal point in the health care community in the past few years because it has been shown to induce the development of tumors, interfere with cellular signaling, and cause damage to organs, tissues, and biomolecules. Although various studies and theories have been proposed, little direct evidence, especially from studies utilizing primary cells in culture, has accumulated to show the mechanism by which the cadmium induced toxicity occurs.

Our studies show that cadmium decreases the level of glutathione in primary rat hepatocytes in a time and concentration dependent manner. In the presence of cadmium, increases in the cytotoxic parameters, lactate dehydrogenase leakage and lipid peroxidation, were observed indicating the presence of oxidative stress. Interestingly, the decrease in the level of cellular glutathione occurred at a time point prior to the observation of cadmium cytotoxicity. Furthermore, the induction of the cytotoxic parameters by cadmium was significantly blocked by various antioxidants. These results suggest that reactive oxygen species, which are generated by depleting cellular glutathione, may play an important role in various cellular events induced by cadmium.
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CHAPTER I

INTRODUCTION

Cadmium and Cadmium Toxicity

Cadmium (Cd) is a common environmental pollutant found in water, air, and soil. Sources of Cd pollution include smelting and mining operations, corrosion of metal-plated iron, disposal of Cd-containing consumer products (e.g., batteries, pigments, and stabilizers), and from fertilization of pastures and croplands with urban sewage sludge. Cd has a resident half-life of 18 - 33 years in human tissue, much longer than other trace metals (Experimental Nutrition, 1988). Due to its extensive use and long biological half-life, the potential health effects of Cd in humans have attracted much attention over the years. The effects of Cd on cytotoxicities, gene regulation, signal transduction, aging, and cell proliferation in either whole animals or cell cultures have been studied and reported. Based on evidence from both experimental and epidemiological investigations, the International Agency for Research on Cancer has classified Cd as a Group 1 carcinogen in both humans and rodents (IARC, 1993).

In animals, the carcinogenicity of Cd has been widely studied and reported. Cd is shown to induce lung tumors in humans and experimental animals. It also induces the tumors of the prostate and testis, and local tumors at various injection
sites in experimental animals (IARC, 1993). It is believed that Cd is not a strong initiator of tumors because it is a weak mutagen (Beyersmann and Hartwig, 1994). Although the carcinogenic mechanism of action is unclear, Cd has been shown to be capable of stimulating the expression of proto-oncogenes such as \textit{c-jun} and \textit{c-myc} in rat L6 myoblast cells (Jin & Ringertz, 1990; Abshire et al., 1996); \textit{c-jun, c-fos, and c-myc} in Osborn-Mendel rat kidney fibroblast (NRK) cells (Tang & Enger, 1993); \textit{c-fos} and \textit{egr-1} in mouse fibroblast cells (Epner & Hershman, 1991); and \textit{c-jun, c-fos, c-myc} and \textit{egr-1} in rat kidney epithelial (LLC-PK1) cells (Matsuoka & Call, 1995). The induction of these proto-oncogenes at the transcription level may be the cause of the Cd-induced malignant transformation of the cells (Jin & Ringertz, 1990; Abshire et al., 1996; Terracio & Nachtigal, 1998).

The involvement of signal proteins in the Cd-mediated proto-oncogene induction has been studied by numerous groups. Protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) are two well-defined signal proteins. PKC regulates the phosphorylation of transcription factors and their responsive genes. Activation of the MAPK pathway is believed to contribute to amplification and specificity of other signals that ultimately regulate cytoplasmic and nuclear events. In mouse fibroblasts, Block et al. (Block et al., 1992) showed that PKC was activated after cells were exposed to Cd. An inhibitor of PKC, H7, significantly blocked the accumulation of \textit{c-myc} mRNA induced by Cd (Tang & Enger, 1993), indicating the possible involvement of PKC in the induction of \textit{c-myc} gene. A study by Wang and Templeton (1997) suggests that Cd induces \textit{c-fos} expression in part by causing a
sustained activation of MAPK independent of its ability to activate PKC in rat mesangial cells (RMC). Although the mechanisms of Cd-induced activation of signal proteins have not been described yet, we now at least know that Cd can modulate cellular signaling, which further affects cytoplasmic or nuclear events.

In addition to the carcinogenic studies, in whole animals Cd also causes damage to organs and tissues. Sarkar et al. (1995) showed that treatment with Cd (0.4 mg/kg body weight, intra-pelvic injection) significantly increased lipid peroxidation (LPO), which is a consequence of the generation of reactive oxygen species (ROS), in rat heart (3 hours), kidney (6 hours), and liver (12 hours). The peroxidative damage noted in these studies may cause injury to cellular components due to the interaction of metal ions with the cell’s organelles. A study by Karmakar et al. (1998) also showed that LPO was elevated in mouse liver after oral Cd exposure (2.5 mg/kg body wt.). Increases in LPO in rat testes (Kojima et al., 1990), rat gastric mucosa (Oner et al., 1994), rat brain (Gill et al., 1989), and rat erythrocytes (Sarkar et al., 1997) after administration of Cd, by either injection or oral supplements, have also been reported.

In cell culture models, Cd is found to be cytotoxic (Frenal et al., 1986), mutagenic (Ochi and Ohsawa, 1983) and transformative (Dipaolo and Casto, 1979). The cytotoxic parameters, LPO and lactate dehydrogenase (LDH), have been shown to increase in a variety of cell types after Cd exposure. LDH leakage is generally considered to be a consequence when cell the membrane is compromised. In a human fetal lung fibroblast cell line (MRC-5 cells), Cd has been shown, in a time (0-20 hours) and dose (0-35 μM of Cd) dependent manner, to increase the level of LPO and
LDH leakage (Yang et al., 1997). This Cd induced increase in LPO and LDH leakage is also observed in a human fetal hepatic cell line (WRL-68 cells) (Bucio et al., 1995), in isolated rat hepatocytes (Martel et al., 1990; Santone et al., 1982), in bovine vascular smooth muscle cells (Kaji et al., 1994), and in LLC-PK1 cells (Wispriyono et al., 1998).

In addition, at the molecular level, Cd is capable of causing DNA single-strand breaks and chromosomal aberrations in various types of cultured mammalian cells (Tsuzuki et al., 1994; Ochi et al., 1983; Ochi & Ohsawa, 1985). The inhibition of DNA repair and synthesis (Chin & Templeton, 1993; Nocentini, 1987), RNA and protein synthesis (Cohen et al., 1991; Hidalgo et al., 1976) by Cd in different types of cells has also been studied and reported.

