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Effect of Antioxidants on Cadmium Induced Oxidative Stress and Signal Transduction

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EFFECT OF ANTIOXIDANTS ON CADMIUM INDUCED OXIDATIVE STRESS AND SIGNAL TRANSDUCTION

by

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A study was done to establish if reactive oxygen species (ROS) participate in the signal transduction pathway through which cadmium induces gene transcription. Cadmium has become a concern in the health care community in the past few years as evidence suggests that cadmium can induce the development of tumors, influence cell signaling and cause damage to organs, tissue, cells and DNA. The mechanism by which cadmium exerts its action on cellular processes or induces toxicity is however unclear although there is evidence to suggest that ROS play a role. Our laboratory is interested in understanding the mechanisms of differentially regulated gene expression in response to the oxidative state of a cell.

Our results show that cadmium activated transcription factor nuclear factor kappa B and heat shock factor transcription factor in a time and concentration dependent manner. Cadmium also activated cadmium response factor binding to the cadmium response element in the heme oxygenase-1 gene. Furthermore, this induction was blocked by antioxidants suggesting involvement of reactive oxygen species. Our results implicate that signal protein p38 mitogen activated protein kinase protein is not only involved in Cd mediated induction of these transcription factor but also in a stress response protein heat shock protein-70.

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CHAPTER I

INTRODUCTION

Cadmium

Cadmium (Cd) is a common environmental pollutant that affects many cellular functions. Sources of Cd pollution include batteries, paint pigments, electroplating smelting and mining operations, corrosion of metal-plated iron, and disposal of Cd containing consumer products (e.g., batteries and pigments) (Stohs et. al., 2000). It is also a byproduct of zinc and lead mining. Recent studies show that cigarette smoke also contains significant amounts of Cd. Because of the widespread nature of Cd it can easily be ingested or inhaled. Between 10-50 percent of cadmium fumes are absorbed through the lungs and approximately five percent of oral cadmium is absorbed through the digestive tract. Smokers absorb 1-2 μg of cadmium per pack of cigarettes, approximately doubling the average exposure of a nonsmoker and doubling the average amount of cadmium found stored in the liver and kidneys. (Jarup et. al., 2002, Jarup et. al., 1998). Soluble cadmium salts accumulate and can result in toxicity to the liver, kidneys, heart, brain, lungs, testes, and central nervous system (Stohs et. al., 2000). Unlike other heavy metals Cd has a long biological half life (10-30 years) (Goyer et. al., 1997; Sugita et. al., 1995). Cd has been classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) and

exposure as been associated with tumors of the lung, prostate, testes and hepatopoietic system (Ercal et. al., 2001).

Although a carcinogen, Cd is not believed to be a strong initiator of tumors because it is weak mutagen (Beyersmann et. al., 1994). To date the carcinogenic mechanism of action is unclear although Cd has been shown to be capable of stimulating the expression of various proto-oncogenes. In various types of cells, the proto-oncogenes affected by cadmium include c-jun, c-fos, egr-1, c-myc and gadd (Beyersmann et. al., 1997; Hsiao & Stapleton et. al., 2004; Misra et.al, 2002). The induction of the transcription of proto-oncogene by Cd might be the cause of malignant transformation of cells involving this metal. Translation products of proto-oncogenes are often engaged in signal pathways that transduce growth regulatory signals from the cell surface to the nucleus (Beyersmann et. al., 2000).

Cd is also able to contribute to hepatotoxicity and nephrotoxicity but the role of Cd in toxicity is unclear. Some evidence suggests that Cd is may induce toxicity by increasing oxidative stress. Since Cd is not a redox-active metal, its oxidant role is not clear (Ercal et. al., 2001), however it may contribute to oxidative stress through increased levels of reactive oxygen species (ROS). Cd is able to deplete reduced glutathione in the cell so it might increase ROS by disturbing redox state of the cell (Hsiao & Stapleton, 2004).

Reactive Oxygen Species

ROS are normally produced as a result of aerobic metabolism in mitochondria. ROS are radicals, e.g. hydroxyl radicals ($OH^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$) and superoxide radicals ($O_2^{\cdot-}$) or reactive non radical compounds such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Superoxide is formed upon one-electron reduction of oxygen mediated by enzymes such as NADPH oxidase or xanthine oxidase or from the respiratory chain. Superoxide dismutase converts superoxide radicals into hydrogen peroxide. Hydrogen peroxide is converted to H_2O by the antioxidant enzyme catalase.

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

$$H_2O_2 + Fe^{++} \rightarrow Fe^{+++} + OH^{\bullet} + OH^-$$

$$H_2O_2 + 2GSH \xrightarrow{GlutathionePeroxidase} 2H_2O + 2GSSG$$

If there are free reduced transitional metals (e.g., ferrous or cuprous ions) available, hydrogen peroxide can be converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) through the Fenton reaction. In a reaction catalyzed by glutathione peroxidase (GSH peroxidase) glutathione is converted to glutathione disulfide, which is recycled back to glutathione by glutathione reductase (GSH reductase) along with NADPH consumption (Figure 1).

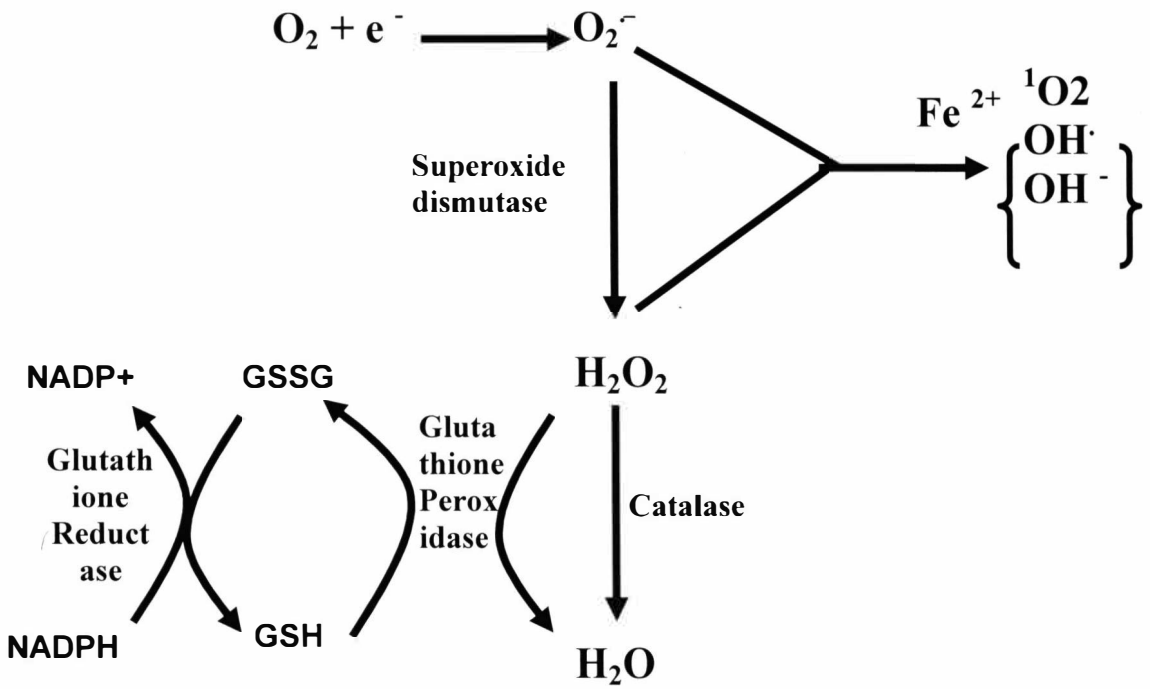


Figure 1. Generation of Reactive Oxygen Species (ROS) and Antioxidant Action.

Cadmium Toxicity and Gene Expression

ROS are capable of damaging biological macromolecules such as DNA, carbohydrates or proteins (Watjen et. al., 2002). To defend itself from the damaging effects of ROS, cells produce free radical scavengers and a variety of antioxidant enzymes to help and deal with the ROS. These antioxidants can either scavenge superoxide and other free radicals or stimulate the detoxification mechanism within the cells resulting in detoxification of free radicals. Antioxidants are of two different types. Examples of enzymatic antioxidants include superoxide dismutase, catalase and reduced glutathione peroxidase, while non enzymatic antioxidants include

vitamin C, vitamin E and reduced glutathione. Under normal conditions these antioxidants are capable of handling ROS, however, when intracellular concentrations of ROS increase beyond the capacity of cell to eliminate them, cells experience a biochemical imbalance termed oxidative stress.

Cd exposure studies have shown that the increase in cellular oxidative stress is associated with DNA damage, increased lipid peroxidation, depletion of sulfhydryls, disturbances in the antioxidant defense system as well as changes in gene expression (Stohs et. al., 2000; Xioa et. al., 2002). These results of Cd exposure rely on initial efficient uptake of the metal into the cell. The major route of Cd influx into the cell is through Ca^{++} channels (Beyersmann et. al., 1997; Stacey et. al., 1980). According to Blazka et. al., 1990, about one-third of Cd entering hepatocytes uses Ca^{++} channels. Partial interaction between Cd and Ca was also described by Souza et al.1997 in that Cd inhibited Ca uptake, but only to a maximum value of ~ 40%. A study by Shaikh et. al., 1995 showed that the transport of cadmium in hepatocytes does not require energy and occurs primarily (80%) by temperature-sensitive processes, i.e., ion channels and carriers, that involve interaction with sulfhydryl groups. These processes generally are thought to transport essential metals like copper, zinc and calcium. The remaining 20% of the cadmium in hepatocytes is transported via a temperature-insensitive process, possibly by diffusion. The relative contribution of various transport pathways available for cadmium uptake is different in each cell type and apparently depends on the morphological and functional differences between the cell membranes (Baker et. al., 2003; Erfurt et. al., 2003; Pham et. al., 2004; Zalups et. al., 2003).

Inside the cell Cd can bind to metal binding proteins known as metallothionein and sulfhydryl groups of proteins. Cd has a high affinity for glutathione (GSH) and so it is able to decrease the levels of reduced GSH in cells. GSH is a tripeptide that has been shown to protect cells against oxidative stress. Thus any alteration in GSH levels (either a decrease or increase) indicates a disturbed oxidative status. When a cell is oxidatively challenged, GSH synthesis increases. As oxidative stress continues GSH synthesis can not efficiently meet the demand which leads to GSH depletion. The decrease in glutathione leads to an increase in ROS (Ercal et. al., 2001). Previous work in our lab has shown that cadmium decreases the intracellular concentration of GSH prior to increase in lipid peroxidation and lactate dehydrogenase (LDH) leakage. The antioxidant N-Acetyl Cysteine (NAC) was able to block the induction of lipid peroxidation and LDH leakage supporting a role for ROS in Cd mediated toxicity (Xu et. al., 2003).

To prevent cadmium-induced intracellular damage, cells can respond by inducing the transcription of genes that increases proteins to help or repair. These proteins can (*a*) bind or chelate the metal to prevent further damage, (*b*) remove reactive oxygen species, (*c*) repair membrane and DNA damage, and (*d*) properly fold or denature unfolded proteins (Liao & Freeman, 1998). Cadmium has been shown to affect the steady-state levels of the mRNAs encoding metallothionein (Hamer et. al., 1986; Chu et. al., 1999; Smirnova et. al., 2000), heme oxygenase (Alam et. al., 2000) and low and high molecular weight heat shock proteins (Wiegant et. al., 1994; Souza et. al., 2004) in various types of cells. Metallothioneins (MTs) are cysteine-rich heavy

metal binding proteins (Blais et. al., 1999). Metallothionein-I and Metallothionein-II have been demonstrated to participate in the detoxification of cadmium (Masters et. al., 1994; Habeebu et. al., 2000; Zheng et. al., 1996) and protection against oxidative stress in mice (Dalton et. al., 1996; Liu et. al., 2004; Chen et. al., 2004; Kuester et. al., 2002). The relationship between Cd resistance and MTs is strong and has been studied in various kinds of cells (Liu et. al., 1991). It has been shown that MTs-null mice are more prone to Cd-induced renal injury compared to wild type, which supports a role for MTs as a protecting agent against Cd toxicity in liver and kidney (Habeebu et. al., 2000). Over expression of MTs by pretreating animals with low doses of Cd (Harstad et. al., 2002 ; Liu et. al., 2004) and zinc (Harsted et. al., 2002; Liu et. al., 2000) and hypoxia (Murphy et. al., 1999) was found to protect animals from subsequent dosages of Cd. They were shown to be protected from both Cd-induced lethality and hepatotoxicity.

Heme oxygenase (HO) is an antioxidant defense enzyme that converts heme to biliverdin, iron, and carbon monoxide and has antioxidant properties against oxidative stress (Martin et. al., 2004). Heme oxygenase catalyzes the rate-controlling step of heme degradation into biliverdin, iron, and carbon monoxide (Maines et. al., 1997). Biliverdin is converted to bilirubin by the action of biliverdin reductase, which can act as an antioxidant. There are two isoforms of HO which have been reported (HO-1 and HO-2) (Takeda et. al., 1994; McCoubrey et. al., 1997). HO-1 and HO-2 share ~ 40% amino acid homology (McCoubrey et. al., 1997). HO-1 has been shown to be induced by the substrate heme as well as metalloporphyrins, heavy metals, interleukin-6,

ultraviolet light, heat shock and a various other chemicals or stress inducers but HO-2 is expressed in a constitutive fashion (Bonkovsky et. al., 2002; Shan et. al., 2000, Takeda et. al., 1994). In addition to MT and HO-1, increases in superoxide dismutase, catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase activities have also been observed following Cd exposure in cultured cells and whole animals (Kostic et. al., 1993, Salovsky et. al., 1992, Xu et. al., 2003). Some studies show that cadmium-activated transcription may occur through specific metal-responsive upstream regulatory elements found in the promoters of cadmium-responsive genes. A study by Chu et. al., 1999 showed that the metal response element-binding transcription factor-1 binds specifically to metal response elements (MRE) and activates MT gene expression in response to both zinc and cadmium. Metal-responsive regulatory elements may include MRE sequences, found in most MT genes or cadmium-responsive elements, as found in the human heme oxygenase gene (Takeda et. al., 1994; Kapturczak et. al., 2003).

The mechanism(s) by which Cd modulates the levels of expression of most of the genes however remains unknown. ROS generated by Cd have also been proposed to act as second messengers and pass signals that might mediate various events in cells like proliferation, apoptosis and gene expression (Beyersmann et. al., 1997; Lander et. al., 1997). These observations suggest that cells have mechanisms to sense reactive oxygen species and induce specific responses (Hardin et. al., 2001; Kwast et. al., 1998).

Oxidative stress has been shown to be involved in the activation of a number of transcription factors including activator protein-1 (AP-1) (Chen et. al., 2000; Ding et. al., 1999), heat shock factor-1 (HSF-1), upstream stimulatory factor (USF) (Li et. al., 1998), nuclear factor kappa B (NF-κB) (Thevenod et. al., 2000; Chen et. al., 2000, 2002; Shi et. al., 2000; Huang et. al., 2000), P53 (Wang et. al., 2000) and metal regulatory transcription factor 1 (MTF-1) (Larochelle et. al., 2001)) in various cell culture models. Activation of these transcription factors can ultimately affect transcription of various genes (Joseph et. al., 2004). Transcription factors are a set of proteins which can either hinder or help the rate of transcription by interacting directly with the DNA strands or by reacting with molecules that interact with RNA polymerase. These transcription factors play a pivotal role in expression of specific stress response genes.

In different studies the activation of a well defined transcription factor AP-1, has been identified to be regulated by ROS (Joseph et. al., 2001). In JB6 cells, AP-1 has been shown to be activated by silica which is able to generate ROS (Ding et. al., 2001). In NIH3T3 and Hela cells heat shock mediated stress induced AP-1 DNA binding involved redox factor-1 signal protein (Diamond et. al., 1999). A study by Hsiao & Stapleton, 2004 showed that Cd induced oxidative stress is able to induce transcription factor AP-1 binding to DNA in rat hepatocytes and can be reduced by using antioxidants. The AP-1 transcription factor is typically composed of c-fos and c-jun proteins. When activated AP-1 is a dimer of either jun-fos or jun-jun and binds to DNA and induces transcription.

The transcription factor NF-κB has also been shown to be activated under oxidative stress. NF-κB is a transcriptional regulator that consists of homo and heterodimers of proteins from the Rel family. NF-κB is found normally in the cytosol as an inactive complex consisting of two subunits (P^{50} and P^{65}, although other members of the Rel transcription factor family may contribute to NF-κB complexes), which are bound to an inhibitory subunit IκB. The interaction of NF-κB with IκB masks the nuclear localization signal and retains NF-κB in the cytoplasm in a latent form. Upon activation of NF-κB, IκB is phosphorylated by IκB kinases. Degradation of IκB unmasks the nuclear localization signal of NF-κB, which allows for translocation of NF-κB from cytosol to nucleus, where it binds to its cognate DNA recognition sequences and increases the transcription of specific genes. ROS intermediates including H_2O_2 are potent activators of NF-κB.

NF-κB activation is implicated in the activation of several stress related genes, e.g. Angiotensin II (Brasier et. al., 1990; Li et. al., 1996), cyclooxygenase-2 in MC3T3-E1 cells (Yamamoto et. al., 1999) and in Human endometrial cancer cells (St-Germain, et. al., 2004), MAP4K1 in B-lymphoma cell lines (Carter et. al., 2002), superoxide dismutase (Cu/Zn SOD) (Rojo et. al., 2004), Mn SOD in Human coronary artery endothelial cells (HCAEC) and human umbilical vein endothelial cells (HUVEC) (Abid et. al., 2004), and Bcl-2 in human prostate carcinoma cells (Catz et. al., 2001). The HO-1 promoter contains a cis acting element named as cadmium response element. A study by Takeda et. al., 1994 showed that this cadmium response element is 8-10 base pair long and specifically responds to Cd, within the HO-1

promoter there is also an AP-1 binding site but it is not directly involved in Cd response. Several studies have identified consensus AP-1 and NF-κB binding elements in the 5'-untranslated region (UTR) of the HO-1 gene as playing critical roles in the induction of the gene by chemical or physical stressors. The HO-1 promoter also has a heat shock factor binding site but the involvement of this binding site is still not clear (Lavrovsky et. al., 1994).

