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**CADMIUM INDUCED GENE EXPRESSION AND SIGNAL TRANSDUCTION  
PATHWAY**

by

**Daisuke Maki**

**A Thesis  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment of the  
requirements for the  
Degree of Master of Science  
Department of Biological Science**

**Western Michigan University  
Kalamazoo, Michigan  
December 2000**

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Daisuke Maki

## CADMIUM INDUCED GENE EXPRESSION AND SIGNAL TRANSDUCTION PATHWAY

Daisuke Maki, M.S.

Western Michigan University, 2000

Cells respond to cadmium (Cd), a common environmental contaminate, by potentially decreasing reduced intracellular glutathione, as well as, increasing the expression of several antioxidant genes. One of these antioxidant genes, glucose-6-phosphate dehydrogenase (G6PDH), is the rate limiting enzyme of the pentose phosphate pathway. This pathway produces NADPH which is used for maintenance of reduced glutathione so, it is hypothesized that Cd should increase G6PDH expression. In this study, we demonstrate that Cd increases the level of G6PDH mRNA in primary rat hepatocytes in culture. In the presence of the antioxidants, N-acetylcysteine (NAC), Trolox, or glutathione monoethyl ester an attenuation of this effect was observed. It has been demonstrated in several instances that Cd can activate genes through signal transduction pathways mediated by stress-response proteins such as mitogen activated protein kinase (MAPK) or c-jun N-terminal kinase (JNK). We also show that Cd activates both MAPK and JNK. In the presence of antioxidants NAC or Trolox, a decrease in activation of MAPK and JNK is observed. Using the well defined MAPK inhibitor, PD098059, we also show that proteins preceding MAPK in this pathway may influence the expression of the G6PDH gene.

## TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	ii
LIST OF FIGURES.....	v
INTRODUCTION.....	1
Oxidative Stress and Reactive Oxygen Species.....	1
Cadmium.....	5
Glucose-6-Phosphate Dehydrogenase.....	9
Oxidative Stress and the MAP Kinase Signal Transduction Pathway.....	11
Objective of Study.....	15
MATERIALS AND METHODS.....	17
Materials.....	17
Primary Rat Hepatocytes Isolation and Maintenance.....	17
Cell Treatment.....	18
Cell Processing.....	19
Western Blot Analysis.....	19
MAP Kinase Assay.....	20
c-jun-N-terminal Kinase (JNK) Assay.....	21
Plasmid DNA Preparation.....	22
DNA Probe.....	22
Isolation and Measurement of mRNA Levels.....	23
Statistical Analysis.....	24

## Table of Contents—continued

RESULTS .....	25
Effect of Cd on G6PDH mRNA Levels in Primary Rat Hepatocytes.....	25
Effect of BSO on G6PDH mRNA Levels in Primary Rat Hepatocytes.....	25
The Effects of Antioxidants on Cd Induced G6PDH mRNA Level .....	26
The Effects of Antioxidants on BSO Induced G6PDH mRNA Level .....	27
Cd Induces MAPK and JNK in Primary Rat Hepatocytes.....	27
Effect of Antioxidants on Cd-Induced MAPK Phosphorylation .....	28
Effect of Antioxidants on Cd-Induced JNK Phosphorylation .....	29
Cd Induced G6PDH Expression is Sensitive to MEK Inhibitor .....	30
DISCUSSION .....	39
APPENDIX	
A. Investigator IACUC Certificate .....	48
BIBLIOGRAPHY .....	50

## LIST OF FIGURES

1. Generation of Reactive Oxygen Species .....	2
2. Detoxification of Hydrogen Peroxide .....	10
3. Mitogen Activated Protein Kinase Signal Transduction Pathway .....	12
4. Cadmium Stimulated G6PDH mRNA Levels.....	31
5. BSO Stimulated G6PDH mRNA Levels.....	32
6. Effect of Antioxidants and MEK Inhibitor PD098058 on Cd Induced G6PDH mRNA Gene Expression .....	33
7. Protective Effect of Antioxidants on BSO Induced G6PDH Gene Expression .....	34
8. Effect of Cd on MAPK Activation.....	35
9. Effect of Antioxidants and MEK Inhibitor on MAPK Activation.....	36
10. Effect of Cd on JNK Activation.....	37
11. Effect of Antioxidant and MEK Inhibitor on JNK Activation.....	38

## INTRODUCTION

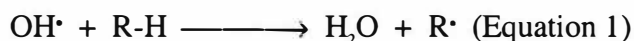
### Oxidative Stress and Reactive Oxygen Species

Although oxygen and its metabolism is essential for life, it also imposes a potential threat to cells because of the formation of partially reduced oxygen species. These partially reduced oxygen species are collectively called reactive oxygen species (ROS). One electron reduction of oxygen produces superoxide whereas a two-electron reduction of oxygen produces hydrogen peroxide. Therefore, electron flow through oxygen utilizing processes such as the mitochondrial electron transfer chain, flavoproteins, cytochrome P450, and oxidases is tightly coupled to avoid partial reduction of oxygen (Freeman et al., 1982). During normal cellular homeostasis, the rate and magnitude of oxidant formation and elimination are balanced. This balance occasionally fails and a condition known as oxidative stress occurs.

Examples of ROS that are formed when the balance fails include superoxide ( $O_2^{\cdot-}$ ), hydroxyl ( $OH^{\cdot}$ ), peroxy ( $RO_2^{\cdot}$ ) and alkoxy ( $RO^{\cdot}$ ) radicals, as well as, certain non-radicals such as, hypochlorous acid (HOCL), ozone ( $O_3$ ), peroxynitrite (ONOO $^-$ ), singlet oxygen ( $^1O_2$ ), and hydrogen peroxide ( $H_2O_2$ ). As shown in Figure 1, transfer of one electron to oxygen leads to multiple reactive oxygen species through the superoxide anion radical,  $O_2^{\cdot-}$ . Superoxide dismutates to hydrogen peroxide ( $H_2O_2$ ) +  $O_2^{\cdot-}$  in a reaction catalyzed by superoxide dismutase (SOD). Further addition of electrons requires cleavage of the bond between the oxygen atoms, a reaction



catalyzed by iron (Fenton Reaction) to form the highly reactive hydroxyl radical ( $\text{OH}^\bullet$ ).  $\text{OH}^\bullet$  reacts instantaneously with any molecule from which it can abstract a hydrogen atom (Equation 1).



Of these,  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  react quickly with only a few molecules, whereas  $\text{OH}^\bullet$  reacts quickly with almost any molecule.

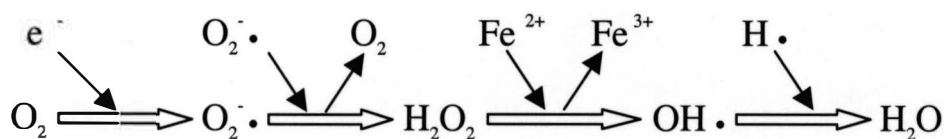


Figure 1. Generation of Reactive Oxygen Species.

In biological systems, the short-lived  $\text{OH}^\bullet$  is one of the most powerful oxidants known and can cause severe damage to DNA, protein, and membrane lipids (Davies et al., 1987).  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  can also cause damage either directly or indirectly by undergoing further reduction to the powerful  $\text{OH}^\bullet$  (Fridovich., 1978). Increased concentration of ROS causes drastic changes in the structure and biochemical properties of many biological molecules including nucleic acids, proteins, and lipids. The oxidation of lipid results in the formation of lipid peroxides through a series of chain reactions. DNA oxidation by ROS causes chemical modifications, including the formation of glycols, hydantoins, deamination products, and rearrangements in the pyrimidine ring which lead to DNA strand breaks (Reid et al., 1993). These molecular lesions can be counter acted only by eliminating the damaged components of the

biological molecules and structures (Cimino et al., 1997). Mutagenesis by ROS could contribute to the initiation of cancer, in addition to being important in the promotion and progression phases. ROS cause structural alternations in DNA such as, base pair mutations, rearrangements, deletions, insertions and sequence amplification (Wiseman et al., 1996). The effect exerted by ROS on proteins is of two kinds: the first, metal ion-catalyzed oxidation of proteins is mainly a site-specific process in which only one or a few amino acids at the metal-binding sites on the protein are preferentially oxidized. Histidine, proline, arginine, and lysine residues have been identified as major targets for oxidation. A second type of effect involves the oxidation of cysteine residues, which within a defined range, can be reversed by enzyme systems that catalyze the reduction of oxidized cysteines (Stadmen, 1993) suggesting that protein oxidation-reduction may be a molecular mechanism by which the cell regulates protein function.

Accumulated evidence suggests that ROS play several physiological roles in either regulating gene expression or cell proliferation (Maki et al., 1992), or by acting as an intracellular second messenger (Sundaresen et al., 1995). In different studies, the activation of a well defined transcription factor, activator protein-1 (AP-1) has been identified to be regulated by ROS. AP-1, which controls expression of cell growth mediators, is a heterodimer comprised of Fos and Jun proteins, which are protein products of *c-fos* and *c-jun* proto-oncogenes, respectively (Rivera et al., 1990). Post-translational modification of AP-1 also plays an important role in the control of AP-1 regulated gene expression. This control is executed in the form of phosphorylation of Fos and Jun proteins by members of the mitogen activated protein

kinase family. Both *c-fos* and *c-jun* genes are induced by agents that promote intracellular ROS accumulation such as  $\text{TNF}\alpha$ , UV irradiation, hydrogen peroxide, and mitogens such as polypeptide growth factors (epidermal growth factor, platelet-derived growth factors, insulin growth factor, etc) as well as insulin (Arrigo, 1999). For example, *c-fos* mRNA has been found to increase in the presence of ROS generated by xanthine oxidase in mouse epithelial JB5 cells and Balb/3T3 cells (Crowfoed et al., 1988; Shibnuma et al., 1988).  $\text{H}_2\text{O}_2$  induced *c-fos* and *c-jun* expression was observed in rabbit lens epithelial cells. (Li et al., 1997). The environmental risk factor asbestos also induces the expression of *c-fos* and *c-jun* proto-oncogenes and this may be due to the generation of ROS (Janssen et al., 1995).

Antioxidants are substances that either directly or indirectly protect cells against adverse effects of drugs, xenobiotics, and carcinogens. Several biologically important compounds have been reported to have antioxidant functions. These include vitamin C, vitamin E,  $\beta$ -carotene, N-acetylcysteine, metallothionein, glutathione, superoxide dismutase, and catalase. These antioxidants can either scavenge superoxide and other free radicals or stimulate the detoxification mechanisms within cells resulting in detoxification of free radical formation. In human keratinocytes, UVB-induced *c-fos* and *c-jun* expression was blocked by N-acetyl cysteine (NAC) (Garmyn et al., 1997). Pretreatment with NAC in bovine chondrocytes significantly attenuated interleukin-1-induced *c-fos* and collagenase gene expression (Lo et al., 1998). In primary hepatocytes, copper and cadmium chloride induced cytotoxicity and malondialdehyde (MDA) indicative of increased

lipid peroxidation formation. This effect was significantly reduced by the ROS scavengers dimethyl sulfoxide, manitol, catalase or SOD, as well as  $\alpha$ -tocopherol succinate (Pourahmad et al., 2000). Thus, a growing body of evidence indicates that transition metals induce oxidative damage to the cell by generating ROS.

### Cadmium

Cadmium (Cd) is a highly toxic heavy metal that can easily form complexes with other metals and elements. Cd is most often encountered in combination with other elements such as oxygen (cadmium oxide), chlorine (cadmium chloride), or sulfur (cadmium sulfide). These compounds that pre-exist in soil are all stable solids that are not water soluble. However, through the interaction with a pollution-formed pH-decreasing agent like acid-rain, the Cd in the soil may be converted into a soluble form. This soluble form becomes available to plants and enters the food chain (Hernandez, 1995).