The studies described above demonstrate LPO to be an early and sensitive consequence of Cd exposure, which might enhance Cd-induced cellular cytotoxicity such as LDH leakage, DNA and chromosome damage. However, the mechanisms by which Cd induces cytotoxicity, regulates intracellular signaling, modulates the expression of specific genes, or inhibits genetic information transfer are still not clear. Previous studies have shown that these events induced by Cd can be prevented in the presence of free radical scavengers and antioxidants, suggesting that reactive oxygen species (ROS) may mediate many of the adverse effects of Cd (Shaikh et al., 1999; Ochi et al., 1987). This conclusion is supported by the finding resistant Chinese hamster ovary (CHO) cells are cross-resistant to high Cd concentrations (Sugiyama, 1994).
Reactive Oxygen Species

Reactive Oxygen Species (ROS) are either free radicals such as, or reactive non-radical compounds such as H₂O₂. ROS are generated inside the cell during oxidative metabolism and various sources of ROS have been identified in living organisms (Figure 1). As shown in Figure 1, the reactivity of molecular oxygen (O₂) increases upon acceptance of one, two, or three electrons to form, respectively, O₂•, H₂O₂, and OH•. Among these ROS, O₂• appears to play a central role as other reactive intermediates are formed via this as an intermediate (Scott, 19987). O₂• is formed upon one-electron reduction of O₂ and is mediated by enzymes such as NADPH oxidase or the respiratory chain.

Figure 1. Formation of Reactive Oxygen Species (ROS). Reaction (1) one-electron reduction; (2) superoxide dismutase; (3) free Fe²⁺, as in Fenton reaction; (4) catalase; (5) x-ray; (6) initiation of LPO.


OH• is the most reactive ROS due to its very high standard electrode
potential, +2.3 V (the standard potential of O₂ is ca. + 0.8 V) (Farr and Kogoma, 1991). It can spontaneously react with most biomolecules at the site where it is generated. The average diffusion distance of OH•, however, is only a few nanometers, thus its effect on any given biomolecule will depend largely upon the location of its formation (Singh & Singh, 1982). In a biological system, as shown in Figure 1, the major concern about OH• is its capability to initiate LPO. This is because LPO can enhance the oxidative damage by increasing the permeability of the cell membrane. This allows some extracellular oxidative agents such as heavy metals to have free access to react with intracellular components.

The reactions of H₂O₂ with organic molecules are not well understood, partly because it reacts quickly with contaminating metals to form more reactive species, which obscure its own role in oxidation reactions. It can also act as a weak oxidizing agent and attack thiol groups of proteins and reduced glutathione (GSH) (Halliwell & Gutteridge, 1990; Wefers & Sies, 1983). Additionally, H₂O₂ will react with reduced iron or copper ions to generate OH• in the Fenton reaction:

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{OH}• + \text{Fe}^{3+} \]

\[ \text{O}_2• + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+} \]

Since H₂O₂ has a reasonable long diffusion distance and can freely pass through a membrane, it is probably able to augment the potential damage induced by OH•.

In a biological system, it is known that there are two distinct mechanisms with regard to metal-induced production of ROS. Transition metals such as iron, copper,
cobalt and vanadium are capable of oxidizing various substrates by a single-electron withdrawal, as shown in Fenton reaction. Whereas, other metals such as nickel, mercury, lead, zinc and Cd can deplete critical sulphydryl groups in a cell, such as the sulphydryl groups of GSH and proteins, to form cation radicals and ROS.

Effect of ROS on Biological System

It is clear that ROS are naturally produced in aerobic cells, arising from a variety of intracellular and extracellular sources. If the concentration of ROS, however, exceeds the capacity of the cellular defense system, it will give rise to an oxidative stress condition. Under this condition, abnormal production of ROS can damage biological macromolecules such as DNA, lipid, and proteins and therefore are currently discussed as pathobiochemical mechanisms involved in the initiation or progression of various diseases (Buttke & Sandstrom, 1994). In addition, these highly reactive molecules are known to regulate many important cellular events, such as gene expression. Lo et al. (1996) reported that ROS was involved in the stimulation of c-fos and c-jun gene expression, which generally are associated with enhanced cell proliferation, in bovine chondrocytes (Lo & Cruz, 1995). The activation of two well-defined transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), has been identified to be regulated by ROS in human T cells (Sen & Packer, 1996). In various types of cells, two important signal proteins, PKC and MAPK, are also reported to be activated when cells are under an oxidative stress condition (Stevenson et al., 1994; Lander et al., 1996; Larsson & Cerutti, 1989). Fialkow et al.
(1994) demonstrated that treatment of cells with either oxidizing agent or H$_2$O$_2$ produced a phosphorylation and activation of MAPK. These studies also provided evidence that pretreatment of cells with antioxidants could inhibit the induction of early gene expression as well as the activation of signal proteins and transcription factors, supporting the hypothesis that ROS may act as a second messengers in cellular signaling. At the molecular level, ROS has been shown to cause DNA single-strand breaks, DNA double-strand breaks, and DNA fragmentation, which are also partly or completely protected by various antioxidants (Li et al., 1994; Yu & Anderson, 1997; Izzotti et al., 1998).

It is well known that ROS generated intracellularly interacts with unsaturated fatty acids to initiate LPO (Huimin et al., 1994), a major factor influencing the breakdown of cell membranes (Sugiyama et al., 1993). LDH, on the other hand, is an intracellular enzyme released into the extracellular environment when cell membranes are compromised (Bonnekoh et al., 1990). Therefore, LPO and LDH leakage are commonly considered as important indicators of cytotoxicity induced by ROS and/or environmental agents. In isolated rat hepatocytes, Caraceni et al. (1994) have shown that the induction of LPO and LDH leakage follows the formation of ROS. Although previous studies using antioxidants suggest the involvement of ROS in Cd-induced cytotoxicity (Shaikh et al., 1999; Ochi et al., 1987), the mechanism by which Cd generates intracellular ROS has not been well elucidated. More and more evidence, however, has accumulated to support that intracellular reduced glutathione (GSH) may be critical in regard to the generation of ROS and subsequent cytotoxicity.
induced by Cd.