Heat shock factor (HSF) is a transcription factor and has been shown to be induced by various kinds of external stimuli (Maroni et. al., 2003; Ovelgonee et. al., 1995). There are four different isoforms of HSF known as HSF-1, HSF-2, HSF-3 and HSF-4. Out of these four forms, HSF-4 is the only one found in humans. HSF-1 is the most inducible one under stress. Some of the well studied downstream transcriptional products of these transcription factors are HSP-27, HSP-70, HSP-90. These proteins act as chaperones and help in the proper folding of the proteins. In cells, HSF-1 is generally found in the cytoplasm as a monomer in inactive state. When cells experience stress, there is aggregation of denatured protein; HSP-70 binds to these proteins and separates from HSF-1, then HSF-1 translocates to the nucleus where it trimerizes and becomes activated. Once activated it then binds to the heat shock element (HSE) and induces transcription. When there is no more need of HSP-70, it translocates to the nucleus and binds to DNA and the HSF-1 trimer complex (Liu et. al., 1996). This HSP-70 binding dissociates the complex and HSF-1 is converted to its monomeric inactive form and translocates back to the cytoplasm (Figure 2).

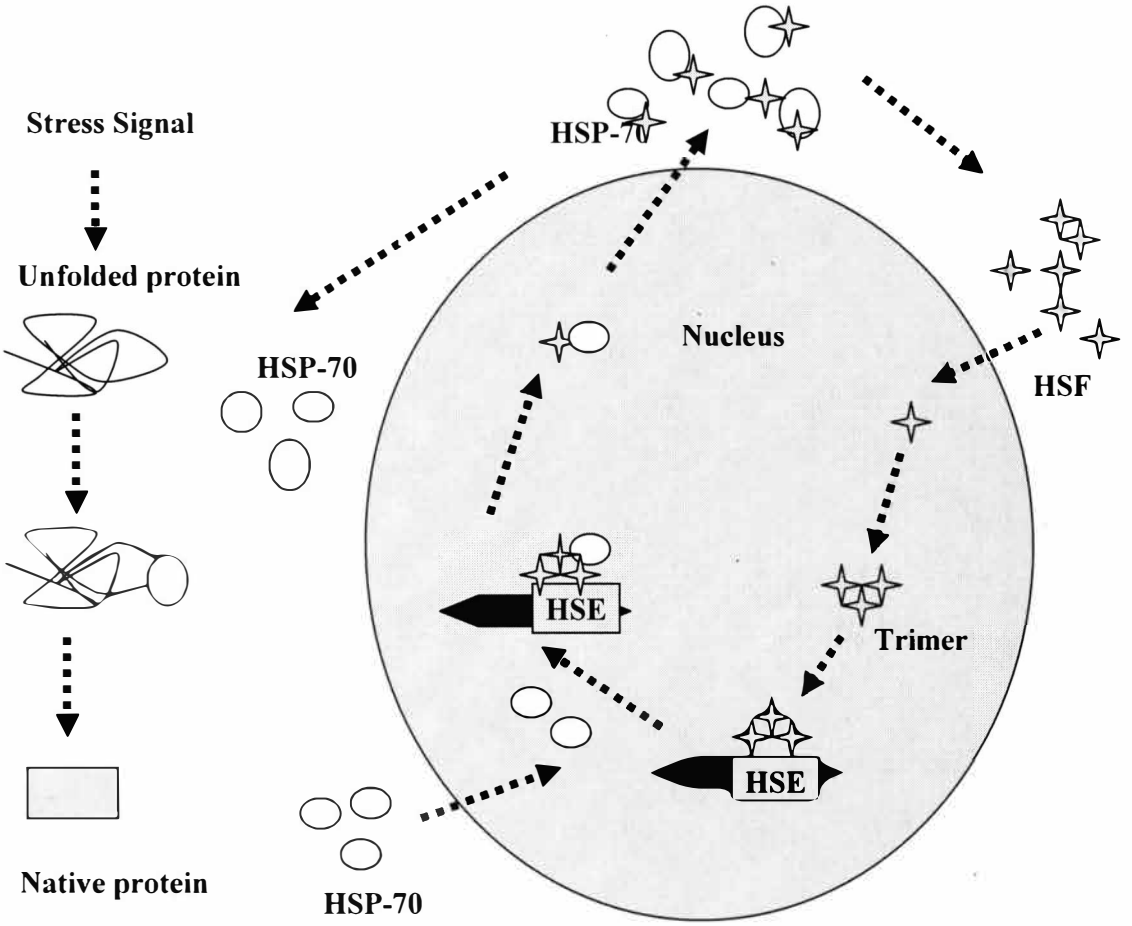


Figure 2: Regulation of Activation of HSF Transcription Factor.

MAPK Signaling Pathway

The mechanism of activation of these transcription factors is still not fully understood. But some evidence suggests involvement of the mitogen-activated protein kinase (MAPK) pathway (Aga et. al., 2004; Bhat et. al., 2002; Jacobs et. al., 2003). The MAP Kinase cascade is a key pathway by which signals are transduced by the cell. MAPK activation is thought to contribute to amplification and specificity of other signals that ultimately regulate cytoplasmic and nuclear events.

MAPKs can phosphorylate specific serine and threonine of target protein substrates and can regulate cellular activities like gene expression (Jonak et. al., 2004), mitosis and apoptosis (programmed death) (Kim et. al., 2004). MAPKs catalyze phosphorylation of substrate proteins which function as a switch to turn on or off the activity of the substrate protein. MAPK pathways can be activated by a wide variety of different stimuli acting through diverse receptor families, including hormones and growth factors that act through receptor tyrosine kinases (e.g., insulin (Wang et. al., 2003), epidermal growth factor (EGF) (Chwieralski et. al., 2004), platelet-derived growth factor (PDGF) (Lubinus et. al.,1994; Yamboliev et. al.,2001) and fibroblast growth factor (FGF) (Upadhyay et. al., 2003), cytokine receptors (e.g., growth hormone) and environmental stresses such as osmotic shock and ionizing radiation and heavy metals (Lau et. al., 2004; Alam et. al., 2000; Jonak et. al., 2004).

To date, three major MAPKs have been identified, namely the extra cellular signal-regulated kinases (ERK1/2, p44/p42), the stress-activated protein kinases1 / c-Jun NH_2-terminal kinases (SAPK / JNK), and the p38 mitogen-activated protein kinases (p38MAPK, stress-activated protein kinase 2). ERK1/2 are mainly (not exclusively) activated by growth factors (Liu et. al., 2005) and are involved in the regulation of mitogenesis (Handra-Luca et. al., 2003). While, JNK and p38MAPK have been associated with cell survival, anti-cytotoxicity, anti-apoptosis (Kim et. al., 2005) and proliferation (Du et. al., 2004). All of the MAPK members are catalytically inactive in unstimulated cells, and are activated in response to the appropriate stimulus by phosphorylation. Catalytic activation of MAPK requires phosphorylation

on threonine and tyrosine residue by a dual specific MAPK kinase (MAP2K/MAPKK/MKK). The MKKs themselves phosphorylated and activated by a serine/threonine kinase that functions as MKK Kinase. JNK is known to be activated by MKK4 and MKK7 (Sundarrajan et. al., 2003). Similarly, p38 is activated by MKK3 & MKK6, whereas ERK is activated by MKK1 and MKK2 (Chuang et. al., 2001). Once activated, MAPK's can then phosphorylate transcription factors (example ATF-2, c-jun, c-myc, NFAT4) and regulate gene expression (Iavarone et. al., 2003; Rolli-Derkinderen et. al., 2003). A study by Wang & Templeton (Wand & Templeton, 1997) suggests that sustained activation of MAPK's in rat mesengial cells by Cd results in induced expression of c-fos. There are multiple MAPK's which are activated by the cell to respond against different sets of stimuli. A study by Du et. al., 2004 showed that JNK plays a key role in cell proliferation and cell cycle progression in KB-3 human carcinoma cell line. Jacobs-Helber et. al., 2000 showed that JNK participates in apoptosis induced by Cd in CL3 lung carcinoma cells. This shows that MAPK pathway proteins can be involved in different physiological functions, sometimes in opposite roles in different cell culture systems. The complexities of the physiological roles of MAPKs may be due partially to MAPK activities differentially regulating genes in various cell types. While transient ERK1/2 activation leads to proliferation, persistent activation tends to mediate growth arrest or differentiation signals (Liu et. al., 2005). In contrast, transient JNK and p38 induction could provide a survival signal, whereas persistent activation induces apoptosis (Kim et. al., 2005; Du et. al., 2004; Liu & Lin et. al., 2005).

Antioxidants

Antioxidants prevent ROS induced tissue damage by preventing the formation of radicals, scavenging them or by promoting their decomposition. Antioxidants are of three types: antioxidants enzymes, chain breaking antioxidants and transitional metal binding proteins. Examples of enzymatic antioxidants are catalase, superoxide dismutase and glutathione peroxidase. Chain breaking antioxidants can either donate or receive an electron from radicals to form a stable product. Transition metal binding proteins (ferritin, transferrin, lactoferrin and ceruloplasmin) act as crucial components of an antioxidant system by sequestering iron and copper so that they are not available for the Fenton reaction and subsequent production of hydroxyl radicals.

Flavonoids are a large group of polyphenolic antioxidants found in many fruits vegetables and beverages such as tea and wine. There is some evidence to suggest that the intake of flavonoids might reduce effect biochemical indices of oxidative damage (Chen et. al., 2004; Serafini et. al., 1996; Stein et. al., 1999). The basic structure of flavonoids contain a flavon nucleus (2-phenyl-benzo-γ-pyrane) consisting of 2 benzene rings combined by an oxygen containing pyran ring. Antioxidant properties of polyphenols or flavonoids arise from their high reactivity as hydrogen or electron donors and from the ability of a polyphenol derived radical to stabilize and delocalize the unpaired electron. Rosemary leaf extract contains various antioxidants like carnosol, carnosic acid and rosmarinic acid. Rosmarinic acid is also found in other herbs like sweet basil, sage and perilla (Schwarz et. al., 1992; Santos-Gomes et. al., 2003). Antioxidant activity of these phytophenols depends upon the

presence of the OH group in the flavon nucleus (Figure 3 A, B & C). Compounds having more hydroxyl groups have more antioxidant activity.

A study by Mace et. al., 1994, showed that rosemary extracts, carnosic acid and carnosol strongly inhibited the phase I enzyme, CYP 450 and induced the expression of the phase II enzyme, glutathione *S*-transferase (GST). Carnosol and carnosic acid are powerful inhibitors of lipid peroxidation in microsomal and liposomal systems and can scavenge peroxyl radicals and hydrogen peroxide (Aruoma et. al., 1992; Aruoma et. al., 1996). Recent studies by Gao et. al., 2005 showed that rosmarinic acid decreased both ROS amount and malondialdehyde. It also showed anti-apoptotic properties by inhibiting caspase-3 (which is a component of an apoptotic pathway) and increasing the mitochondrial membrane potential. Various studies have shown that carnosol, carnosic acid and rosmarinic acid possess antioxidant properties and anticancer properties in various model systems (del Bano et. al., 2003; Fiander et. al., 2000; Haraguchi et. al., 1995; Huang et. al., 2005; Lo et. al., 2002; Matsingou et. al., 2003; Minnunni et. al., 1992; Offord et. al., 1997 & 1995). N-Acetyl Cysteine (NAC) is a thiol containing antioxidant which has the property of being both a cellular reducing agent and a precursor of GSH synthesis. Aruoma et. al., 1989 showed that NAC reacts with hydroxyl radical and hydrogen peroxide and exhibits antioxidant properties. As potent antioxidants these compounds may be especially important in protecting against human diseases involving oxidative stress.

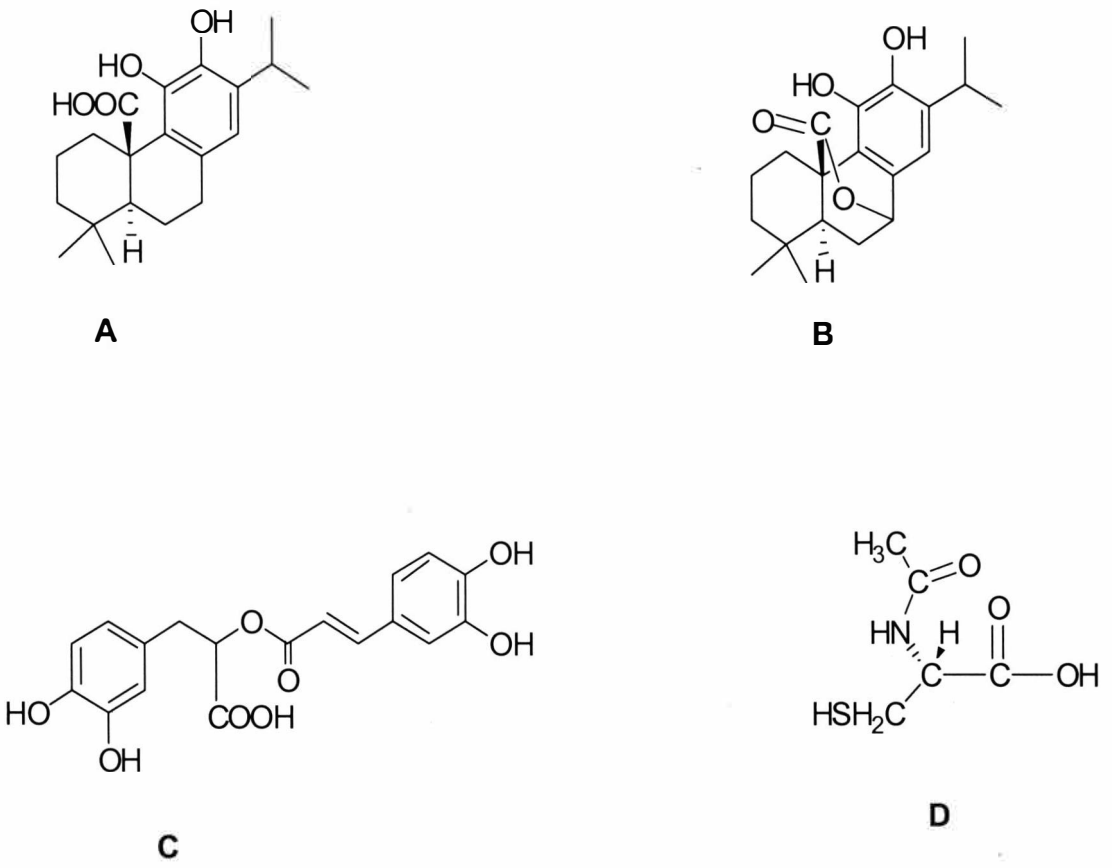


Figure 3: Strucrure of Antioxidants. A. Carnosic Acid, B. Carnosol, C. Rosmarinic acid, D. N-Acetyl cysteine

Source: www.sigma-aldrich.com & www.agscientific.com

Objectives of Study

There have been several hypothesis mechanisms proposed for the mechanism involved in Cd induced gene expression but still it is not fully understood. The hypothesis based on evidence to date, is that prior to the generation of cytotoxicity, Cd through oxidative stress manipulates activation of transcription factors and various signal pathways to affect gene expression. To test this hypothesis, I (a) Investigated whether Cd is able to induce the activation of stress related transcription factors (HSF, NF-κB) & binding of CRF or nuclear proteins to CRE from HO-1. (b) Investigated the role of ROS in this activation of transcription factors using the antioxidants carnosol, carnosic acid, rosmarinic acid and NAC. (c) Determined whether the activation of transcription factors affected the amount or activity of downstream proteins. (d) Checked if the antioxidants were able to affect downstream proteins. (e) Determined whether Cd or ROS were able to affect MAPK signal cascade proteins and investigated whether the activation of MAPK signal proteins were required or involved for the activation of transcription factors.

We used H4IIE (rat liver cells) for this study. The liver is a major target organ for the detoxification of various kinds of metals. Cd is accumulated mainly in the liver (Beyersmann et. al., 1997) so it is a physiologically relevant model. To study the activation of transcription factors we used luciferase reporter system. In this method cells were transiently transfected the cell with a plasmid which contains a binding site for transcription factor linked to the luciferase gene sequence. One plasmid contains binding sites for one kind of transcription factor. When cells are stressed with Cd if

the transcription factors are activated, they will bind to the binding site and induce the expression of luciferase. Generally luciferase is not present in mammalian cells so all the luciferase quantified corresponds to the transcriptional gene product generated by binding of transcription factors. The Luciferase amount is quantified by chemiluminescent reaction using a luciferase substrate. Antioxidants carnosol, carnosic acid and rosmarinic acid were generously provided by Kalsec Inc. These antioxidants were extracted from rosemary extract. NAC is a commonly used antioxidant and is a precursor of reduced GSH in the cell.

CHAPTER II

MATERIAL AND METHODS

Cell Culture

Rat Hepatoma (H4IIE) cells were obtained from American Type Tissue Culture. Delbucco's Modified Eagle Media (DMEM) F-12 (Gibco) containing 5% fetal calf serum was used for growth and maintenance of cells. Cells were incubated at 37^0C in a humidified atmosphere supplemented with 5% CO_2. In a 25 cm^2 culture flask, at approximately 80-90% confluence, 1.0 mL of trypsin (Hyclone Laboratories) was added and the cells were split into tissue culture flasks. Typically for experiments, cells were plated either in six well dishes or 60 mm tissue culture dishes. Once the cells reached 70% confluence and prior to experiments, cells were serum starved for at least 6 hours.

Cell Treatment

Cells were treated with cadmium chloride ($CdCl_2$) (Fisher Scientific) at the following concentrations: 0 (no addition, NA), 1, 2, 4, 5 or 10 μM for various times 1, 2, 3, or 6 hours. $CdCl_2$ was dissolved in water and stock solution of 1μg/μl was prepared and used throughout the study. For the studies involving antioxidants, cells were pretreated for 30 minutes with either 0.5μg/ml carnosol, 0.5μg/ml carnosic acid, 0.5μg/ml rosmarinic acid (Gift of Kalsec, Inc.) or 10mM N-acetylcysteine (NAC)

(Sigma) followed by a media change prior to the addition of $CdCl_2$. For inhibitor studies cells were pretreated with 20μM SP600125 (Calbiochem), 25μM PD098059 (Promega), or 10μM SB203580 (Promega) for 1 hour and then media was changed and $CdCl_2$ stock solution was added.

Cell Processing

Treated cells were processed by removing the media and washing twice with cold phosphate buffered-saline (PBS) solution. A 1X reporter lysis buffer (Promega) (400μl for each 60mm dish) was added at room temperature for 15 minutes to initiate lysis. Cells were then scraped from the wells/plates and the cell suspension was tipped at an angle and allowed to sit for an additional 10 minutes. The cell lysate was collected in eppendorf tubes for either the luciferase reporter assay, protein assay or western blot protein analysis and then centrifuged at 10,000 rpm for 1.0 minute at 4^0C. Supernatants were then transferred to eppendorf tubes and immediately stored at -20^0C.