During the past several decades, Cd abundance in soil, air, and water has increased due to its widespread industrial use (Waalkes et al., 1992). Cd is widely used in electroplating and galvanizing, as a color pigment in paints, and in batteries. It is a byproduct of zinc and lead mining and smelting (Bagchi et al., 1995). Not only have groups of workers in these industries been exposed to Cd, but contamination of the general population is increasing. The itai-itai disease in Japan, characterized by osteomalacia and renal tubular malfunction, has been attributed to Cd in irrigation water (Hamond et al. 1986). Recent studies have shown that the internalization of

soluble Cd salts into an animal's physiological system can accumulate and lead to serious tissue and organ damage. In particular, accumulation in liver, kidneys, brain, lungs, heart, testes, and the central nervous system have been noted.

Recent attention has focused on the role that Cd may play in carcinogenesis, leading the International Agency for Research on Cancer to conclude that Cd is classified as a category 1 carcinogen in humans (IARC 1993). In animals, oral CdCl<sub>2</sub> induces malignant prostatic tumors in rats (Waalkes *et al.*, 1995) and myoblasts treated with Cd become transformed to give rise to malignant sarcomas when injected into nude mice (Waalkes *et al.*, 1996). The mechanism by which Cd causes these diverse pathologies is, however, not well understood. The process of tumor development requires the mutation of critical genes (initiation), stimulation of proliferation of committed cells (promotion), and transformation to malignant growth (progression). Cd is not a strong initiator because it is only a weak mutagen (Rossman *et al.*, 1992; Beyersmann and Hartwig, 1994). However, Cd has been shown to stimulate the expression of various types of genes in a number of different cells, including cellular proto-oncogenes, which generally associate with enhanced cell proliferation (Harris, 1991). Cd induces *c-fos*, *c-jun*, *c-myc*, and *egr-1* in LLC-PK1 cells (Matsuoka and Call, 1995); *c-fos*, *c-jun* and *c-myc* in NRK cells (Tang and Enger, 1993); *c-fos* and *egr-1* in Swiss 3T3 cells (Epner and Herschman 1991), *c-jun* and *c-myc*, but not *c-fos* in L6 myoblasts (Jin and Ringertz, 1990); and *c-fos* and *c-jun* in PC12 cells (Hechtenberg *et al.*, 1996). *c-jun* and members of the *c-fos* gene family

are immediate early-response genes involved in cell cycle and are implicated in the response of cells to oxidative stress (Angel et al., 1991).

It is possible that Cd activated transcription occurs through specific metal-responsive upstream regulatory elements found in the promoters of Cd responsive genes. These may include the well characterized metal response element sequences found in most metallothionein genes (Searle et al., 1990), or the Cd-responsive element found in the human heme oxygenase genes (Takeda et al., 1994). To effect gene expression and activate transcription factor, Cd may influence signal transduction pathways. Both protein kinase C (PKC) and mitogen activated protein kinase (MAPK) are key enzymes in signal transduction pathways that control cellular growth and differentiation. Cd has been shown to affect the activities of protein kinase C, c-AMP-dependent protein kinase, and calmodulin (Wang et al., 1998). In one report, it has been suggested that Cd-induced transcription of *c-fos* and *c-jun* is mediated via protein kinase C and calmodulin (Beyersmann et al., 1997). In addition, the induction of *c-fos* by Cd in rat kidney LLC-PK1 cells was blocked by the PKC inhibitor, H7 (Matsuoka and Call, 1995). Inhibition of *c-fos* induction in mesangial cells by a MEK inhibitor suggests that sustained activation of MAPK is responsible in part for the effect of Cd (Templeton et al, 1998).

Protein denaturation, LPO, and DNA strand breaks are major forms of intracellular damage associated with Cd exposure. In whole animals, low doses of Cd (4.4mg/kg/day, intra-pelvic injection) induce hepatic and brain mitochondrial, microsomal lipid peroxidation, excretion of urinary lipid metabolite, and hepatic

nuclear DNA-single strand breaks in female Sprague-Dawley rats (Bagchi et al., 1997). Karamakar et al. (1998) also showed that oral administration of Cd (2.5mg/kg/day) triggered induction of LPO and inhibition of reduced glutathione mediated glutathione S-transferase catalyzed detoxification. Cd also induced DNA single-strand breaks (Ochi and Osawa, 1983) and chromosomal aberrations (Ochi et al., 1984), and mutation at the HPRT locus. A study by Lopez-Ortal et al, (1999) also showed that DNA strand breaks occur in a human fetal hepatic cell line after Cd exposure. LPO and lactate dehydrogenase (LDH) has also been shown to increase in various types of cells by Cd exposure. In general, LDH leakage is considered as a result of cell membrane compromise. In rat primary hepatocytes, Cd has been shown to induce LPO and LDH leakage in a time and dose dependent manner (Xu., 1999). This Cd induced increase in LPO and LDH leakage is also observed in isolated rat hepatocytes (Fariss., 1991) and in human fetal hepatic cell line (Bucio et al., 1995). Proposed mechanisms by which Cd induces these events involve the metal binding to reduced cysteine residues generating reactive oxygen species. One important cysteine molecule that might be involved in all of this is glutathione (Abe et al., 1994).

Glutathione, an intracellularly synthesized tripeptide,  $\gamma$ -glutamylcysteinylglycine, is found in all mammalian tissues but is especially high in concentration in the liver. It has been considered as a first line of defense against oxidative damage caused by a wide variety of drugs and chemicals. Glutathione exists in either an oxidized (GSSG) and reduced (GSH) form, and the GSSG : GSH ratio can indicate the cellular redox balance. GSH, which can act as both an antioxidant

and a metal-chelating agent, plays an important role as a first line of cellular defense against Cd (Singhal et al., 1987). A study by Karmakar et al (1998) showed that GSH was decreased in mouse liver after oral Cd exposure. Decreased GSH in rat kidneys (Stajn et al., 1997), C6 glioma cells (Cookson et al., 1996), and rat glomerular mesangial cells (Chin et al., 1993) after Cd exposure has also been reported. Therefore, more evidence has accumulated to support that glutathione is an early inducible mechanism of protection from Cd toxicity.

### Glucose-6-Phosphate Dehydrogenase

The intracellular redox potential is determined by the concentration of oxidants and reductants. A critical modulator of redox potential is NADPH, the intracellular reductant in all cell types. Glucose-6-phosphate dehydrogenase (G6PDH: EC 1.1.1.49) is the key regulatory enzyme of the pentose phosphate pathway. It controls the flow of carbon through this pathway and produces reducing equivalents in the form of NADPH for both biosynthetic reactions and maintenance of the cellular redox state. It has been traditionally thought that G6PDH was a typical “housekeeping” enzyme, present in most tissues and multicellular organisms at relatively consistent levels, however, G6PDH is subject to tissue-specific regulation by hormonal and nutritional factors. For example, insulin and glucocorticoids are primary hormones in upregulating expression of G6PDH in the liver. Carbohydrate also influences G6PDH activity, as well as, gene expression in animals and hepatocytes in culture (Kletzien et al., 1994). In recent years, it has been found that



G6PDH gene expression is also sensitive to a variety of oxidants such as  $\text{H}_2\text{O}_2$ , thioacetamide and asbestos. (Izawa et al., 1998; Díez-Fernández et al., 1996).

As shown in Figure 2, G6PDH participates in the detoxification of elevated concentrations of  $\text{H}_2\text{O}_2$ . During the detoxification of  $\text{H}_2\text{O}_2$ , GSH is oxidized to GSSG by the enzyme GSH peroxidase (1). The reduction of GSSG to GSH is catalyzed by GSSG reductase (2), which utilizes NADPH as the reducing potential. G6PDH produces NADPH by catalyzing glucose-6-phosphate to 6-phospho-gluconate (3).

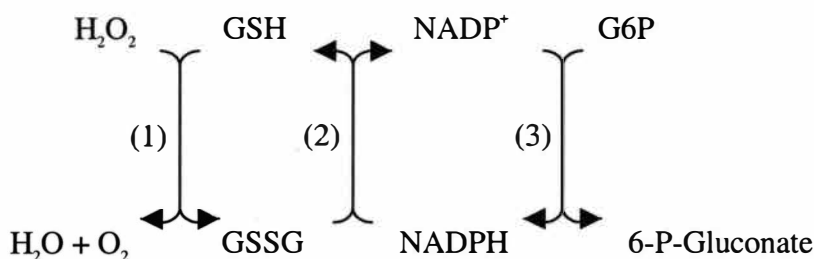


Figure 2. Detoxification of Hydrogen Peroxide. Reaction (1) catalyzed by GSH peroxidase: (2) GSH reductase: (3) G6PDH.

A study by Pandolfi et al. (1995), using G6PDH- deficient cell lines, showed that other sources of NADPH do not adequately replace the lack of NADPH production by G6PDH. The G6PDH-deficient cells had decreased growth rates and cloning efficiencies and were highly sensitive to oxidative stress compared with cells expressing endogenous levels of G6PDH. Thus, G6PDH is critical for NADPH production and is the principal source of NADPH in the mammalian cell. Interestingly, a metabolic deficiency, G6PDH deficiency syndrome, is a common genetic disorder that can result in haemolytic anemia and enhanced oxidant sensitivity

of erythrocytes. In the absence of this enzyme, a deficient individual is not able to provide sufficient amounts of NADPH to form reduced glutathione to prevent oxidative damage to the red blood cells.

Recent studies suggested that ROS generated by oxidative stress may also regulate G6PDH in mammals. In primary rat hepatocytes, diquat and ethanol have been shown to induce hepatic G6PDH, and its expression is modulated by free radicals during oxidative stress (Cramer et al., 1993). In COS-7 cells, the G6PDH inhibitor 6-aminonicotinamide potentiated H<sub>2</sub>O<sub>2</sub>-mediated cell death. Over expression of G6PDH increased resistance to H<sub>2</sub>O<sub>2</sub>-induced cell death (Tian et al., 1999). Indeed, *Saccharomyces cerevisiae* strains which contained mutation in the G6PDH genes were sensitive to H<sub>2</sub>O<sub>2</sub> that specifically depleted the intracellular pool of GSH (Izawa et al., 1998).

#### Oxidative Stress and the MAP Kinase Signal Transduction Pathway

ROS have been observed to influence molecular and biochemical processes and directly cause some of the changes observed in cells during differentiation, aging, and transformation (Allen et al., 1989; Sohal et al., 1990). Extracellular signaling molecules such as growth factors and cytokines induce changes in cell behavior via complex signal mechanisms that involve transmission of this signal from the plasma membrane to the nucleus (Allen et al., 2000). These signals are relayed to their targets by the means of various components of the signal transduction pathway including receptors, couplers, effectors, second messengers, protein kinases, and phosphoproteins. Eventually, these signaling processes lead to the induction of

biological activities such as gene expression, cell growth, muscle contraction, and neurotransmission.