Glutathione and Cellular Redox Status

As discussed above, ROS have a number of adverse effects when their production exceeds the capacity of the cellular defense system. It is not surprising, therefore, that the cell must maintain a strong and effective defense system against the threat of oxidative toxicity. Virtually all aerobic organisms have evolved complex defense and repair mechanisms to mitigate the damaging effects of ROS (McCord & Fridovich, 1988; McCord et al., 1971). They function through either enzymatic or non-enzymatic pathways. Among those non-enzymatic antioxidants, GSH has become one of our interests.

GSH (commonly referred to as reduced glutathione), a tripeptide (L-γ-glutamyl-L-cysteinylglycine), is synthesized intracellularly (Figure 2). GSH is synthesized from glutamate, cysteine, and glycine by the consecutive action of γ-glutamylcysteine synthetase and GSH synthetase (reaction 1 and 2). The breakdown of GSH is catalyzed by γ-glutamyltranspeptidase, which catalyzes transfer of the γ-glutamyl moiety to acceptors – the amino acids, cysteine and glutamine, and GSH itself (reaction 7). The balance between glutathione in the reduced (GSH) and oxidized (GSSG) form maintains the sulfhydryl groups of intracellular proteins in their correct oxidation states (reaction 3). Intracellular GSH is also converted to GSSG by GSH peroxidase, which catalyzes the reduction of H₂O₂ and other peroxides (reaction 4). In addition, GSSG is also formed by scavenging of free radicals by GSH
Figure 2. Summary of Glutathione Metabolism (see text). Reaction (1) γ-glutamylcysteine synthetase: first step of GSH synthesis; (2) glutathione synthetase; (3) thiol transferase: modulation of protein thiol-disulfide balance; (4) glutathione peroxidase: peroxide detoxification; (5) glutathione reductase: regeneration of GSH from GSSG; (6) glucose-6-phosphate dehydrogenase: generation of NADPH-cellular reducing equivalent; (7) γ-glutamyl transpeptidase: transfer of the γ-glutamyl moiety to amino acids.

(Meister and Anderson, 1983). Reduction of GSSG to GSH is mediated by the widely distributed enzyme GSSG reductase that uses NADPH (reaction 5). NADPH is produced in the first step of the pentose phosphate pathway, which is catalyzed by glucose-6-phosphate dehydrogenase (G6PDH) (reaction 6).

GSH has been implicated to function in cytoprotection against Cd toxicity, although the mechanism by which GSH plays this role has not been well defined. An explanation for these protective properties of GSH was proposed by Stacey who observed reduced cellular levels of Cd after addition of the GSH to isolated hepatocytes (Stacey, 1986). This may be due in part to the formation of a GSH-Cd chelate complex (Iguchi et al., 1991; Kang, 1992).

Since GSH is considered a key player in cellular defense mechanism, the studies in which the cellular levels of GSH are experimentally decreased or increased appear to be important to elucidate the specific functions of this molecule. L-Buthionine-(S, R)-sulfoximine (BSO) is a well known, specific inhibitor of γ-glutamylcysteine synthetase, and its administration to animals or cell culture media turns off cellular GSH synthesis effectively (Griffith & Meister, 1979; Griffith, 1982). BSO does not react with GSH, and there is no evidence that the sulfoximine moiety itself exerts toxicity. Mårtensson et al. (1991) demonstrated a model for endogenously produced oxidative stress by BSO. In this model, cellular GSH is depleted by administration of BSO to rats, and oxidative damage is observed in various tissues.

Alternatively, increases in cellular levels of GSH can be achieved by administration of glutathione monoethyl ester (GSH-E) to animals or cell culture
media. Previous studies have shown that injection or oral administration of GSH-E to mice or rats leads to substantial increases in the GSH levels in the liver, kidney, spleen, pancreas, and heart, indicating that this compound is transported and then hydrolyzed intracellularly to GSH (Puri and Meister, 1983; Anderson et al., 1985). In the human lymphoid cell lines CEM, HSB, and MOLT, as well as the normal skin fibroblast cell line CRL-150, and patient skin fibroblast cell lines GM 3878 and GM 3877 (in which GSH synthetase is deficient), intracellular GSH levels are significantly increased after cells are treated with GSH-E (Wellner et al., 1984). These studies also show that the intracellular GSH levels are rapidly restored by GSH-E even after the animals or cultured cells are pre-treated with BSO. GSH-E can therefore protect the cells from oxidative damage induced by BSO depletion of GSH (Singhal et al., 1987). Since GSH itself is not significantly taken up by tissues, GSH-E provides the most direct and convenient means available for increasing the intracellular GSH concentration of many tissues and cell types (Kang, 1992).

Cd, the metal used in our studies is capable of depleting cellular GSH. In whole animal studies, the cellular levels of GSH are significantly decreased in liver and kidney after injection or oral administration of Cd to mice (Kawata & Suzuki, 1983; Nehru & Bansal, 1997; Karmakar et al., 1999). In isolated rat hepatocytes, a time- and dose-dependent decrease in cellular level of GSH and an increase in cellular level of LPO were observed after cells were treated with 10-100 µM of Cd (Müller, 1986). Cellular GSH levels are markedly elevated in Cd-resistant cell lines when compared to their parental cells (Chubatsu et al., 1992; Li et al., 1994; Hatcher et al.,
1995), suggesting that GSH is valuable for protecting cells against Cd-induced damage. Depletion of cellular GSH by BSO pretreatment enhances Cd-induced cytotoxicity such as LPO and LDH leakage in various types of cells (Kang & Enger, 1987; Prozialeck & Lamar, 1995). Results from these previous studies therefore support the involvement of depletion of GSH in Cd-induced cytotoxicity.

Objective of Study

Although various theories have been proposed, the cellular mechanisms involved in Cd toxicity are still not well understood. The objective of this study is therefore to understand the mechanism behind Cd cytotoxicity. This is carried out in this study by: (a) investigating the cytotoxic effect of Cd on primary rat hepatocytes in particular by measuring LPO and LDH leakage; (b) estimating the effect of Cd on cellular GSH level and cellular redox state; and (c) assessing the protective effect of antioxidants, and GSH repletion, on Cd-induced cellular damage. Our hypothesis for this study is that Cd toxicity is mediated by ROS, which is generated by depletion of cellular GSH. This increase in ROS can serve as a second messenger to activate other cellular or physiological responses such as activation of signal proteins that mediate gene expression.