Determination of Protein Content

Total protein content of the sample was measured using the Micro BCA Protein Assay Kit (Pierce). First bovine serum albumin (Sigma) stock solution was prepared at 1mg/mL by dissolving bovine serum albumin in water. In a micro titer plate standard solution from 1~ 20μL (1~ 20μg) was used and final volume of the standards was adjusted and diluted to 130μl with H_2O. For the sample, 10μL of the sample was diluted to 130μL with H_2O and each sample was duplicated. Working

Reagent (WR) was prepared fresh by mixing BCA reagent A (MA) 25 parts, BCA reagent B (MB) 24 parts and BCA reagent C (MC) 1 part. 130µL WR was added to each sample, the plate was covered with aluminum foil, agitated to mix for 1-2 minutes and incubated at 37°C for 2 hours. After the plate was cooled to room temperature for 5 min, the protein was quantified by measuring the absorbance at 550nm on a Benchmark micro plate reader (Bio-rad).

Immunoprecipitation

A total of 200µg of protein was used for immunoprecipitation. Heat shock protein-70 (HSP-70) (Stressgen) polyclonal antibody was diluted (1µL antibody + 49µL PBS). One micro liter of the diluted antibody was added to each sample and incubated overnight at 4°C on shaker. In the morning a 50% agarose beads (Upstate) slurry was washed five times with PBS and the pellet was collected. Finally, an equal amount of PBS was added to the pellet and a 50% slurry was prepared. For each sample 30µL of this slurry was added and then again incubated for 3 hours at 4°C with shaking. Samples were centrifuged at 5,000-6,000 rpm for 30 seconds and pellet was collected. The pellet was then washed five times with 500µL PBS. Sodium dodecyl sulfate (SDS) sample buffer (Invitrogen), water and DTT (50mM) were added to each sample and centrifuged at 14,000 rpm for 2 minutes at 4°C. Then samples were heated for 10 min at 70 °C. After this the samples were cooled on ice and stored at -20 °C for further use.

Western Blot Analysis

Ten micro liter of immunoprecipitated sample was subjected to electrophoresis at 100V for 90 minutes with a running buffer (Invitrogen). For HSP-70 protein 4-12% Nu-Page Bis Tris gels (Invitrogen) were used and for p38 proteins 10% Tris-Glycine gels (Invitrogen) were used for electrophoresis. Five micro liter of protein marker (Cell signaling) was used and 2µl of sample buffer was loaded in the empty lanes. Proteins were then transferred to a PVDF membrane (Immobilin, Millipore, Bedford, MA) using a standard semidry electro blotting apparatus (Invitrogen, CA). After blocking with 5% non-fat dry milk, the blots were incubated overnight at 4°C with antibodies (1: 100,000) dilution directed against HSP-70 or p38 (Cell signaling). 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, P^H-7.6) incubated with horseradish peroxidase-conjugated anti-rabbit IgG, (1:2000) dilution, and anti-biotin antibody, (1: 1000 dilution), for 1 hour at room temperature. The membranes were washed as above, and the bands detected by chemiluminescence (HRP-Western Detection Kit, Cell Signaling Technology, Inc.). Developed blots were exposed to Kodak X-OMAT autoradiography film and exposure time was varied depending on the signal intensity. Blots were quantified using scanning densitometry (NIH Image) and graphed in percent increase of no addition samples.

Plasmid DNA Preparation

A mercury pathway profiling system kit (K2049-1) was purchased from Clontech. E.Coli bacteria (DH5a) were transformed with either Heat shock element (HSE) containing binding site for heat shock factor (HSF), nuclear factor kappa B (NF-κB) or PTAL plasmid. These plasmids are a circular piece of DNA containing a binding site for transcription factor, and a promoter linked to the luciferase gene. The reporter plasmid containing the human heme oxygenase promoter linked to luciferase (PH-HOL) was a gift from Dr. Shibahara lab, Japan (Takeda et. al., 1994) and was transformed into the bacteria. These bacteria containing the reporter plasmid were plated on Luria-Bertani (LB) plates containing 50μg/mL of the antibiotic ampicillin and then incubated overnight. The next day a single colony was used to inoculate 10 ml of Luria-Bertani media containing ampicillin (50μg/mL) and grown for 8-10 hours at 37^0C with vigorous shaking. Five milliliters of this media was used to further inoculate 500 ml of LB media with the antibiotic ampicillin. This was incubated overnight at 37^oC with vigorous shaking. Plasmid preparation was carried out according to the manufacturer protocol using Qiagen® Plasmid Mega Kit Purification System. The purity and yield of the plasmid was determined using agarose gel electrophoresis and OD 260, respectively.

Transfection

H4IIE cells, seeded in 6 well plates were cultured until 70% confluent, serum starved for 6 hours, and subsequently transiently transfected using Lipofectin ™

(Invitrogen). Ten micro liter (10μg) per well for a total of 60μL (60μg) of lipofectin was incubated in a small Erlenmeyer flask with 1mL of serum free DMEM media for 15 min prior to addition of plasmid DNA. This ensures swelling of the lipid prior to incubation with DNA. Plasmid DNA 3μg/well for a total of 18μg was added to the lipid for an additional 15 minutes. Additional serum free media (~ 11.1 mL) was added to this mixture and resuspended several times to ensure adequate mixing. Two milliliters of this transfection mixture was then added to each well of the six well plate. Cells were incubated with this transfection mixture for 13-15 hours in an atmosphere of 5% CO_2 and 95% air. After incubation, cells were washed once with serum free media and 2.0 ml of serum free media was added for experimentation. Cells were then treated as indicated in results.

Luciferase Activity Determination

Luciferase enzyme activity was determined using a Luciferase Assay System from Promega. Cell lysate was prepared and analyzed for luciferase activity using a Packard Instruments 1600TR liquid scintillation counter. Luciferase activity is determined by chemiluminiescence using luciferase substrate luciferin. Luciferin produces light photons when mixed with luciferase. These light photons were detected by a scintillation counter. The number of photons corresponds to the amount of luciferase in the sample. Increased light output indicated more luciferase in the sample. Luciferase substrate was purchased from Promega and prepared according to the manufacture's protocol. Then 100μL of Luciferase substrate was used to measure

the background. For samples 20μL of lysate and 100μL of luciferase substrate was mixed to measure luciferase activity. The data was normalized to the total cell protein and is expressed as percent of control +/- Standard error of the mean (S.E.M.).

Statistical Analysis

The results are expressed as the mean +/- S.E.M. Statistical significance was evaluated by a Student T-test or one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test ($p<0.05$). ANOVA is a procedure which allocates the variation in a process and determines if it is significant different compare to other treatment.

CHAPTER III

RESULTS

Effect of Cd on HSF Transcription Factor Activation

To examine the effect of Cd on activation of HSF transcription factor, H4IIE cells were serum starved and then transiently transfected with HSE plasmid containing HSF transcription factor binding site. If HSF transcription factor is activated by Cd or any other metal, then when cells are stressed with Cd, HSF transcription factor will bind to HSE and induce the transcription of luciferase. Luciferase activity is then determined using the luciferase substrate. Transfected cells were treated with $CdCl_2$ at concentrations of either 0 (NA), 1.0, 2.0, 4.0, 5.0 or 10.0 μM for 6 hours or with 4μM for 1, 3 or 6 hours. The cells were then processed and the cell lysate was collected and luciferase activity was measured. The results show that there is a significant increase in luciferase activity with increased Cd treatment. Cd treatments of 4.0, 5.0 and 10.0 μM for 6 hours showed significant increase of 17, 28 and 11 fold, respectively compared to NA. This increase was dependent on concentration (Figure 4) and time of incubation (Figure 5). This indicates that Cd influences the HSE-Luc reporter construct in a concentration and time dependent manner. The HSF activation was decreased with 10μM Cd suggesting of either a transient response or a decrease in activation due to an increase in toxicity. Indeed

previous data from our lab shows that 10μM Cd can increase parameters of cytotoxicity.

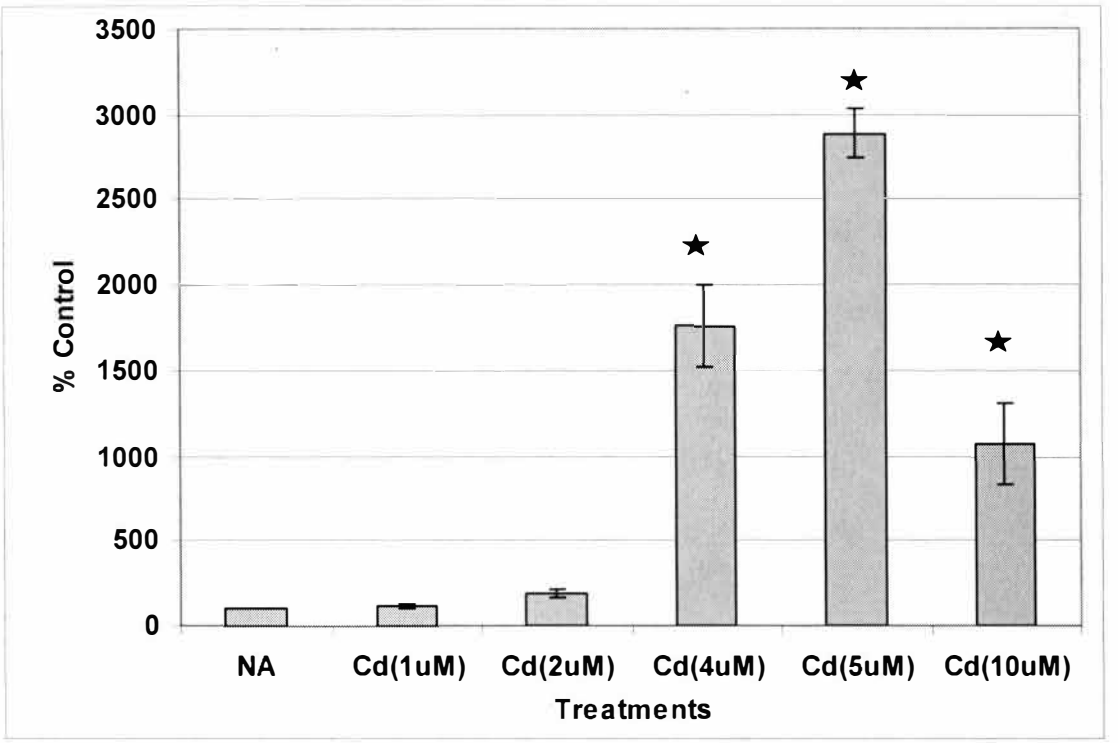


Figure 4: Effect of Cd on HSF Transcription Factor Activation in a Concentration Dependent Manner. H4IIE (Liver cells) were transfected with a luciferase reporter plasmid containing HSF response element. Transfected cells were then incubated with either 0 (NA), 1.0, 2.0, 4.0, 5.0 or 10.0 μM for 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=5). Statistical analysis was performed by Student t-test. ★ = Significant stimulation compared to NA ($P<0.05$).

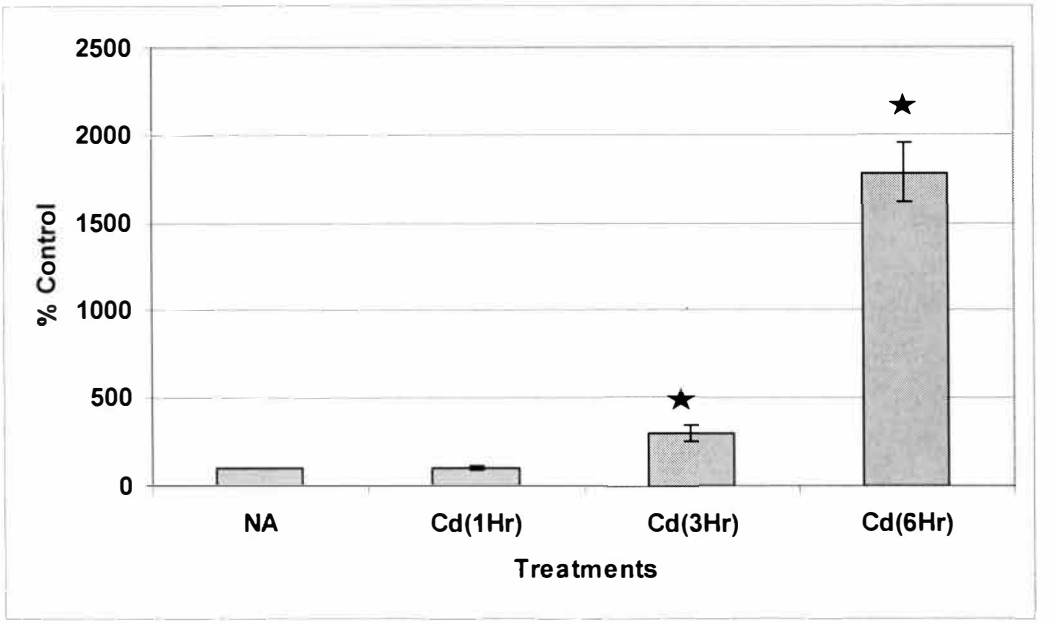


Figure 5: Effect of Cd on HSF Transcription Factor Activation in a Time Dependent Manner. H4IIE (Liver cells) were transfected with a luciferase reporter plasmid containing HSF response element. Effect of Cd on HSF Transcription Factor Activation in a Time Dependent Manner Transfected cells were then incubated with 4μM for 1, 3 or 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=8). Statistical analysis was performed by Student t-test ($P<0.05$). ★ = Significant stimulation compared to NA.

Effect of Cd on NF-κB Transcription Factor Activation

To examine the effect of Cd on NF-κB transcription factor, H4IIE cells were cultured and serum starved for 6-8 hours and then transiently transfected with NF-κB reporter plasmid. This plasmid contains a binding site for the NF-κB transcription factor. When NF-κB transcription factor is activated it binds to the NF-κB binding site on the plasmid and induces the transcription of luciferase. For this experiment

cells were treated with $CdCl_2$ at concentrations of either 0 (NA), 1.0, 2.0, 4.0, 5.0 or 10.0 μM for 6 hours or with 4μM for 1, 3 or 6 hours. The cells were then processed and cell lysate was collected as described in material and methods and luciferase activity was measured.

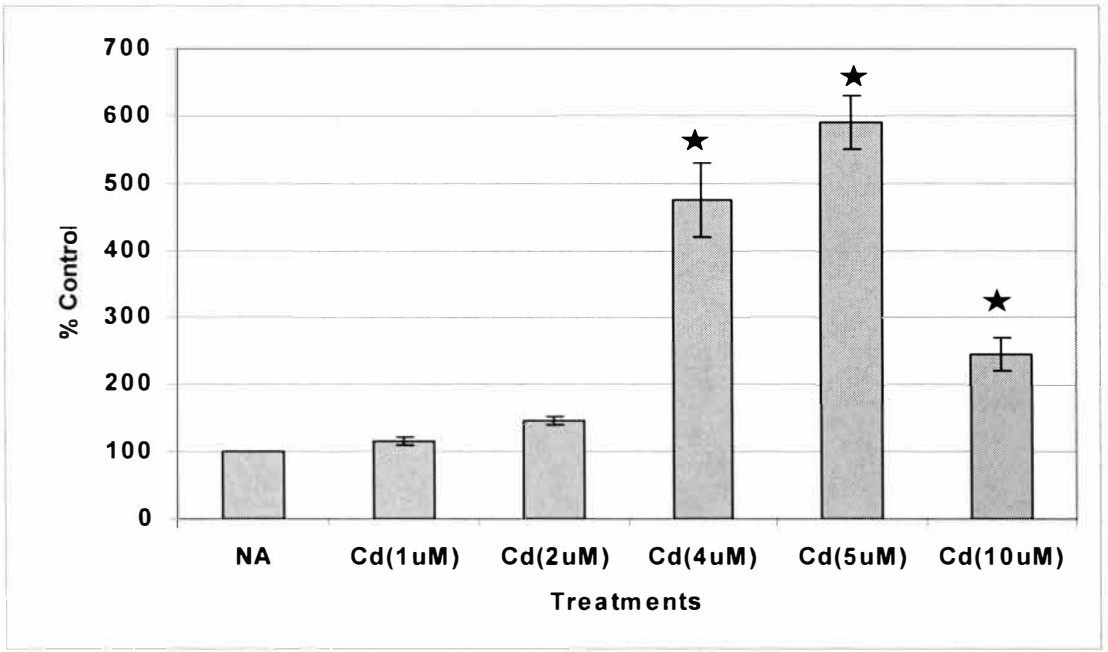


Figure 6: Effect of Cd on NF-κB Transcription Factor Activation in a Concentration Dependent Manner. H4IIE (Liver cells) were transfected with a luciferase reporter plasmid containing NF-κB binding site. Transfected cells were then incubated with either 0 (NA), 1.0, 2.0, 4.0, 5.0 or 10.0 μM for 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=4). Statistical analysis was performed by Student t-test. ★ = Significant stimulation from NA.

The results show that with 1μM or 2μM treatment there is a little increase in NF-κB activation but this increase was significantly different from control with 4μM

and 5μM treatment. NF-κB activation decreases with 10μM treatment. Ten micro molar Cd is similar to that observed with HSF activation. There was a time dependent increase in the activation of NF-κB for 1, 3 and 6 hours with 4μM Cd. These data points show that Cd is able to increase NF-κB activation in a concentration and time dependent manner (Figure 6 & 7).

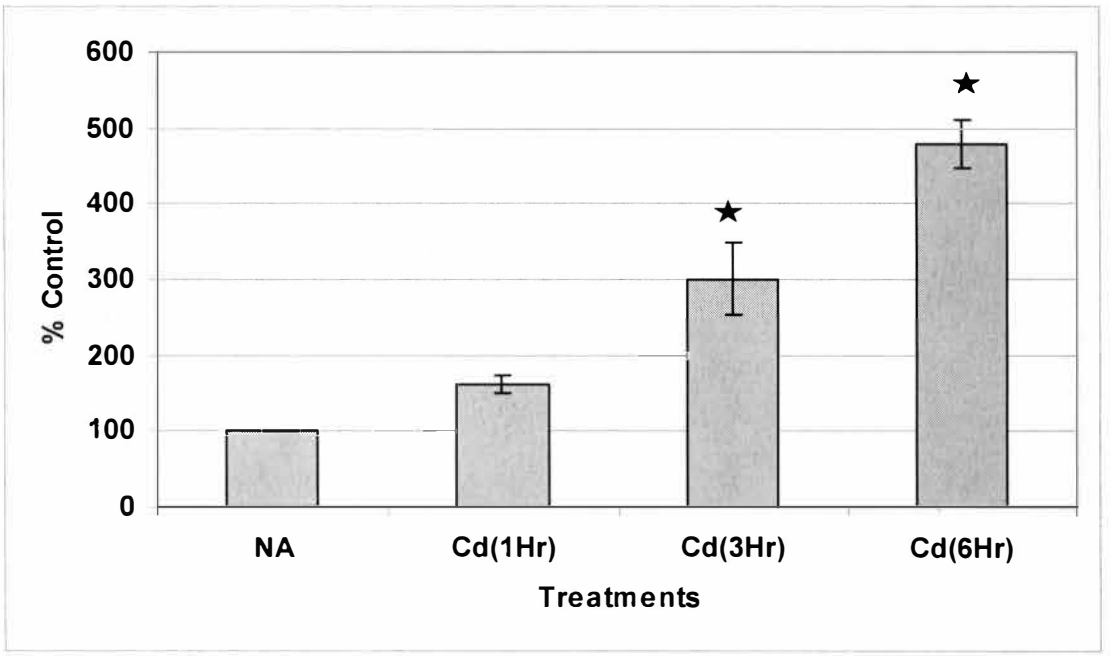


Figure 7: Effect of Cd on NF-κB Transcription Factor Activation in a Time Dependent Manner. Liver cells were transfected with a luciferase reporter plasmid containing NF-κB binding site. Transfected cells were then incubated with 4μM for 1, 3 or 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=4). Statistical analysis was performed by Student t-test ($P<0.05$). ★ = Significant stimulation compared to NA.