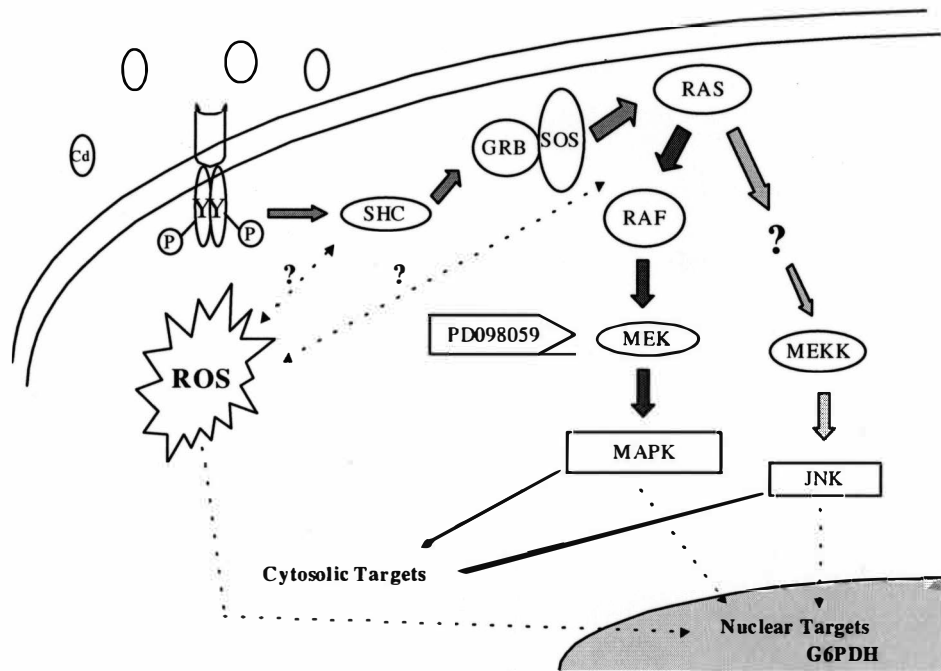


Figure 3. Mitogen Activated Protein Kinase Signal Transduction Pathway.

Recently, oxidation-reduction reactions have gained attention as important chemical processes that regulate signal transduction pathways. Although many pathways are known to be redox-sensitive, none have been more thoroughly examined in this regard than the MAP kinase signal transduction pathway. The MAP kinase family constitutes a superfamily of proteins that includes the extracellular signal regulated kinases (ERKs), c-jun NH<sub>2</sub> terminal kinase and stress activated protein kinase (JNKs /SAPKs) as well as p38 MAP kinase. All of the MAPK

members are catalytically inactive in unstimulated cells, and are activated in response to the appropriate stimulus by phosphorylation on both threonine and tyrosine. This phosphorylation is carried out by a dual specificity MAPKK (MAP kinase kinase or MEK), which in turn is activated through phosphorylation by MAPKKK (MAP kinase kinase kinase or MAP3K) (Feurstein et al., 2000).

Some of the early down stream components of this MAPK signaling pathway have been identified (Figure 3). A Signal typically begins at the growth factor receptor. Ligand binding to receptor causes autophosphorylation of the receptor's tyrosine residue creating docking sites that recruit the adapter protein Grb-2 (growth factor receptor bound-2) through its SH2 (Src homology 2) domain. This binding results in a conformational change in Grb-2, and its SH-3 domain binds to SOS (son of seventhless). The phosphorylated Grb-2/SOS complex binds to SHC (Src homology and collagen) and the multiprotein complex activates membrane-bound Ras-GDP by converting it to its GTP-bound form (Allen et al., 1999). A study by Rao showed that  $H_2O_2$  induced phosphorylation of the epidermal growth factor receptor which then formed a complex with SHC-Grb2-SOS (Rao, 1996). The first kinase downstream of Ras is the serine/threonine protein kinase Raf-1. The GTP-bound form of Ras stimulates translocation of Raf-1 to the plasma membrane, where it is phosphorylated (Geilen et al., 1996). Once activated, Raf-1 phosphorylates serines in the catalytic site of MAP kinase kinase (MEK). MEK then activates members of the MAPK family, which are dual specificity (serine/ threonine and tyrosine) kinases that regulate down stream responses to a broad range of mitogenic, apoptotic, and differentiation-inducing stimuli.

2'-amino-3'-methoxyflavone (PD098059) is potent inhibitor of MAPK pathway in a variety of cell types. A study by Chin et al. (1998) showed that TNF- $\alpha$  induced MAPK activation was inhibited in the presence of PD098059 in cultured macrophages. In addition, pretreatment of the human intestine 407 cells with PD098059 significantly attenuated hypo-osmotic stress induced MAPK activity (Van der Wijk et al., 1998).

A number of different studies have shown that oxidants stimulate tyrosine kinase activity and increase tyrosine phosphorylated proteins. A study by Chen et al. observed that naphthaquinone stimulated protein tyrosine phosphorylation in rat liver plasma membrane, and proposed that ROS may participate in this quinone stimulation of phosphorylation (Chen et al., 1986). H<sub>2</sub>O<sub>2</sub> was found to stimulate cytosolic protein tyrosine phosphorylation in different cell lines (Zick et al., 1990). Additionally H<sub>2</sub>O<sub>2</sub> treatment of tracheal myocytes successively stimulated protein kinase C, Raf-1, and MAPK pathways, leading to activation of Erk 1 and 2, serine/threonine kinases of the MAPK superfamily important in transduction of mitogenic signals to the nucleus (Abe et al., 1994; 1998). In Jurkat T-cells, lowering intracellular GSH induced tyrosine phosphorylation presumably by influencing ROS levels (Staal et al., 1994). In NIH3T3 cells, a study by Stevenson et al. (1994) showed that threonine / tyrosine phosphorylation of MAPK and subsequent kinase activity were found to be stimulated by H<sub>2</sub>O<sub>2</sub> and ionizing radiation, as well as phorbol esters. This increased MAPK activity by phorbol esters was blocked by the presence of the antioxidant, N-acetyl cysteine, suggesting the possible role of ROS in the mechanism of activation.

Another serine/threonine kinase in the MAPK family, the stress activated protein kinase SAPK/JNK, has been shown to be phosphorylated in response to diverse extracellular stimuli, including UV irradiation, pro-inflammatory cytokines and certain mitogens. The JNKs phosphorylate specific sites on the amino terminal trans-activation domain of transcription factor c-Jun, an important component of transcriptional activator AP-1. Phosphorylation of these sites stimulates the ability of c-Jun to activate transcription of specific target genes. Accumulated evidence has also shown that activation of JNK is mediated by intracellular ROS. For example, interleukin 1 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) activated JNK was blocked by the antioxidant NAC in bovine chondrocyte (Lo et al., 1996). In PC12 cells, the auto-oxidized dopamine-induced JNK activation was prevented by NAC (Kang et al., 1998). Cd, the metal used in our studies, has also been found to activate JNK in LLC-PK1 cells (Matsuoka et al., 1998).

### Objective of Study

Although various theories have been proposed, the cellular mechanisms involved in Cd induced gene expression and the activation of signal proteins are still not clear. Therefore, the objective of this study is to elucidate how Cd induces signal transduction pathways that result in increased gene expression. The hypothesis is that Cd, which is capable of generating ROS in a cell, mediates its effect on signal proteins and gene expression through this mechanism. To test this hypothesis, I (a) investigated whether or not Cd could induce G6PDH gene expression, (b)

investigated whether or not ROS played a role in this induction of G6PDH, (c) investigated whether or not Cd and/or ROS were capable of activating signal proteins and if this activation was required for increase the in gene expression, and d) investigated whether or not induction of G6PDH by ROS required MAPK activation. The studies were carried out in primary hepatocytes, which provided us with an excellent Cd responsive model.

## METHODS

### Materials

MAP Kinase Assay and SAPK/JNK Assay Kit were purchased from New England Biolabs. Waymouth's MB 752/1 media was purchased from Gibco BRL, together with the TRIzol®. Bovine serum albumin (BSA), hyaluronadase, N-Acetyl Cystein, and trypsin inhibitor were from Sigma. Collagenase D was purchased from Boehringer Mannheim. Rat tail collagen, type 1, was from Becton Dickinson Labware. The plasmid mini prep kit was obtained from Promega, whereas the Multiprime Labeling Kit and  $\alpha$ -P<sup>32</sup>[dCTP] were purchased from Amersham. L-buthione-(S, R)-sulfoximine (BSO) and Trolox were obtained from Aldrich. PD-98059 was purchased from BIOMOL Research Laboratories, Inc. Cadmium chloride (CdCl<sub>2</sub>) was from Fisher Scientific, while all other chemicals were purchased from Sigma-Aldrich Corporation and were of the highest grade available.

### Primary Rat Hepatocytes Isolation and Maintenance

The hepatocytes were isolated from 8–10 week old male Sprague-Dawley rats (Harlan Sprauge-Dawley, Indianapolis, IN) which were food deprived 48 hours prior to the isolation procedure. Water was given ad libitum without restriction. The rat was



anesthetized with pentobarbital (45 mg/ Kg), and the liver was then first perfused via the portal vein with a perfusion solution containing 0.148 M NaCl, 0.01 M HEPES, 0.017 M fructose, 0.49 mM EGTA, 6 ml of 0.5 %phenol red, and 6 unit/ml heparin in water. Then a digestion solution containing 100 µg /ml collagenase D, 93 unit/ml hyaluronidase, 160 unit/ml trypsin inhibitor, and 0.2% BSA in water was administered. The partially digested liver was forced through 4 layers of sterile gauze as described by Stapleton et al (1993). The isolated hepatocytes were washed three times with Waymouth's MB 752/1 media containing 0.5% BSA. The viability of the cells was checked with a hemocytometer using the trypan blue dye exclusion method. Cells of greater than 85% viability were then plated on sterile 60mm culture plates coated with collagen and maintained at 37 °C in a humidified environment of 5% CO<sub>2</sub> and 95% air. After 4 hours, the cells were washed with BSA free media and allowed to incubate overnight before treatment began.

### Cell Treatment

Primary hepatocytes were treated with cadmium chloride (Cd) at the following concentrations: 0 (no addition, NA), 1, 2, 4, 6µM for either 6 or 24 hours. In separate experiments, the primary hepatocytes were also treated with 0.2mM BSO for 6 and 24 hours. For the studies using antioxidants, the primary hepatocytes were pretreated for 2 hours with either 0.5mM Trolox (water soluble form of Vitamin E), 10 mM N-acetylcysteine (NAC), or 2.0 mM glutathione ethyl ester (GSH-E) prior to the addition of Cd or BSO.

## Cell Processing

The treated primary rat hepatocytes were first processed by removing the media and washed twice with ice-cold phosphate-buffered-saline (PBS) solution. The cells were then lysed by adding 1 ml of 1x cell lysis buffer from the MAPK assay kit that contained 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerolphosphate, 1 mM sodium vanadate, and 1  $\mu$ g leupeptin incubated for 5 minutes, and then scraped off the plates. The samples were sonicated 4 times at 25 % of output power for 5 seconds( Sonic Dismembrator 50, Fisher Scientific) and microfuged for 10 minutes at 4 °C. The supernatants were transferred to a new eppendorf tube and used for either the MAP kinase or c-jun N-terminal kinase assay as described below. The total protein content of the sample was measured using the Lowry method (Lowry et, al., 1951).

## Western Blot Analysis

A total of 200  $\mu$ g of protein was immunoprecipitated and 20  $\mu$ l of this sample was subject to electrophoresis at 100 mV for 2 hours. Proteins were then transferred to a PVDF membrane (Immobilon-P, Milipore, Bedford, MA) using a standard semi-dry electroblotting apparatus (NOVEX, San Diego, CA). After blocking with 5% (w/v) non-fat dry milk, the blots were incubated overnight at 4°C with antibodies (1 : 1000) dilution, directed against phosphorylated forms of Elk/ p44/42 and SAPK/JNK. Blots were washed three times with 0.1% Tween-20 in Tris-buffered

saline (0.02 M Tris base and 0.14 M NaCl in water) incubated with horseradish peroxidase-conjugated anti-rabbit IgG, (1 : 2000) dilution, and anti-biotin antibody, 1 : 1000 dilution, for one hour at room temperature. The membranes were washed as above, and the bands detected by chemiluminescence (HRP-Western Detection Kit, New England Biolab, Inc.). Developed blots were exposed to Hyper film (Amersham Life Science, IL) and bands were quantified using scanning densitometry.