The liver is known as a major detoxification organ for many heavy metals including Cd. It is considered a main target organ of acute inorganic Cd accumulation. Much of the work to date on Cd induced cytotoxicity in liver has been carried out in in vivo models. If solid mechanistic questions are to be addressed, a
more manipulatable model needs to be considered. Thus, primary rat hepatocytes in
culture, a well-defined model system, was chosen for our present study. Primary cells
can be maintained in a chemically defined, serum-free medium, and their responses to
external agents generally mimic those observed \textit{in vivo}. Additionally, a cell culture
model allows us to dissect out the mechanism of interest. The primary rat hepatocytes
in culture are therefore the best model for our present study, the objective of which is
to define the mechanism involved in Cd toxicity. Characterization of cellular
responses to environmental agents, such as Cd, and/or oxidative stress is essential for
understanding the physiologic consequences of these agents, as well as for the
development of new therapeutic venues to defend and/or adapt to oxidative injury.
CHAPTER II

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Waymouth’s MB 752/1 medium was purchased from Gibco BRL (Grand Island, NY). Rat tail collagen, type 1, was from Becton Dickinson Labware (Bedford, MA) and Collagenase D was purchased from Boehringer Mannheim (Indianapolis, IN). L-buthionine-(S, R)-sulfoximine (BSO) and Trolox were obtained from Aldrich (Milwaukee, WI). The Glutathione Assay Kit and the Lipid Peroxidation Assay Kit were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). The Micro BCA Protein Assay kit was from Pierce (Rockford, IL). Cadmium chloride (CdCl₂) and Metaphosphoric acid (MPA) were from Fisher Scientific (Pittsburgh, PA), while all other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO) and were of the highest grade available.

Primary Rat Hepatocyte Isolation and Maintenance

Hepatocytes were isolated using the collagenase and hyaluronidase perfusion method as described by Elliget and Koloja (Elliget and Koloja, 1983), and modified by Stapleton et al. (Stapleton et al., 1993). Male rats, 8-10 weeks old, were food-
deprived 48 hours prior to the isolation procedure. Water was given ad libitum without restriction. The rat was anesthetized with an intra-pelvic injection of 0.35 cc of pentobarbital (50 mg/ml). The rat liver was perfused via the portal vein with a pre-perfusion solution containing 6 unit/ml Heparin, and then a digestion solution containing 0.2% Bovine Serum Albumin (BSA) Fraction V, 93 unit/ml hyaluronidase, 160 unit/ml trypsin inhibitor, and 100 unit/ml collagenase D. The isolated liver was then rinsed with digestion solution and forced through 4 layers of sterile gauze. The resulting cell suspension was centrifuged 3 times at 500 rpm for 3 minutes at 4°C, and each time the pellets were washed with cold Waymouth’s MB 752/1 medium supplemented with 5% BSA. Cell viability was determined with a hemocytometer by using the trypan blue dye exclusion method. Cell populations with a viability greater than 85% were used in this study. The cells were then plated on collagen-coated 60 mm Falcon 3002 culture dishes. The cells were maintained in 4.0 ml Waymouth’s MB 752/1 medium containing 5% BSA under a humidified environment of 5% CO₂ and 95% air at 37°C. After 4 hours, the cells were washed with 1.0 ml BSA free Waymouth’s MB 752/1 medium, and then incubated in 4.0 ml BSA free medium overnight.

**Cell Treatments**

Primary hepatocytes were treated with Cd at the following concentrations: 0 (no addition, NA), 0.1, 0.5, 1.0, 2.0, 4.0 and 6.0 µM for 6, 12, 24, or 48 hours. In separate experiments, the primary hepatocytes were also treated with BSO at the
concentrations of 0 (NA), 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mM for either 6, 12, 24, or 48 hours. For the studies using antioxidants, the primary hepatocytes were pretreated for 2 hours with either 0.5 mM Trolox (water soluble vitamin E), 2.0 mM glutathione ethyl ester (GSH-E), or 10 mM N-acetylcysteine (NAC) prior to the addition of Cd or BSO.

Determination of Protein Content

Protein content of the cell sample was measured either by using the method of Lowry et al. (1951), or by using the Micro BCA Protein Assay Kit (Pierce). The procedure for processing cells for protein assays will be discussed in the following sections concerning the specific assays.

The Micro BCA Protein Assay kit contains Micro BCA Reagent A (MA), Reagent B (MB), and Reagent C (MC). Briefly, before use, 25 parts of MA and 24 parts of MB are mixed with 1 part of MC to make the BCA Working Reagent (WR). Into a microtiter plate, 10-50 µl of sample was diluted to 150 µl with H2O. 150 µl of WR was then added, and the plate was covered and agitated for 1-2 minutes. The microtiter plate was incubated at 37°C for 2 hours, and then cooled to room temperature. The protein amount in the cell sample was quantitated by measuring the absorbance at 562 nm.

GSH Assay

Intracellular GSH levels were determined by using the Glutathione Assay Kit
(Calbiochem), which includes a 12 mM solution of chromogenic reagent in 0.2 N HCl (R1), 30% NaOH (R2), and buffer (Solution 3: 200 mM potassium phosphate, pH7.8, containing 0.2 mM diethylene triamine pentaacetic acid and 0.025% LUBROL). The measurement of GSH is based on reactions between GSH and chemical reagents that produce a chromophoric thione with a maximal absorbance at 400 nm.

After incubation, the primary hepatocytes were washed twice with cold phosphate-buffered-saline (PBS). The cells were then scraped in 1.0 ml of cold PBS and centrifuged at 500 rpm for 5 minutes at 4°C. The pellets were resuspended in 500 μl of 5% MPA, and then homogenized by means of a Teflon pestle. The homogenate was centrifuged at 3,000 × g for 10 minutes at 4°C, and 60 μl of the resulting supernatant was transferred into an appropriate well of a microtiter plate. 120 μl of buffer (Solution 3) and 10 μl of solution R1 were then added. The total thiol content, mercaptans, were measured by the absorbance at 356 nm. After which, 10 μl of solution R2 was immediately added and mixed thoroughly. The microtiter plate was incubated in the dark at room temperature for 10 ~ 15 minutes. The GSH levels were determined by measuring absorbance at 400 nm. Both total mercaptan and GSH levels were then normalized to μmol per μg of protein in the sample (μmol/μg protein).

Evaluation of Lipid Peroxidation

Malondialdehyde (MDA) is in many instances the most abundant individual aldehyde resulting from LPO, thus the measurement of MDA provides a convenient
index of LPO (Esterbauer & Cheeseman, 1990). The Lipid Peroxidation Assay Kit takes advantage of a chromogenic reagent (R1) that reacts with MDA to yield a stable chromophore with maximal absorbance at 586 nm.