Effect of Cd on PH-HOL Plasmid

To evaluate the effect of Cd on other stress related transcription factors, the plasmid PH-HOL was used. PH-HOL plasmid has a part of the promoter region from human heme oxygenase-1 (HO-1) gene, which has a Cd response element (CRE) in it. This CRE is linked to the luciferase gene. Nuclear proteins bind to this Cd response element and initiate the transcription of HO-1 genes. These nuclear proteins are yet to be identified and because these proteins bind to CRE we refer to these nuclear proteins as cadmium response factor (CRF) throughout this thesis. If the Cd is able to activate CRF then it will bind to CRE and will initiate the luciferase transcription. For this experiment, cells were first transfected with plasmid DNA and then after 13-16 hours of incubation were treated with Cd. Cells were treated with $CdCl_2$ at concentrations of either 0 (NA), 1.0, 2.0, 4.0, 5.0 or 10.0 μM for 6 hours or with 4μM for 1, 3 or 6 hours. After the incubation time period treated cells were processed and cell lysate was collected and luciferase activity was measured. An increase was observed in the luciferase activity with Cd treatments. This increase was concentration and time dependent with regard to the amount of Cd and exposure time (Figure 8 & 9).

Effect of Cd on PTAL Plasmid

An additional control plasmid PTAL was also used. PTAL plasmid does not have any transcription factor binding site or Cd response element in it. So when cells transfected with this plasmid are treated no activation should be observed with Cd

HSF, NF-κB and CRF transcription factors are activated in response to Cd treatment. If increased luciferase activity with PTAL plasmid is not observed, this would

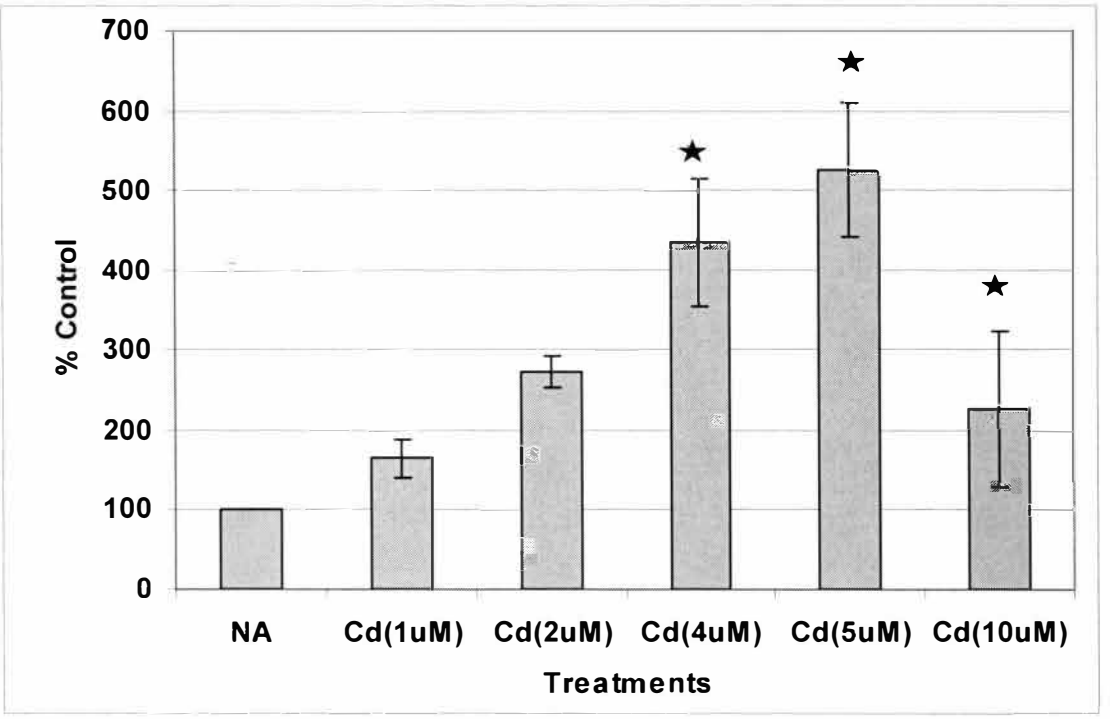


Figure 8: Effect of Cd on PH-HOL Plasmid in a Concentration Dependent Manner. H4IIE (Liver cells) were transfected with a luciferase reporter plasmid containing CRE. Transfected cells were then incubated with either 0 (NA), 1.0, 2.0, 4.0, 5.0 or 10.0 μM for 6 hours. The cells were then processed and luciferase activity was determined. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=3). Statistical analysis was performed by Student t-test ($P<0.05$). ★ =Significant stimulation compared to NA.

indicate that the observed increase in luciferase activity with the other plasmids was specific to specific transcription activation and was not due to non-specific binding to plasmids. First cells were serum starved and transiently transfected with the PTAL plasmid. Then cells were treated with either 0 (NA) or 5.0 μM for 6 hours of $CdCl_2$.

After 6 hours of incubation, cells were processed and luciferase activity was determined. As expected there was no significant difference between no addition treatment and Cd treatment with PTAL plasmid (Figure 10). But with HSE, NF-κB and PH-HOL plasmid there was a significant increase with 5.0μM Cd treatment. These results show that HSF, NF-κB and CRF were activated in response to Cd treatment.

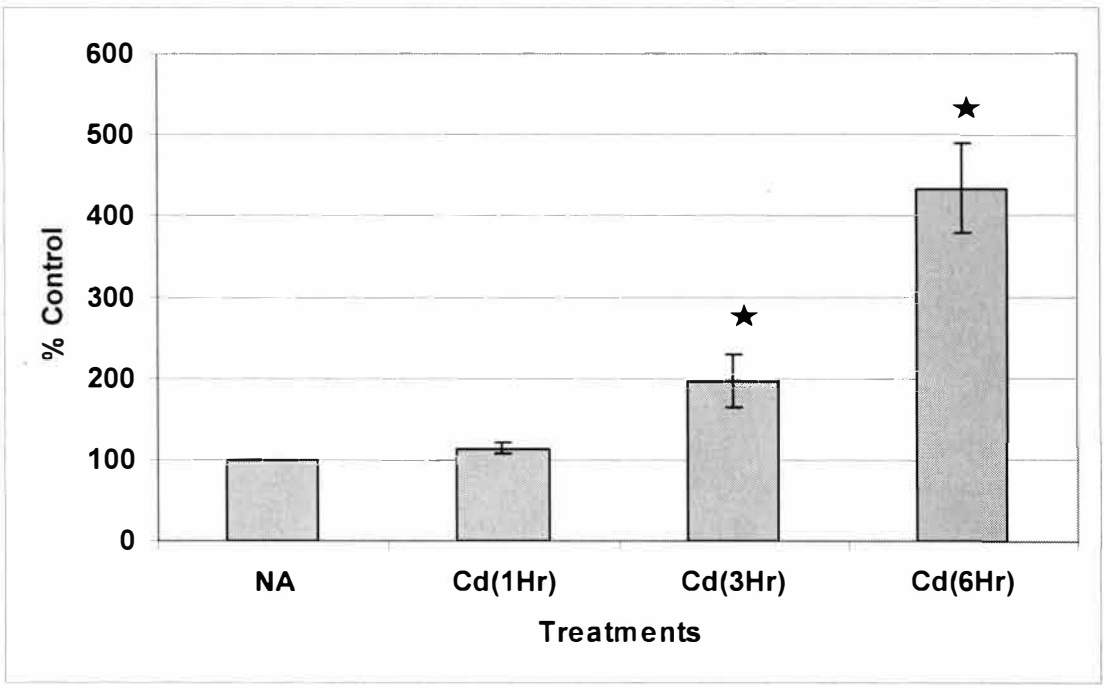


Figure 9: Effect of Cd on PH-HOL Plasmid in a Time Dependent Manner. H4IIE (Liver cells) were transfected with a luciferase reporter plasmid containing CRE. Transfected cells were then incubated with 4μM for 1, 3 or 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=4). Statistical analysis was performed by Student t-test ($P<0.05$). ★ = Significant stimulation compared to NA.

Effect of Antioxidants on Cd Induced Transcription Factors

To elucidate whether ROS played a role in the Cd mediated increase in transcription factor activation, the effect of various antioxidants was evaluated. These antioxidants can scavenge the free radical species or help the cell to defend itself against cytotoxicity associated with ROS. Carnosol, carnosic acid and rosmarinic acid are natural antioxidants and have been shown to inhibit lipid peroxidation, which is a consequence of oxidative stress (Aruoma et. al., 1992). These antioxidants also have been shown to increase phase II enzymes like glutathione peroxidase and superoxide dismutase. For these experiments, the cells were first pretreated with the antioxidants carnosol (csol), carnosic acid (caacid), rosmarinic acid (racid) or NAC for 30 minutes. After this media was removed from the plates and cells were treated with $CdCl_2$ for 6 hours at 5μM. The concentration used for carnosol, carnosic acid, rosmarinic acid antioxidants was 1.36μM (0.5μg/ml) and for NAC, 10mM was used. Cells were then processed and cell lysate was collected for each treatment and then luciferase activity was determined.

HSF: HSF transcription factor was activated and was decreased upon antioxidant treatment. Use of NAC reduced the activation by 90% and rosmarinic acid reduced it by 60%. Whereas carnosol and carnosic acid decreased the activation by about 40%. From these results NAC is the more potent antioxidant when compared to the others (Figure 11). These results also suggest that HSF activation by Cd is mediated through ROS.

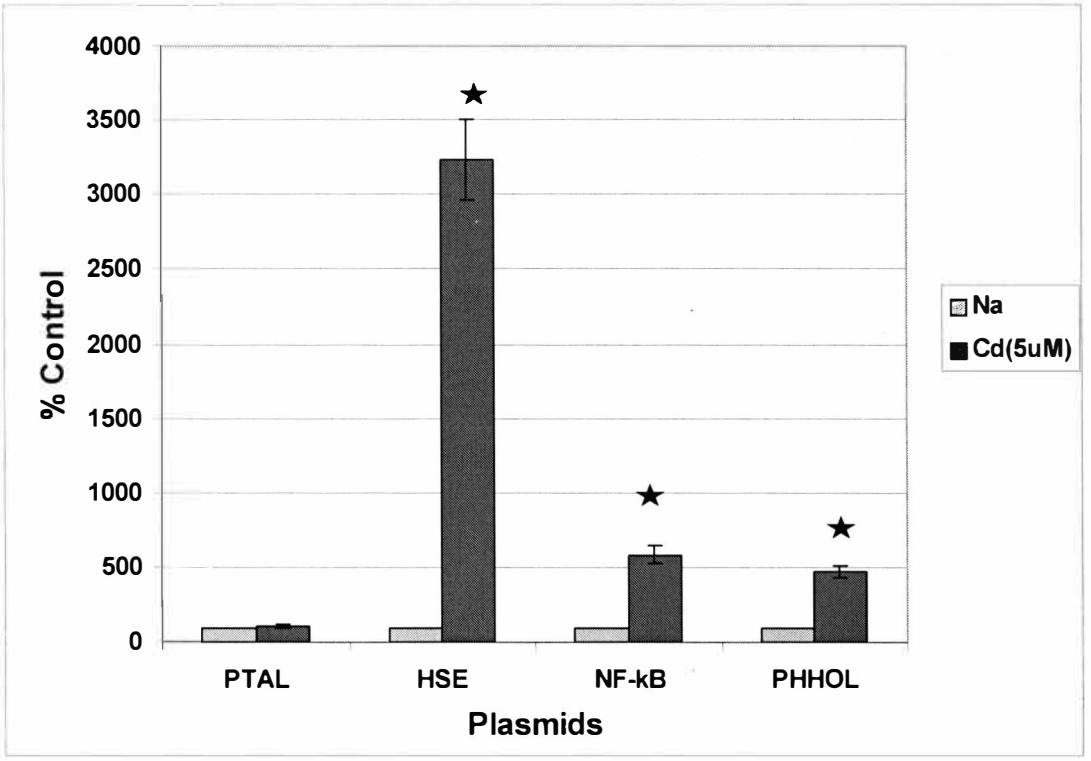


Figure 10: Effect of Cd on PTAL Plasmid. First the cells were transiently transfected with control plasmid (PTAL) or HSE or NF-κB or PH-HOL plasmid. After that cells were treated with 5μM Cd for 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$) ($n=5$). ★ =Significant stimulation compared to NA.

NF-κB: With cells transfected with NF-κB plasmid and then treated with antioxidants we found that NAC and carnosol decreased the Cd activation by about 80 and 70 % respectively. Rosmarinic acid and carnosic acid were only able to decrease the activation by about 40%. So, for regulation for NF-κB transcription

factor NAC and carnosol were the most effective at preventing Cd induction (Figure 12).

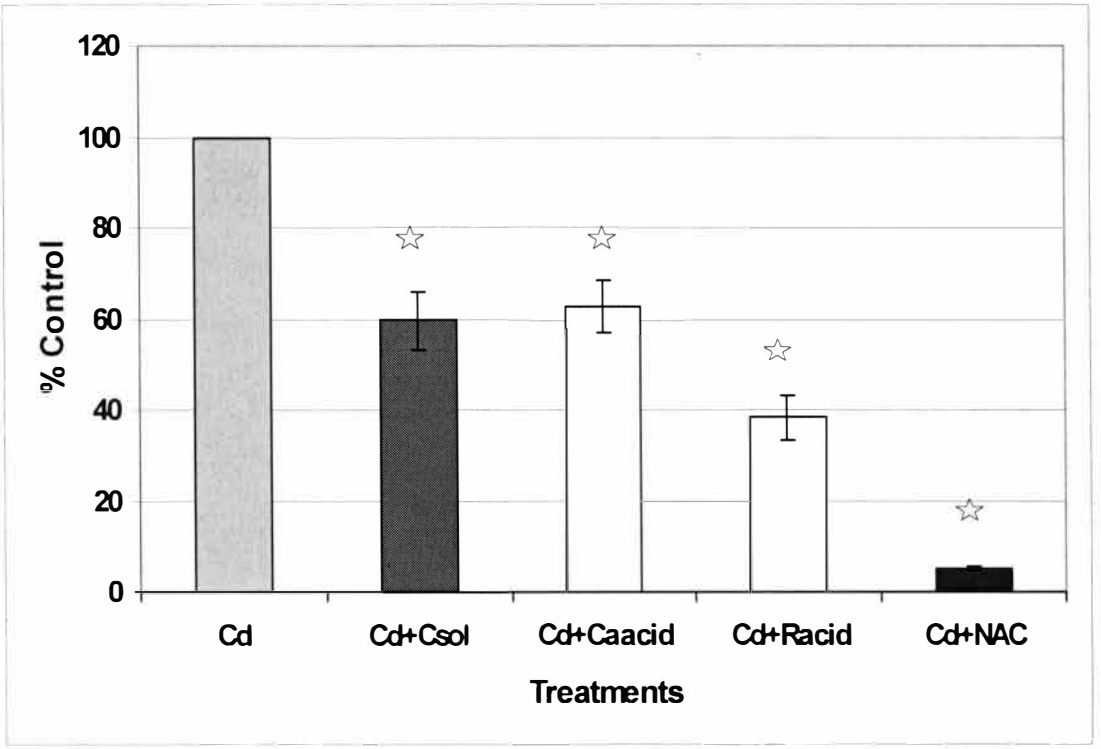


Figure 11: Effect of Antioxidants on Cd Induced HSF Transcription Factor. H4IIE cells were pretreated with antioxidants for 30 minutes. Then 5.0μM of Cd was appropriately added and cells were incubated for 6 hours. The cells were then processed and luciferase activity was measured. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=17~19). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ☆ = Significant inhibition compared to Cd.

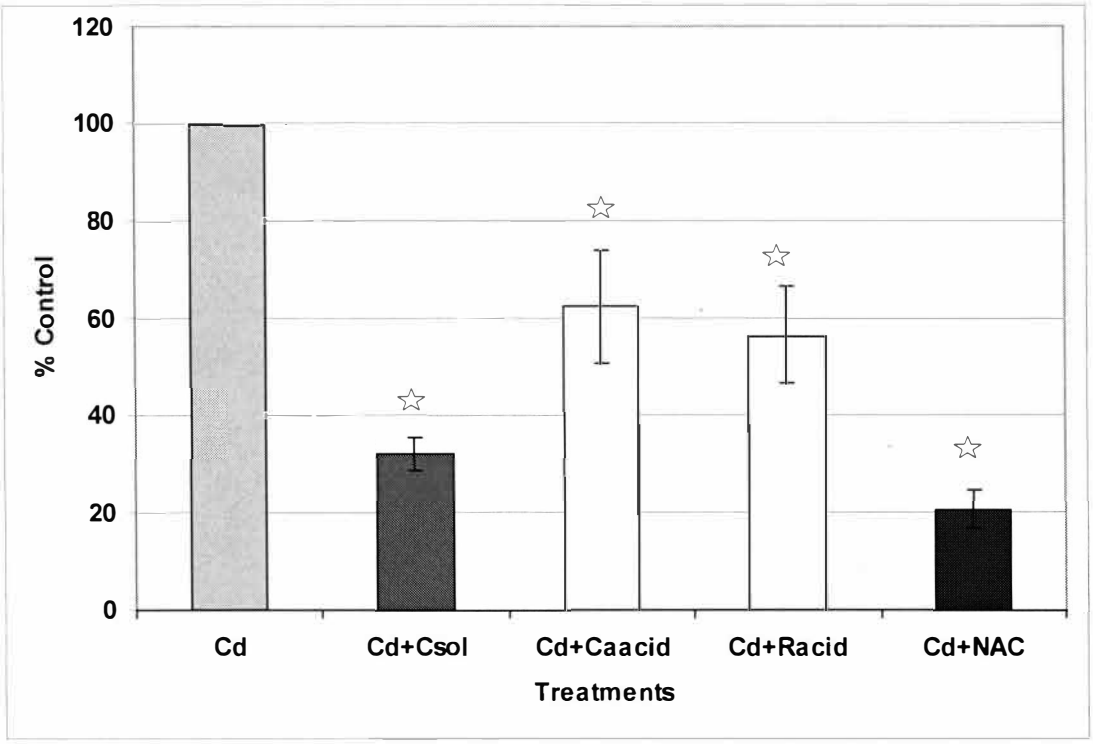


Figure 12: Effect of Antioxidants on Cd Induced NF-κB Transcription Factor. H4IIE cells were pretreated with antioxidants for 1 hour. Then 5.0μM of Cd was appropriately added and cells were incubated for 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=10~14). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ☆ = Significant inhibition compared to Cd.