### MAP Kinase Assay

One microgram of the phospho-specific p44/42 MAP Kinase Monoclonal Antibody was added to 200  $\mu$ g of total protein and incubated with gentle rocking overnight at 4°C. The immunocomplex was precipitated by incubating with 20  $\mu$ l of a 50 % protein-A-agarose slurry in lysis buffer as mentioned above, for 2 hours at 4 °C. The pellet was washed twice with 500  $\mu$ l of cell lysis buffer and then twice with 500  $\mu$ l of kinase buffer that contained 25 mM tris, 2 mM DTT, 5 mM  $\beta$ -glycerophosphate, 0.1 mM sodium vanadate, and 10 mM magnesium chloride. The pellet was suspended in 50  $\mu$ l of kinase buffer supplemented with 100  $\mu$ M ATP and 1  $\mu$ g ELK 1 fusion protein. After incubation for 30 minutes at 30 °C, the reaction was terminated by adding 25  $\mu$ l of 3x SDS sample buffer that contained 187.5 mM Tris, 6 % SDS, 30 % glycerol, and 125 mM DTT. The sample was then boiled for 5 minute, vortexed and centrifuged for 2 minutes at 14,000 rpm. 20  $\mu$ l of the sample was subjected to SDS-PAGE at 100mV for 2 hours. Western blot analysis was carried out as described above using phospho-specific Elk1 antibody as a primary antibody. Proteins were

detected on hyperfilm using phototope-HRP Western detection kit provided by New England Biolab, Inc. Scanning densitometry of the exposed film was used to quantify kinase activity.

#### c-jun-N-terminal Kinase (JNK) Assay

JNK was immunoprecipitated from 200  $\mu$ g total protein lysate by incubating with the 2  $\mu$ g c-jun fusion protein beads, which selectively pull down JNK from cell lysate, at 4 °C overnight. The samples were microfuged for 2 minutes at 4 °C. The pellet was washed twice with lysis buffer and twice with kinase buffer. The pellet was suspended in 50  $\mu$ l of kinase buffer supplemented with 100  $\mu$ M ATP. After incubation for 30 minutes at 30 °C, the reaction was terminated with 3 x SDS sample buffer. The samples were boiled for 5 minutes, vortexed and centrifuged for 2 minutes. 20  $\mu$ l of the sample was subjected to SDS-PAGE at 100 mV for 2 hours. Western analysis was carried out as described above using phospho-specific c-Jun antibody, 1 : 1000 dilution. Proteins were detected using phototope-HRP western detection kit provided by the manufacture and exposed to hyper film. Scanning densitometry of developed film was used to quantify kinase activity.

## Plasmid DNA Preparation

The *E.Coli* bacteria containing the plasmid with the Glucose-6-phosphate dehydrogenase cDNA was plated on Luria- Bertani (LB) agar plates containing 50 µg/ml of the antibiotic ampicillin. A single colony was used to inoculate 10 ml of LB media containing 50 µg/ml ampicillin and grown overnight at 37 °C in a shaking incubator. The plasmid DNA was then isolated using the Wizard® plus SV Miniprep DNA purification system which is based on alkaline lysis method (Promega, WI). Visualization of the purified DNA using gel electrophoresis and the OD 260/280 nm absorption ratios confirmed the purity.

## DNA Probes

The following probes were used in Northern Blot analysis: the 1.6 Kb EcoRI G6PDH cDNA subcloned into, the 1.8kb HindIII chicken  $\beta$ -actin cDNA subcloned into pBR322 (gift from Dr. Cleveland, the Johns Hospkins University). The G6PDH and  $\beta$ -actin cDNA inserts were purified by digestion with the appropriate restriction endonucleases and separating the insert by gel electrophoresis. DNA restriction fragments of interest were excised from the gel, minced into very fine pieces with a scalpel and placed into microcentrifuge tubes. Molecular biology grade phenol (no more than 0.5 ml per tube), pH 7.6 (Fisher Scientific) was added to each tube and vortexed thoroughly. The agarose-phenol mixture was placed for 10 min at -80 °C, centrifuged for 10 min at 12000 rpm (room temperature) and the upper phase

collected in a new microcentrifuge tube. The final concentration of 100 mM NaCl and 2x volume of ice-cold 100 % ethanol were added, mixed, and the tubes placed at  $-20^{\circ}\text{C}$  overnight. The tubes were microcentrifuged at  $4^{\circ}\text{C}$  for 25 min in order to precipitate DNA. The remaining DNA was re-suspended in  $20\mu\text{l}$  water. The cDNA for both G6PDH and  $\beta$ -actin were labeled with [ $^{32}\text{P}$ -dCTP] by random priming (Amersham, IL).

#### Isolation and Measurement of mRNA levels

Total RNA was isolated with TRIzol® Reagent according to the manufacturer's instruction (Life Technologies, Grand Island, NY). The work area was decontaminated with RNase ZAP (Invitrogen) and all glassware was baked in order to avoid RNase contamination. The concentration of RNA was determined by absorbance at 260 nm. Total RNA ( $20\mu\text{g}$ ) was separated on a 1 % agarose / 2.2 M formaldehyde gel and transferred to "GeneScreen" (NEN /DUPONT) in 20X SSC buffer containing 0.3 M sodium citrate and 3 M NaCl in DEPC water. Hybridization was performed using the method as described by Berg et al (1995). After a 12 hr hybridization the membranes were washed with 2X SSC/1% SDS at  $48^{\circ}\text{C}$  and  $50^{\circ}\text{C}$  for 15 min each and 1X SSC/1% SDS at  $53^{\circ}\text{C}$  for 15 min. The membranes were exposed to autoradiography at  $-70^{\circ}\text{C}$  with Kodak XAR-5 film and intensifying screens before developing. Quantification of data was by densitometry. The amount of G6PDH mRNA was normalized to the control probe,  $\beta$ -actin.

## Statistical Analysis

The results are expressed as the mean  $\pm$  S.E.M. of N number of animals used for each experiment. Statistical significance was evaluated by the one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test ( $p < 0.05$ ).

## RESULTS

### Effect of Cd on G6PDH mRNA Levels in Primary Rat Hepatocytes

To examine the effect of Cd on G6PDH gene expression, primary rat hepatocytes were treated with CdCl<sub>2</sub> at concentrations of either 0 (NA), 1.0, 2.0, or 4.0  $\mu$ M for 6 hours. The cells were processed, total RNA was isolated and Northern analysis was carried out. The results show that a significant increase in the mRNA level for G6PDH was observed in the presence of Cd. This increase was concentration dependent (Figure 4). When the incubation time was extended to 24 hours for the 1.0  $\mu$ M concentration, maximum 4 fold induction in G6PDH mRNA was observed (Figure 6).

### Effect of BSO on G6PDH mRNA Levels in Primary Rat Hepatocytes

Previous studies in our laboratory showed that intracellular glutathione levels decreased in a concentration and time dependent manner when primary hepatocytes were treated with BSO. Additionally, increases in lipid peroxidation and lactate dehydrogenase leakage were observed in BSO treated cells (Xu., 1999). BSO is a potent and specific inhibitor of the intercellular glutathione precursor enzyme,  $\gamma$ -glutamylcysteine synthetase. BSO has been shown to inhibit GSH biosynthesis both *in vivo* and *in vitro* (Griffith & Meister, 1979; Griffith, 1982).



To examine whether or not the increase in G6PDH gene expression by Cd is affected by the intracellular GSH concentration, we treated the primary rat hepatocytes with BSO. As shown in Figure 5, incubation of the primary rat hepatocytes with 0.4mM BSO alone resulted in a 4 fold increase in increased G6PDH mRNA levels. This increase in G6PDH mRNA is comparable to the increase observed in the presence of Cd.

#### The Effects of Antioxidants on Cd Induced G6PDH mRNA Level

To elucidate whether or not ROS played a role in the Cd mediated increase in G6PDH expression, we evaluated the effect of antioxidants. To accomplish this we pretreated the primary hepatocytes for 2 hours with one of the following antioxidants, NAC, Trolox, or GSH-E. NAC is an effective free radical scavenger and has been shown to also affect intracellular glutathione levels (Lo et al., 1996). Trolox, a water soluble form of Vitamin E, is a potent peroxyl radical scavenger and GSH-E is a reduced form of GSH that hydrolyzes to GSH intracellularly. The levels of G6PDH mRNA in the presence of Cd were reduced with antioxidant treatment (Figure 6). Treatment with antioxidants alone did not show significant effect on the levels of G6PDH mRNA (data not shown). Treatment with NAC shows significantly reduced G6PDH mRNA levels by approximately 95% as compared to Cd alone. Whereas treatment with Trolox also significantly reduced Cd induced G6PDH mRNA levels by approximately 90% Treatment with GSH-E reduced Cd induced G6PDH mRNA levels by approximately 76%.

## The Effects of Antioxidants on BSO Induced G6PDH mRNA Level

BSO has been shown to influence ROS levels because of its ability to deplete intracellular glutathione. A study by Xu (1999) showed that BSO-induced LPO or LDH leakage was significantly blocked by the addition of either Trolox, GSH-E, or NAC in the primary rat hepatocytes. To further confirm that ROS are involved in mediating G6PDH expression, we investigated the effect of antioxidants on BSO treated cells. Before addition of BSO, we pretreated the primary hepatocytes for 2 hours with either NAC, Trolox, or GSH-E. Then, primary hepatocytes were treated for 24 hours with 0.4 mM BSO and G6PDH mRNA level was measured. Similar to Cd, BSO significantly increased G6PDH gene expression and the antioxidants, NAC, Trolox, or GSH-E significantly attenuated the level of mRNA (Figure 7). Treatment with antioxidants alone did not show significant effect on the levels of G6PDH mRNA (data not shown). Cells treated with NAC showed significant reduction of G6PDH mRNA levels by approximately 70%. Cells treated with Trolox also showed significant reduction of G6PDH mRNA levels by 65%. Whereas cells treated with GSH-E showed reduction of G6PDH mRNA levels by approximately 55%.

## Cd Induces MAPK and JNK in Primary Rat Hepatocytes

It is known that cells commonly use multiprotein kinase cascades to signal information from the cell membrane to the nucleus. Several conserved signaling pathways related to the mitogen activated protein kinase (MAPK) pathway have been shown to be involved in allowing cells to respond to normal developmental signals, as

well as, signals produced under stressful conditions (Perrimon N. et al., 1999). Stress-activated protein kinase / c-Jun N-terminal kinase (JNKs / SAPKs) are MAPK-related protein kinases that are involved in several cellular events, including growth, differentiation, and apoptosis. JNKs are activated in response to UV irradiation, osmotic shock, and treatment with tumor necrosis factor-  $\alpha$  (Zhuang et al., 1997). Thus, elucidation of whether or not Cd induces MAPK and JNK phosphorylation may be an important indicator of when cells are under stress or damage. After primary hepatocytes were treated for 6 hours with either 1.0  $\mu$ M or 4.0  $\mu$ M Cd, Dose dependent increase in phosphorylation of both MAPK and JNK was observed with Cd treatment. The level of MAPK and JNK phosphorylation increased about 3 to 9 fold over NA (Figure 8 and 10).