After incubation, the primary hepatocytes were washed twice with cold PBS. The cells were then scraped off the plate using 400 µl of autoclaved distilled water and transferred into 1.5 ml Eppendorf tubes. The cells were lysed by repetitive (4-5 times) freeze/thaw in liquid nitrogen, and 200 µl of the resulting cell lysate was then transferred into 10 ml glass test tubes. The remaining cell lysate was centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was collected to determine the protein content in the samples by using the Micro BCA Protein Assay kit as described above.

In a glass test tube, 650 µl of diluted R1 (1:4 with distilled water) was added to the 200 µl of cell lysate and vortexed for 4-5 seconds. Afterwads, 150 µl of 12 N HCl was added and again vortexed for 4-5 seconds. The glass test tube was then closed with a tight stopper and incubated at 45°C for 60 minutes. After incubation, the cell lysate was cooled on ice for 10 minutes. The amount of LPO was evaluated by measuring the absorbance at 586 nm. The final value of MDA was normalized to µmol per µg of protein in the sample (µmol/µg protein).

Measurement of Lactate Dehydrogenase Activity

Lactate Dehydrogenase (LDH) is an intracellular enzyme that is released into the extracellular environment when cell membranes are compromised (Bonnekoh et
al, 1990). The measurement of the LDH activity in cell culture media is therefore one of the most common assays used in determination of cytotoxicity. The LDH assay was performed using the method of Wroblewski & LaDue (Wroblewski & LaDue, 1995), which is based on the reduction of pyruvic acid to lactic acid catalyzed by LDH in the presence of NADH.

After incubation, 1.0 ml of media was obtained from the primary hepatocytes treated as described above. The media was used to quantitatively estimate the amount of LDH released by the cells. In a cuvette, 50 µl of cell culture media and 33.5 µl of 0.02 M pyruvic acid were added to 900 µl of 0.1 M potassium phosphate buffer (pH 7.4). After which, 16.4 µl of 14.0 mM NADH was added to initiate the reaction. Immediately, the LDH activity was measured spectrophotometrically at 340 nm for 180 seconds. The rate of decrease of optical density representing the rate of oxidation of NADH was taken as the measurement of LDH activity. The final value of LDH activity is expressed as units per minute per µg of protein in the sample (units/min/µg protein).

Statistical Analysis

The results are expressed as the mean ± S.E.M. of N numbers of animals used for each experiment. The differences between non-treated and treated primary hepatocytes were evaluated by one-tailed Student’s t-test. The comparisons within groups were performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. Statistical significance was tested at $p < 0.05$. 
CHAPTER III

RESULTS

Cd Decreases Intracellular GSH Levels in Primary Rat Hepatocytes

To examine the effects of Cd on intracellular GSH levels, primary rat hepatocytes were treated with CdCl₂ at concentrations of 0 (NA), 0.1, 0.5, 1.0, 2.0, 4.0 and 6.0 µM and incubated for either 1, 6, 12, 24, or 48 hours. No significant effect on intracellular GSH levels by Cd in the short-term incubation and low-concentration treatment groups was observed (Figure 3). However, a significant decrease in GSH levels was observed at 6 hours after the primary hepatocytes were treated with 4.0 and 6.0 µM of Cd. When the incubation time was extended to 12, 24, and 48 hours, a more pronounced decrease in GSH levels was noted at various concentrations of Cd. Overall, the Cd-induced decrease in intracellular GSH levels was both concentration- and time-dependent.

The Effects of BSO on Intracellular GSH Levels

BSO is a potent and specific inhibitor of γ-glutamylcysteine synthetase and its administration to animals or addition into tissue culture media inhibits GSH biosynthesis. The addition of BSO causes a marked depletion of cellular GSH levels (Griffith & Meister, 1979; Griffith, 1982). Studies on the effects of GSH depletion
Figure 3. Effects of Cd on Intracellular GSH Levels. Primary rat hepatocytes were treated for 1 ~ 48 hours with 0 ~ 6.0 µM Cd. Data represent the mean ± S.E.M. (N = 4 ~ 6). One-tailed Student’s t-test was used for statistical analysis. Significant difference from the NA as described in text (p < 0.05).

Legend.  
-•- = 1 hour treatment,  ---◊--- = 6 hour treatment,  
-□- = 12 hour treatment,  --- Δ--- = 24 hour treatment,  
-○- = 48 hour treatment

might be an important tool to reveal the role of GSH in cellular defense system. To demonstrate the effects of BSO on the intracellular GSH levels and to examine whether or not the decrease in GSH levels by Cd is due to the inhibition of GSH biosynthesis, we treated the primary hepatocytes with BSO alone or with both BSO and Cd. As expected, the primary hepatocytes treated with BSO alone also showed
Figure 4. Effects of BSO on Intracellular GSH Levels. Primary rat hepatocytes were treated for 6 ~ 48 hours with 0 ~ 0.8 mM BSO. Data represent the mean ± S.E.M. (N=5). One-tailed Student's t-test was used for statistical analysis. Significant difference from the NA as described in text (p < 0.05).

a concentration- and time-dependent decrease in intracellular GSH levels (Figure 4). A significant decrease in cellular GSH level was observed at 6 hours after the primary hepatocytes were treated with 0.8 mM BSO. In the 12-hour treatment groups, the intracellular GSH levels were significantly reduced with concentrations of BSO at 0.05 mM and higher. In 24- and 48-hour groups, a significant decrease in GSH levels occurred at all concentrations of BSO tested. Interestingly, addition of BSO (0.4 mM)
Figure 5. Addition of Cd and BSO Together Has Additive Effects on Intracellular GSH Levels. Primary rat hepatocytes were treated for 6 ~ 48 hours with both Cd (0 ~ 6.0 µM) and BSO (0.4 mM). Data represent the mean ± S.E.M. (N = 4 ~ 6). One-tailed Student’s t-test was used for statistical analysis. All data, except for 0.5 µM Cd at 6 hours, are significant from NA (ρ < 0.05).

and Cd (0 ~ 6.0 µM) together showed an additive effect in decreasing intracellular GSH levels compared to the effect of either Cd or BSO treatment alone (Figure 5). For instance, BSO (0.4 mM) alone reduced intracellular GSH by 10.63% (Figure 4), and
Cd (4.0 µM) alone decreased GSH level by 11.62% (Figure 3) after a 6 hour incubation. During a similar incubation period, however, addition of both BSO (0.4 mM) and Cd (4.0 µM) additively decreased intracellular GHS by 28.80% (Figure 5). After 12 hours of treatment, a 33.54% decrease in GSH levels was induced by 0.4 mM BSO (Figure 4), while a 21.27% decrease by 2.0 µM Cd (Figure 3) was observed. Together, BSO (0.4 mM) and Cd (2.0 µM) (Figure 5) decreased GSH levels more than either agent alone.