CRF: As shown, Cd activates CRF (Nuclear proteins). To find out if CRF activation involves ROS or not liver cells were transfected with PH-HOL plasmid and then pretreated with antioxidants for 30 minutes. Results were obtained after antioxidant treatment and 5μM Cd was added for 6 hours. Results indicated that NAC pretreatment reduced the activation by 40%, rosmarinic acid and carnosol reduced it

by 35% and carnosic acid by 20% (Figure 13). These results were different from the other transcription factors in that, the antioxidants appear to be less potent against the Cd induction of CRF.

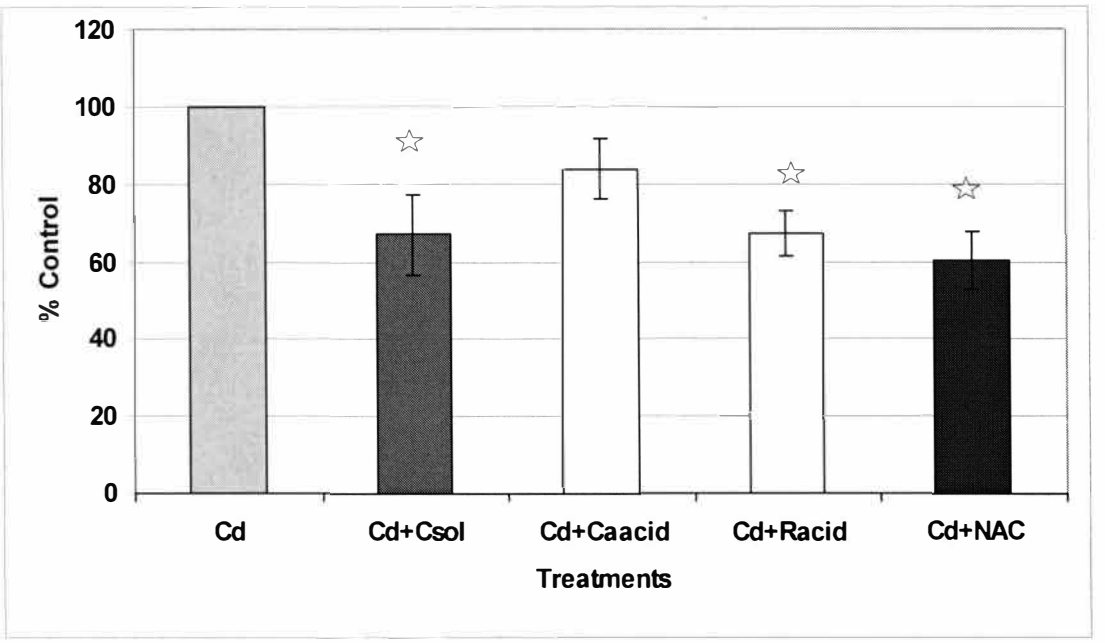


Figure 13: Effect of Antioxidants on Cd Induced CRF Transcription Factor. H4IIE cells were pretreated with antioxidants for 1 hour. Then 5.0uM of Cd was appropriately added and cells were incubated for 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=10~12). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ☆ = Significant inhibition compared to Cd.

PTAL: Cells were transfected with the control plasmid and subsequently pretreated with antioxidants and Cd. There was no observed difference between NA, Cd treatment plus antioxidants treatments (Figure 14). This control experiment

showed that the effect of Cd on the reporter plasmids was specific and that the luciferase activity was due to activation of the indicated transcription factors.

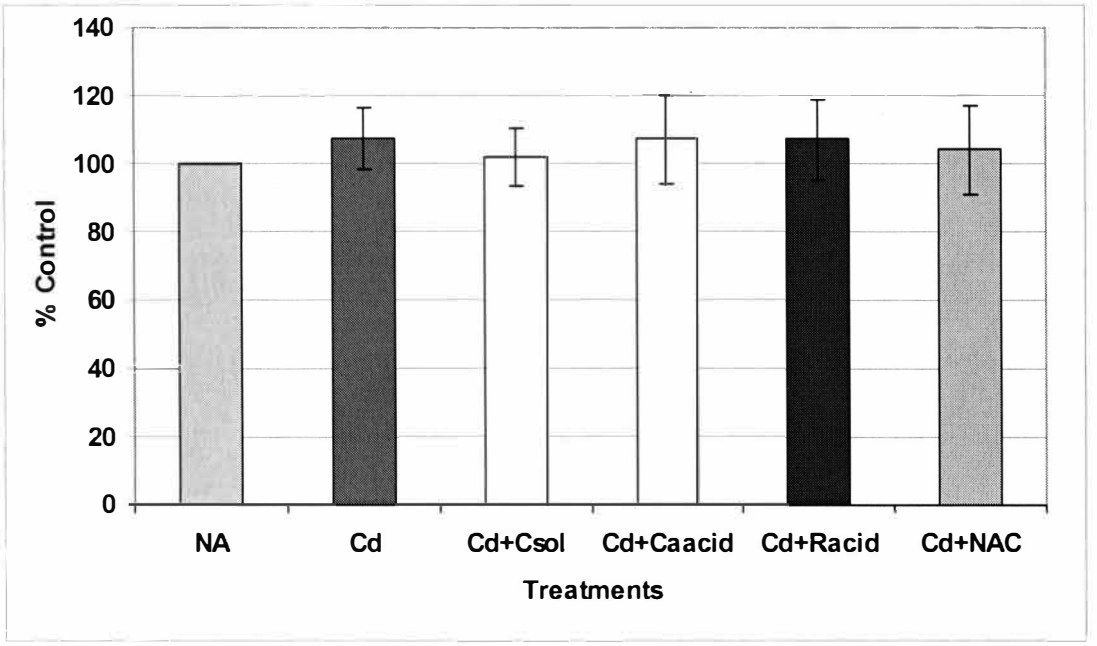


Figure 14: Effect of Antioxidants on Cd Induced PTAL Plasmid. H4IIE cells were pretreated with antioxidants for 1 hour. Then 5.0μM of Cd was added and cells were incubated for 6 hours. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$).

Effect of Antioxidants on Transcription Factors

An additional control experiment with antioxidants alone was also performed. Cells were transiently transfected with HSE, NF-κB or PH-HOL plasmids and then

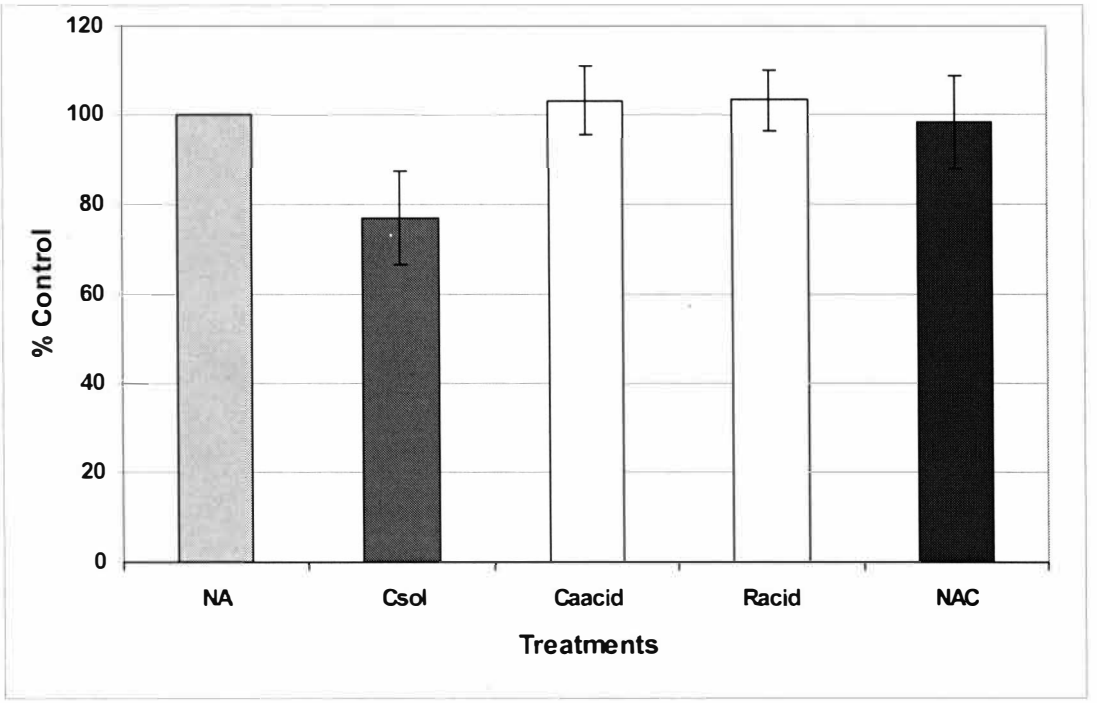


Figure 15: Effect of Antioxidants on HSF Transcription Factor. H4IIE cells were transfected with a luciferase reporter plasmid containing HSF binding site and then pretreated with 0.5μg/ml of antioxidants carnosol (Csol), carnosic acid (Caacid), rosmarinic acid (Racid) and 10mM N-acetyl cysteine (NAC) for 30 minutes. The cells were then processed and luciferase activity in presence of substrate luciferin was measured using a scintillation counter. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=6~8). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$).

treated with either carnosol, carnosic acid, rosmarinic acid (0.5μg/ml) or NAC (10mM) for 30 minutes. After 30 minutes, the media was changed. After 6 hours cells were lysed and processed. There was no significant difference between NA and any of the antioxidant treatments for HSF or NF-κB transcription factors (Figure 15 & 16).

PH-HOL plasmid also did not show any difference between NA treatment and antioxidant treatment alone (Figure 17).

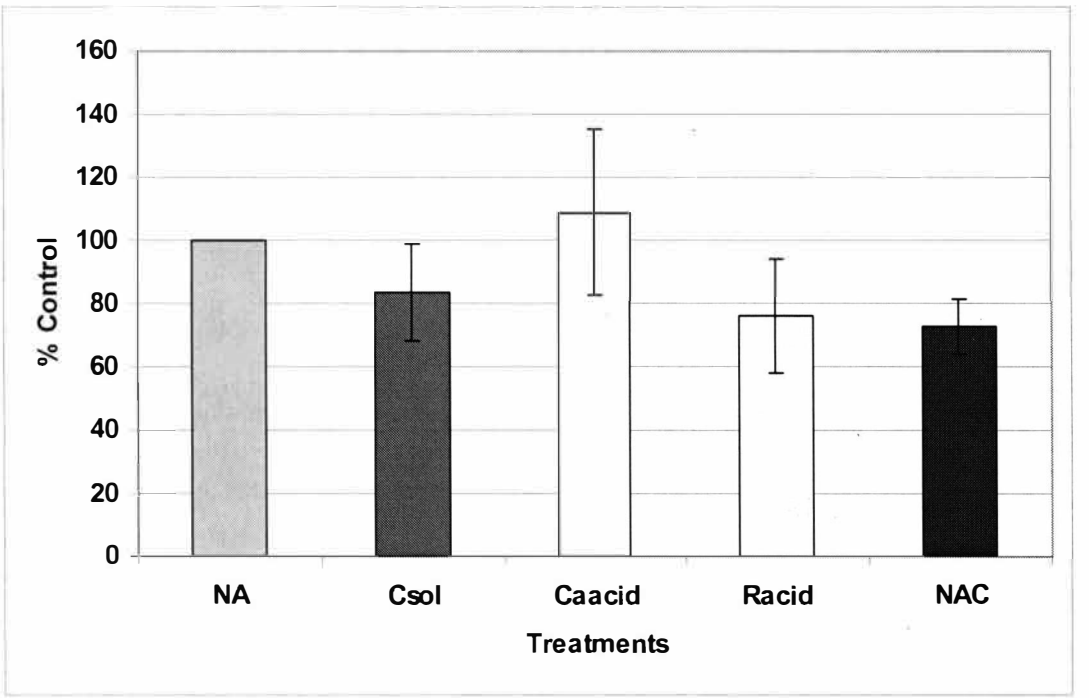


Figure 16: Effect of Antioxidants on NF-κB Transcription Factor. H4IIE cells were transfected with a luciferase reporter plasmid containing NF-κB binding site and then pretreated with 0.5μg/ml of antioxidants carnosol (Csol), carnosic acid (Caacid), rosmarinic acid (Racid) and 10mM N-Acetyl cysteine (NAC) for 30 minutes. The cells were then processed and luciferase activity was determined. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=7). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$).

Effect of Cd on Amount of HSP-70

Heat shock protein-70 (HSP-70) is a chaperone protein. It helps in proper folding of proteins. HSP-70 is a downstream gene product of HSF binding to DNA.

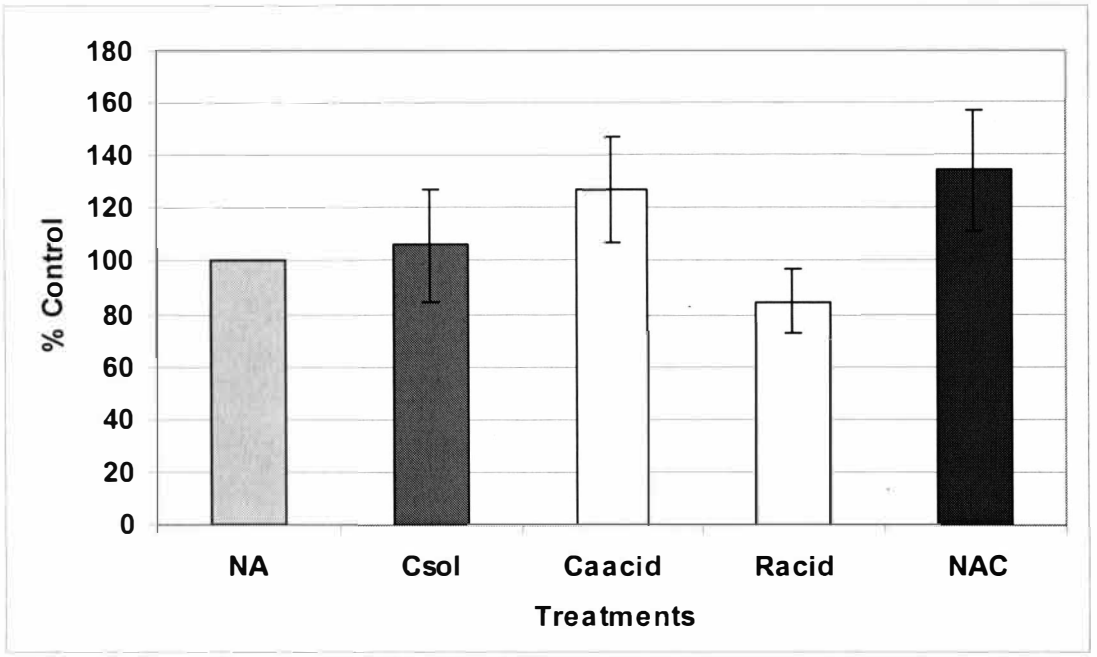


Figure 17: Effect of Antioxidants on CRF Transcription Factor. H4IIE cells were transfected with a luciferase reporter plasmid PH-HOL and then pretreated with 0.5μg/ml of antioxidants carnosol (Csol), carnosic acid (Caacid), rosmarinic acid (Racid) and 10mM N-Acetyl cysteine (NAC) for 30 minutes. The cells were then processed and luciferase activity was determined. Activities were normalized to total cell protein and reported as percent of control +/- S.E.M (n=7). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$).

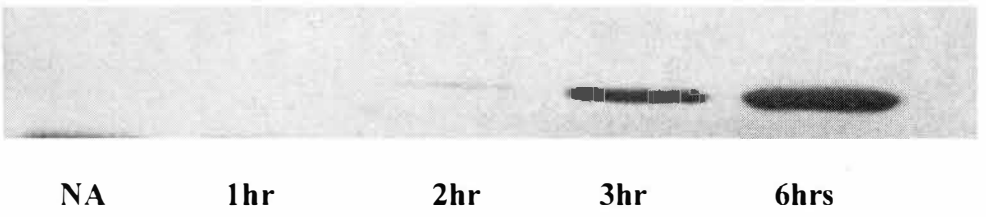
Results show that HSF is activated upon Cd treatment. To check whether this activation is able to induce some of the heat response genes or not levels of HSP-70 were quantified using western blot technique. To estimate the amount of HSP-70 protein in cells, first the cells were treated with 4μM $CdCl_2$ for NA, 1, 2, 3, 6 or 12 hours. After the desired treatments, cells were lysed and processed and cell lysate was collected for each sample. Ten micro liters of immunoprecipitated sample was

electrophoresed in a polyacrylamide gel and then incubated with primary and secondary antibodies. The membrane was washed and then exposed to film. Blots were quantified with densitometry using NIH software. There was a significant increase in the amount of HSP-70 as the treatment time with Cd increased. This shows that HSP-70 increases in a time dependent manner when treated with $CdCl_2$ (Figure 18). These results are comparable with the previous HSF luciferase reporter system results (Figure 5).

Effect of Antioxidants on Cd Induced HSP-70 Amount

Since HSP-70 protein levels increased in response to Cd, it may be expected that antioxidants were able to effect the increase in HSP-70. To check the effect of antioxidants on HSP-70, the cells were first pretreated with the different antioxidants for 30 minutes, then media was removed and treated with 4μM $CdCl_2$ for 6 hours. After 6-hour incubation, cells were lysed and processed and cell lysate was collected. Cell lysate was immunoprecipitated with HSP-70 antibodies and subjected to electrophoresis. Proteins were transferred onto the membrane and then the membrane was exposed to the film. Film was quantified with densitometry. Results show that antioxidants were able to reduce the amount of HSP-70. NAC decreased the amount of HSP-70 by 80% compared to the amount with Cd treatment. Rosmarinic acid reduced HSP-70 by about 40%, while carnosol and carnosic acid reduced the effect of Cd by approximately 20%. The effect of antioxidants on HSP-70 amount (Figure 19) parallels the same trend observed with the transcription factor HSF (Figure 11).

A.



B.