#### Effect of Antioxidants on Cd Induced MAPK Phosphorylation

Recent studies have shown that ROS are recognized as the intercellular signaling molecules in several cellular responses such as the activation of NF $\kappa$ B or plant systemic acquired resistance (Khan and Wilson. 1995). If it is true that Cd induces intracellular ROS and this mediates signals, then removing ROS should significantly decrease the phosphorylation of signal proteins. To determine whether or not ROS mediate MAPK phosphorylation, we pretreated the primary hepatocytes for 2 hours with either NAC or Trolox before Cd was added As seen in Figure 9, both antioxidants have reduced MAPK phosphorylations. Treatment with antioxidant, as well as, MEK inhibitor PD098059 alone did not show significant effect on MAPK

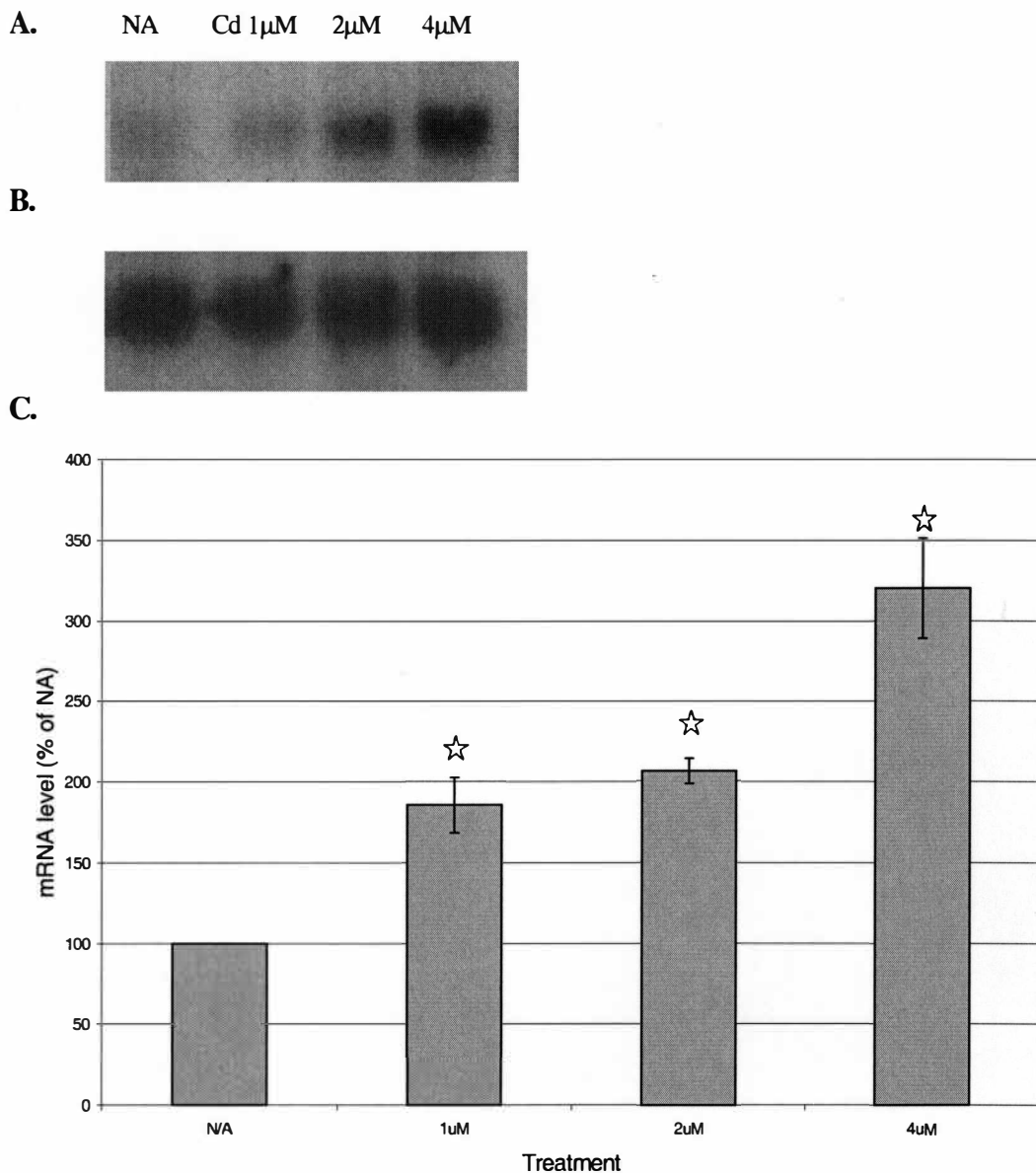
phosphorylation levels (data not shown). Treatment with NAC shows significantly reduced Cd-induced MAPK phosphorylation levels by approximately 86%. Treatment with Trolox also significantly reduced Cd-induced MAPK phosphorylation levels by approximately 77%. PD098059 has been proven to be an effective inhibitor of MAPK activation in a variety of cell types. PD 098059 was pretreated the primary hepatocytes for 30 minutes before Cd was added. Treatment with PD098059 also significantly reduced MAPK phosphorylation level by approximately 80%.

#### Effect of Antioxidants on Cd- Induced JNK Phosphorylation

Although Cd activated both MAPK and JNK, the pathways involved in their activation are different. JNK is a group of serine / threonine kinases structurally related but distinct from ERK or other MAPK families. Unlike the ERKs, JNKs are activated by two Ha-Ras related GTP binding proteins of the Rho family, Rac and Cdc42Hs. Thus, we investigated whether or not JNK phosphorylation occurs in a different manner from MAPK phosphorylation. Treatment with antioxidant, as well as, MEK inhibitor PD098059 alone did not show significant effect on JNK phosphorylation levels (data not shown). Treatment with NAC significantly reduced Cd-induced JNK phosphorylation levels by approximately 90%. Treatment with Trolox also significantly reduced Cd-induced JNK phosphorylation by approximately 90%. However, PD098059 reduced Cd-induced JNK phosphorylation levels by approximately 37%. Thus, PD098059 did not significantly reduce Cd-induced JNK phosphorylation (Figure 11).

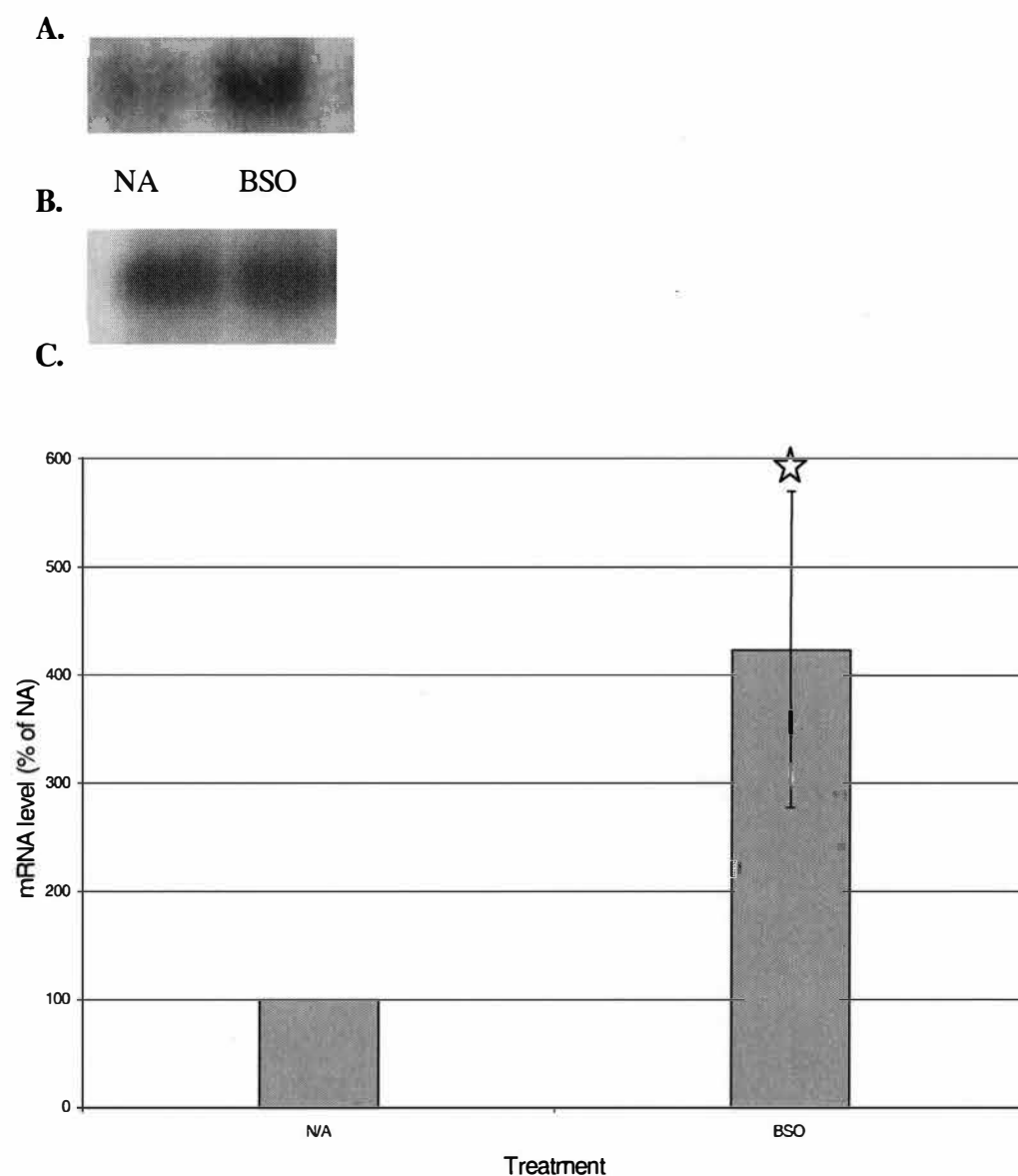
### Cd-Induced G6PDH Gene Expression is Sensitive to MEK Inhibitor

After establishing the importance of ROS in the Cd-induced G6PDH gene expression and signal transduction proteins, the role of the MAPK pathway in the regulation of G6PDH gene was determined. To elucidate whether or not the MAPK pathway is involved in the Cd-induced G6PDH expression, we pretreated the primary hepatocytes for 30 minutes with PD098059 before the addition of Cd. The cells were processed, total RNA was isolated and Northern analysis was carried out. As seen in Figure 6, PD098059 effectively attenuated Cd-induced G6PDH mRNA levels by approximately 58% suggesting that the MAPK pathway is important in Cd induced G6PDH expression.



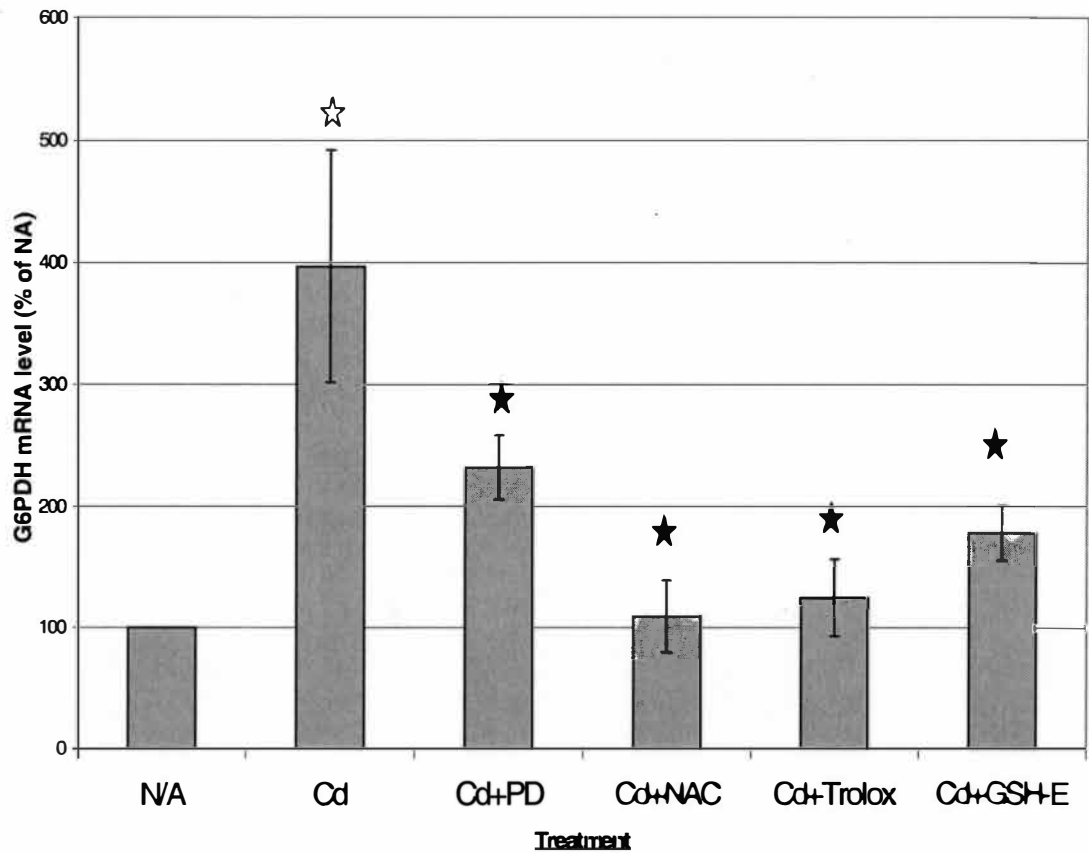
Legend. **A.** G6PDH mRNA Levels. **B.**  $\beta$ -Actin as Internal Control.  
 ☆= Significant Stimulation ( $p < 0.05$ ).