Cd Induces LPO and LDH Leakage in Primary Rat Hepatocytes

It has been reported that LPO, which is a consequence of the accumulation of reactive oxygen species (ROS) (Caraceni et al., 1994; Huimin et al., 1994), is a major factor influencing the breakdown and turnover of the membrane (Sugiyama et al., 1993). LDH is released to the extracellular environment when the cellular membrane is compromised (Bonnekoh et al., 1990). Thus, both LPO and LDH leakage are considered as cytotoxic parameters to measure when cells are under stress or damaged. As shown in Figure 6 and 7, significant increases in both LPO levels and LDH leakage were observed at 24 and 48 hours after primary hepatocytes were treated with various concentrations of Cd. Twelve hours after incubation, the MDA levels indicative of LPO significantly increased in the presence of 4.0 and 6.0 µM Cd (Figure 6), whereas the LDH leakage significantly increased in the presence of 6.0 µM Cd (Figure 7). No obvious effect on LPO level or LDH leakage was observed at 6 hours after primary hepatocytes were treated with any of the Cd concentrations.
Figure 6. Exposure to Cd Induces the Accumulation of MDA. Primary rat hepatocytes were treated for 6 ~ 48 hours with 0 ~ 6.0 µM Cd. Data represent the mean ± S.E.M. (N = 4 ~ 6). One-tailed Student’s t-test was used for statistical analysis. Significant difference from the NA as described in text ($p < 0.05$).
Figure 7. Cd Increases the Extracellular LDH Activity. Primary rat hepatocytes were treated for 6 ~ 48 hours with 0 ~ 6.0 µM Cd. Data represent the mean ± S.E.M. (N = 6). One-tailed Student’s t-test was used for statistical analysis. Significant difference from the NA as described in text (p < 0.05).
Effect of Antioxidants on Cd Induced LPO and LDH Leakage

If it is true that the Cd cytotoxicity is mediated by depleting GSH and/or accumulating ROS, then restoring cellular GSH level and/or removing the ROS should significantly diminish the Cd toxic effects. GSH-E, in which the glycine carboxyl group of GSH is esterified, has been shown to be taken up by several tissues and intracellularly hydrolyzed to GSH (Puri & Meister, 1983; Wellner et al., 1984; and Anderson et al., 1985). Because GSH itself is not taken up by the cells, GSH-E is therefore very important in replacing the cellular GSH experimentally. Trolox, a water-soluble form of vitamin E, is a potent peroxyl radical scavenger, which terminates the chain reaction of LPO. NAC has both GSH and GSH-independent antioxidant properties, and has been shown to protect cells from oxidative damage (Yan et al., 1995).

To determine whether or not Cd toxicity is associated with the depletion of GSH and/or the accumulation of ROS, we pretreated the primary hepatocytes for 2 hours with either GSH-E, NAC, or Trolox before Cd was added. As expected, the levels of cellular GSH in GSH-E treated primary hepatocytes were about 50% higher than that in control cells after a 24-hour incubation. Treating the hepatocytes with 2.0 mM GSH-E restored cellular GSH to normal in Cd-treated cells (Figure 8).

As shown in Figure 6 and 7, Cd is capable of inducing LPO and LDH leakage. After primary hepatocytes were treated for 24 hours with 4.0 μM Cd, the levels of cellular MDA and the extracellular activity of LDH were about double that over NA
The pretreatment of GSH-E, NAC, and Trolox, however, significantly blocked the Cd induced increase in LPO level (Figure 9) and LDH leakage (Figure 10). Together, these results suggest that ROS may be involved in the cytotoxicity of Cd.

Figure 8. Effect of GSH-E on the Cellular GSH Levels. Primary rat hepatocytes were pretreated for 2 hours with either nothing (NA) or 2.0 mM of GSH-E. Then 4.0 µM of Cd was appropriately added and cells were incubated for 24 hours. Data represent the mean ± S.E.M. (N = 3 ~ 4). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test (p < 0.05). * indicates significantly different from NA. ** indicates significantly different from 4.0 µM of Cd.
Figure 9. Protective Effect of Antioxidants on Cd Induced LPO. Primary rat hepatocytes were pretreated for 2 hours with either nothing (NA) or antioxidants. Then 4.0µM of Cd was appropriately added and cells were incubated for 24 hours. Data represent the mean ± S.E.M. (N = 3 ~ 5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test (p < 0.05). ★ indicates significantly different from NA. ★★ indicates significantly different from 4.0 µM of Cd.
Figure 10. Protective Effect of Antioxidants on Cd Induced LDH Leakage. Primary rat hepatocytes were pretreated for 2 hours with either nothing (NA) or antioxidants. Then 4.0µM of Cd was appropriately added and cells were incubated for 24 hours. Data represent the mean ± S.E.M. (N = 4 ~ 6). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test (p < 0.05). * indicates significantly different from NA. † indicates significantly different from 4.0 µM of Cd.
BSO Has an Effect Similar to That of Cd on LPO Levels and LDH Leakage