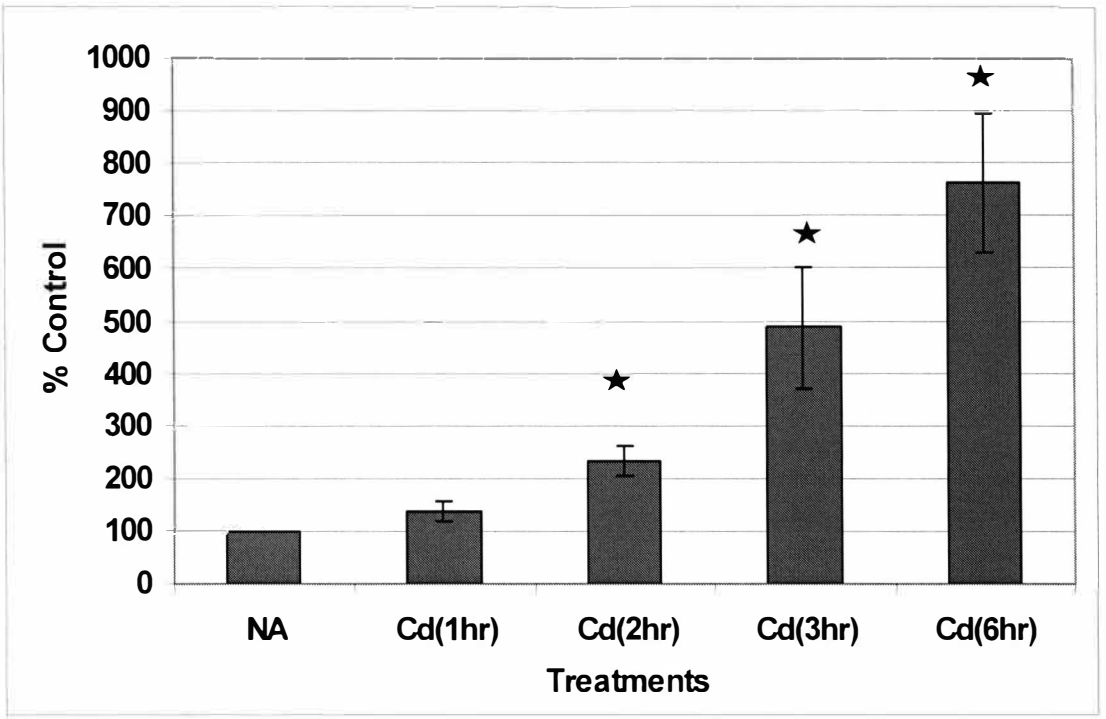
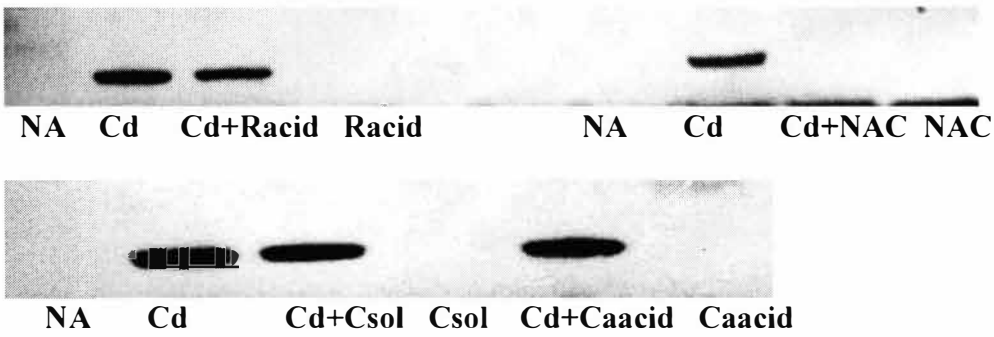


Figure 18: Effect of Cd on HSP-70 Amount. A. Western blot showing HSP-70 amount for each treatment. B. Quantitation of HSP-70 blots. H4IIE cells were treated with 4.0μM Cd for 1 ~ 6 hours. The cells were lysed, processed and immunoprecipitated with HSP-70 antibodies. A western blot was run with the samples and blots were obtained. Data represents the mean +/- S.E.M (n=6). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ★ = Significant stimulation compared to NA.

A.



B.

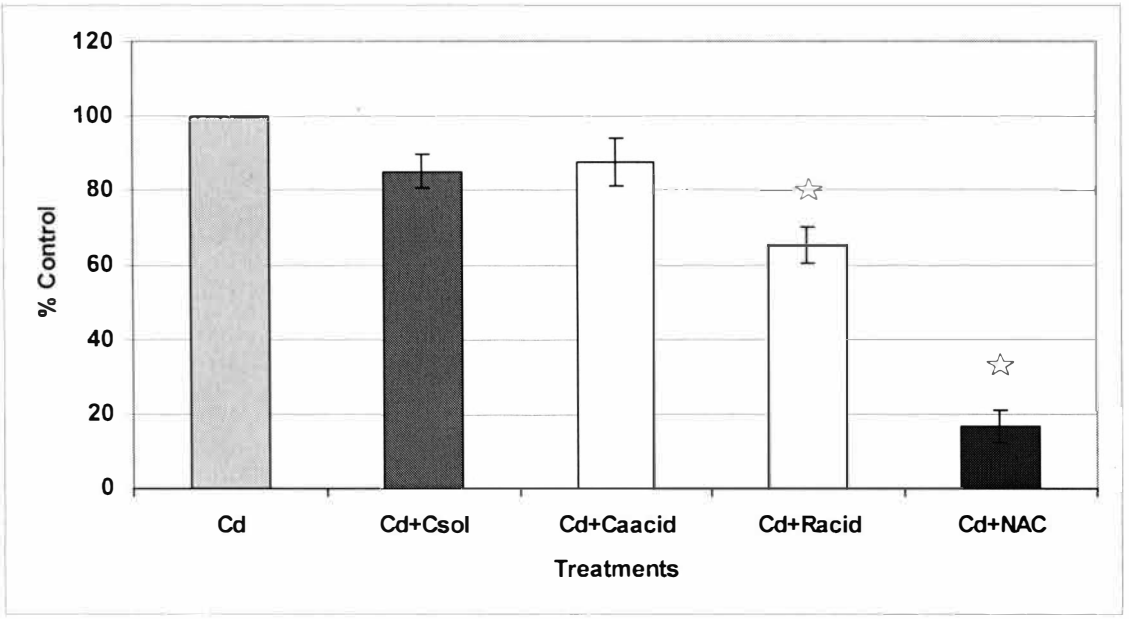


Figure 19: Effect of Antioxidants on Cd Induced HSP-70 Amount. A. Western blot showing HSP-70 amount for each treatment. B. Quantitation of HSP-70 blots. H4IIE cells were pretreated with antioxidants and then with 5.0μM Cd for 6 hours. The cells were lysed, processed and immunoprecipitated with HSP-70 antibodies. A western blot was run with the samples and blots were obtained. Data represents the mean +/- S.E.M (n=5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ☆ = Significant inhibition compared to Cd.

Effect of MAPK Protein Inhibitors on Cd Induced HSF Activation

To find out which signal proteins might be involved in transmitting the signal to activate the HSF transcription factor, different MAPK signal proteins inhibitors were used. MAPK are critical components of signal transduction pathways and mediate the cellular response to numerous extra cellular stimuli, ranging from growth factors to environmental stress. There are three main components of MAPK pathway, ERK1/2, JNK and p38. In general, activation of the ERK1/2 cascade leads to cell proliferation, differentiation, and enhanced cell survival after cellular stress. On the other hand, activation of the JNK and p38 cascades are usually associated with enhanced apoptosis and production of inflammatory cytokines. MAPKs also play a major role in regulation of transcription factor activity in response to various stress stimuli (Karin et. al., 1995). MAPKs have been implicated in phosphorylation and activation of a diverse array of transcription factors in mammals and yeasts (Herskowitz et. al., 1995; Hill et. al., 1995). They are phosphorylated and become activated. Once activated, MAPK can translocate from the cytoplasm to the nucleus, leading to phosphorylation of several transcription factors and altered regulation of gene expression. To evaluate the relative role of the MAPK family members, chemical inhibitors were used. In these experiments SP600125 was chosen an inhibitor for JNK, PD098059 for ERK1/2 and SB203580 for p38 proteins. The cells were transfected with HSF plasmid and then pretreated with inhibitors for 1hour. After incubation with inhibitors, media was replaced and cells were treated with 4μM $CdCl_2$ for 6 hours. After the treatment, the cells were lysed and processed and lysate

was collected to run luciferase assays on these samples. Results indicated that with both the inhibitors of JNK and ERK1/2 there was a decrease in HSF activation by about 20%. But with the p38 inhibitor it was reduced to almost 50% of the Cd treated cells. This suggests a possible role for p38 in the activation of HSF transcription factor (Figure 20).

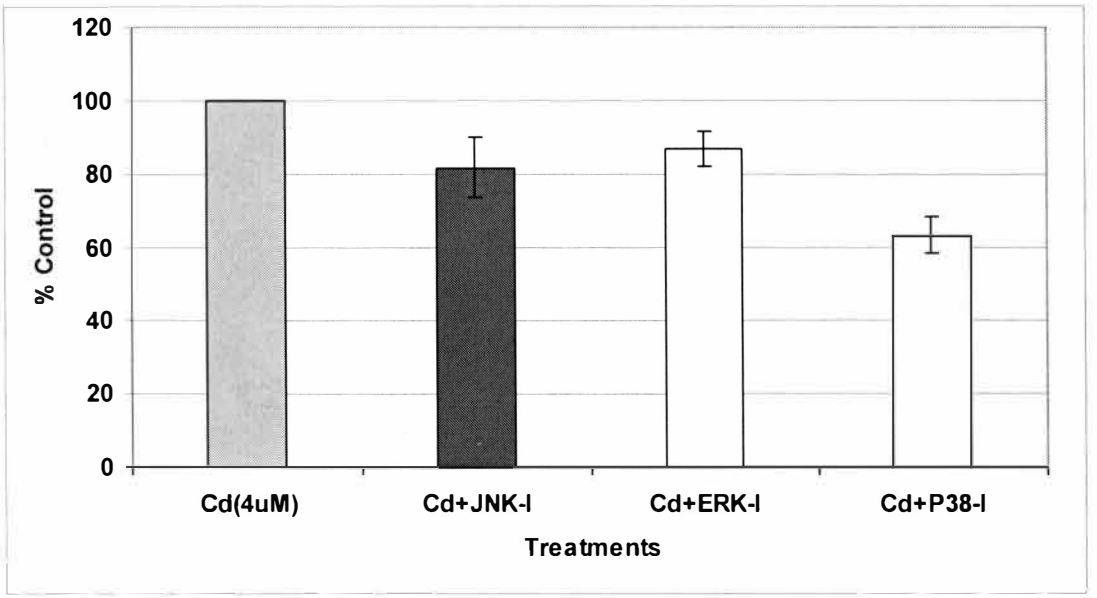
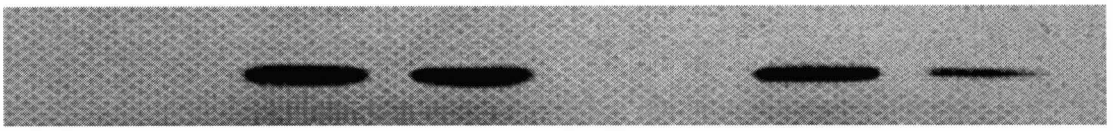


Figure 20: Effect of MAPK Protein Inhibitors on Cd Induced HSF Activation. Cells were transfected with HSE plasmid and then pretreated with MAPK inhibitors SP600125 for JNK, PD098059 for ERK1/2, and SB203580 for p38 inhibitors for 1 hour. After pretreatment with inhibitors media was changed and 4μM Cd was added. After 6 hours cells were processed and luciferase activity was determined as indicated in material and methods. Data represents the mean +/- S.E.M (n=8). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ☆ =Significant inhibition compared to Cd.

Effect of MAPK Inhibitors on Cd Induced HSP-70 Amount

To further confirm that MAPK signal proteins are involved in HSF activation. The effect of inhibitors on the amount of HSP-70 was also examined. For this experiment first cells were pretreated with JNK, ERK1/2 and p38 inhibitors for 1 hour and then media removed and then treated with 4μM $CdCl_2$ for 6 hours. After the treatment cells were processed and cell lysate was collected for each sample. Two hundred micrograms of protein was immunoprecipitated with HSP-70 antibodies and then subjected to electrophoresis. Proteins were then transferred onto the membrane and this membrane was incubated with primary and the secondary antibodies. The membrane was exposed to film and this film was quantified to determine the amount of protein. The amount of Cd induced HSP-70 protein was reduced by 40 % when treated with p38 inhibitor but was not significantly affected by either the inhibitors of JNK and ERK1/2. This is further evidence to support an involvement of p38 signal protein in the signal transduction pathway of HSF activation and subsequent effect on the amount of HSP-70 (Figure 21).

A.



NA Cd Cd+ JNK-I Cd+ERK-I Cd+P38-I

B.

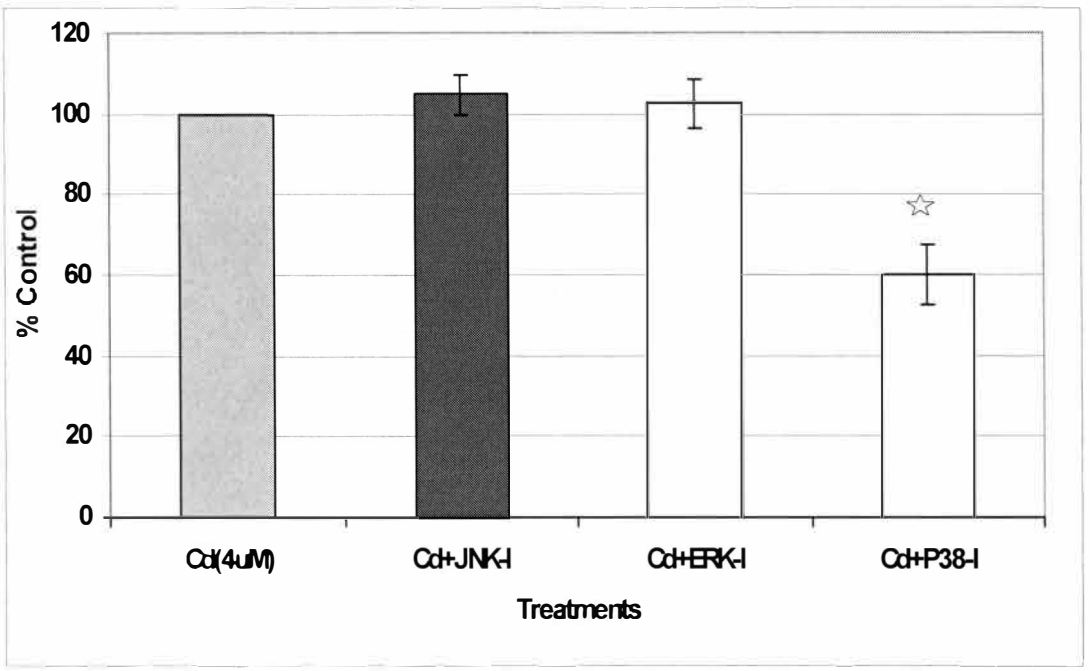
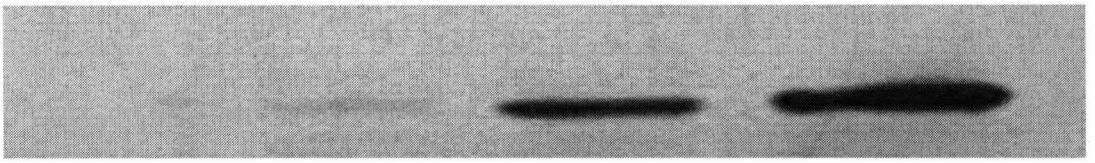


Figure 21: Effect of MAPK Inhibitors on Cd Induced HSP-70 Amount. A. Western blot showing HSP-70 amount for each treatment. B. Quantitation of HSP-70 blots. H4IIE cells were pretreated with inhibitors for 1 hour and then with 4.0μM Cd for 6 hours. Cells were lysed, processed and immunoprecipitated with HSP-70 antibodies. Western blot was run with the samples and blots were obtained. Data represents the mean +/- S.E.M (n=5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ☆ = Significant inhibition compared to Cd.

Effect of Cd on Phosphorylated P38 Amount

Cells commonly use signal protein cascades to pass a message from the cell surface to the nucleus. The MAPK signal cascade is one of the major signaling pathways in the cell. There are three main protein family members JNK, ERK1/2 and p38, through which signals are transmitted. In previous studies from our lab, the activity of both JNK and ERK1/2 was increased by Cd (Xu et. al., 2003; Hsiao & Stapleton, et. al., 2004) but we had not investigated the effect of Cd on p38. The phosphorylated form of p38 is the active form of this signal protein. Previous studies have suggested that p38 might be involved in HSF activation (Hung et. al., 1998; Maroni et. al., 2003). Thus, we tested whether Cd would increase the activity of p38 in the cell. To study the effect of Cd on p38, cells were exposed to 4uM $CdCl_2$ for either 1, 3 or 6 hours and after treatment cells were processed and cell lysate was collected. We used western blot analysis to estimate the amount of phosphorylated p38 as described in material and methods. There was an approximate 2-fold increase in the phosphorylated form of p38 when exposed for 1 hour to Cadmium at 4μM. When the exposure time was increased to 6 hours there was approximately a 7-fold increase in the phosphorylated form of p38. As expected, an increase in phosphorylated p38 was dependent on the time of incubation with Cd (Figure 22).

A.



NA Cd (1hr) Cd (3 hr) Cd (6hr)

B.