Figure 4. Cadmium Stimulated G6PDH mRNA Levels. **A.** & **B.** Northern blot showing G6PDH and  $\beta$ -Actin gene expression. **C.** Quantitation of G6PDH mRNA levels. ( $p < 0.05$ ). Primary rat hepatocytes were treated 6 hours with 0 ~ 4.0  $\mu$ M Cd. Data represent the mean  $\pm$  S.E.M. (N=5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test ( $p < 0.05$ ).



**Legend.** **A.** G6PDH mRNA Levels. **B.**  $\beta$ -Actin as Internal Control.  
 ☆ = Significant Stimulation ( $p < 0.05$ ).

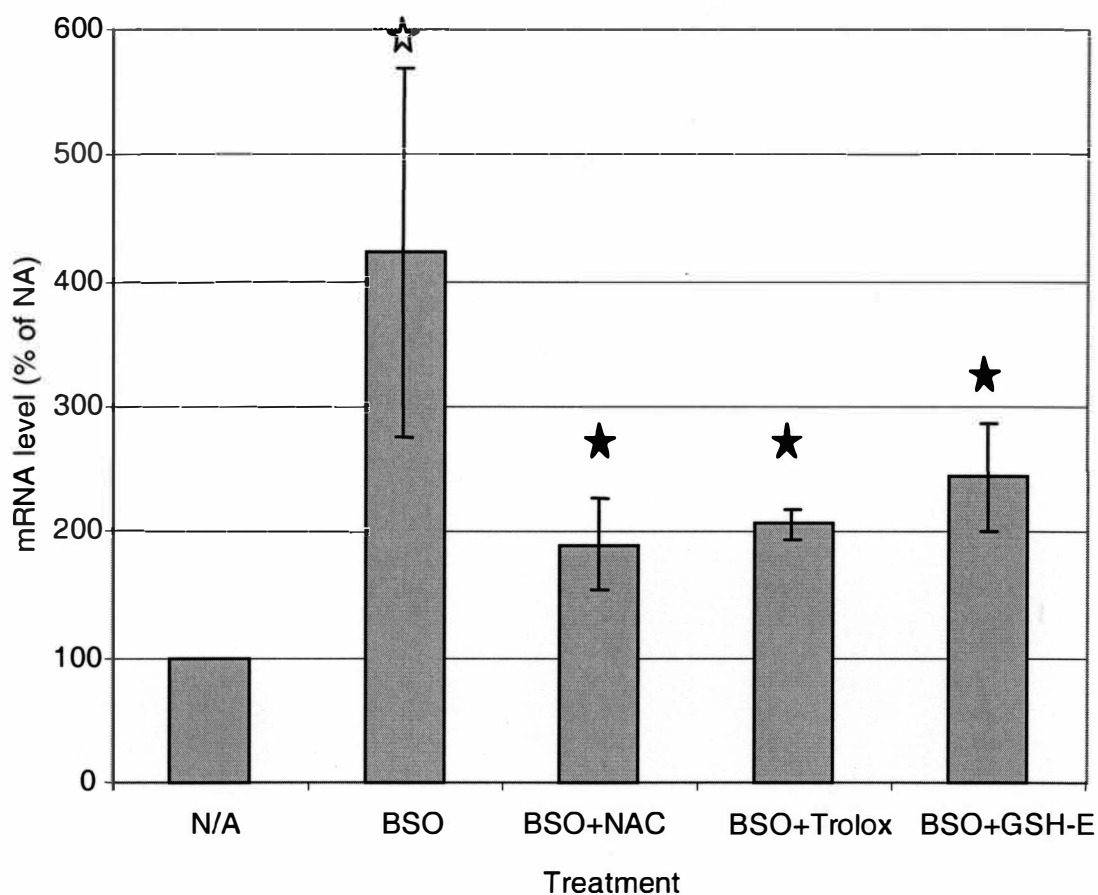
**Figure 5.** BSO Stimulated G6PDH mRNA Levels. **A. & B.** Northern blot showing G6PDH and  $\beta$ -Actin gene expression. **C.** Quantitation of G6PDH mRNA levels. Primary rat hepatocytes were treated 6 hours with 0.4mM BSO. Data represent the mean  $\pm$  S.E.M. ( $N=5$ ). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test ( $p < 0.05$ ).



Legend. ☆= Significant Stimulation ( $p < 0.05$ ).  
 ★=Significantly Different from 1.0  $\mu\text{M}$  of Cd ( $p < 0.05$ ).

Figure 6. Effect of Antioxidants and MEK Inhibitor PD098059 on Cd Induced G6PDH Gene Expression. Primary rat hepatocytes were pretreated for 2 hours with antioxidants or 30 minutes with PD098059. Then 1.0  $\mu\text{M}$  of Cd was appropriately added and cells were incubated for 24 hours. Data represent the mean  $\pm$  S.E.M. (N= 4). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test ( $p < 0.05$ ).





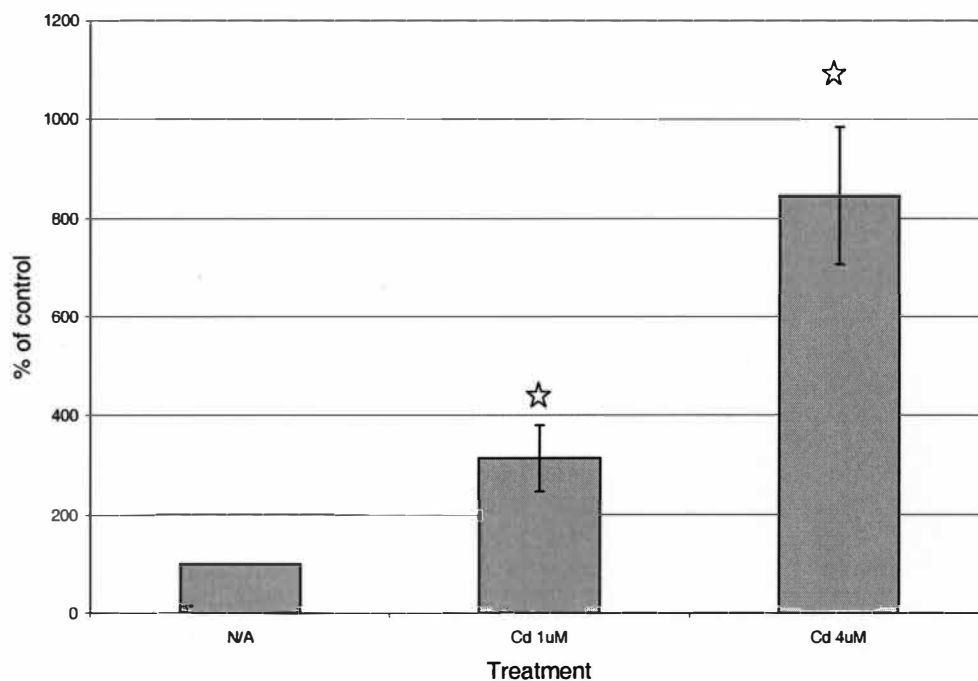
Legend. ☆ = Significant Stimulation ( $p < 0.05$ ).  
 ★ = Significant Inhibition ( $p < 0.05$ ).

Figure 7. Protective Effect of Antioxidants on BSO Induced G6PDH Gene Expression. Primary rat hepatocytes were pretreated for 2 hours with antioxidants. Then 0.4mM BSO was appropriately added and cells were incubated for 24 hours. Data represents the mean  $\pm$  S.E.M. ( $N = 4 \sim 5$ ). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test ( $p < 0.05$ ).

A.

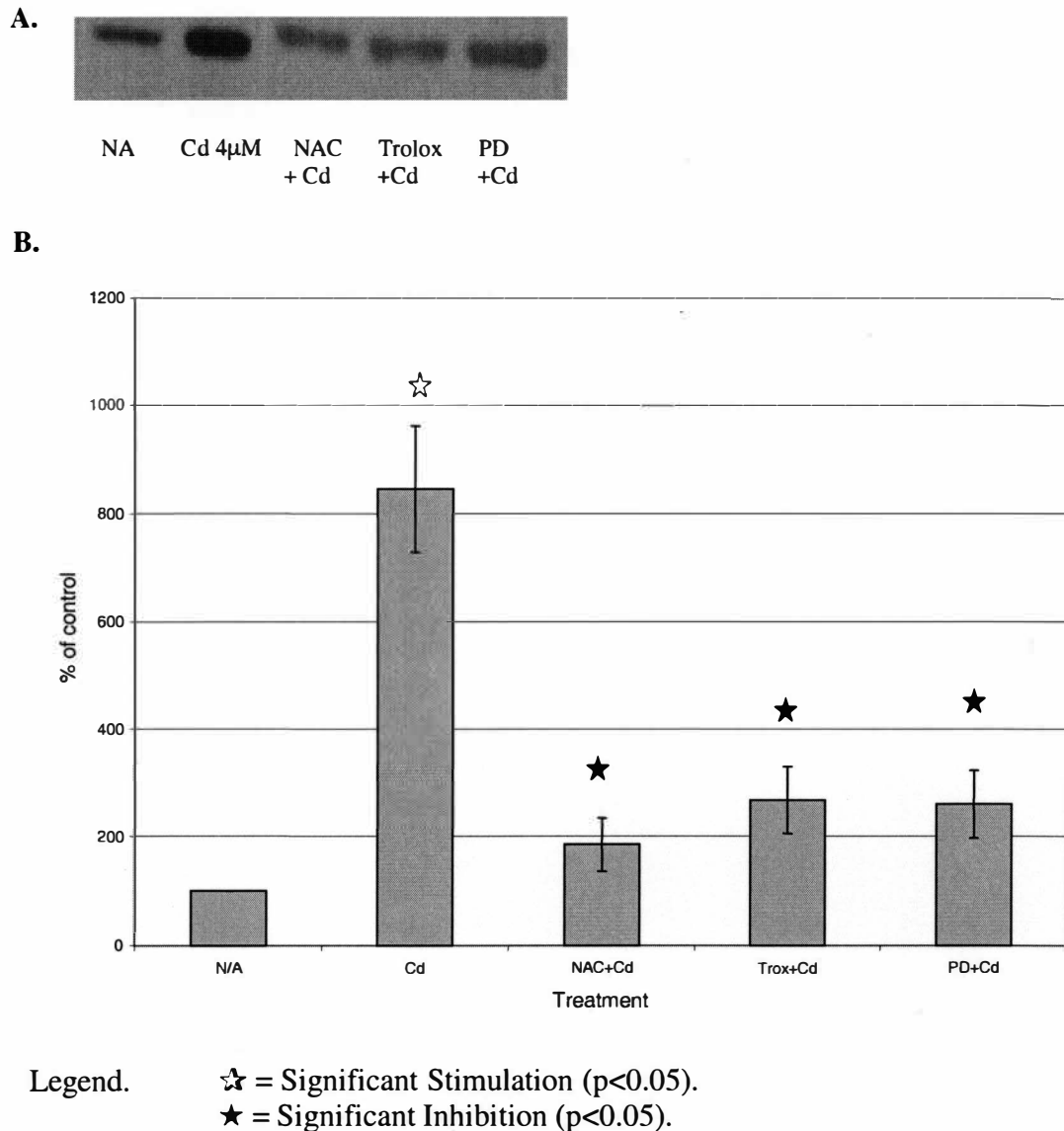
NA      Cd 1 $\mu$ M      Cd 4 $\mu$ M

B.