Because of its capability to deplete cellular GSH, BSO is believed to generate ROS. It is therefore widely used as a model for endogenously produced oxidative stress (Martensson et al., 1991; Skaper et al., 1997; Vornov et al., 1998). To further confirm that the toxicity of Cd is mediated by ROS, we investigated the effect of BSO on LPO level and LDH leakage. The primary hepatocytes were treated for 24 hours with 0.4 mM BSO and LPO level and LDH leakage were measured. Similar to Cd, BSO remarkably increased the cellular MDA and caused significant increases in LDH leakage (Figure 11 and 12). All agents tested, Trolox, GSH-E, and NAC, significantly blocked the induction of LPO level by BSO, although the level of MDA in the BSO and NAC together treated group was still higher than NA (Figure 11). The increase in LDH leakage by BSO was also significantly blocked by the addition of either Trolox, GSH-E, or NAC (Figure 12).
Figure 11. Effect of BSO and Antioxidants on the Levels of LPO. Primary rat hepatocytes were pretreated for 2 hours with either nothing (NA) or antioxidants. Then 0.4 mM of BSO was appropriately added and cells were incubated for 24 hours. Data represent the mean ± S.E.M. (N = 3 ~ 5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test (p < 0.05). ★ indicates significantly different from NA. ★★ indicates significantly different from 0.4 mM of BSO.
Figure 12. Effect of BSO and Antioxidants on the LDH Leakage. Primary rat hepatocytes were pretreated for 2 hours with either nothing (NA) or antioxidants. Then 0.4 mM of BSO was appropriately added and cells were incubated for 24 hours. Data represent the mean ± S.E.M. (N = 5 ~ 7). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test (p < 0.05). ★ indicates significantly different from NA. ★★ indicates significantly different from 0.4 mM of BSO.
CHAPTER IV

DISCUSSION

Cd has become a health concern due to its accumulation as an environmental pollutant and its capability to induce cellular damage to organs and tissues. Previous studies have shown that administration of Cd to either whole animals or to cell culture media induces oxidative stress tissues and cells. There is little evidence, however, to show the interrelationship between the end result of Cd cytotoxicity and intracellular ROS build up. GSH and metallothionein (MT) are two of the most important thiol-rich molecules known to be essential in the protection against the deleterious effects of the Cd (Bast et al., 1991; Sato & Bremner, 1993). MT has been shown to bind Cd and certain other heavy metals with high affinity (Vallee & Ulmer, 1972; Vallee, 1979). Cellular levels of MT are generally low, but are increased markedly upon administration of Cd and certain other metal ions. In regard to Cd toxicity, Singhal et al. (1987) hypothesized that while Cd-induced MT synthesis may play an important role in Cd detoxification, its synthesis may be too slow to protect animals against initial tissue damage caused by excess Cd ingestion. Thus, they concluded that in the absence of significant levels of MT, GSH provides a first-line of defense against Cd toxicity.

GSH constitutes more than 90% of the intracellular non-protein thiol pool, which is in turn required for maintaining the reduced environment of a cell including
reduced thiol groups on proteins, enzymes, and amino acids. It also serves to protect the cells against oxidative damage as it conjugates with compounds of exogenous and endogenous origin (Meister & Anderson, 1983; Meister, 1983). As shown in Figure 2, GSH itself serves as a cofactor of glutathione peroxidase, which neutralizes a wide variety of ROS including H$_2$O$_2$. Thus, a decrease in cellular levels of GSH may cause an accumulation of ROS, thereby mediating oxidative damage of the cells.

In the present study, a time- and concentration-dependent decrease (20-50%) in cellular GSH levels was observed after primary rat hepatocytes were exposed to a concentration range of Cd from 0-6.0 µM (Figure 3). BSO, an inhibitor of GSH biosynthesis, also caused a significant decrease in cellular GSH levels in a time- and concentration-dependent manner (Figure 4). Addition of both Cd (0-6.0 µM) and BSO (0.4 mM) together yielded an additive effect on the depletion of GSH (Figure 5), suggesting that the depletion of cellular GSH by Cd is not through the inhibition of GSH synthesis, but rather through the formation of a GSH-Cd chelate complex, as proposed by Iguchi et al. (1991). Perrin and Watt (1971) have shown that the viability of such a complex is favored by the very high affinity that Cd has for GSH. This affinity of Cd to form a complex with GSH has also been demonstrated in vitro (Perrin & Watt, 1971).

The maintenance of cell membrane integrity is essential for cell survival. Certain heavy metals such as Cd are known to diminish the viability of isolated hepatocytes by inducing LPO (Stacey et al., 1980; Muller, 1986). This increase in LPO enhances cellular membrane permeability (Bonnekoh et al., 1990), as indicated
by LDH leakage. Therefore, LPO and LDH leakage are commonly considered parameters of cytotoxicity. Previous studies have suggested that Cd toxicity is involved in both cell membrane destruction (Muller, 1983) and disturbance of various cellular metabolic processes (Vallee & Ulmer, 1972). However, it is unclear whether or not metabolic disturbances precede membrane damage (Muller & Ohnesorge, 1982). Thus, assessment of the cellular functional integrity might be a tool to detect early cellular injuries. Our results show that the primary rat hepatocytes exposed to Cd for either 24 or 48 hours result in a 1.5 to 5.0 fold increase in cellular LPO level (Figure 6) and a 1.3 to 2.1 fold increase in LDH leakage, respectively (Figure 7). A significant decrease, however, in cellular GSH levels was observed after primary hepatocytes were treated for 6 hours with 4.0 and 6.0 µM Cd, or for 12 hours with various concentrations of Cd tested. After 24 and 48 hours, a more pronounced decrease in GSH levels was noted at various concentrations of Cd tested (Figure 3). The results of this time study provide direct evidence that depletion of cellular GSH occurs at the time point prior to the observed cellular damage as assessed by LPO levels and LDH leakage. These results suggest that the depletion of GSH by Cd may be the cause of such damage.

It appears that GSH is very important as a first line of defense against Cd toxicity. As discussed above, Cd has a high affinity for SH-containing ligands. The increased Cd toxicity was observed after depletion of GSH is probably due to the formation of GSH-Cd chelation. Our results show that administration of GSH-E, a method used to increase GSH intracellularly, significantly elevates the intracellular
levels of GHS in normal or Cd-treated primary rat hepatocytes (Figure 8). Additionally, pretreatment of GSH-E completely prevents the induction of LPO and LDH leakage by both Cd and BSO (Figure 9-12). These data strongly suggest that although Cd and BSO may share different mechanisms to deplete intracellular GSH levels, the consequence of depleting cellular GSH is the same: generation of intracellular ROS, which will further trigger cytosolic and nuclear events.

