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The Zinc Induction of Heme Oxygenase-1 Gene Promoter In Mouse Hepa Cells

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THE ZINC INDUCTION OF HEME OXYGANASE-1 GENE PROMOTER IN MOUSE HEPA CELLS

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DEDICATION

To my mother and father. May their souls rest in peace.

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AKT	AK (mouse strain) transform (serine threonine protein kinase).
AP-1:	Activator protein-1
ATF:	Activator of transcription
BTB/POZ:	Broad complex, Tramtrack, and Brick a Brac/ poxvirus and zinc finger
CAT:	Catalase
C/EBPB:	CCAAT/enhancer-binding protein B
Cd:	Cadmium
CHO:	Chinese hamster ovary
CNC/bZIP:	Cap'N'Collar/basic leucine zipper
CREB:	CyclicAMP response element binding protein
CTR:	C-terminal region
CS:	Conserved sequence
DGR:	Double glycine region
DMSO:	Dimethyl sulfoxide
DNM:	Dominant negative mutant
EMSA:	Electromobility shift assay
ERK1/2	Extracellular signal-activated protein kinase 1/2
ETC:	Electron transport chain
y-GCS:	gamma-glutamylcysteine synthetase
CPX:	Glutathione peroxidase
GR:	Glucocorticoid receptor
GSH:	Glutathione

GST:	Glutathine-S-transferase
HEK293:	Human embryonic kidney 293
hmox-1	Heme oxygenase-1 gene
HO:	Heme oxygenase
HS:	Heat shock
HSE:	Heat shock element
HSF:	Heat shock factor
HSP:	Heat shock protein
ISRE:	Interferon-stimulation response element
IVR:	Intervening region
JNK:	c-Jun N-terminal kinase
Keap1:	Kelch-like ECH-associated protein 1
LPS:	Lipopolysaccharide
MEF:	Mouse embryonic fibroblast
MT:	Metallothionein
MyT1:	Myelin transcription factor1
NES:	Nuclear export sequence
NQO1:	NAD(P)H:quinine oxidoreductase
NF-E2:	Nuclear factor-erythroid 2
NLS:	Nuclear localization signal
Nrf-2:	NF-E2 related factor
15d-PGJ2:	15-deoxy-Δ12,14-prostaglandin J2
PKC	Protein kinase C

PMSF:	Phenylmethylsulfonyl fluoride
P38	Phosphatase -38
PI-3K	Phosphatidylinositol 3-kinase
PPAR/RX/R:	Peroxisome proliferators-activated receptor/retinoid X receptor
RD:	Regulatory domain
RIPA:	Radioimmune precipitation assay
ROS:	Reactive oxygen species
RNAi:	Ribonucleic acid interference
shRNA:	small hairpin ribonucleic acid
SOD:	Superoxide dismutase
STAT5:	Signal transducer and activator of transcription
StRE:	Stress Response Element
tBHQ:	Tert-butylhydroquinone
TNF-V:	Tumor Necrosis Factor-alpha
TPA:	12-O-tetra-decanoylphorbol-13-acetate
TRX:	Thioredoxin
UV:	Ultra-violet
WCE:	Whole cell extract
Zn:	Zinc

ABSTRACT

Heme oxygenase-1 (HO-1) catalyzes the initial and rate-limiting reaction in heme catabolism (yielding equimolar quantities of iron, carbon monoxide, and biliverdin), which is further metabolized to bilirubin by biliverdin reductase. HO-1, an important enzyme with antioxidant and cytoprotective properties, functions against oxidative stress generated in response to heme and to various stress stimuli, such as zinc. The cytoprotective function of HO-1 is dependent on both the activities of the reaction products and on the inducibility of the heme oxygenase gene (*hmox-1*), which is regulated primarily at the level of gene transcription by two distal enhancers, E1 and E2. Characterization of the enhancers led to the identification of multiple stress response elements (StREs) that are necessary and sufficient for *hmox-1* gene regulation by almost all inducers tested. Studies from this laboratory identified Nrf-2 as a dominant regulator that mediates activation of *hmox-1* via the StRE motifs by several inducers, including zinc.

Accumulating evidence from work done in our lab suggested that Nrf-2 was partly regulated by subcellular compartmentalization in the cytoplasm and transported to the nucleus upon stimulation by stress agents to activate target genes like *hmox-1*. Investigation of the subcellular compartmentalization and cytoplasmic-nuclear transport of Nrf-2 in Hepa cells revealed that Nrf-2 is a highly labile protein that is rapidly and specifically degraded by the ubiquitin-proteasome pathway, and that zinc stimulation results in Nrf2 stabilization. Furthermore, transactivation of E1 luciferase reporter activity was not completely abolished when all three StREs were mutated, suggesting that other transcription factors and cis-acting DNA elements may be involved in *hmox-1* regulation. Examination of the *hmox-1* sequence revealed other conserved regions within E1. My analysis of the conserved sequences (CS) led to identification of a functional heat shock element (HSE) in the 5' end of E1that had no response to zinc.

Findings suggest ERK1 plays an important role in inducer-dependent regulation of *hmox-1* by zinc through its action (phosphorylation of Nrf-2 and cooperation between the StRE and Nrf-2 induce *hmox-1*). In conclusion, targeting Nrf-2-ERK1 individually or together allows for controlled regulation of *hmox-1* expression and illuminates new avenues of research and strategies for therapeutic intervention and treatment in diseases involving oxidative stress and HO-1 expression.

1. INTRODUCTION

1.1 Zinc and the Environment

Humans are exposed to zinc from numerous industrial and environmental sources, mainly through ingestion. In industry, zinc is used in steel coating and in making dry batteries. Zinc, as a pollutant from industry, exists in two forms: as solid dust and in water. We ingest zinc into our bodies through drinking water and eating seafood (King and Keen, 1999; Lonnerdal, 2000). Ingested, or absorbed, zinc is stored in soft tissues, especially in the liver and kidney (Ho and Ames, 2002). The recommended daily allowance (RDA) of zinc is 15 milligrams/day for males and 12 milligrams/day for females (King and Keen, 1999).

It is well known that micronutrients in the diet, such as zinc, can influence human development. Zinc deficiency can lead to immune-suppression, delayed sexual maturation, and poor vision. Increased levels of zinc can lead to anemia because of harmful effects on red blood cells (Berg and Shi, 1996; Salgueiro et al., 2000). Also, exposure to zinc in the workplace contributes to progression of renal diseases (Wittman, and Hu 2002)

1.2 Effects of Zinc on DNA Repair

Recent studies have demonstrated that ultraviolet (UV) radiation exposure increases production of reactive oxygen species, which has harmful effects on the skin. The defense mechanism that humans have developed is to a pathway in which glutathione, selenium, and zinc are utilized in the cellular action against oxidative damage by UV. Thiols and selenium protect cells against UV radiation with a synergistic interaction through an increase in glutathione peroxidase (CPX) activity (Beani, 2001).

Zinc supplementatioalso has anti-carcinogenic activity in human colon cells exposed to the well–known carcinogen, cadmium (Cd). In this situation, a DNA repair activity known to play an important role in colon cancer can be reversed by zinc supplement, which acts on the

DNA mismatch repair (MMR) (Lutzen et al., 2004). This property of zinc has been explained by the inhibition of mismatch DNA repair of small misalignments and base-base mismatches (Beyersmann and Hechtenberg, 1997; Hartwig and Schwerdtle, 2002; McMurray and Tainer, 2003; Lutzen et al., 2004). The zinc finger protein ZEC, which is strongly expressed in the testis, is involved in DNA damage recognition during nucleotide-excision repair (Chen et al., 1995; Asmuss et al., 2000). Hartwig and Kopera demonstrated that this inhibition of xeroderma pigmentosum, complementation group A (XPA) is due to a substitution of protein-bound zinc with cadmium (Hartwig and Schwerdtle, 2002; Kopera et al., 2004; Stewart, 2005).

All the previously mentioned studies demonstrated that zinc directly or indirectly plays a vital role in the DNA repair system.

1.3 Zinc Effects on Apoptosis

A recent study showed that zinc, by down-regulating p53 activity, through inhibition of the ERK1/2 downstream signaling pathways promoted apoptosis in epithelial breast cancer cells by depolarizing the mitochondrial membrane. (Ostrakhovitch and Cherian, 2005).

After exposure to isomeric Zn(II) N-methylpyridylporphyrins (ZnTM-2(3,4)-PyP4 +), colon adenocarcinoma cells showed morphological changes consistent with both necrotic and apoptotic death sequences, depending on the presence of oxygen (Al-Mutairi et al., 2007).

. Zinc has been shown to induce apoptosis in several animal cells. For example, in a study of rat hippocampal neurons, the protective effect of growth inhibitory factor (GIF) against zinc-induced neuronal death was demonstrated.(Liu et al., 2004)

In a similar study using PC12 cells, intracellular accumulation of Zn, induced by the synergy of zinc and pyrithione, leads to an increase in apoptosis (Seo et al., 2001).. However, exposure to zinc does not always increase apoptosis. In a different study, zinc appears to have

anti-apoptitic activity on SH-SY5Y neuroblastoma cells by an ERK1/2 signal that reduces the mitochondria membrane potential effects (An et al., 2005).

In 2005, Zago et al. showed that, in zinc-deprived conditions using IMR-32 cells in a model for neuronal cells, there are two ways in which zinc acts. First, zinc acting on ERK1/2 and p38, induces apoptosis by increasing the N-terminal kinase (JNK). This is due to zinc deprivation. In other words, zinc negatively regulates JNK . Secondly; the supplementation of zinc has no effect on apoptosis. From these studies, zinc can induce apoptosis. Since Erk inhibition leads to release of the apoptosis-induced factor (AIF), the inhibition of ERK can lead to apoptosis via nuclear relocation after depolarizing of the mitochondrial membrane. This will inhibit the p53 expression and decrease cell life.

1.4 Zinc Effects on Redox Homeostasis

Recent studies of a colon-derived cell line showed that increasing intracellular zinc concentration has a protective effect on colon mucosa. In another study, Martelli and Moulis (2004) demonstrated that increases of both zinc and cadmium act on the thiol group and iron regulatory proteins (IRP). This will increase the level of iron and, subsequently, improve the function of erythrocytes through what is known as repairing of ion homeostasis (Blazovics et al., 2004).

Hao and Maret (2005), demonstrated that maintaining zinc homeostasis is critical in neurological disease where the zinc and zinc donor/acceptor pair (metallothionein/thionein), respectively, play the major role in maintaining the zinc homeostasis, based on the fact that zinc has no inert redox activity.

From these findings, zinc supplementation,alone has no direct effect on distribution, and redistribution play a critical role in redox homeostasis.

1.5 Reactive Oxygen Species (ROS), Oxidative Stress, and Disease

Zinc enters the body primarily through ingestion and is stored in soft tissues, especially in the liver and kidneys. Among stress response genes are those encoding metallothionein (MT), heat shock proteins (HSP), proteins involved in oxidative stress, and glutathione (GSH) and related proteins (Stewart, 2005). Induction of these stress response genes is considered a cell-protective mechanism (Ho and Ames, 2002; Stewart, 2005).

Zinc, typical of other stress-related agents, stimulates transcription factors that interact with binding domains in the StRE. In addition, zinc acts on the signal transduction pathways by activating cellular protein kinases, which "results in increased phosphorylation of transcription factors leading to the activation of target genes" (Stewart, 2005). Therefore, zinc stimulates the transcription factor activity.