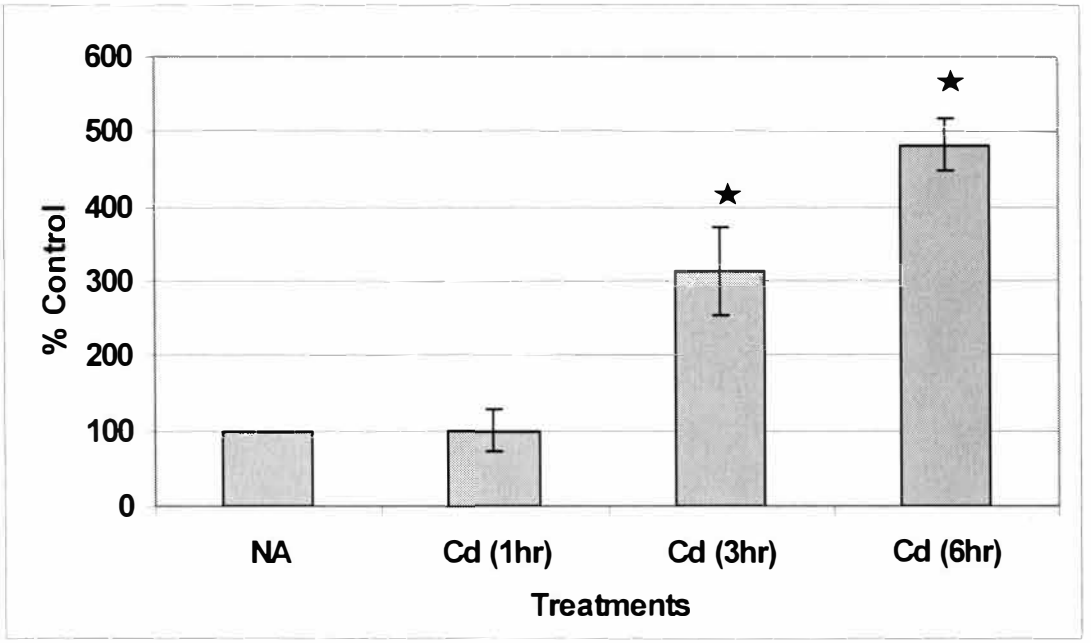
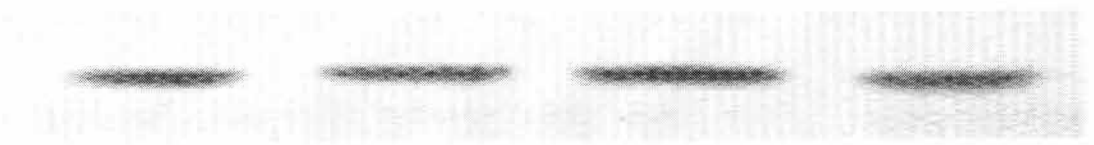


Figure 22: Effect of Cd on Phosphorylated P38 Amount. A. Western blot showing phosphorylated p38 amount for each treatment. B. Quantitation of phosphorylated p38 blots. H4IIE cells were treated with 4.0μM Cd for 1 ~ 6 hours. Cells were lysed, processed and immunoprecipitated with phosphorylated p38 antibodies. Western blot was run with the samples and blots were obtained. Data represents the mean +/- S.E.M (n=6). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$). ★ = Significant stimulation compared to NA.

Effect of Cd on Total p38 Amount

Since a time dependent increase in the phosphorylated form of p38 with Cd was observed, it was important to determine whether this increase was due to increased in phosphorylation or the amount of protein. Total p38 is the total amount of p38 in active form and inactive form present inside the cell. For this experiment, cells were treated with 4uM of $CdCl_2$ for either 1, 3 or 6 hours and after treatment, cells were processed and cell lysate was collected. We used western blot analysis to estimate the total p38 as described in material and methods. Blots were obtained and quantified by densitometry using NIH software. We found that there is no increase in the amount of total p38 between NA and any other treatment with Cd (Figure 23). This experiment showed that there was no increase in total amount of p38 but there was a change in the form of p38. When cells are experiencing oxidative stress, more and more p38 is converted from its inactive (non-phosphorylated) to its active phosphorylated form to transmit the signals to such receivers as the transcription factor HSF.

A.



NA Cd (1hr) Cd (3 hr) Cd (6hr)

B.

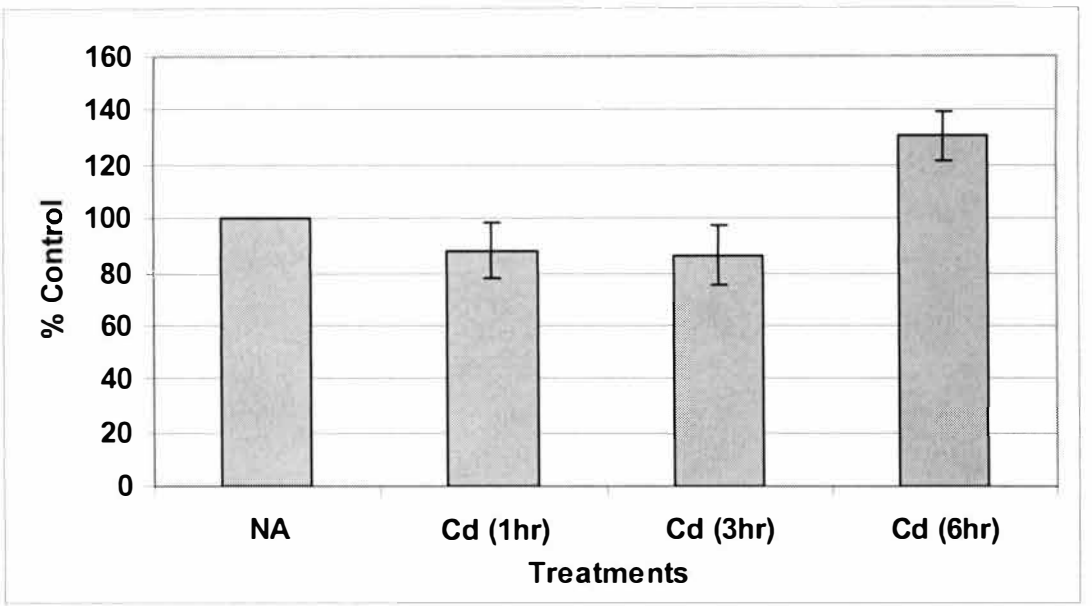


Figure 23: Figure 22: Effect of Cd on Total P38. A. Western blot showing total p38 amount for each treatment. B. Quantitation of total p38 blots. H4IIE cells were treated with 4.0μM Cd for 1 ~ 6 hours. Cells were lysed, processed and immunoprecipitated with total p38 antibodies. Western blot was run with the samples and blots were obtained. Data represents the mean +/- S.E.M (n=3). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman Keuls test ($P<0.05$).

CHAPTER IV

DISCUSSION

Reprogramming of gene expression has emerged as an important mechanism used by cells to respond and to adapt to adverse environmental changes or contact with toxic substances. This reprogramming of gene expression to an adverse condition is commonly referred to as a stress response. The stress response can be induced by a variety of stressors that come from numerous sources including contaminated air, water, soil and food. These stressors include heat shock, UV light, oxidizing agents, organic chemicals, transition and heavy metals (e.g. lead, arsenic, mercury and chromium); sulfhydryl agents, hypoxia, anoxia, ethanol and diesel exhaust (Alam et. al., 2000; Bauer et. al., 2002; Murphy et. al., 1999).

The effects of these stressors have been demonstrated in a number of studies in a variety of systems. For example, a study by Sanchez-Moreno et. al., 2003 showed that ethanol induced neuron cell damage by formation of free radicals and production of pro-inflammatory molecules. Heat shock induced cellular injury and cell death is thought to be due to the generation of ROS as well as alterations in intracellular antioxidant capacity (Diamond et. al., 1999; Freeman et. al., 1990). Iordanov & Magun, 1999, showed that UV light elicits biological responses such as

cytotoxicity, mutagenesis, carcinogenesis and causes oxidative damage to diverse cellular substrates. They also demonstrated that free radicals might be involved in the toxicities caused by UV light exposure in Rat-1 cells. Acrylonitrile is a chemical which is present in auto exhaust, cigarette smoke and synthetic paints. It is a carcinogen and can cause lung cancer. Acrylonitrile also induces oxidative stress by depleting GSH and forming of cyanide, which can inhibit the antioxidant enzyme superoxide dismutase in rats (Pouyatos et. al., 2005). When diesel fuel burns in an engine, the resulting exhaust is made up of soot and gases representing thousands of different chemical substances which can be inhaled and deposited in the lungs. Particles in exhaust carry cancer causing substances known as polynuclear aromatic hydrocarbons which can cause DNA adduct formation and induce mutagenesis through oxidative stress. The potency and is related to their bio-activation in vivo by cytochrome P-450 1A1 leading to production of electrophilic and reactive metabolites (Baulig et. al., 2003). Diesel and car exhaust also contain various transition metals also (e. g. lead, Cd and aluminum) which can be inhaled and induce free radical formation by redox cycling. A study by Lai et. al., 2005 showed that highway toll station workers exposed to traffic exhaust showed increased oxidative DNA damage compared to office workers. Exposure of human subjects to whole diesel exhaust also results in induced oxidative stress (Li et. al., 2003; Mudway et. al., 2004; Pourazar et. al., 2005). Quinones another example of an environmental contaminant are found in herbicides and pesticides. These chemicals can leach into the soil and can contaminate soil, food and water. People can be exposed to quinones by injesting

contaminated food and water. Quinones are carcinogenic and induce mutations, cytogenetic damage, cell transformation and formation of DNA adducts (Anderson et. al., 1994). Again oxidative stress appears to play an important role as studied by Sagai et. al., 1993; Squadrito et. al., 2001 showed that quinones can generate oxidative stress through redox cycling and are responsible for the production of superoxide and hydroxyl radicals.

Recent studies indicate that transition metals act as catalysts in the oxidative reactions of biological macromolecules (Beyersmann et. al., 2002; Ding et. al., 1999; Ercal et. al., 2001; Järup et. al., 2002; Lau et. al., 2004; Liu et. al., 2004). Therefore a number of studies have tried to link the toxicities associated with these to oxidatively damaged tissues. Redox active metals such as iron, chromium and copper undergo redox cycling whereas redox inactive metals, such as lead, cadmium, mercury and others deplete cells of major antioxidants, particularly thiol containing antioxidants and enzymes (Ercal et. al., 2001). Oxidative stress, altered gene expression, elevated lipid peroxidation and enhanced glutathione S-transferase was observed in mice liver in response to chronic exposure to arsenic (Xie et. al., 2004). Chromium has also been shown to induce oxidative stress by increasing superoxide radical thereby causing damage in the liver and brain cells (Bagchi et. al., 2002). Arsenite can also induce ROS formation by activation of radical producing system in the cells. Arsenite at low levels has been found to activate NADH oxidase and produce superoxide radicals, which can increase DNA strand breaks in vascular smooth muscle cells (Lynn et. al., 2000). A study by Gurer-Orhan et. al., 2004 showed that workers exposed to high

concentrations of lead had significantly increased catalase and glucose-6-phosphate dehydrogenase (G6PDH) activities, and decreased blood glutathione: glutathione disulfide ratios compared to the non exposed workers. Cd is a redox inactive metal which has been shown to disturb the redox homeostasis in the cell however, little is known about its mechanism of action in particular on regulation of gene expression. Thus, we used Cd as a model to study the effects of metals on gene expression. The liver is the main detoxifying organ in the body. It is the first organ in the body to filter the blood, so it is very susceptible to damage by contaminants. Cd mainly accumulates in the liver and can cause serious changes to its histology and function (Brzoska et. al., 2003; Mitsumori et al., 1998; Stohs et. al., 2000). We used liver cells as our model cell system which provides us with a good cell model to study the effects of Cd on gene expression.

The purpose of this study was to examine how Cd, could alter gene expression and to identify which key players are involved in this process. The mechanism behind Cd mediated gene expression is not yet fully understood. Cd is a well known environmental toxicant and has been shown to be associated with prostate and lung cancer as well as heart diseases (Stohs et. al., 2000; Tang et. al., 2003; Tomera, et. al., 1994). As the literature suggests one possible mechanism of Cd toxicity might be through ROS (Chin & Templeton, 1993; Hsiao & Stapleton, 2004). Previous studies have shown that Cd is able to induce various ROS and can induce oxidative stress (Ercal et. al., 2001; Stohs et. al., 2000). For example Cd treatment induced generation of hydrogen peroxide radicals in BY-2 cells (Piqueras et. al., 1999) as well as resulted

in cell death mediated by ROS in rat lung epithelial cells (Hart et. al., 1999). Since Cd is a redox inactive metal, it can not induce the ROS by the Fenton reaction therefore other mechanisms must exist. Evidence from the literature indicates that Cd may possibly upset the antioxidant defense system. Studies have indicated that Cd can disturb the redox balance by depleting the GSH level in the cell (Chin et. al., 1993; Kostic et. al., 1993; Xu. et. al., 2003). A study by Pourahmad et. al., 2003 and Wang et. al., 2004 showed that Cd induces ROS by disrupting the electron transport complex function in hepatocytes.

Since ROS is harmful whether or not it is produced by redox cycling or the electron transport system, aerobic organisms must maintain redox homeostasis to survive. This challenge becomes increasingly difficult to meet when cells are exposed to environmental oxidants that increase ROS production, such as heavy metals, redox active chemicals, and hyperoxia (Dalton et. al., 1999; Sohal et. al., 1990). Under conditions that increase ROS beyond basal levels, cells evoke a series of protective responses that include modulation of the gene transcription machinery to regulate gene expression to deal with this increase in ROS. In eukaryotes, transcription factors are key players in the regulation of gene transcription. Transcription factors bind to the RNA polymerase or elsewhere on the gene and induce or repress transcription of genes. When a cell needs more transcription product, the factor is activated and helps increase the production of the required protein.

Various kinds of external stimuli have been shown to affect transcription factor activation. Activation of transcription factors have been shown to be regulated

by the redox state of the cell (Qu et. al., 2005). For example heat shock and the transition metal lead, activate HSF-1 and NF-κB (Ramesh et. al., 1999). Buthionine-sulfoximine (blocks selectively GSH by inhibiting γ-glutamyl cysteine synthetase), nitrofurantoin (causes formation of superoxide anion and hydrogen peroxide by redox cycling and finally lowers GSH concentration) and phorone (decreases the concentration of GSH through the action of GSH S-transferase) are chemicals, which can cause liver oxidative stress. Rats when treated with these three chemicals showed activation of AP-1, hypoxia inducible factor-1 and signal transducer and activator of transcription factor mediated by oxidative stress (Tacchini et. al., 2002).

A study by Ahn et. al., 2003 and Nishizawa et. al., 1999 showed that HSF is activated in response to both heat and hydrogen peroxide in pheochromocytoma cells. In another study with LLC-PK1 cells, HSF and HSF-DNA binding is activated in response to glutathione depletion, increased cellular calcium, oxidative stress and lipid peroxidation by the alkylating agent iodoacetamide in (Liu et. al., 1996). In H35 rat hepatoma cells Cd increases binding of HSF to HSE in a concentration and time dependent manner mediated by oxidative stress (Ovelgonne et. al., 1995). These studies indicate that activation of HSF largely depends upon the redox status of the cell. NF-κB activation is observed in response to a wide variety of agents that include cytokines, inorganic compounds, chemotherapeutics, oxidants, etc (Baeuerle et. al., 1998; May et. al., 1998). Li et. al., 2000 showed that NF-κB is activated by ciprofibrate and this activation is mediated by reactive oxygen species in a hepatoma cell line. NF-κB activation or over expression has been shown to be associated with

tumorigenesis and metastasis, suggesting a role for this transcription factor in the regulation of cell cycle progression (Guttridge et. al., 1999).

The CRE was identified in the HO-1 promoter. Nuclear proteins bind to this CRE and activate the transcription of HO-1 gene. Unfortunately the identities of the nuclear proteins that bind to CRE are unknown (Takeda et. al., 1994). Since this CRE has protein bound to it in response to Cd, this protein(s) can be referred to as CRF. The binding of CRF to CRE increases in response to oxidative stress induced by Cd in HeLa cells. This increase is specific to Cd as there is no effect of other stressors like sodium arsenite, cobalt protoporphyrin, hemin and zinc (Takeda et. al., 1994; Takeda et. al., 1995).

We choose to study the effect of Cd on the activation of NF-κB, HSF & CRF because each has been shown to be activated under oxidative stress by various external stimuli (Pahl et. al., 1999; Janssen-Heininger et. al., 2000). To investigate the effect of Cd on the activation of these important transcription factors, various concentrations of Cd were used at different time points. Results show that Cd activated all three transcription factors NF-κB, HSF & CRF in a concentration and time dependent manner. These results support the literature that HSF (Nishizawa et. al., 1999), NF-κB (Li et. al., 2000), CRF (Kapturczak et. al., 2003; Takeda et. al., 1994) is activated in response to stressors capable of generating oxidative stress.

Next we investigated whether the activation of these transcription factors was through ROS. The antioxidant NAC is a precursor of reduced GSH and has been shown to scavenge ROS in vascular smooth muscle cells (Meister et. al., 1983;

Neuhauser et. al., 1986; Rao et. al., 1999). A study by Voskoboinik et. al., 1998 showed that GSH synthesis is dependent on NAC in GSH-depleted human umbilical vein smooth muscle cells. Additionally Kyaw et. al., 2004 showed that NAC can modulate the cellular GSH. When they treated vascular smooth muscle cells with buthionine-SR-sulfoximine (BSO) as an inhibitor of γ-glutamyl-cysteine synthetase GSH was decreased, which was replenished back when cells were treated with NAC. This evidence shows that NAC can act as a GSH precursor in these cells. Apart from being a precursor for GSH, NAC can also scavenge hydroxyl radical and hydrogen peroxide (Aruoma et. al., 1989). Carnosol, carnosic acid and rosmarinic acid are antioxidants which have the capability to decrease lipid peroxidation and scavenge various free radicals (Haraguchi et. al., 1995; Wiegant et. al., 1994). A study by Singletary et. al., 1996 showed that supplementation of rosemary extract in the diet of rats resulted in a 3.5- to 4.5-fold increase in liver glutathione-S-transferase and a 3.3- to 4.0-fold increase in liver NAD(P)H-quinone reductase (QR) activities compared to controls. Glutathione S-transferase (GST) and NAD(P)H-quinone reductase are phase II enzymes and have been suggested to be candidates to protect cells against free radical damage. Pretreating cells with rosmarinic acid significantly increased cell viability and decreased the apoptosis rate induced by H_2O_2 which shows the protective effect of rosmarinic acid towards ROS (Gao et. al., 2005). A study by Offord et. al., 1997 showed that rosmarinic acid decreases activation of cytochrome P450 which can induce free radical production through the degradation of xenobiotic compounds. They also showed that carnosol affected expression of the phase II enzyme

glutathione-S-transferase and NAD(P)H: quinone reductase. These enzymes help to the cell to battle against ROS.

We used all four (carnosol, carnosic acid, rosmarinic acid and NAC) of these antioxidants to evaluate their potential to reduce the effect of Cd. Out of these four, NAC was most effective when compared to the other antioxidants in our experiments. The other antioxidants however were still able to exhibit protective effects but not as effective as NAC. Our results with NAC are comparable with previous findings that showed Cd induction of NF-κB decreased when proximal tubule cells were pretreated with NAC (Thevenod et. al., 2000). Carnosol and rosmarinic acid appear more effective compare to carnosic acid to diminish the effect of Cd on HSF, NF-κB and CRF transcription factors. However for HSF, rosmarinic acid is more effective than Carnosol. The literature supports a potential for different potencies of these antioxidants towards different free radical species. For example study by Aruoma et. al., 1992 showed that carnosol is a better hydroxyl radical scavenger compared to carnosic acid. Frankel et. al., 1996 showed that rosmarinic acid has a greater antioxidant capacity compared to carnosol and carnosic acid. Kang et. al., 2003 demonstrated that rosmarinic acid inhibited activation of transcription factor nuclear factor of activated T cells but does not affect AP-1 induced by t-cell antigen receptor. This evidence suggests that antioxidants may act through different mechanisms thereby showing different effectiveness towards a variety of stress inducers.