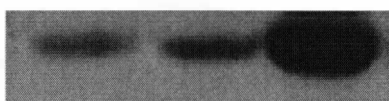
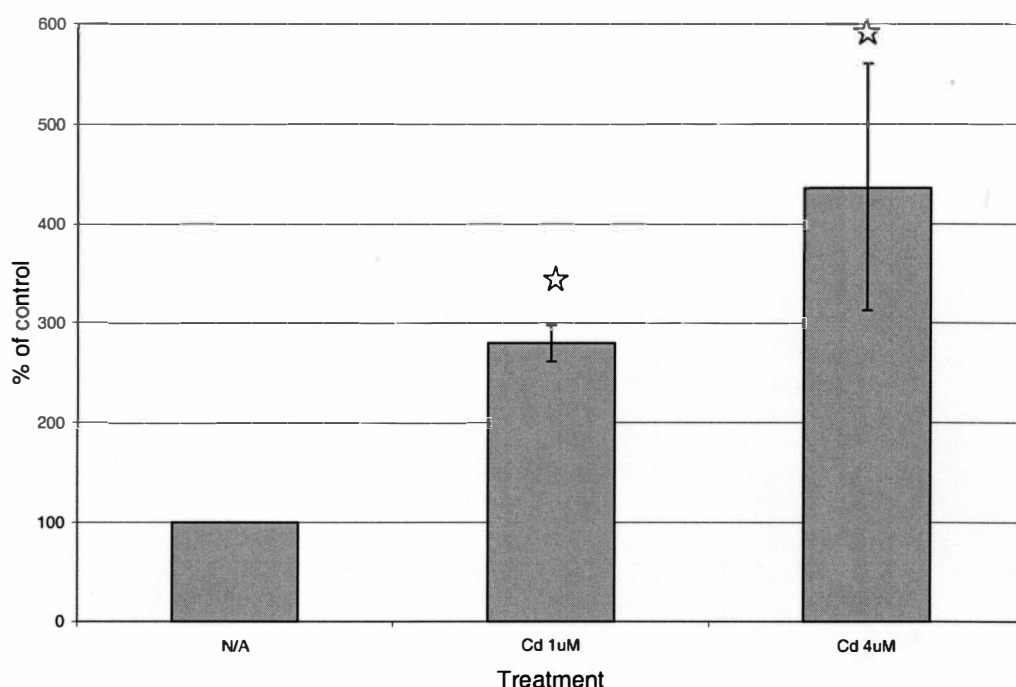


Legend. ☆ = Significant Stimulation.

**Figure 8.** Effect of Cd on MAPK Activation. **A.** Western blot showing MAPK phosphorylation. **B.** Quantitation of MAPK phosphorylation levels. Primary rat hepatocytes were treated for 6 hours with 0 ~ 4.0 $\mu$ M Cd. Data represent the mean  $\pm$  S.E.M. (N = 6). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test ( $p < 0.05$ ).



**Figure 9.** Effect of Antioxidants and MEK Inhibitor on MAPK Activation. **A.** Representative western blot showing MAPK phosphorylation. **B.** Quantitation of MAPK phosphorylation levels. Primary rat hepatocytes were pretreated for 2 hours with antioxidants or 30 min for MEK inhibitor PD098059. Then, 4.0 $\mu$ M Cd was appropriately added and cells were incubated for 6 hours. Data represent the mean  $\pm$  S.E.M. ( $N = 5$ ). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test ( $p < 0.05$ ).

**A.**NA    Cd 1 $\mu$ M    Cd 4 $\mu$ M**B.**

Legend.      ☆ = Significant Stimulation ( $p < 0.05$ ).

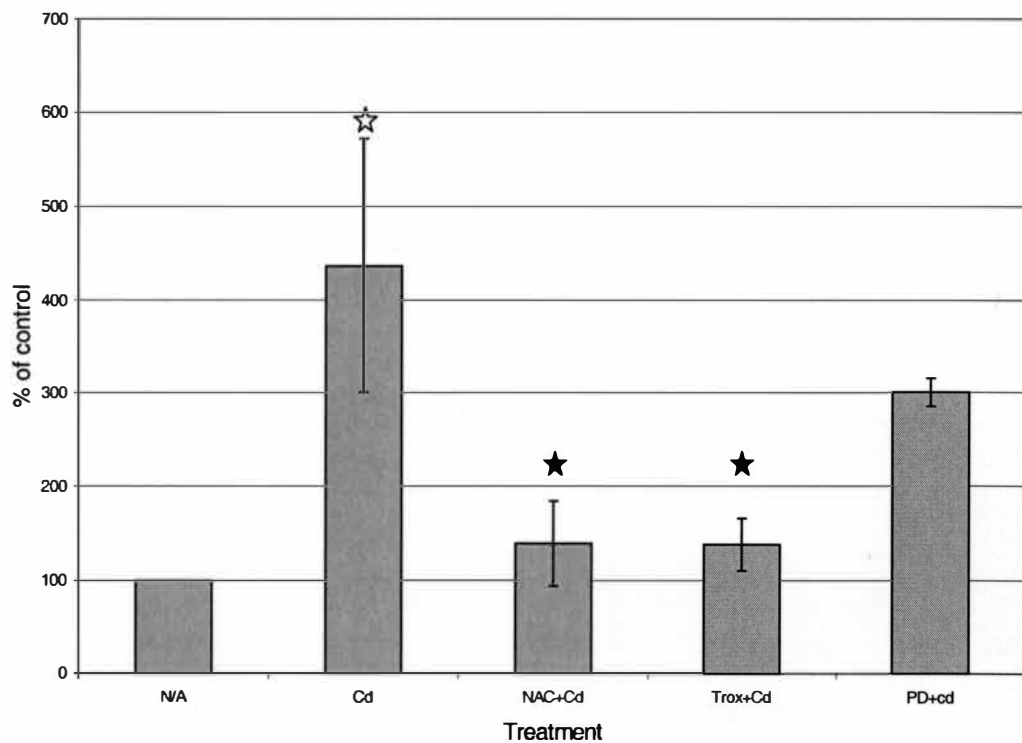
**Figure 10.** Effect of Cd on JNK Activation. **A.** Representative western blot showing JNK phosphorylation. **B.** Quantitation of JNK phosphorylation levels. Primary rat hepatocytes were treated for 6 hours with Cd (0 ~ 4.0 $\mu$ M). Data represent the mean  $\pm$  S.E.M. (N = 5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test ( $p < 0.05$ ).

A.



NA Cd 4 $\mu$ M NAC Trolox PD  
+Cd +Cd +Cd

B.



Legend.

☆ = Significant Stimulation ( $p < 0.05$ ).★ = Significant Inhibition ( $p < 0.05$ ).

Figure 11.

Effect of Antioxidant and MEK Inhibitor on JNK Activation.

**A.** Representative western blot showing JNK phosphorylation.

**B.** Quantitation of JNK phosphorylation levels. Primary rat hepatocytes were pretreated for 2 hours with antioxidants or 30 min for MEK inhibitor PD098059. Then 4.0 $\mu$ M of Cd was appropriately added and cells were incubated 6 hours. Data represent the mean  $\pm$  S.E.M. ( $N = 5$ ). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test ( $p < 0.05$ ).

## DISCUSSION

The purpose of this research was to investigate whether or not Cd, which is capable of generating ROS in a cell, effects signal proteins and gene expression, in particular, the expression of G6PDH. Cd, a ubiquitous environmental contaminant, has been shown to induce cellular damage to both organs and tissues by causing the accumulation of intracellular ROS (Ossola et al., 1995). However, the precise molecular mechanism responsible for the Cd-induced generation of ROS and resulting oxidative stress is not entirely clear. Several different studies have suggested that acute Cd-induced toxicity may be due to the initial depletion of intracellular GSH and then the subsequent increase in oxidative stress (Sarker et al., 1996). Rana and Verma (1996) showed that co-administration of GSH with Cd in rats not only restricted Cd uptake but also decreased MDA levels which are representative of lipid peroxidation. Although Singhal et al. (1987) showed that metal ions induced metallothioneine (MT) biosynthesis, considerable toxicity produced by Cd occurred before effective levels of MT were established. A study in our laboratory (Xu., 1999) showed that Cd induced both concentration- and time- dependent decreases in intracellular GSH levels. This depletion occurred prior to the increase in cytotoxicity, suggesting that GSH is indeed needed to protect the cell. Thus, it appears plausible that GSH provides a first-line of defense against Cd toxicity.

G6PDH, a key enzyme of the pentose phosphate pathway, plays an important role in protecting the cell by providing the major cellular source of NADPH.

GSH is maintained in the reduced form in a cell by glutathione reductase, which uses the NADPH. Because of this physiological role of G6PDH, it has been suggested that it is part of the defense against oxidative injuries (Salvemini et al., 1999). Therefore, it is important to understand the molecular mechanism by which Cd, an agent that might influence the oxidative state, regulates G6PDH gene expression.

We hypothesized that changes in the oxidative state of the cell, as influenced by changes in GSH concentration, affect the expression of the redox sensitive gene, G6PDH. In the present study, we observed a concentration-dependent increase (1.8-3.5 fold) in G6PDH expression after primary rat hepatocytes were exposed to Cd in a concentration range of 0 – 4.0  $\mu$ M for 6 hours (Figure 4). A previous study from our laboratory showed that Cd diminished cellular GSH levels after 6 hours in high concentration (4.0 and 6.0  $\mu$ M), or various concentration (0.1- 6.0  $\mu$ M) after 24 and 48 hours in primary rat hepatocytes (Xu, 1999). Therefore, decreases in intracellular GSH concentration may influence the expression of G6PDH gene.

If as mentioned earlier, GSH plays an important role in maintaining the redox state, and this redox state influences G6PDH expression, then treating cells with agents known to either lower or raise intracellular GSH should effect G6PDH. BSO, a specific inhibitor of GSH biosynthesis, was chosen as the GSH depleting agent. In cortical cultures exposed to 0.5 mM BSO for 24 hours, the total GSH content was significantly reduced (Bridges et al, 1991). A study by Usami et al. (1999) showed that BSO modified selenium embryotoxicity in cultured rat embryo at a concentration causing depletion in embryonic GSH. In earlier studies, our laboratory showed that

BSO caused significant decreases in cellular GSH levels (Xu,1999). Primary rat hepatocytes exposed to 0.4 mM BSO for 24 hours resulted in a significant increase (4-fold) in G6PDH gene expression level (Figure 5). This increase is similar to the one observed after cells were treated with Cd.