ROS generation (either endogenous or exogenous) results of normal cellular respiration, inflammation, infection, tissue damage, and in response to stress-promoting stimuli, such as cadmium (Ambrosone, 2000; Cadenas and Davies, 2000; Salvemini and Cuzzocrea, 2002; Stewart, 2005). Radical derivatives of molecular oxygen can interact with metals to form ROS (Halliwell et al., 2000; Nordberg and Arner, 2001). ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\bullet$), are generated and degraded by all aerobic organisms (Figure 1.1) (Fang et al., 2002; Mates, 2000). In his dissertation, Stewart (2005) states:

> An increase in ROS formation leads to disruption of cellular homeostasis because of damage to cell components: i.e., protein modification, lipid peroxidation, membrane disruption, and DNA damage (including DNA strand breakage, DNA base modification, and DNA-protein cross-linkage) (Dean et al., 1997; Nordberg and Arner, 2001; Olinski et al., 2002; Marnett et al., 2003). In an environment with sufficient antioxidants, cells detoxify ROS and repair the cellular damage However, a continuing imbalance between ROS production and detoxification results in cellular oxidative stress (Mates, 2000; Davis et al., 2001) (Figure 1.2). Many human pathological conditions such as Alzheimer's

> disease, ischemia/reperfusion injury, diabetes mellitus, atherosclerosis, hypertension, acute-respiratory distress syndrome, and cancer have been associated with oxidative stress (Nordberg and Arner, 2001; Cho et al., 2002; Olinski et al., 2002). (p. 2)

1.6 Oxidative Stress – The Cellular Response

In study by Alam et al ,demonstrated , \ that zinc has direct effects on changes multiple cellular processes that although largely secondary to the perturbation of redox homeostasis, result in ROS production and cellular oxidative stress (Martindale and Holbrook, 2002; Stewart, 2005). Stewart (2005) also stated:

> To maintain cellular homeostasis and counter ROS damage, cells use both exogenous and endogenous antioxidants to detoxify ROS and break the chain of free radical formation. Exogenous mechanisms include vitamins and plant-derived phenolic antioxidants. Endogenous mechanisms include the expression of a select set of genes that encode cytoprotective proteins (Wild et al., 1999; Fang et al., 2002). (p. 13)

Superoxide, hydrogen peroxide, and other peroxides, including antioxidant molecules and enzymes that are direct scavengers, such as reduced GSH, glutathione peroxidase (CPX), Cat, MT, and SOD (Figure 1.2) are effective defenses against ROS damage . (Dharkshinamoorthy and Jaiswal, 2000; Dharkshinamoorthy et al., 2000; Mates et al., 2000; Nordberg and Arner, 2001; Stewart, 2005). A continuous ROS imbalance results in toxic amounts of ROS and oxidative stress, requiring detoxification or removal of toxins by induced antioxidant enzymes, e.g., NAD(P)H:quinine oxidoreductase (NQO1), -glutamylcysteine synthetase (y-GCS), thioredoxin (TRX), and glutathione S-transferase (GST) (Stewart, 2005). These antioxidant enzymes metabolize ROS and toxic compounds into forms that are readily excreted by the cell (Figure 1.2) (Itoh et al., 1997; Moinova and Mulcahy, 1999; Wild et al., 1999; Mates, 2000; Kim et al., 2003).

Recent studies have identified heme oxygenase-1 (HO-1) as another inducible enzyme with antioxidant and cytoprotective functions (Choi and Alam, 1996; Alam and Cook, 2003).

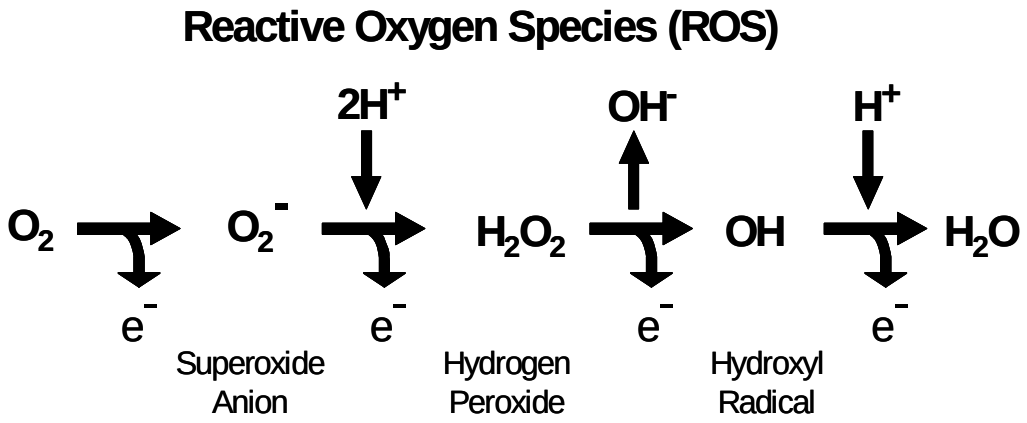

Figure 1.1 Generation of ROS. the products of heme–catalyzed reactions. The peroxyl and alkoxyl radicals species may account for the peroxyl radical generation during decomposition catalysed by heme.

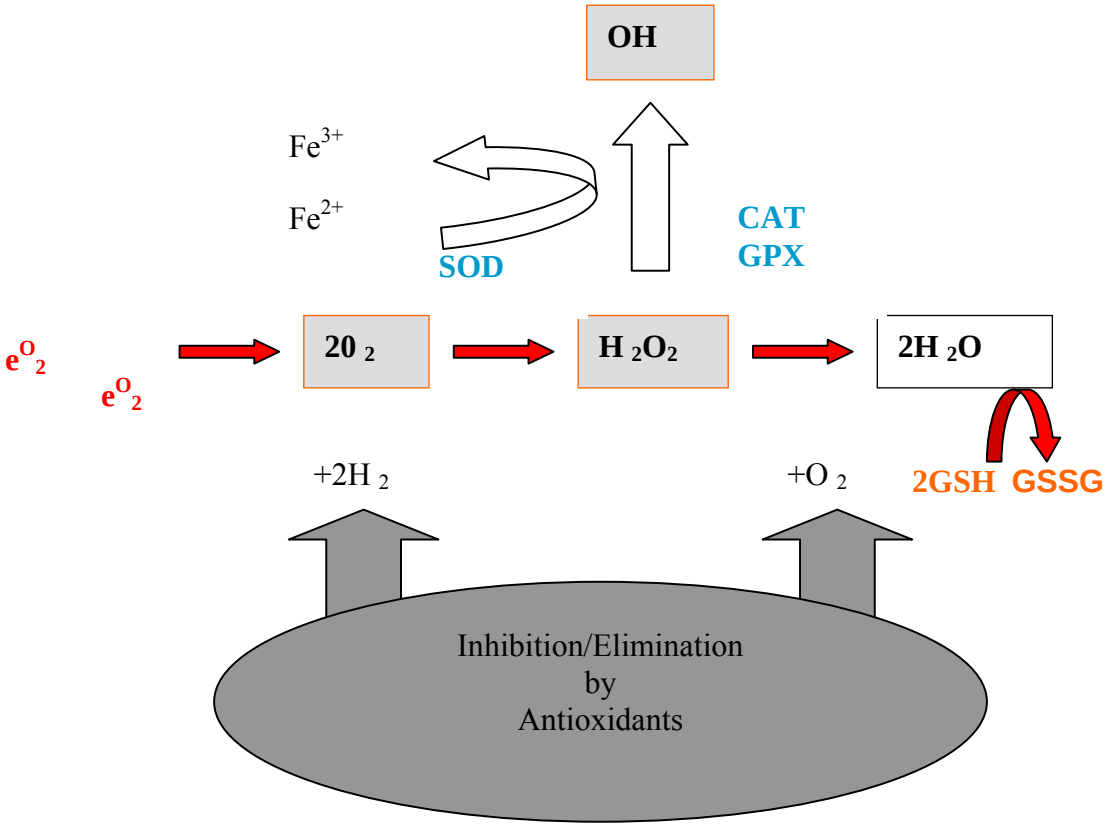

Figure 1.2 Detoxification of ROS by Antioxidant Enzymes. ROS are generated during cellular aerobic metabolism and/or when cells are exposed to various stress conditions (UV light, gamma radiation, environmental pollutants such as metals and xenobiotics, inflammatory cytokines, enzymatic disorders). ROS are radicals like superoxide anion and the hydroxyl and peroxyl radicals, and also include hypochlorite ion (OCl^-) and hydrogen peroxide (H_2O_2). They play important roles both in homeostatic mechanisms and pathogenic processes (IMAGE FROM *www.meduniwien.ac.at/.inner-med-1/krebsforschung/science/staff/academy/../imgs/elbling-fig1.gif*)

1.7 Heme Oxygenase-1 and Cellular Homeostasis

From the discovery of the HO-1 protein in the 1960s until recently, it was thought that the essential biological function of HO-1 was heme metabolism (Choi and Alam, 1996; Stewart, 2005). Heme, a compound containing iron, facilitates vital cellular functions by functioning as the prosthetic group of proteins such as, hemoglobin, myoglobin, oxydemoglobin, cytochromes, catalase, guanylate cyclase, and nitric oxide synthase (Maines, 1988; Schwartsburd, 2001; Stewart, 2005). Hemoproteins deliver oxygen and enable mitochondrial respiration and signal transduction (Maines, 1988; Nath et al., 1996; Schwartsburd, 2001; Stewart, 2005). When cells are destroyed or damaged, lipophilic heme is released from its linkage with such proteins. Free heme ─with its reactive iron ─ acts as a pro-oxidant and generates ROS, causing DNA damage, protein denaturation, and lipid peroxidation (Vincent, 1989; Balla et al., 1991; Nath et al., 1996; Stewart, 2005). HO-1 regulates intracellular concentrations of heme in major tissues via the turnover of heme-binding proteins. Therefore, the highest concentrations of HO-1 are found in the spleen, liver, and kidneys, performing the critical function of hemoglobin-heme degradation during the turnover of erythrocytes (Maines, 1988; Choi and Alam 1996; Poss and Tonegawa, 1997a, 1997b; Stewart, 2005).

As Stewart (2005) wrote:

> HO-1 enzymes catalyze the initial and rate-limiting step in the oxidative cleavage of b-type heme molecules to yield equimolar quantities of iron, carbon monoxide, and biliverdin IX*a*, which is then metabolized further to bilirubin by biliverdin reductase (Figure 1.3) (Choi and Alam, 1996; Elbirt and Bonkovsky, 1999; Montellano, 2000; Otterbein and Choi, 2000). Two enzymatically active isoforms of HO, HO-1 and HO-2, encoded by distinct genes, have been identified and localized to the endoplasmic reticulum (Maines, 1997; Choi and Alam, 1996; Alam and Cook, 2003). (p. 18)

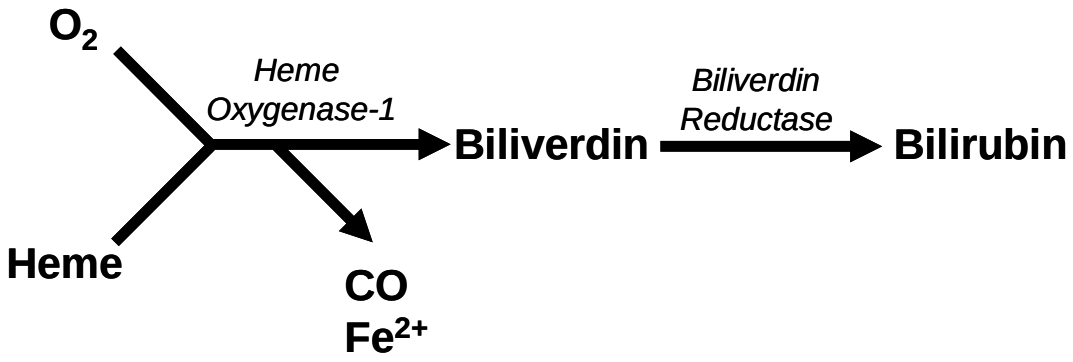

Figure 1.3 Heme Degradation Pathway. Heme Oxygenase-1 (HO-1) degrades heme into iron, biliverdin (BVR), and carbon monoxide (CO). Biliverdin reductase (BVR) converts biliverdin into bilirubin.