Transcription factors upon activation bind to DNA and initiate transcription that can lead to increased amount of protein. Therefore the effect of Cd on heat shock

proteins was investigated. HSF transcription factor binds to a heat shock element in the promoter region of a gene to increase heat shock proteins. There are two different kinds of heat shock proteins; constitutive heat shock proteins that remain constantly expressed and inducible heat shock proteins that are increased when a cell is undergoing stress. In normal cells inducible heat shock proteins are rarely expressed. There are various kinds of inducible heat shock proteins including HSP-27, HSP-40, HSP-60, HSP-70/72 and HSP-90. All these proteins are chaperone proteins and help in the proper folding of the proteins. The most abundant HSPs are part of the family whose molecular mass is around 70 ~ 72 k Da and referred to as HSP-70s. The expression of HSPs in particular HSP27 and HSP-70/72 have been shown to enhance the survival of mammalian cells exposed to numerous types of stimuli that induce stress and apoptosis. HSPs enhance the survival of cells exposed to oxidative stress (Arrigo et. al., 1998). In our system, antioxidants were able to block the Cd mediated increase of both HSF and HSP-70 suggesting that the antioxidant affects factors upstream to HSF. Also there was a significant difference in the potency of the antioxidants to prevent Cd's effect. The results with antioxidants on the effect of Cd on HSP-70 mirrored the effect with HSF in that both NAC and rosmarinic acid were most effective however carnosol and carnosic acid failed to show a significant inhibition. Hung et. al., 1998 showed that treatment of 9L rat brain tumor cells with Cd results in the induction of HSP-70. In another study by Nishizawa et. al., 1999 revealed that HSF binding activity to HSE and mRNA of HSP-70 is increased in response to stress which is decreased when treated with antioxidant catalase. These

results compare favorably with our results which show that Cd is able to increase HSF and inducible HSP-70 which is inhibited by the action of antioxidants (Figure 18).

There is evidence from the literature to suggest that HSP-70 can interact with IκB kinase (IKK) complex and thereby can inhibit NF-κB activation. Our results show that there was an increase in HSP-70 but surprisingly this was accompanied by activation of NF-κB. A study by Chen & Ross, 2004 showed that HSP-70 induced by heat shock inhibited NF-κB activation induced by angiotensin. They showed that HSP-70 interacts with IKKa and suppress NF-κB / IκB complex dissociation. Under non- stressed conditions NF-κB is bound to IκB inhibitor but when the cell senses stress it is phosphorylated by IKK complex and NF-κB is detached from IκB inhibitor. NF-κB then translocates to the nucleus and induces transcription. There are three known subunits in the IKK complex: two protein kinases (IKK-α, IKK-β) and a structural/regulatory subunit (IKK-γ). HSP-70 binds IKK-γ to hamper IKK and inhibit NF-κB activation (Chen & Arrigo, 2004). There is some evidence that other heat shock proteins could also down-regulate IKK activity. It has recently been shown that Hsp27 binds IKK-ß and inhibits NF-κB activity (Park et al. 2003). The situation becomes more complex because Hsp90 can also bind IKK-α and IKK-β to form part of IKK complex (Chen et al. 2002). But binding of Hsp90 increased NF-κB activation mediated by tumor necrosis factor. Therefore, although heat shock down-regulates NF-κB activity, this is likely due to a complex interaction between Hsp70–IKK- γ and Hsp27–IKK-β to down-regulate IKK activity, whereas Hsp90–IKK-α and Hsp90–IKK-β interactions up-regulate IKK activity. Because heat shock down-regulates NF-

κB activation, the effects of Hsp70 and Hsp27 on down-regulation of NF-κB must overwhelm the up-regulation of NF-κB by Hsp90 (Ran et. al., 2004) indicating that possibly proteins are involved in this series of events. Thus, more studies are needed in this area to find out what is going on in the system.

Since it had been shown that Cd affects transcription factors it seemed logical to hypothesize that signal proteins, which modulate gene expression are also affected by Cd. There are a number of studies which show that the MAPK cascade is involved in the signaling of stress responsive genes. There are three main family members of this pathway that have been shown to be activated under various stress situations and include JNK, ERK1/2 and p38. Activation of ERK1/2 is generally associated with cell proliferation whereas JNK and p38 are activated during stress response (Hung et. al., 1998). A study by Chuang et. al., 2001 showed that Cd activated JNK through a MKK-7 dependent pathway and reduced ERK1/2 in a CL3 lung carcinoma cell line. Another study by Son et. al., 2001 showed decreased cellular GSH content caused by sulfur amino acid deprivation that potentiated the cytotoxicity induced by cadmium and this potentiation of cytotoxicity resulted from activation of ERK1/2 in conjunction with p38 kinase or JNK. H_2O_2 treatment also induced activation of MAPK protein especially ERK1/2, which plays an important role in cell survival following oxidant injury (Guyton et. al., 1995). Activation of ERK1/2 by the heavy metals Cd and Cu was shown by Jonak et. al., 2004. It has been reported that p38 and ERK signaling pathways can differentially participate in the activation of HSF-1,

which leads to the induction of HSP70 by cadmium in rat brain tumor cells (Hung, et. al., 1998).

To find out that what signal proteins might be involved in the activation of the transcription factor HSF by Cd, we used different protein kinase inhibitors with distinct substrate specificities. SB203580 belongs to a group of pyridinyl imidazole compounds, which specifically bind at or near the ATP binding pocket of p38 and inhibits this kinase (Baeza-Raja, et. al., 2004). SB203580 specifically inhibits the cadmium-induced responses of p38 MAPK and phosphorylation of HSP25 in kidney cells (Hirano, et. al., 2005). PD98058, on the other hand is a flavonoid and a potent inhibitor of MAPK-ERK1/2 kinase. PD98058 selectively binds to and inactivates MEK, inhibiting both the phosphorylation and activation of downstream substrate ERK1/2 (Longuet et. al., 2005). No substrate other than ERK1/2 has been identified for MEK thus it is appropriately used to inhibit ERK1/2 (Cuenda et. al., 1999; Page et. al., 2004). SP600125 is an ATP competitive inhibitor and specifically inhibits JNK activation (Heo. et. al., 2004). Hung, et. al., 1998 showed that 100μM Cd induced HSF activation in rat brain tumor cells which was blocked by PD98058 and SB203580. HSF activation by 60μM Cd however was blocked by only PD98058 which shows that p38 and ERK1/2 pathway differentially participate in HSF activation which leads to induction of HSP-70 by Cd. A study by Chaung et. al., 2000 showed that Cd activated JNK, ERK1/2 and p38 in lung carcinoma tumor cells and that both PD98059 the inhibitor of ERK1/2 and SB202190, the inhibitor of p38 decreased the toxic consequences induced by Cd. Kim et. al., 2005 showed that both

JNK and p38 mediated the activation of HSF-1 in NIH3T3 cells induced by Cd. This evidence shows that JNK, ERK1/2 and p38 can be activated alone or together in response to stress depending on the cell culture system. Results from this study showed that administration of the p38 inhibitor significantly reduced HSF activation and HSP-70 amount. Whereas JNK & ERK1/2 inhibitors failed to show any significant inhibition to HSF activation and HSP-70 amount induced by Cd. Our data suggest an involvement of p38 in the activation of HSF and transcription of HSP-70 induced by Cd. These results support the other reports in the literature that show MAPK signal proteins involvement in Cd induced HSF activation and HSP-70 expression (Hung et. al., 1998; Souza et. al., 2004).

Since the results of this study support the role of p38 in activation of HSF & HSP-70 by Cd, we needed to confirm that Cd was indeed able to induce p38. Phosphorylated p38 is the active form of p38. Results from western blot analysis indicated a time dependent increase in the phosphorylated form of p38 but there was no difference in total p38 amount in the cell indicating that the activity of p38 measured with Cd. These results are comparable with study which indicates that p38 is stimulated by Cd in promocytic cells (Galan et. al., 2000). Evidence exists to indicate that antioxidants can reverse the induction of JNK-MAPK by Cd (Hsiao & Stapleton, 2004). A next step might be to investigate the effect of antioxidants on p38MAPK activation.

In conclusion the results of the present study support the hypothesis that Cd induces stress related transcription factors (HSF, NF-κB and CRF) through a change

in the redox state of the cell. This activation was dependent on the concentration and time of incubation with Cd. Antioxidants decreased the activation of transcription factors and HSP-70 suggesting the involvement of ROS. Cd also activated the p38 MAPK signal protein, and this signal protein appears to be involved in the activation of HSF and HSP-70 in liver cells in culture.

Appendix A

Abbreviations Used in This Thesis

Cd: Cadmium

ROS: Reactive oxygen species

$O_2^{\cdot}$: Superoxide radical

$OH^{\cdot}$: Hydroxyl radical

SOD: Superoxide dismutase

GSH: Reduced glutathione

GSSG: Oxidised glutathione

H_2O_2: Hydrogen peroxide

LDH: Lactate dehydrogenase

NAC: N-Acetyl Cysteine

MT: Metallothionein

HO-1: Heme oxygenase-1

MRE: Metal response element

AP-1: Activator protein-1

HSF: Heat shock factor

HSE: Heat shock element

NF- kB: Nuclear factor – kappa B

IkB: Inhibitor kB

IKK: IkB kinase

MTF-1: Metal regulatory transcription factor-1

HSP-70: Heat shock protein-70

MAPK: Mitogen activated protein kinase

ERK: Extra cellular signal-regulated kinase

JNK: c-Jun NH_2-terminal kinase

CRF: Cadmium response factor

CRE: Cadmium response element

PH-HOL: Plasmid human-heme oxygenase luciferase

Appendix B

Example of Statistical Analysis

I - Analysis of Variance (ANOVA)

Cd	Cd+Carnosol	Cd+Carnacid	d+Roseacid	CD+NAC
100.0000	41.75084175	47.13804714	22.22222222	16.83501684
100.0000	23.98989899	58.33333333	61.61616162	18.83910387
100.0000	17.37089202	70.18779343	105.0505051	19.9592668
100.0000	50	146.9483568	27.70833333	12.26415094
100.0000	25.10638298	40.8839779	54.16666667	7.681940701
100.0000	19.36170213	66.92913386	85.04531722	20.28985507
100.0000	32.21360896	56.69291339	85.04531722	22.22222222
100.0000	37.38156761	25.11111111	29.58199357	47.98387097
100.0000	27.69461078	48	37.62057878	19.35483871
100.0000	46.25748503			

N	10	10	9	9	9
Mean	100	32.11269902	62.24718522	56.45078841	20.6033629
SE	0	3.582662117	11.53025821	9.932660285	3.742035128
Variance	0	128.3546784	1196.521689	887.919663	126.0254421

Before performing Factorial analysis of variance , I will do a Hartley's test for Homogeneity of variance

H0: All variances are equal H1: All variances are not equal

Fcal = Max variance/ Min variance

Fcal = 1196/126
9.49206349

df1 = # of treatment groups, df2 = (n-1) where n is # of observation within a treatment group. If N is unequal then use larger N.

Fcrit (.05, df1 = 5, df2 = 9) = 3.48

The Fcal > Fcrit, therefore accept H1.

Therefore, the data need to be transformed before the ANOVA can be performed

Data Transformation

LOG(SQRT(X))				
Cd(5uM)	Cd+Carnosol	Cd+Carnacid	Cd+Roseacid	CD+NAC
1	0.810332618	0.836685793	0.673393743	0.613106778
1	0.69001421	0.882958397	0.89484732	0.63753012
1	0.61991106	0.923130795	1.010699072	0.650072292
1	0.849485002	1.083582367	0.721305202	0.544318744
1	0.699892075	0.805776572	0.866866055	0.442735475
1	0.643471767	0.912807602	0.964825203	
1	0.754019691	0.876764388	0.964825203	
1	0.786328755	0.699932965	0.735513719	
1	0.721197633	0.840620619	0.787712736	
1	0.832591008			

N	10	10	9	9	5
Mean	1	0.740724382	0.873584389	0.846665362	0.577552682
SE	0	0.024954225	0.034438205	0.040676305	0.038341006
Variance	0	0.006227134	0.010673909	0.014891056	0.007350164

Fcal for transformed data = .01489/0.0063
= 2.36349206 <3.48
The ANOVA can be performed on this transformed data

LOG(SQRT(X))				
Cd(5uM)	Cd+Carnosol	Cd+Carnacid	Cd+Roseacid	CD+NAC
1	0.810332618	0.836685793	0.673393743	0.613106778
1	0.69001421	0.882958397	0.89484732	0.63753012
1	0.61991106	0.923130795	1.010699072	0.650072292
1	0.849485002	1.083582367	0.721305202	0.544318744
1	0.699892075	0.805776572	0.866866055	0.442735475
1	0.643471767	0.912807602	0.964825203	
1	0.754019691	0.876764388	0.964825203	
1	0.786328755	0.699932965	0.735513719	
1	0.721197633	0.840620619	0.787712736	
1	0.832591008			

N	10	10	9	9	5
Mean	1	0.740724382	0.873584389	0.846665362	0.577552682
SE	0	0.024954225	0.034438205	0.040676305	0.038341006
Variance	0	0.006227134	0.010673909	0.014891056	0.007350164
Sum Y	10	7.40724382	7.862259498	7.619988254	2.887763408
Sum Y^2/N	10	5.4867261	6.868347157	6.45158011	1.6678355
Sum Y^2	10	5.542770303	6.953738433	6.570708556	1.697236155
total N	43				
# Treatments	5				
Total Sum Y	35.777255				
Treat Sum	30.4744889				
Total Sum Y^2	30.7644534				
C.F.	29.7677203				

Source	SS	DF	MS	F	Fcrit
Treatment	0.70676854	4	0.176692136	23.15559089	2.3
Error	0.28996458	38	0.007630647		
Total	0.99673312	42			

Ho: all the means are same H1: At least one mean is different

Treatment Fcalc> Fcrit , therefore we reject H0 and accept H1

ANOVA Conclusion : At least one mean is different

II Student-Newman-Keuls Test for Multiple Comparisons

Pooled SE(with unequal N) = SQRT(MS ERROR/((2*N1*N2)/(N1+N2)))

Pooled SE =	cdvs cd+csol	cd vs cs+cacid	cd vs cs+roseacid	cd vs cs+NAC
	0.02762363	0.028380577	0.028380577	0.033831894

Pooled SE =	cd+csol/cd+cacid	cd+csol/cd+racid	cd+csol/cd+NAC
	0.02838058	0.028380577	0.033831894

Pooled SE =	cd+cacid/cd+ra	cd+cacid/cd+nac
	0.02911786	0.034452714

Pooled SE = cd+racid/cd+nac / 0.03445271

LSR common	0.02762363				
df Error =	38				
Range	2	3	4	5	6
q(.05,79)	2.86	3.44	3.79	4.04	4.23
LSR*q	0.07900357	0.095025272	0.10469354	0.111599447	0.116847936

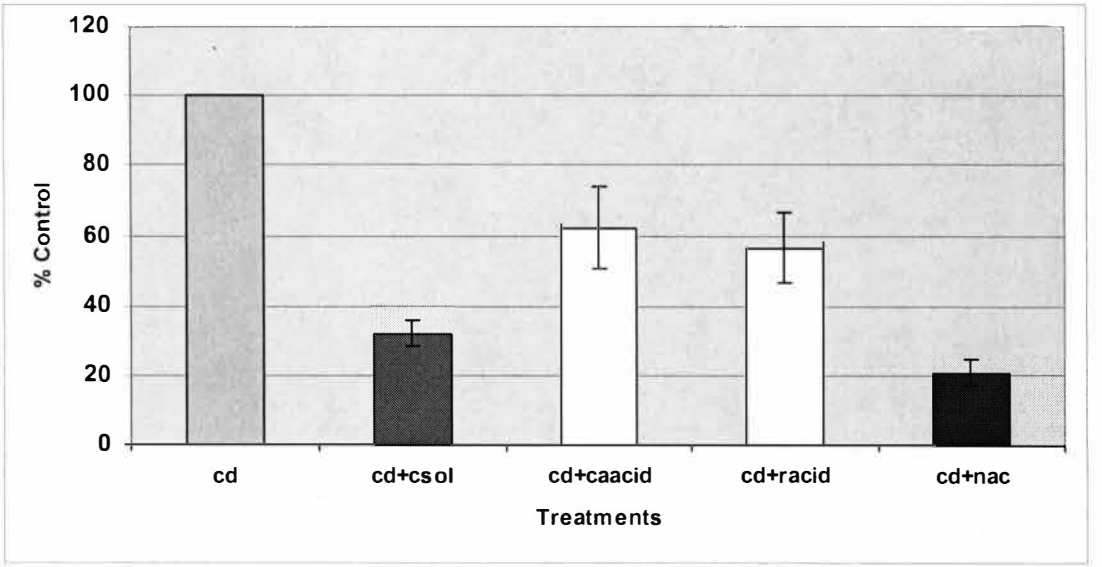
Means(lowest to highest)	cd+nac	cd+csol	cd+racid	cd+caacid	cd
	0.57755268	0.740724382	0.846665362	0.873584389	1

cd vs cs+csol			0.10469354	
cd vs cs+cacid	0.08116845			
cd vs cs+roseacid		0.097629186		
cdvs cd+nac				0.13668085
cd+csolvscd+cacid		0.097629186		
cd+csolvscd+racid	0.08116845			
cd+csol/cd+nac			0.128222877	
cd+cacid/cd+racid	0.08327707			
cd+cacid/cd+nac			0.130575786	
cd+racid/cd+nac		0.118517336		

	2	3	4	5	6
LSR*q	0.079003569	0.095025272	0.10469354	0.111599447	0.11684794

		cd	cd+caacid	cd+racid	cd+csol	cd+nac
		1	0.873584389	0.846665362	0.740724382	0.57755268
cd	1	0	0.126415611	0.153334638	0.259275618	0.42244732
cd+caacid	0.87358439		0	0.026919027	0.132860007	0.29603171
cd+racid	0.84666536			0	0.10594098	0.26911268
cd+csol	0.74072438				0	0.1631717
cd+nac	0.57755268					0

	Mean	SE
cd	100	0
cd+csol	32.11269902	3.582662117
cd+caacid	62.24718522	11.53025821
cd+racid	56.45078841	9.932660285
cd+nac	20.6033629	3.742035128



From this antioxidants Carnosol, Carnosic acid, Rosemarinic acid and NAC are significantly different from Cd and the most effective is NAC.

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