Glutathione monoethyl ester (GSH-E) pretreatment, on the other hand, is commonly used to replenish intracellular GSH. GSH-E is readily transported into cells and is converted by intracellular enzyme activity to glutathione (Anderson et al., 1985). A study by Xu (1999) showed that administration of GSH-E significantly elevates the intracellular level of GSH in normal or Cd-treated primary rat hepatocytes. Shinghal et al. (1987) showed that administration of GSH-E protected against Cd toxicity in BSO treated mice. To investigate whether or not we could neutralize the effect of Cd or BSO, we replenished GSH in the cell by using GSH-E. Our results showed that both Cd- and BSO-induced G6PDH gene expression was normalized in the presence of GSH-E (Figure 6 and 7). Therefore, the depletion of GSH by Cd or BSO modulates the expression of G6PDH.

As discussed above, exposure of primary rat hepatocytes to Cd initially results in a significant decline in GSH. This decline could be caused by the conjugation of GSH with Cd. Alternatively, GSH could be depleted by interaction with the ROS formed as a result of Cd exposure. Gubin et al. (2000) showed that intracellular GSH was depleted by 50 %, using 50  $\mu$ M diethyl malate (GSH scavenger). This build up of ROS was believed to be responsible for an increase in heat shock protein levels. These investigators concluded that this GSH depletion impedes the glutathione peroxidase-related detoxification of hydrogen peroxide and lipid hydroperoxides, the



major consequence being the accumulation of hydroxyl radicals. In yeast, the increased fluorescence of extracts from cells exposed to Cd for 3 hours in the presence of the free radical sensitive reporter compound dichlorofluorescein diacetate (DCFHDA) indicated that free radicals were generated following Cd exposure (Brennan et al., 1996).

Generation of ROS in a cell can be a cause for a variety of damages such as, lipid peroxidation and DNA strand breaks. It is a well known fact that xenobiotic-generated ROS interacts with the unsaturated fatty acids to initiate peroxidation which is a major factor influencing the breakdown and turnover of biomembranes (Sugiyama et al., 1993). Hydroxyl radicals cause structural alternation in DNA, e.g. base pair mutations, rearrangements, deletions, insertions and sequence amplification (Wiseman et al., 1996). The elevated level of LPO stimulates mitochondrial respiration, which is an important source of ROS (Halliwell et al., 1989). A study by Shaikh et al. (1999) showed that co-administration of antioxidants, NAC and vitamin E with Cd significantly reduced cytotoxic parameters of LPO and LDH leakage in rat liver cells. A study by Xu (1999) showed that Cd induced LPO and LDH leakage in primary rat hepatocytes was blocked with the presence of either antioxidant NAC or Trolox. It was concluded from this study that Cd induced cytotoxicity is mediated by ROS, which is generated by depletion of GSH. GSH levels were diminished before the cytotoxic parameters significantly increased. N-acetyl cysteine, in addition to its antioxidant properties, can be used to supplement intracellular GSH. NAC is effective in this regard because cells are able to deacetylate NAC and utilize the liberated cysteine to support GSH biosynthesis. Gong et al., (1996) showed that NAC

pretreatment significantly attenuated both Cd and BSO induced MT expression in rat lung epithelial cells.

We show that both the Cd- and BSO- depletion of GSH and induction of G6PDH gene expression is normalized in the presence of NAC and Trolox (Figure 6 and 7). These results are in good agreement with the study by Abe et al.(1994) showing that treatment of WISH cells with 1.5 and 30mM NAC significantly reduced both HSP 70 and MT mRNA levels in cells exposed to 50 $\mu$ M Cd. Our results from the experiments with the antioxidants NAC and Trolox support the idea that induced ROS are involved in Cd induced G6PDH expression. NAC traps free radicals, particularly the hydroxyl radicals (OH $\cdot$ ). Trolox, a water-soluble form of vitamin E, is a powerful free radical scavenger, particularly the hydroxyl radical (OH $\cdot$ ) and peroxy radical (ROOH $\cdot$ ). The protective effects of both NAC and Trolox against oxidative damage by Cd have been widely studied. Shaikh et al., (1999) showed that co-administration of antioxidants, NAC or vitamin E with Cd controlled Cd-induced LPO and protected the animals against hepatic as well as renal toxicity. Kamata et al., (1996) showed that nerve growth factor induced *c-fos* expression was blocked by the presence of NAC in PC-12 cells. Cd-induced expression of heat shock proteins 90, 70, and 27 was significantly attenuated by pretreatment with NAC in the A549 human lung cell-line (Gaubin et al., 2000). Thus, it seems plausible that generation of intracellular ROS triggers the expression of G6PDH because GSH is depleted.

Since we have shown that the expression of G6PDH gene is effected by Cd, it seemed logical to hypothesize that signal proteins, which modulate gene expression, may also be effected by Cd. Mammalian cells respond to extracellular stimuli by

activating signaling cascades (Minden et al., 1997). The MAPK pathway proteins including ERK, JNK, and p-38 and have been implicated as integral in the transduction of both stress and mitogen stimuli that culminates in the phosphorylation of nuclear factors and the transcriptional activation of downstream genes (Seger and Krebs, 1995). Masuya et al. (1998) showed that the Cd induced-heme oxygenase expression was mediated by MAPK / extracellular signal regulated kinase (ERK) in HeLa cells. In other studies, Cd induces phosphorylation of JNK in LLC-PK<sub>1</sub> or sustained activation of MAPK that correlated with the induction of *c-fos* in rat mesangial cells (Wang et al., 1998). Our results show that incubation with Cd for 6 hours increases the level of phosphorylation of both MAPK and JNK in a dose-dependent (1-4.0 $\mu$ M) manner (Figure 8 and 10). Because of its role in activation of stress related genes, we hypothesized that MAPK pathway may play a pivotal role in Cd-induced expression.

Since Cd increases the phosphorylation of these signal proteins then this induction may be through ROS. A study by Lo et al., (1996) showed that both IL-1 and TNF- $\alpha$  induced JNK activity was blocked by the presence of antioxidants, NAC and ascorbic acid in bovine articular chondrocytes. Wilmer et al., (1997) showed that IL-1 induced both ERK and JNK activation and this was blocked by the presence of NAC in human mesangial cells. In our studies, pretreatment of the cells with either NAC or Trolox normalized the Cd-induced phosphorylation of both MAPK and JNK (Figure 9 and 11). These results suggest that indeed ROS may be involved in the activation of MAPK and JNK by Cd.

Although Cd activated both MAPK and JNK, the pathways involved in their activation are distinct. Previous studies have suggested that the Ras→ Raf → MEK → MAPK pathway is activated by growth factors. However, activation of JNK is thought to be through the Ras → Rac → MEKK→ JNKK→ JNK pathway (Minden et al., 1997). While Raf is critical for MAPK activation in response to Ras, Raf does not appear to have any direct role in the JNK activation pathway (Minden et al., 1994).

Since we have showed that ROS has an effect on both MAPK and JNK activation, we tried to elucidate whether or not ROS directly effected an upstream protein of the MAPK pathway using the well-characterized inhibitor PD098059. PD098059 selectively binds to and inactivates MEK, inhibiting both the phosphorylation and activation of the down stream substrate MAPK (Pang et al., 1995). No substrate other than MAPK has been identified for MEK (Cuenda 1999). Hung et al., (1998) showed that 60  $\mu$ M Cd induced MAPK and this was significantly suppressed by PD098059. In good agreement with these findings, our result showed that administration of PD098059 significantly reduced Cd-induced MAPK activation but not JNK activation (Figure 9 & 11). Thus, Cd induced MAPK activation appeared to be involved MEK.

It is not yet clear how Cd induced ROS may lead to activation of JNK upstream proteins, Rac and cdc42HS. One possibility is that the lipid kinase phosphoinositide-3-kinase (PI3 kinase) may play a role. The catalytic subunit of PI3 kinase, p110, binds to, and is activated by Ha-Ras, in a GTP dependent manner (Minden et al., 1997). Recent work has suggested that overexpression of a

constitutively active mutant of p110 can lead to Rac activation (Reif et al., 1996). Cd has been reported to rapidly elevate the concentration of intracellular  $\text{Ca}^{2+}$  which is an important second messenger for the regulation of JNK activity. It has been reported that Cd increases inositol triphosphate and then triggers  $\text{Ca}^{2+}$  mobilization via a reversible interaction with an external site “Cd receptor” on the cell surface (Smith et al., 1989). Our laboratory have some evidence to suggest that Cd also activates PI3 kinase in primary rat hepatocytes. It is not clear, however, whether PI3 kinase activation by Cd is involved in activation of the JNK pathway in our system.

We have demonstrated the activation of MAPK and JNK occur upon Cd treatment in primary rat hepatocytes. Both of these signal proteins can lead to the activation of transcription factors. Some of the most intriguing substrates are nuclear transcription factors that are thought to be phosphorylated after MAPK's translocation to the nucleus. One of the first reported transcription factors to serve as a substrate for the MAPK was *c-jun*, a member of AP-1 transcription factor. The AP-1 transcription factor exists either as a heterodimer consisting of *c-jun* and *c-fos* or as a homodimer of *c-jun* protein. The induction of AP-1 and its subsequent ability to bind to DNA have both been shown to be sensitive to the change in the cellular redox status. Increased *c-jun* and *c-fos* expression, AP-1 synthesis, and AP-1 binding activity have been shown to occur following Cd exposure. AP-1 binding activity has been directly correlated with an elevation in GST gene expression (Bergelson et al., 1994). There is the evidence that the promoter region of G6PDH gene contains three putative AP-1 binding sites (Rank et al., 1994). A study by Wang (1998) showed that Cd can cause

sustained induction of *c-fos* in part through sustained activation of MAPK in rat mesangial cells.

We employed PD098059 to investigate whether or not the activation of MAPK pathway is necessary for Cd-induced G6PDH expression. Our results show that PD098059 caused a marked decrease in the induction of G6PDH by Cd, but did not eliminate it completely (Figure 6). These results suggest that activation of MAPK by Cd may be linked to G6PDH expression. In our cells in culture, Cd increased MAPK activity by 3 hours. This was sustained for at least 6 hours, consistent with the time course of G6PDH mRNA accumulation. Since the MAPK pathway mediates activation of AP-1 transcription factors, a next step might be to look at whether or not the induction of G6PDH by Cd could be through the activation of AP-1.

In conclusion, the results obtained from the present study support our original hypothesis: Cd induces oxidative stress in the cell by generating intracellular ROS, which in turn activates signal transduction pathways that cause the increase in expression of the antioxidant enzyme, G6PDH.

**Appendix A**  
**Investigator IACUC Certificate**

10/91

Date Received \_\_\_\_\_  
 IACUC Number 97-07-03  
 First Renewal Request 07/20/98  
 Second Renewal Request 07/15/99

**WESTERN MICHIGAN UNIVERSITY**  
**YEARLY RENEWAL FORM APPLICATION TO USE**  
**VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING**

GENERAL INFORMATION: Fill in all appropriate information

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


\_\_\_\_\_  
 Co-Principal/Student Investigator Department Campus Phone

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:

  
 Principal Investigator/Instructor

7/20/99  
 Date

\_\_\_\_\_  
 Co-Principal/Student Investigator  
 (If PI not a faculty member)

\_\_\_\_\_  
 Date

**INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL**

  
 IACUC Chairperson

7/28/99  
 Date

PLEASE MAIL COMPLETED APPLICATION TO:  
 Research Compliance Coordinator  
 Western Michigan University  
 327E Walwood Hall  
 Kalamazoo, MI 49008  
 (616) 387-8293

**NOTE:** It is the responsibility of the Principal Investigator to obtain the signature of any Co-Principal/Student Investigators



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