Recent studies have convincingly shown that HO-1 is also a cytoprotective enzyme that acts as both an antioxidant and an anti-inflammatory, and also has a role in cell signaling and cellular homeostasis in response to injury and oxidative stress (Choi and Alam, 1996; Alam and Cook, 2003; Stewart, 2005). Stewart (2005) deduced these roles as a result of several observations about the HO-1 inducers and the by-products of heme degradation:

> 1) In addition to the substrate heme, HO-1 expression is induced by a variety of stress conditions and agents including ultraviolet irradiation (UV), hyperthermia, lipopolysaccharides (LPS), inflammatory cytokines, heavy metals, hydrogen peroxide, hypoxia, hyperoxia, endotoxins, oxidized low-density lipoproteins, and cell and tissue injury (Choi and Alam, 1996; Maines, 1997; Montellano, 2000; Otterbein and Choi, 2000; Alam and Cook, 2003).
> 2) These and other stimuli generate cellular oxidative stress by producing ROS and/or modifying GSH levels (Abraham et al., 1995; Choi and Alam, 1996).
> 3) Heme degradation by HO-1 eliminates a pro-oxidant heme and simultaneously generates products that are antioxidants, biliverdin and bilirubin (Stocker, 2004).
> 4) The release of heme-iron, which acts as an effective catalyst in the generation of ROS, induces the synthesis of the iron-sequestering protein ferritin and reduces the level of transferring receptor (TfR), which is involved in the uptake of iron (Ward et al., 1984; Choi and Alam, 1996; Arosio and Levi, 2002).
> 5) CO, a diffusible gas, functions as a neural messenger and has vasodilatory, antiproliferative, and anti-inflammatory properties (Choi and Alam, 1996; Maines, 1997; Otterbein and Choi, 2000). Thus, HO-1 activation appears to be a general response against oxidative stress in cells and tissues caused by various stressful stimuli and conditions. (p. 18)

HO-1 functions as an anti-oxidative, an anti-inflammatory, and a cytoprotective enzyme, as observed in multiple studies utilizing both animal and cell culture models of injury or disease (Choi and Alam, 1996; Stewart, 2005). For instance, Nath et al. (1996) showed that, in rats, release of heme from myoglobin using glycerol resulted in kidney failure and death; whereas induction of HO-1 by preinfusion of hemoglobin avoided kidney failure and a reduced mortality (Nath et al., 1998; Stewart, 2005). In yet another experiment, rats pretreated with hemoglobin had increased survival after a lethal injection of LPS, which coincided with increased HO-1 expression (Otterbein et al., 1995; Stewart, 2005). Stewart (2005) also stated:

> Induction of HO-1 by a sublethal dose of UVA protects human skin fibroblasts against a subsequent lethal dose of UVA, whereas administration of anti-sense oligonucleotides directed against HO-1 transcripts attenuated the cytoprotective response (Vile et al., 1994). In an *in vitro* cell study, ectopic expression of HO-1 in cultured endothelial cells provided protection against heme and hemoglobin toxicity (Abraham et al., 1995). Furthermore, neurons over-expressing HO-1 resisted oxidative stress-induced cell death, and over-expression of HO-1 inhibits tumor necrosis factor-alpha (TNF-α) induced apoptosis (Stocker et al., 1990; Goodman et al., 1997; Poss and Tonegawa, 1997a, 1997b; Petache et al., 2000). (p. 19)

Although the protective function of HO-1 has been documented in the oxidative stress-mediated injury of cellular and animal models, the biological importance of HO-1 is most evident in the physiological abnormalities (i.e., growth retardation, anemia, leukocytosis, and tissue iron deposition) observed in mice and a single human with HO-1 deficiency (Choi and Alam, 1996; Poss and Tonegawa, 1997a, 1997b; Kawashima et al., 2002; Stewart, 2005). Also, mating between heterozygous HO-1 mice results in partial prenatal lethality of the HO-1 deficient mice; homozygous mating pairs do not yield viable litters (Stewart, 2005).

Furthermore, when endotoxin was administered to HO-1 deficient mice, the result was an increase in liver injury and mortality; (Montellano, 2000). Likewise, HO-1 deficient embryo fibroblasts cells were hypersensitive to heme, H_2O_2, cadmium, and hydrogen peroxide treatment

and exhibited increased intracellular ROS production, which indicates that HO-1 expression is important in the defense against oxidative stress(Choi and Alam, 1996; Maines, 1997).

1.8 Regulation of the *hmox*-1 Gene

As previously stated, in addition to heme and the various extracellular agents listed above, HO-1 expression is also upregulated under certain physiological conditions, such as exercise and pregnancy, or the pathophysiological conditions associated with increased ROS production (inflammation, sepsis, ischemia-reperfusion injury, atherosclerosis, diabetes mellitus, AIDS, and Parkinson's and Alzheimer's diseases) (Choi and Alam, 1996; Pilegaard, 2000; Cosso et al., 2001; McLaughlin et al., 2003; Stewart, 2005). Although not all agents or conditions have been tested, accumulating data supports the idea that HO-1 induction by most, if not all, stimuli is regulated at the level of gene transcription (Choi and Alam, 1996; Alam and Cook, 2003; Stewart, 2005).

The work in our lab on *hmox-1* gene has been most extensively done on the mouse. The coding portion of the mouse *hmox-1* gene is approximately 7 kilobase pairs (kbp) in length. It is organized into 5 exons and 4 introns (Figure 1.4) (Alam et al., 1994; Stewart, 2005). Functional analyses of the mouse *hmox-1* promoter enabled the identification of two 5' distal enhancers, E1 and E2, located approximately -4 and -10 kbp, respectively, upstream from the transcription start site (Figure 1.4) (Stewart, 2005). These distal enhancers exhibited both basal level and inducer-dependent transcriptional activities (Alam and Zhining, 1992; Alam et al., 1994; Alam et al., 1995; Choi and Alam, 1996; Stewart, 2005).

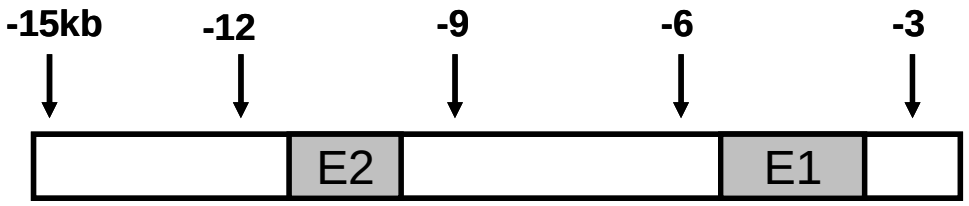

Figure 1.4 Enhancer elements (E1, E2) localization within the hmox-1 Gene. Proximal Promoter (PP) cis-elements localized within the hmox-1 gene PP and enhancer regions (E1 and E2). Exons are designated by rectangles; open segments indicate 5' and 3' untranslated regions. +1, transcription start site. Numbers are in base pairs.

The mapping of the E1 and E2 enhancers identified a dominant *cis*-acting element, called the stress response element (StRE), present in multiple copies within each enhancer (Stewart, 2005). The StRE's importance resulted from its role in *hmox-1* induction in response to most agents tested, including $CdCl_2$, $HgCl_2$, 12-O-tetra-decanoylphorbol- 13-acetate (TPA), hyperoxia, heme, LPS, and H_2O_2 (Choi and Alam, 1996; Alam and Cook, 2003; Stewart, 2005). The sequence, location, and number of the StREs between the mouse, rat, and human *hmox-1* genes were conserved, further pointing to the importance of these elements (Alam and Cook, 003; Alam et al., 2004: Stewart, 2005).

Stewart (2005) wrote:

> Sequence comparison and mutation analyses have identified the minimal consensus StRE as the 10-bp sequence (T/C)GCTGAGTCA (Prestera et al., 1995; Choi and Alam, 1996; Inamdar et al., 1996). The consensus closely resembles the consensus binding site for various families of transcription factors, including AP-1, CREB/ATF, Maf, Cap'N'Collar-basic-leucine zipper (CNC-bZIP), and Bach families of transcription factors, all of which belong to the bZIP class of sequence-specific DNA-binding proteins (Table 1.1). This similarity suggested that one or more members of these families of proteins may function as StRE-binding proteins and inducer-dependent *hmox-1* activators. (p. 20)

1.9 Nrf-2 Regulation of the *hmox*-1 Gene

During a previous study in our lab using various experimental strategies, Nrf-2 (NF-E2 related factor 2) ─ a member of the CNC-bZIP family ─ was identified as the dominant regulator

of *hmox-1* gene activation in response to multiple inducers (Stewart, 2005). Those inducers included heme, cadmium, cobalt, curcumin, 15-deoxy-Δ12,14 – prostaglandin J2 (15d-PGJ2), and arsenite in multiple cell types, including fibroblast, hepatoma, human mammary epithelial, and Chinese hamster ovary (CHO) cells (Alam et al., 1999; Alam et al., 2000; Gong et al., 2001; He et al., 2001; Gong et al., 2002a, 2002b; Balogun et al., 2003: Stewart, 2005).

Many of the agents listed above increased the StRE-binding activity of Nrf-2, as studied by electrophoretic mobility shift assays (EMSA) (Stewart, 2005). Also, transfection of fibroblast cells with candidate transcription factors showed that members of only the CNC/bZIP family of proteins effectively activated an E1 luciferase reporter construct; Nrf2- displayed the strongest activity (Alam et al., 1999; Stewart, 2005).

Stewart (2005) wrote:

> Conversely, overexpression of the dominant negative mutant (DNM) form of Nrf2 inhibits E1 activity and endogenous HO-1 mRNA expression in the above cell types in response to various inducing agents, including cadmium, 15d-PGJ2, heme, arsenite, and cobalt (Alam et al., 1999; Alam et al., 2000; Gong et al., 2001; He et al., 2001; Gong et al., 2002a, 2002b). Additional studies (Ishii et al., 2000) demonstrated that macrophages derived from Nrf-2-deficient mice exhibit reduced *hmox-1* gene activation by several stimuli, including cadmium. In contrast, overexpression of Nrf-2 in several cell lines increased the expression of cytoprotective genes, including *hmox*-1 (Alam et al., 1999; Kim et al., 2003; Shih et al., 2003). These observations suggest that Nrf-2 is an essential regulator of *hmox-1* gene activation during the adaptive response to oxidative stress. (pp. 24-25)

1.10 Structural Characteristics of Nrf-2 (NF-E2):

in head and neck development in drosophila (Mohler et al., 1991). Stewart (2005) also stated that

The transcription factors that regulate activation of stress-responsive genes encoding proteins with antioxid oxidative stress. Our laboratory studies have identified Nrf-2 as a key stress-responsive transcription factor (Alam et al., 1999; Stewart, 2005). Nrf-2, similar to other members of the CNC-bZIP family, contains a Cap'N'Collar (CNC) domain "homologous to a region within the fruit fly homeotic selector protein encoded by the CNC gene" (Stewart, 2005), which is involved ant and cytoprotective activities are central to the response to cellular

Table 1.1 Transcription Elements Similar to StRE[1]