The assessment of the involvement of ROS in Cd toxicity in these cells was achieved by using antioxidants and BSO. Administration of BSO to animals and cell culture media has proved to be a useful model to study oxidative stress endogenously (Matensson et al. 1991). Antioxidants are agents that can prevent or delay the effect of oxidation of biological substrates. Antioxidants fall into two mechanistic groups: those which interrupt the radical chain reaction, the “chain-breaking” (CB) antioxidants, and those known as the “preventive” antioxidants that inhibit the formation of free radicals from their unstable precursors and in particular the hydroperoxides (Scott, 1965; Scott, 1993). The CB antioxidants can be further subdivided into chain-breaking hydrogen or electron donors (CB-D) to peroxyl or hydroxyl radicals, and hydrogen or electron acceptors (CB-A) from carbon-centered radicals (Figure 13) (Scott, 1997). Trolox, a water-soluble analog of vitamin E, belongs to the subdivision of CB-D. It is a potent scavenger of hydroxyl radical and peroxyl radicals (Davies et al., 1988), and thus a terminator of the chain reaction of LPO. The protective effects of Trolox against oxidative damage in several different cell lines have been studied and reported (Wu et al., 1990; Forrest et al., 1994).
Importantly, no evidence has shown that Trolox interferes in the synthesis and/or metabolism of GSH.

\[
\begin{align*}
R^* & \rightarrow ROO^* \\
\downarrow A^* & \rightarrow \text{Chain-Breaking Acceptor (CB-A)} \\
RA & \rightarrow ROOH + A^* \\
\text{\textgreater=C=C< + AH} & \rightarrow \text{Chain-Breaking Donor (CB-D)}
\end{align*}
\]

Figure 13. Chain-Breaking Mechanisms of Antioxidant Action.


Because of the instability and consequent radical generating capacity under a wide range of environmental conditions, hydroperoxides are probably the most important ROS. The removal of hydroperoxides, including \( \text{H}_2\text{O}_2 \), without the formation of free radicals therefore constitutes a critical role of preventive antioxidants mechanism. Naturally occurring preventive antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and certain metal chelating agents. As discussed earlier, GSH serves as a cofactor of glutathione, and thus plays an essential role in cellular defense mechanism against oxidative stress.

On the other hand, the thiol-containing antioxidant NAC has the property of
being both a cellular reductant (Yan et al., 1995) and a precursor of GSH synthesis (Weinander et al., 1994). These properties of NAC have also been proposed by Han and coworkers (Han et al., 1997). In their studies, NAC was utilized to protect against glutamate-induced, oxidative cytotoxicity. They observed that low doses (<100 µM) of NAC did not have the protective effects in the presence of BSO, an inhibitor of GSH synthesis. At higher concentration (>500 µM), however, NAC provided partial protection against glutamate cytotoxicity even in GSH synthesis-arrested cells. These results supported that NAC not only has the proglutathione property (low concentration), i.e. it serves as a precursor of GSH biosynthesis, but also serves as an antioxidant scavenger (high concentration).

In our studies, pretreatment of 0.5 mM Trolox or 10 mM NAC completely blocked the induction of LPO and LDH leakage by Cd (Figure 9 and 10). Trolox pretreatment also dramatically repressed the increased LPO level and LDH leakage induced by BSO. The increased LPO level and LDH leakage induced by BSO, however, in NAC pretreated cells is still significantly higher than in control cells, even though it is markedly lower than in the cells treated with BSO alone (Figure 11 and 12). These results suggest that the protective effect of Trolox may be through a ROS scavenging mechanism, whereas, NAC may have proglutathione property as well as GSH-independent antioxidant / cellular reductant properties. This is concluded in part because NAC exhibits full protection against oxidative damage in the cells with a functional GSH synthesis system, and partial protection in the cells where the GSH synthesis is inhibited by BSO. These data from the studies using
antioxidants and BSO are also suggestive of the involvement of ROS in Cd cytotoxicity.

In conclusion, the results obtained from the present study support our original hypothesis: Cd toxicity in liver is mediated by intracellular ROS, which is generated by depletion of GSH.
Appendix A

Abbreviations Used in This Thesis
BSO: L-buthionine- (S, R)-sulfoximine

Cd: cadmium

GSH: reduced glutathione

GSH-E: glutathione ethyl ester

H₂O₂: hydrogen peroxide

LDH: lactate dehydrogenase

LPO: lipid peroxidation

MAPK: mitogen-activated protein kinase

MDA: malondialdehyde

NAC: N-acetylcysteine

O₂⁻: superoxide radical

OH⁻: hydroxyl radical

PKC: protein kinase C

ROO⁻: peroxyl radical

ROS: reactive oxygen species

Trolox: water soluble vitamin E analogue
Appendix B

Protocol Clearance From the Institutional Animal Care and Use Committee
GENERAL INFORMATION: Fill in all appropriate information

Susan Stapleton                      CHEM                      7-2853
Principal Investigator/Instructor    Department               Campus Phone

N/A                                  N/A                       N/A
Responsible Faculty Member           Department               Campus Phone
(If PI not faculty member)

Title of Project/Course: Regulation of Gene Expression by Insulin, Insulin-Mimetics and Other Metals

PRINCIPAL INVESTIGATOR/INSTRUCTOR DECLARATION

I assure that I have obtained IACUC approval prior to implementing this project and that there are no changes in the protocol submitted in the original application to use vertebrate animals for research or teaching. I understand that if at any time changes are made in the use of animals as described in the original application, a letter or amended protocol must be filed for review. I assure that the activities do not unnecessarily duplicate previous experiments.

Signatures:

[Signature]
Principal Investigator/Instructor  7/14/98

[Signature]                     N/A
Responsible Faculty Member       Date
(If PI not a faculty member)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

[Signature]                     7/25/98
IACUC Chairperson                 Date

PLEASE MAIL COMPLETED APPLICATION TO:
Research Compliance Coordinator
Western Michigan University
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(616) 387-8293
BIBLIOGRAPHY


Kaji, T., Ohkawara, S., Inada, M., Yamamoto, C., Sakamoto, M., & Kozuka, H.,
Alteration of glycosaminoglycans induced by cadmium in cultured vascular smooth muscle cells. Archives of Toxicology 68:9, 560-565.


Karmakar, R., Roy, S, & Chatterjee, M., (1999). The effects of cadmium on the hepatic and renal levels of reduced glutathione, the activity of glutathione S-transferase and gamma glutamyl transpeptidase. Journal of Environmental Pathology, Toxicology, and Oncology 18:1, 29-35.


phosphate dehydrogenase activity and mRNA levels in primary rat hepatocytes in culture. Biochimie 75, 971-976.


Tang, N., & Enger, M. D., (1993). Cd-induced c-myc mRNA accumulation in NRK-49F cells is blocked by the protein kinase inhibitor H7 but not HA 1004, indicating that protein kinase C is a mediator of the response. Toxicology 81, 155-164.