Element	**Consensus Sequence**	**Canonicol Binding Proteins**	**Reference**
StRE	TGCTGA GTCAC		Choi and Alam, 1996
NF-E2 binding site	TGCTGA GTCACCCT	CNC-bZIP/Maf heterodimers	Andrews, 1998
Maf binding site	TGCTGA GTCAGCA TGCTGACGTCAGCA	Maf	Kataoka et al., 1994
ARE: antioxidant response element	GCNNN CTCACC T		Rushmore et al., 1991
TRE: TPA response element	TGA GTCA C	AP-1 (Fos/Jun)	Karin et al., 1997
CRE:cAMP response element	TGACGTCA	CREB/ATF	Hai et al., 1999

[1] Stewart, D. (2005). Mechanism of *Hmox-1* induction by cadmium. Unpublished doctoral dissertation, Louisiana State University, New Orleans. (p. 24)

> Nrf2 also contains a potent N-terminal transcription activation domain (TAD), a nuclear localization signal (NLS), a nuclear export signal (NES), and two conserved structural motifs, the C-terminal leucine dimerization (LZ) and basic DNA-binding domains (DBD) (Figure 2.1) (Alam et al., 1999; Alam et al., 2004; Shen et al., 2004; Jain et al., 2005). The dimerization domain consists of a heptads repeat with leucine residues every seventh position, the leucine zipper that is necessary for polypeptide dimerization, and an adjacent DNA-binding domain enriched in basic amino acids (Kouzarides and Ziff, 1988; Struhl, 1989). (p. 28)

The chief structure within the leucine zipper region of Nrf-2 prevents self-dimerization. Therefore, Nrf-2 heterodimerizes predominantly with: small, Maf proteins (MafF, MafG, andMafK); other bZIP proteins, including c-Jun of the AP-1 family; and ATF-4 (activator of transcription 4) (Venugopal and Jaiswal, 1998; Nguyen et al., 2000; He et al., 2001, Alam and Cook, 2003), a member of the ATF/CREB subfamily (Venugopal, 2001; Stewart, 2005).

1.11 Physiological Significance of Nrf-2

New data indicate that Nrf-2 acts as a primary regulator of the adaptive response to oxidative stress (Alam and Cook, 2003; Alam et al., 2004; Stewart, 2005). Recent studies show that Nrf-2 regulates the inducible expression of genes encoding enzymes with cytoprotective functions such as NQO1, GST, TRX, y-GCS, ferritin H, and HO-1 (Venugopal and Jaiswel, 1998; Wild et al., 1999; Jeyapaul and Jaiswal, 2000; Dhakshinamoorthy et al., 2000a, 2000b; Pietsch et al., 2003; Alam and Cook, 2003; Stewart, 2005). These enzymes work together as a cellular defense apparatus that "scavenges ROS, detoxifies electrophiles, and xenobiotics," (Stewart, 2005) and maintains intracellular redox homeostasis (Thimmulappa et al., 2002; Lee et al, 2003; Shih et al., 2003).

Nrf-2-deficient mice develop normally, but are extremely sensitive to butylated hydroxytoluene, develop acute-respiratory distress syndrome: are prone to acetaminophen hepatotoxicity; and present an increased burden on benzo[a] pyrene-induced gastric neoplasia (Chan et al., 2001; Enomoto et al., 2001; Ramos-Gomez et al., 2001; Stewart, 2005).

In addition, Stewart (2005) stated that:

> . . . macrophages derived from such mice "exhibit reduced resistance to toxic electrophiles (Ishii et al., 2000). Consistent with this idea, deletion of the Nrf-2 gene in mice shows a substantial decrease in antioxidant gene expression accompanied by an increased susceptibility to carcinogenesis (Ishii et al., 2000; Ramos-Gomez et al., 2001; Enomoto et al., 2001; Lee at al., 2003). These studies indicate that Nrf-2 provides an important mechanistic link between oxidative stress and antioxidant gene expression in supporting cell survival and cellular homeostasis. (p.31)

1.12 Nrf-2 Activity Regulation

There is quite a bit known about the regulation of Nrf-2 target genes, such as *hmox-1*, but the "mechanisms by which stress agents regulate Nrf2 activity are not well characterized'" (Stewart, 2005). Laboratory evidence indicates that Nrf2 activity is, at least partially, regulated at the level of subcellular compartmentalization (Itoh et al., 1999; Dinkova-Kostova et al., 2002; Sekhar et al., 2002; Zipper and Mulcahy, 2002; Stewart, 2005). Nrf-2 exists normally in an inactive form in the cytoplasm by binding to a protein, Keap1 (Kelch-like ECH-associated protein 1) (Itoh et al., 1999; Dhakshinamoorthy and Jaiswal, 2001; Kobayashi et al., 2002; Sekhar et al., 2002)..

After Nrf-2 has been stimulated by oxidative stress promoting agents, it is liberated from Keap 1 suppression. This allows Nrf-2 to transfer to the nucleus where it activates target genes, by a currently uncharacterized mechanism, but one that perhaps involves PKC-mediated phosphorylation of Nrf-2 (Huang et al., 2002; Stewart, 2005). This mechanism is similar to the cytoplasmic-nuclear transport mechanism of transcription factors NF-κB and p53 (Crook et al., 1998; Kau and Silver, 2003; Stewart, 2005).

Another mechanism of regulation may occur at the level of DNA binding. The association and DNA-binding of Nrf-2 Jun dimers requires a cytosolic factor, which has, to date, remained uncharacterized (Venugopal and Jaiswal, 1998; Jaiswal, 2000; Jeyapaul and Jaiswal,

2000; Stewart, 2005). Whether this factor is responsive to oxidative stress and how it regulates nuclear activity remain to be seen (Stewart, 2005). Stewart (2005) also stated that:

> Recent studies by Kwak et al. (2002) have described a positive feedback mechanism in which *Nrf-2* autoregulates its own expression via an antioxidant response element-like sequence within the Nrf2 gene in response to 3H-1,2 –dithiole-3-thione, an anti-carcinogen. (p. 32)

The goal of this research is to investigate the subcellular characteristics and cytoplasmic-nuclear transport of Nrf-2 in Hepa cells. It will be observed if various HO-1 inducers actually increase the steady-state level of Nrf-2. In the present study, an alternative mechanism of Nrf-2 regulation is described.

1.13 Summary and Rationale for Studies

In summary, ROS produced during cellular metabolic activity, or in response to exogenous agents, are detrimental to cellular structures as they damage lipids, proteins, and DNA. Two environmental pollutants, the cadmium and zinc, are toxic and carcinogenic. Zinc alters multiple cellular processes, and the generation of ROS and oxidative stress. Disruption of cellular activities by excessive ROS leads to an adaptive response involving the coordinate induction of a select set of genes that encode proteins with antioxidant and cytoprotecitve functions. HO-1 has been identified as an important inducible enzyme with antioxidant and cytoprotective activities that function against oxidative stress in response to various stress stimuli.

The cytoprotective function of HO-1 is dependent not only on the activities of the reaction products but on the induciblity of HO-1, which is regulated primarily at the level of gene transcription by two distal enhancers E1 and E2. Identification and analysis of the enhancers led to the discovery of multiple StREs that are necessary and sufficient for *hmox-1* gene regulation by almost all inducers. Additionally, studies from our laboratory identified Nrf-

2, a CNC-bZIP transcription factor, as a dominant regulator that mediates inducer-dependent regulation of the *hmox-1* gene via the StRE motifs by several inducers, However, the mechanism of how Nrf-2 is regulated is in terms of zinc is not completely understood. I will study the role of the Nrf-2 in the HO-1 induction by zinc ..

In the following chapters, supporting experiments will be discussed where zinc induces the *hmox-1* gene promoter in a dose-dependent manner by different approaches, such as luciferase assay, mRNA blotting, and electrophilic mobility shift assay (EMSA).

It will be shown that the increase in the *hmox-1* induction has a profound positive impact on the increase of the StREs, which have multiple copies in the E1 region of *hmox-1*. It will be demonstrating that there is strong Nrf-2 binding and that this binding is a perquisite for HO-1 expression.

Furthermore, in Chapter Three, it will be shown which signal transductions are activated by supplementation of zinc and which one is responsible for HO-1 upregulation. A model will be described that links Nrf-2 and ERK1 in the mechanism by which zinc increases the HO-1 expression.

2. INDUCTION OF THE HEME OXYGENASE-1(*hmox*-1) GENE IN RESPONSE TO ZINC IS MEDIATED BY THE STRESS RESPONSE ELEMENTS (StREs) AND NUCLEAR FACTOR-ERYTHROID 2 RELATED FACTOR-2 (Nrf-2)

2.1 Introduction

The liver is the primary organ of zinc metabolism, which can lead to liver damage (Bailey and Cunningham, 2002).,Contribution of mitochondria to oxidative stress associated with liver disease. Oxidative stress, and more precisely, the generation of excessive ROS (such as hydroxyl ethyl free radicals, hydroxyl radicals, and superoxide anions), inducing cellular damage by targeting various molecules, such as proteins, lipids, and DNA. It has been suggested that ROS are produced during zinc metabolism through different metabolic pathways. For example, Palmiter et al. (2004) demonstrated that phenolic oxidants activate Mt1 transcription by a zinc-dependent mechanism, which involves MTF-1 binding to metal regulator elements in the Mt1 gene promoter.(Bi et al., 2004). In another pathway, Cui et al. (2003) demonstrated that certain transcription factors are involved in the regulation of apolipoprotein A-I (apoAI) expression in human hepatoblastoma Hep G2 cells. Ho (2003) demonstrated that supplementation of zinc to C6 cells increases oxidative DNA damage and induce DNA-repair protein expression. Important downstream signals leading to proper DNA repair are lost without zinc.

At the cellular level, what happens to zinc after it gets to the nucleus of the cells? Regardless of the of the kind of MAPK signal(s), it is generally well known that the main function of all kinds of MAPKs is to tightly control the activity of transcription factors in order to regulate the expression of a large number of specific genes (Murphy et al., 2006).

In study by Alam et 2003 demonstrated that the *hmox*-1 gene is activated in response to heavy metals treatment (including cadmium and 15-deoxy-$\Delta^{12,14}$ –prostaglandin J_2) in several

cell types, including Hepa cells, by the transcription factor Nrf-2 via StREs in the E1 enhancer (Alam et al., 2000; He et al., 2001; Stewart et al., 2003). However, the mechanism by which zinc induces HO-1 was not studied in detail. Stewart et al. (2003) wrote:

> In these studies, additional work demonstrated that two 5' distal enhancer regions mediate mouse *hmox*-1 gene activation in response to multiple inducers including heme, cadmium, and 15-deoxy-$\Delta^{12,14}$ –prostaglandin J_2 (Alam et al., 2000, Gong et al., 2002a, 2002b; Balogun et al., 2003). Additionally, from our previous lab work, ectopic expression of an Nrf-2 dominant negative mutant (DNM) did not completely inhibit activation of the *hmox-1* gene or the E1-controlled luciferase gene by cadmium in several cell types, including Hepa cells (Alam et al., 1999; Alam et al., 2000; He et al., 2001). Furthermore, Ishii et al. (2000) have observed that Nrf-2-deficient macrophages are still capable of partial induction of HO-1 protein and mRNA expression when treated with cadmium, suggesting that other transcription factors and *cis*-acting DNA elements may be involved in *hmox-1* gene regulation. (p. 23)

One potential element, the StRE, was identified in the mouse *hmox-1* gene by Takeda et al. (1994) in Hepa cells. This (StRE) resides in the mouse *hmox-1* E1 locus. Conversely, the StREs within the E1 loci of the human , rat, and mouse genes were conserved with respect to number, relative location, and primary structure (Alam et al., 2000; He et al., 2001; Stewart et al., 2003). Specifically, the E1 sequence of the mouse, rat, and human *hmox-1* genes exhibited 55% overall sequence conservation (Alam and Cook, 2003; Stewart et al., 2003). This level of sequence identity suggests that other elements within E1 may contribute to gene activation. In this chapter, evidence will be provided that StREs and/or HSEs are necessary for this regulation. It will also be shown that StREs have multiple motifs in the E1 region when they bind to transcription factor Nrf-2, which activates the mouse *hmox-1* gene in response to zinc (Stewart, 2003).

2.2 Nrf-2 Structural Characteristics

Previous studies by Alam et al. (1999) showed that Nrf-2 is an important transcription factor with a primary role in the response to cellular oxidative stress. Transcription factors

regulate activation of stress responsive genes, encoding proteins with antioxidant and cytoprotective activities. Nrf-2 is a member of the CNC-bZIP family. This family contains a Cap'N'Collar (CNC) domain similar in structure to a region within the fruit fly homeotic selector protein encoded by the CNC gene involved in head and neck development in *Drosophila.* This is common among the CNC-bZIP family (Mohler et al., 1991). Nrf-2 also contains many domains; for example, a potent N-terminal transcription activation domain (TAD); a nuclear localization signal (NLS); a nuclear export signal (NES); and two conserved structural motifs, the C-terminal leucine dimerization (LZ) and basic DNA-binding domains (DBD) **(**Figure 2.1**)** (Alam et al., 1999; Alam et al., 2004; Shen et al., 2004; Jain et al., 2005; Stewart, 2005).

Stewart (2005) wrote:

> The dimerization domain consists of repetitions of leucine residues every seventh position; the leucine zipper, which plays an important role in polypeptide dimerization; and an adjacent DNA-binding domain enriched in basic amino acids (Kouzarides and Ziff, 1988; Struhl, 1989). The primary structure within the leucine zipper region of Nrf-2 precludes self-dimerization; therefore, Nrf2 heterodimerizes primarily with small Maf proteins (MafF, MafG, and MafK), and other bZIP proteins including c-Jun of the AP-1 family and ATF-4 (activator of transcription 4), a member of the ATF/CREB subfamily (Venugopal and Jaiswal, 1998; Nguyen et al., 2000; He et al., 2001, Alam and Cook, 2003). Other mammalian CNC family members include p45, Nrf-1 (NF-E2 related factors), Nrf-3, Bach1, and Bach2 (Chan et al., 1996; Oyake et al., 1996; Andrews, 1998). (p. 28)

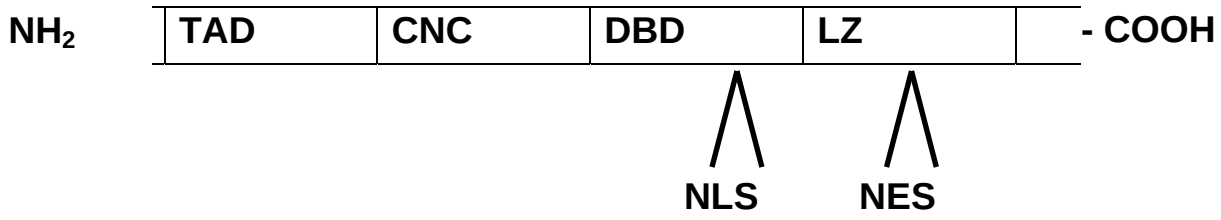

Figure 2.1 A Presentation of Nrf-2 Domains. *NF-E2(Nuclear factor-erythroid*) domain Six highly-conserved regions, referred to as Neh1 to Neh6 (Nrf-2-ECH homology. N-terminal transcription activation domain (TAD); Cap'N'Collar (CNC), a nuclear localization signal (NLS), C-terminal leucine dimerization (LZ) and basic DNA-binding domains (DBD).

Studies by Alam et al. (1999) showed that Nrf-2 is an important transcription factor with a primary role in the response to cellular oxidative stress. Transcription factors regulate activation of stress responsive genes, encoding proteins with antioxidant and cytoprotective activities. Nrf-2 is a member of the CNC-bZIP family. This family contains a Cap'N'Collar (CNC) domain similar in structure to a region within the fruit fly homeotic selector protein encoded by the CNC gene involved in head and neck development in *Drosophila.* This is common among the CNC-bZIP family (Mohler et al., 1991). Nrf-2 also contains many domains; for example, a potent N-terminal transcription activation domain (TAD); a nuclear localization signal (NLS); and two conserved structural motifs, the C-terminal leucine dimerization (LZ) and basic DNA-binding domains (DBD) **(**Figure 2.1**)** (Alam et al., 1999; Alam et al., 2004; Shen et al., 2004; Jain et al., 2005; Stewart, 2005).

2.3 Nrf-2 Physiological Importance

The efficacy of Nrf-2 was discovered from the accumulating studies that implicated it as a key regulator of the adaptive response to oxidative stress (Alam and Cook, 2003; Alam et al., 2004; Stewart, 2005). Additional studies in the past several years have convincingly demonstrated the regulation of the inducible expression of gene encoding enzymes with cytoprotective functions such as NQO1, GST, TRX, y-GCS, ferritin, and *hmox-1* with Nrf-2 (Venugopal and Jaiswal, 1998; Wild et al., 1999; Jeyapaul and Jaiswal, 2000; Dhakshinamoorthy et al., 2000a, 2000b; Alam and Cook, 2003; Pietsch et al., 2003). These enzymes work, in concert, as a cellular defense tool that scavenges ROS, detoxifies electrophiles and xenobiotics, and maintains intracellular redox-homeostasis (Thimmulappa et al., 2002; Lee et al., 2003; Shih et al., 2003).

Although, Nrf-2-deficient mice exhibit normal development, they are highly sensitive to butylated hydroxytoluene and suffer from acute respiratory distress syndrome, are susceptible to acetaminophen hepatotoxicity, and exhibit an increased burden of benzo[a]pyrene-induced gastric neoplasia (Chan et al., 2001; Enomoto et al., 2001; Ramos-Gomez et al., 2001).

Furthermore, Ishii et al. (2000) demonstrated that macrophages derived from such mice exhibit reduced resistance to toxic electrophiles. Likewise, deletion of the Nrf-2 gene in mice results in a significant decrease in antioxidant gene expression with an increased susceptibility to carcinogenesis (Ishii et al., 2000; Enomoto et al., 2001; Ramos-Gomez et al., 2001; Lee et al., 2003). These studies indicate that Nrf-2 performs an important role in bridging the gap between oxidative stress and antioxidant gene expression in supporting cell survival and cellular homeostasis.

2.4 Nrf-2 Activity Regulation

Although much is known about the regulation of Nrf-2 target genes, including *hmox-1*, the ways in which stress agents regulate Nrf-2 activity are not specifically characterized in terms of zinc. Evidence from several laboratories suggests that Nrf-2 activity is, in part, regulated at the subcellular compartmentalization level (Itoh et al., 1999; Dinkova-Kostova et al., 2002; Sekhar et al., 2002; Zipper and Mulcahy, 2002;). Under normal conditions, Nrf-2 exists in an inactive form, sequestered in the cytoplasm in part ─ or fully ─ as a consequence of binding to a cytoskeleton-associated protein Keap1 (Kelch-like ECH-associated protein 1) (Itoh et al., 1999; Dhakshinamoorthy and Jaiswal, 2001; Kobayashi et al., 2002; Sekhar et al., 2002). After it is stimulated by oxidative stress promoting agents, Nrf-2 is freed from Keap1 suppression, which allows Nrf-2 to translocate to the nucleus and activate target genes by an, as yet, uncharacterized mechanism, but one by which it may be assumed that involvement of PKC-mediated

phosphorylation of Nrf-2 (Huang et al., 2002). This method of regulation is somewhat similar to the cytoplasmic-nuclear transport mechanism of transcription factors NF-κB and p53 (Crook et al., 1998; Kau and Silver, 2003).

Another mode of regulation may be at the level of DNA binding. The association of motif-DNA-binding of Nrf-2-Jun dimmers has been shown to require a cytosolic factor yet to be characterized (Venugopal and Jaiswal, 1998; Jaiswal, 2000; Jeyapaul and Jaiswal, 2000). Whether such a factor is responsive to oxidative stress and how it regulates nuclear activity are not known. Studies by Kwak et al. (2002) have recently described a positive feedback mechanism in which Nrf-2 autoregulates its own expression via an antioxidant response element–like sequence, which shows sequence similarity to StRE, within the *nrf*-2 gene in response to 3H-1, 2-dithiole-3-thione, an anti-carcinogen.

Since other studies have revealed that Nrf-2 plays important roles in HO-1 induction by heavy metals (Alam et al., 2003), this chapter will investigate how these transcription factors may be regulating this mechanism. Treatment of Hepa cells with increasing doses of $ZnSO_4$ caused greater expression of Nrf-2 . Since HO-1 production is primarily controlled by transcriptional regulation, it will be examined how Nrf-2 adjusts HO-1 expression in Hepa cells. By using EMSAs performed using oligo 5'-ATCCCCGGAACAGGAGAAGGCCTTTTTCAAGAGAAAAGGCCTTCTCCTGTTCCTTTTTGGAA -3' and 5'-AGCTTTTCCAAAAAGGAACAGGAGAAGGCCTTTTCTCTTGAAAAAGGCCTTCTCCTGTTCCGGG-3, it will be demonstrated that direct binding of *hmox*-1 cis-regulatory elements by transcription factors shows Nrf-2) definitely contributing to the regulation of HO-1.

Experiments were performed to study the effects of treating the Hepa cells with heat shock, Cd, As, and zinc. Zinc caused the formation of DNA-protein complexes. I will also show the effect of zinc on the Hepa cells containing the reporter construct, pHO-15luc and wild-type Hepa cells (both treated with Nrf-2), and control shRNA. Then I will show that the basal and zinc-induced transcription after measuring luciferase assays in the cells containing pHO-15luc.

2.5 Experimental Procedures

2.5.1 Materials

Tissue culture media and gentamicin were from Invitrogen (Carlsbad, CA), and fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). Restriction endonucleases and other DNA-modifying enzymes were purchased from either Invitrogen or New England Biolabs (Beverly, MA). Zinc sulfate was purchased from Sigma Inc. with 99.0% purity and kept at room temp.. Oligonucleotides were synthesized by IDT, Inc. (Coralville, IA) and radiochemicals were obtained from MP Biomedicals, Inc. (Irvine, CA). Antibodies against HSF-1, and β-Actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), whereas antibodies against HSP70, HO-1, and HSF-1 were acquired from StressGen Biotech. Inc. (Victoria, Canada). Phosphatase inhibitors were purchased from Calbiochem (San Diego, CA)

2.5.2 Plasmids

Plasmid pHO-15luc was constructed by cloning a 15-kbp promoter fragment from the mouse *hmox-1* gene (Alam et al., 1994) into the luciferase reporter gene vector pSKluc (Alam et al., 2000). Derivatives of pHO-151uc have been described previously (Gong et al., 2002a, 2002b). A 3' extension of *hmox-1* enhancer 1 (E1), $E1^{+}$ was generated by ligating the 268 bp E1 fragment (Sac1 and Xbal ends) to a double-stranded oligonucleotide (prepared by annealing 5'-

CTAGAAAGGCACAGAAGTTTCTTGTTTAATTTTA-3' and 5'-CTAGTAAAATTAAACAAGAAACTTCTGTGCCTTT-3') with Xbal and Spel ends. The $E1^+$ fragment was subsequently cloned between the Sac1 and Spel sites of pΔ44luc, a luciferase reporter vector containing the *hmox-1* core promoter from -44 to +73, including the TATA BOX, to generate $pE1^+$-luc. Plasmid $pE1^+$ (HSEmut)-luc a derivative of $pE1^+$-luc with a mutant HSE, was created in a similar manner using the complementary oligonucleotides 5'-CTAGAAAGGCACAGAAGTTTCTTGTTTAATTTTA-3' and 5'-CTAGTAAAATTAAACAAGAAACTTCTGTGCCTTT-3' (mutant residues underlined).

The StRE mutant and the StRE and HSE double mutant of $E1^+$, $pE1^+$ (StREmut)-luc and, respectively, were constructed in an analogous manner, except that the base E1 fragment was derived from pE1M789-luc in which the three StREs are mutated.

Plasmid pSUPER/ Nrf-2 (192) expresses a small hairpin RNA (shRNA) targeting mouse Nrf-2 transcript and was constructed by cloning the complementary oligonucleotides, 5'-ATCCCCGGAACAGGAGAAGGCCTTTTTCAAGAGAAAAGGCCTTCTCCTGTTCCTTTTTGGAA -3' and 5'-AGCTTTTCCAAAAAGGAACAGGAGAAGGCCTTTTCTCTTGAAAAAGGCCTTCTCCTGTTCCGGG-3', into the pSUPER vector (Oligoengine). Plasmid pCMV/B-gal, encoding the *E. coli* B-galactosidase gene, was kindly provided by Dr. Ping Wei (Louisiana State University Medical Center,New Orleans, LA).

2.5.3 Cell Culture

Normal mouse hepatoma (Hepa) cells, Hepa stable transfectants harboring pHO-151uc ,or pSUPER/ Nrf-2 [192] ,and transformed mouse embryonic fibroblast (MEF) cells from wild-type and HSF-1 knockout BALB/c mice were cultured in a humidified atmosphere (95% air, 5%

CO_2) at 37°C in Dulbecco's modified Eagle's medium containing 0.45% glucose, 10% fetal bovine serum, and 50 μg/ml gentamicin.

The media for MEF cells was additionally supplemented with 10 mM nonessential amino acids and 0.1 mM B-mercaptoethanol. Cells were routinely passaged every 3-4 days.

2.5.4 Transfection, Treatment, and Reporter Enzyme Assays

Transient transfection of Hepa cells were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Briefly, Hepa cells were seeded (~7 x 10^4/well of 24-well plate) and transfected 20 to 24 h later with a plasmid DNA mixture consisting of 0.1 μg of the experimental luciferase reporter construct and 0.1 μg of pCMV/B-gal.

Between 20 to 24 h after the start of transfection, the culture medium was replaced with serum-free medium. The cells were cultured for another 24 h prior to treatment with chemical agents for 5 h in serum-free medium buffered with 10 mM Hepes (pH 7.3) at 37°C. Hepa/pHO-151uc stable transfectants were cultured for 48 h prior to serum-starvation and treated as described above. Additional information is provided in the figure legends.

2.5.5 Transfection and Reporter Enzyme Assays

Briefly, for transient transfection, Hepa cells were seeded (~7 x 10^4/ well of 24-well plate; ~3 x 10^5 /35-mm plate) and transfected 20 to 24 h later with a plasmid DNA mixture consisting of 0.1 up of the experimental luciferase reporter constructs and 0.1 ug of pCMV/B-gal, or for RNA inteference experiments, 50 ng pΔ44luc/ME1+, 50 ng of pCMV/B-gal, and 100 ng of the appropriate shRNA construct, subjected to heat shock (43°C) for 0.5 h in complete media, followed by a 5.5-h recovery period at 37°C.

2.5.6 RNA Isolation and Blot Analyses

Hepa cells were seeded (1 x 10^6 cells/60-mm plate); cultured for 40 to 48 h, first in complete medium and then, for an additional 24 h in serum-free medium; and then treated with the appropriate agents for 3 h in serum-free medium buffered with 10 mM Hepes (pH 7.0-7.6) (Sigma). Total RNA was isolated by the acid-guanidine-thiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987); Northern or dot blot analysis was carried out as previously described (Alam et al., 1989; Alam et al., 2000). Briefly, for RNA dot blot analysis, 5 ug of total RNA was transferred to a Zeta-Probe (Bio-Rad) nylon membrane using a Minifold l Filtration Manifold (Schleicher & Schuell) and for Northern blot analysis, 10 up of total RNA was fractioned on a denaturing formaldehyde-agarose (1%) gel (Maniatis et al., 1982) and transferred to a Zeta-Probe nylon membrane according to the manufacturer's recommendations. p_{32}-radiolabelehybridization probes were generated by random priming of the rat HO-1 or ribosomal protein S3 cDNA fragments. Vacuum-baked filters were incubated for 4 to 16 h at 45°C with 10 ml of hybridization solution: 3X SSPE (1X SSPE = 0.15 M NaCl, l mM EDTA; 10 mM sodium phosphate buffer; and pH 7.4) containing 40% (v/v) deionized formamide, 7% (w/v) sodium dodecyl sulfate (SDS), and 200μ g/ml denatured salmon sperm DNA. The filters were subsequently incubated with hybridization buffer containing the labeled DNA probe (specific activity: 2-10 x 10^8 cpm/ug, 1-10 x 10^7 cpm/ml solution) for 24 to 48 h at 45°C.

After hybridization, the filters were washed with solutions of 2 X SSC (1 X SSC = 0.15 M NaC1, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 30 minutes and 0.1 X SSC, 0.1% SDS at 50°C (for heterologous probes) or 65°C (for homologous probes) for 60 min. The blots were then exposed to a phosphor-imaging screen for 12 to 72 h at room temperature. HO-1 hybridization signals were detected and quantified using a

phosphorimaging device (Packard Instruments). After signal quantification, the membranes were striped and re-hybridized to the ribosomal protein S3 probe. Relative mRNA levels were calculated after correcting for the RNA loading by normalizing the primary hybridization signal with the ribosomal protein S3 signal.

2.5.7 Protein Isolation and Blot Analyses

Hepa, MEF/*hsf-1*$^{+/+}$ (2 x 10^5 cells/60-mm plate) and MEF/*hsf-1*$^{-/-}$ (4 x 10^5 cells/60-mm plate) cells were seeded, cultured, and treated with chemical inducers as described above for RNA isolation, except that cell treatment, unless otherwise indicated, was carried out for 5 hours. Cells were subjected to hyperthermia (43°C) in complete media for 0.5 h. Where indicated, induction were added 30 min prior to addition of inducers. Western blot analyses were carried out using either whole cell extracts (WCE) or nuclear extracts. were electrophoresed on 4-12% gradient SDS-PAGE gels (Invitrogen), and proteins were transferred to polyvinylidene difluoride membranes. Antibodies were used at the concentrations recommended by the manufacturers and antigen detection was carried out using the ECL Plus Western blotting system (Amersham Biosciences) according to the manufacturer's recommendation.

2.5.8 Electrophoretic Mobility Shift Assay

Hepa cells (1 x 10^6/100-mm plate) were seeded and cultured as described above for RNA isolation. Cells were treated with vehicle (water) or 80 μM $ZnSO_4$ for 5 h in serum-free medium or subjected to hyperthermia (43°C) for 0.5 h in complete medium. In the preparation of cell extracts, the probe used for EMSA reactions was a double-stranded oligonucleotide corresponding to the *hmox*-1 E1$^+$ HSE, 5'-GGCCGT GAAA GTTCT AGAAAGGCACAGAAGTTCTT GTTTA-3', (sequences corresponding to the consensus HSE are underlined). In antibody supershift assays, 1 (2 μg) of normal rabbit 1gG or specific rabbit

polyclonal antibodies against HSF-1 or Nrf-2 (Santa Cruz Biotech, Inc.) was added to the reaction mixture at room temperature 20 min prior to electrophoresis. Signals were detected and quantified using a phosphorimager (Packard Instruments).

2.5.9 Preparation of Nuclear Extracts

Hepa cells (5 x 10^6) were washed three times with cold, 8.5% phosphate-buffered saline and harvested by centrifugation at 1100 rpm for 10 min. The cell pellet was carefully resuspended in 3-pellet volumes of cold buffer containing 20 mM HEPES, pH 7.0, 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1% Nonidet-40, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4. The homogenate was then centrifuged at 500 x *g* for 20 min, and the nuclear pellet was resuspended in 5-pellet volumes of cold buffer containing 10 mM HEPES, pH 8.0, 25% glycerol, 0.1 M NaCl, 0.1 mM EDTA, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4. After centrifugation at 500 x *g* for 20 min, nuclei were resuspended in 2-pellet volumes of hypertonic cold buffer containing 10 mM HEPES, pH 8.0, 25% glycerol, 0.4 M NaCl, 0.1 mM EDTA, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4. The nuclei were then incubated for 30 min at 4°C . Nuclear debris was removed by centrifugation at 900 x *g* for 20 min at 4°C. One part of the supernatant was resolved by SDS-PAGE and submitted to immunoblot analysis using anti-Nrf-2 . The rest of the supernatant was used for electrophoretic mobility shift assay (EMSA). Protein concentration was determined with the Dc protein assay kit (Bio-Rad).

2.6 Results2.6.1 HO-1 Expression Is Upregulated in a Dose-Dependent Manner by Exposure to Zinc

HO-1 regulation by zinc was evaluated. Hepa cells were assayed for baseline and zinc-stimulated HO-1 protein and mRNA levels. Dose-dependent HO-1 production was observed after administration of increasing amount of $ZnSO_4$ (Figure 2.2A). HO-1 mRNA levels were also dependent on the amount of zinc exposure (Figure 2.2A). Both protein and mRNA expression were highest around the same concentrations of $ZnSO_4$, about 80 μM. Hepa cells were also transfected with pHO-15luc, and luciferase activity was quantified (Figure 2.2C.). Expression of the reporter construct in the transfected cells was augmented with increasing concentrations of $ZnSO_4$. Luciferase activity was induced more than 25-fold at its peak response.

2.6.2 Enhancer Regions, Along with StREs Located Within, are Required for Zinc-Induced Activation

Constructs of pHO-15luc were made with deleted sequences, including the E1 and E2 enhancer regions located upstream from the transcription start site on *hmox-1*. These regions contain motifs that are used as binding sites for transcription factors in other types of HO-1 induction (Alam et al., 2000; He et al., 2001; Stewart et al., 2003). These plasmids were transfected into Hepa cells and the cell extracts were assayed for reporter activity (Figure 2.3). Deletion of either region alone significantly decreased luciferase activity induced by zinc, while deletion of both regions ablated the activity. Mutation of an unrelated region, B, did not affect transcription of the reporter. Previous studies have shown similar importance of these regions using different inducers such as proteasome inhibitors (Stewart et al., 2003).

Further studies were carried out using cells transfected with plasmid constructs containing mutated motifs within E1. These constructs included three mutated StREs, a mutated HSE, or both ,But E2 doesn't contain StrE ,and HSE which can be tested together this the reason I used E1 in this experiement.(Figure 2 4). Baseline levels of reporter transcription remained

relatively the same. Upon treatment with zinc, however, reporter expression was significantly altered. Cells that contained the nonfunctioning StREs demonstrated almost no luciferase activity, whereas cells with no HSE and intact StREs had normal zinc-induced HO-1 responses. These results differ from those seen in HO-1 induction by cadmium, which require the HSE (Stewart et al., 2003).

2.6.3 HO-1 Expression Caused by Zinc Exposure Is Dependent upon the Transcription Factor Nrf-2

To analyze whether Nrf-2 protein is expressed in the cytoplasm and then transported to the nucleus during oxidative stress, experiments similar to those described above were performed, except that cell extracts were separated into cytoplasmic and nuclear fractions. As shown in Figure 2.5**,** treatment of Hepa cells with 20-30 uM of $ZnSO_4$ increased the level of Nrf-2 protein in the nucleus within 1 h. However, Nrf-2 was not observed in the whole cell extract, even in uninduced cells (Figure 2.6A), suggesting an alternative mechanism for Nrf-2 protein expression, but one that could still require cytoplasmic-nuclear transport.

2.6.4 HO-1 Expression Caused by Zinc Exposure Is Dependent upon the Transcription Factor Nrf-2 and Independent of HSF-1

Since other studies have revealed that Nrf-2 and HSF-1 play important roles in HO-1 induction by heavy metals (Alam et al., 1999; Alam et al., 2000; He et al., 2001), these transcription factors may regulate this mechanism. Treatment of Hepa cells with increasing doses of $ZnSO_4$ caused greater expression of Nrf-2 (Figure 2.6A). As HO-1 production is primarily controlled by transcriptional regulation, Nrf-2 adjusted HO-1 expression in Hepa cells was examined. EMSAs were performed using olig5’GATCTTTTATGCTGTGTCATGGTTT3’, which showed the formation of specific DNA-protein complexes. The movement of these structures was slowed

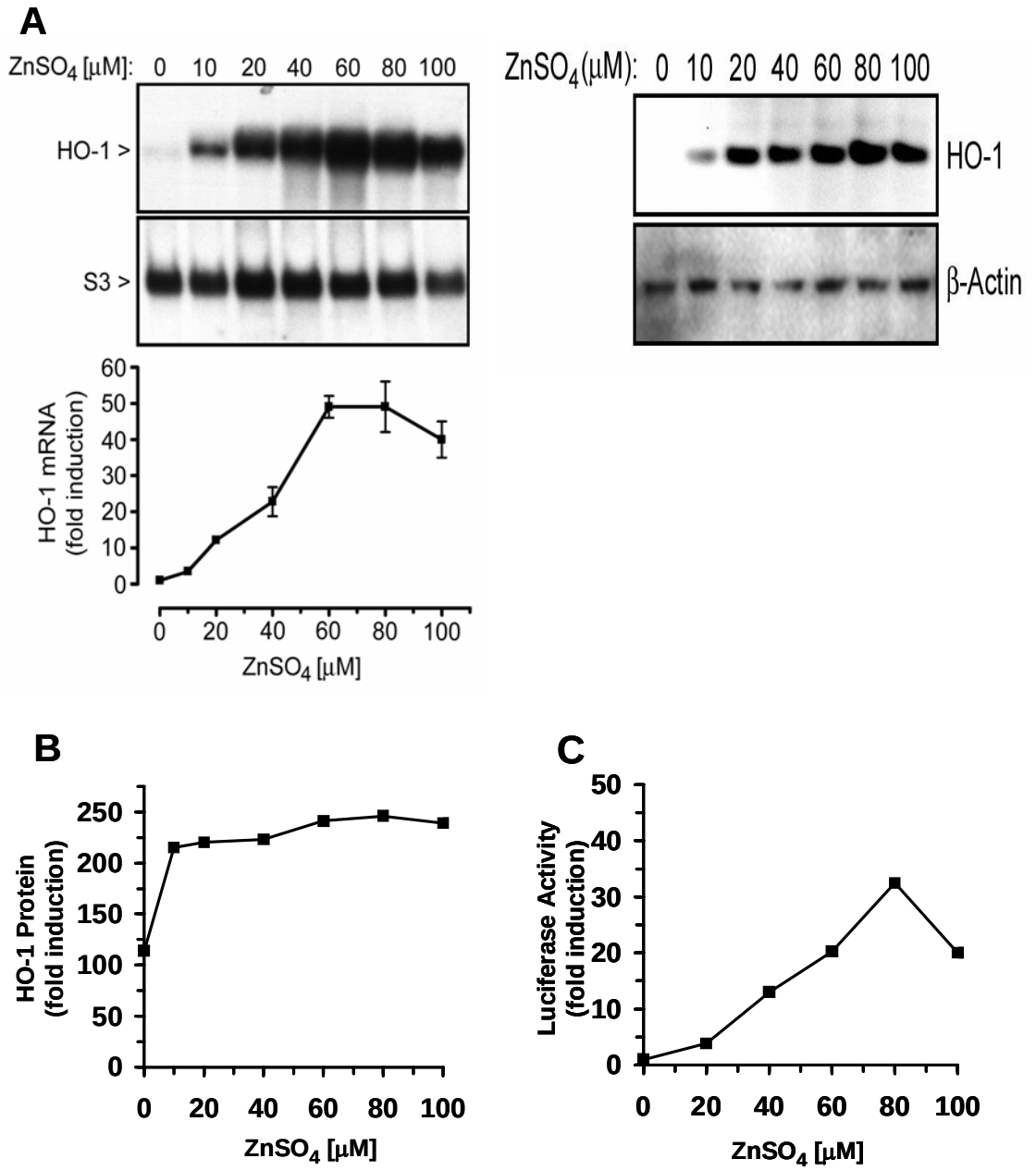

Figure 2.2 Treatment with $ZnSO_4$ Causes Protein Synthesis and in mRNA and HO-1. The effects of zinc on the protein levels of HO-1 .Hepa cells were maintained for 24 h in low serum medium and then stimulated with $ZnSO_4$ (0, 20, 40, 60, 80, or 100μM). **B** Hepa cells were incubated overnight,and HO-1 m RNA was normalized to S3m RNA ,and ratio HO-1/S3 m RNA are expressed as arbitrary densitometric unites under the blots and represented the average The right panel with anti-HO-1 antibody is **a** dose-dependent semiquantitative reverse mean ± SE (n=3) p> 0.05 .transcription –PCR showing dose-dependent induction of HO-1 by zinc determination of HO-1 m RNA levels in the presence of $ZnSO_4$ and analyzed, as shown, under experimental procedure **C.** The indicated cells were transfected with a DNA mixture consisting of 3 μg of pE1-luc and 2μg p CMV-gal transfection. Induction and enzyme assay were carried out as described under experimental procedure. mean ± SE (n=6) p <0.01.

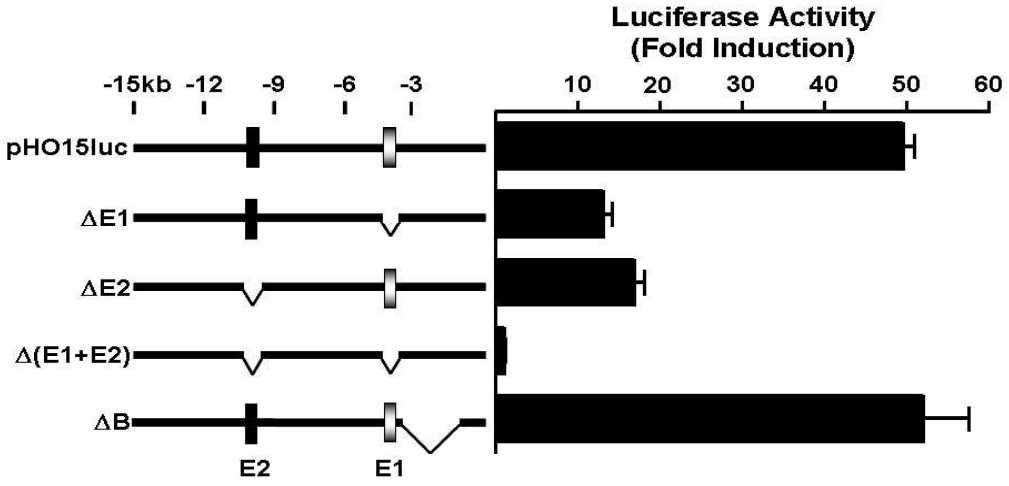

Figure 2.3 The E1and E2 regions of pHO-15luc are required for the activation of pHO-15luc Expression in cells treated with $ZnSO_4$ (80μM). Hepa cells were transfected with pHO-15luc,incubated for 5h at 37°C, as well as mutants of the plasmid containing deleted E1, E2, E1/E2, and B sequences. Cells were then treated with $ZnSO_4$ and the extracts were measured for luciferase activity relative to unstimulated pHO-15luc expression. Data are shown as the fold increase above the unstimulated expression (mean ± SE, n=6 ,$p<0.01$).

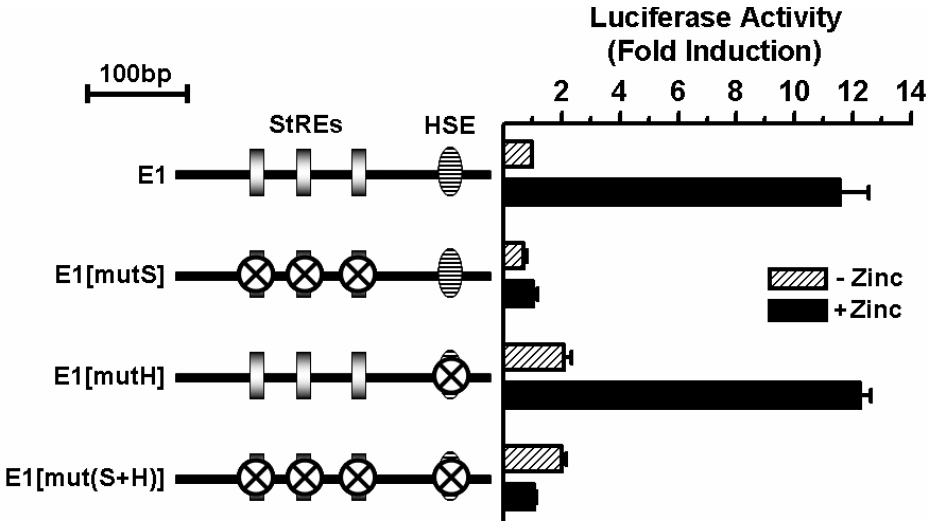

Figure 2.4 StREs are Required Alone. Within region, E1 is required in $ZnSO_4$ in the induction of *hmox*-1. Mutant constructs of E1 *hmox*-1 were made by deleting the three StRE sequences, the HSE sequence, or the three StRE and the HSE sequences. Hepa cells were then treated with $ZnSO_4$. (80 μM) for 5 h at 37°C. Cell extracts were then assayed for luciferase activity relative to unstimulated pHO-15luc expression and shown as a fold increase (mean ± SE, n=5,$p<0.01$).

by the addition of anti-Nrf-2 antibody, meaning Nrf-2 is binding regions on *hmox*-1 (Figure 2.6 B). Direct binding of *hmox-1* cis-regulatory elements by transcription factors showed that Nrf-2 was definitely contributing to the regulation of HO-1.

Similar experiments were performed looking at HSF-1 in zinc-induced HO-1 production. HO-1 was produced by Hepa cells when treated with cadmium, arsenite, and zinc (Figure 2.7A). However, unlike the other inducers, zinc did not cause greater expression of phosphorylated HSF-1. These data are congruent with earlier results showing that HSE is not required for zinc-induced HO-1 expression. Further EMSAs were completed with oligo (Figure 2.7B). Treating the cells with heat shock, Cd, and As caused the formation of DNA-protein complexes, but zinc exposed cells did not.

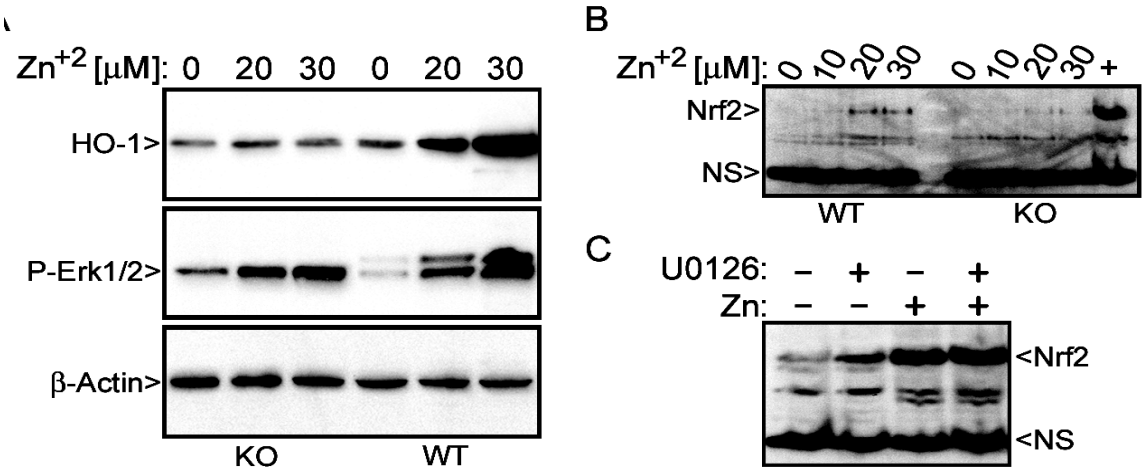

Figure 2.5 HO-1 Induction by $ZnSO_4$ in Mouse Embryonic Fibroblasts is Expressed Through an Nrf-2-Dependent Mechanism (A, B) or Hepa cells treated with U0126 ERK1/2 inhibitor(C). A, B. Wild-type (WT) and ERK1 knockout (KO) MEF cells were exposed to the indicated concentration of $ZnSO_4$ for 4 h. Western blot analysis was carried out as described in "Experimental Procedures" using whole cell (A, 20 μg protein/lane) or nuclear (B, 30 μg protein/lane) extracts and antibodies directed against the indicated antibodies. Cadmium-treated Hepa cell extracts were used as a positive control for Nrf-2 (+). **C**. Hepa cells were treated with 0 (-) or 10 μM (+) U0126 for 1 h prior to treatment with 0 or 80 μM $ZnSO_4$ for an additional 4 h. Western blot analysis was carried out using nuclear extracts (30 μg protein/lane) and anti-Nrf-2 antibodies. NS, non-specific. Nrf-2 *(*NF-E2): Nuclear factor-erythroid.

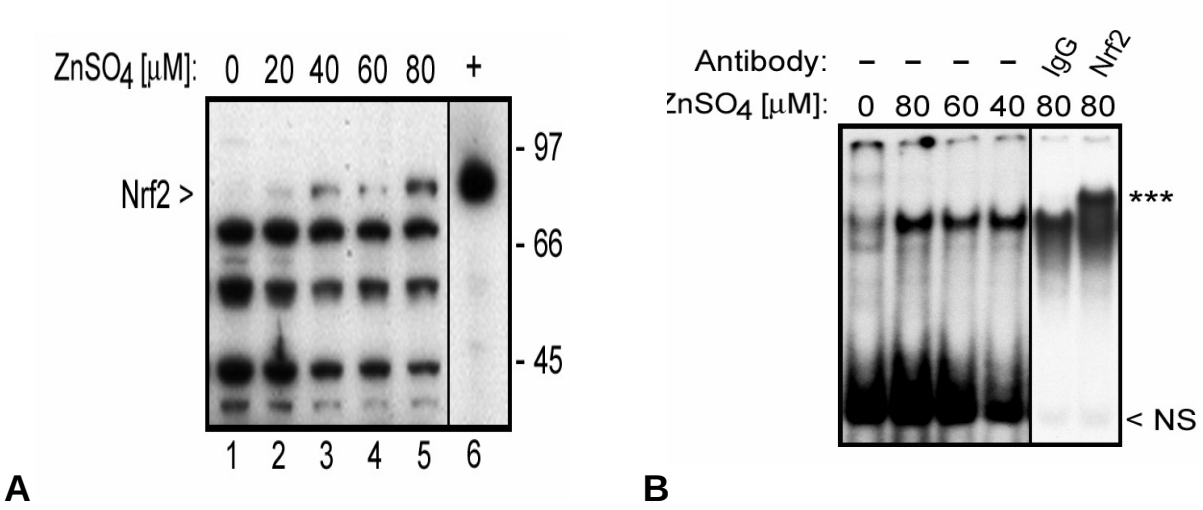

Figure 2.6 Zinc Stimulates Nrf-2 Protein Expression (A) and StRE Binding Activity (B) in Hepa cells. A. Cells were exposed to the indicated concentration of $ZnSO_4$ for 3 h. Western blot analysis was carried out as described in "Experimental Procedures" using whole cell extracts (30 μg protein/lane) and anti-Nrf-2 antibodies. The Nrf-2 positive control protein (+, lane 6) was generated by transfection of an Nrf-2 expression plasmid into Hepa cells. B. EMSA reactions using oligonucleotides corresponding to the mouse HO-1 sStRE and nuclear extracts from cells treated with 0, 40, 60, or 80 μM $ZnSO_4$ the last two lanes correspond to cell extracts from zinc-treated cells preincubated without(-)or with(+) anti-Nrf-2 antibody.The *arrow head* points to the position of the complex that was not formed in the presence of anti-Nrf-2 antibodystars indicate specific binding..”

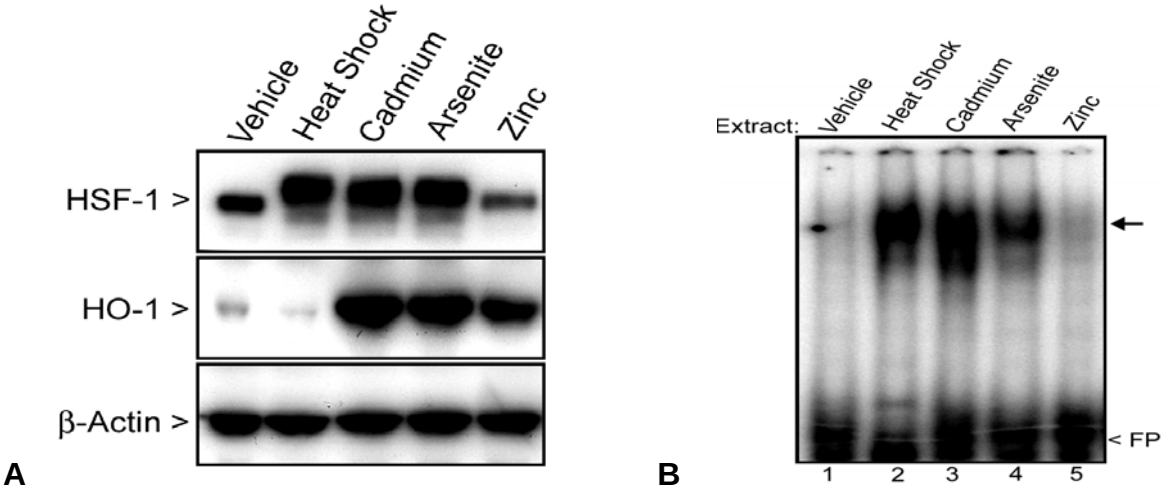

Figure 2.7 Heat shock elements HSE is not important in HO-1 induction by Zinc .in Transiently and Stably Transfected Cells. A.Hepa cells were either treated with a vehicle 50 μM $CdCI_2$, 20 μM $NaAsO_3$ or 80μM $ZnSO_4$ for 5 h, or exposed to 43°C heat shock for 30 min. Whole cells exctracts (20 g/lane) were subjected to immunoblot analysis for the indicated proteins. B.EMSA reactions were carried out using the mouse E1+ HSE probe and extracts prepared from Hepa cells that were either treated with vehicle, 50 μM $CdCI_2$, 20 μM $NaAsO_3$, or 80 μM $ZnSO_4$ for 3 h, or exposed to a 43°C heat shock for 30 min. The HSE:HSF complexes are marked by an arrow. FP, free probe.

2.6.5 Nrf-2 Knockdown Diminishes HO-1 Accumulation in Response to Zinc

To more directly assess the role of Nrf-2 in zinc-mediated HO-1 induction, stable transfectants of Hepa cells expressing a short hairpin RNA (shRNA) that targeted the Nrf-2 trranscript for RNAi mediated degradation were generated. The efficacy and specificity of the Nrf-2 shRNA were demonstrated by the significantly reduced accumulation of Nrf-2 protein, in response to zinc, relative to that observed in cells expressing the control shRNA, and by the lack of effect on the expression Nrf-2 Figure.2.8A). Importantly, Nrf-2 knockdown caused a significant reduction (>90%) in zinc-mediated HO-1 induction, demonstrating a role of Nrf-2 in such regulation.

2.6.6 Induction of HO-1 Expression by Zinc in Mouse Embryo Fibroblasts Lacking HSF-1

Experiments were performed looking at HSF-1 in zinc-induced HO-1 production. HO-1 was produced by Hepa cells when treated with cadmium, arsenite, and zinc. However, unlike the other inducers, zinc did not cause greater expression of phosphorylated HSF-1. These data are congruent with earlier results showing that the HSE is required for zinc-induced did not reduce HO-1 expression. Further EMSAs were completed with oligo (Figure 2.7b). Treating the cells with heat shock, Cd, and As caused the formation of DNA-protein complexes, but zinc exposed cells did not.

Cells containing the reporter construct, pHO-15luc, and wild-type Hepa cells treated with Nrf-2, and control shRNA (Fig. 2.8A). The basal and zinc induced transcription was then measured by luciferase assays in the cells containing pHO-15luc. RNAi-mediated knockdown of Nrf2 diminishes HO-1 production,. The normal Hepa cells were also treated with two different Nrf-2 shRNA constructs, Nrf-2[192] Fig. 2.8B

As in Hepa cells, zinc treatment elicited a robust and dose-dependent accumulation of HO-1 protein in WT MEF cells (Fig.2.9). In KO MEF cells, however, both the basal and zinc-induced levels of HO-1 were affected, although not totally ignored. By comparison, loss of HSF-1 completely abrogates induction of Hsp70 as previously reported (Hatayama et al. 1992; Hatayama et al., 1993; Hatayama and Hayakawa, 1999).

Thus, while induction of Hsp70 in MEF cells is entirely dependent on HSF-1, induction of HO-1 is completely dependent on this transcription factor. The phenotype of the MEF cells was confirmed by zinc-mediated inactivation of HSF-1 (i.e., reduced mobility) in WT cells and the absence of HSF-1 in KO cells. Furthermore, targeted deletion of HSF-1 does not affect zinc inducibility of Nrf-2. This result suggests that the reduction of HO-1 induction in KO cells is an effect is independent on the HSF-1. HSF-1 is dependent on HSP70 expressionn of Nrf-2 activity and at the same time provides acceptable mechanism for the residual expression of HO-1. and Nrf-2[437]. The whole cell protein extracts were then treated with shRNAs and were also assayed for Nrf-2 and HO-1 protein expression. The Nrf-2[192] treated cells showed diminished Nrf-2 and HO-1 expression, while the cells transfected with Nrf-2[437] showed no Nrf-2 and significantly less HO-1 (Figure 2.8 A and B)

The role of HSF-1 in HO-1 regulation in mouse embryo fibroblasts (MEF) derived from wild-type (hsf+/+) and HSF-1 knockout (hsf-/-) mice was investigated (Figure 2.9).

2.7 Discussion

2.7.1 The Major Finding of This Work

The major findings of this study are: zinc is a potent inducer of both endogenous HO-

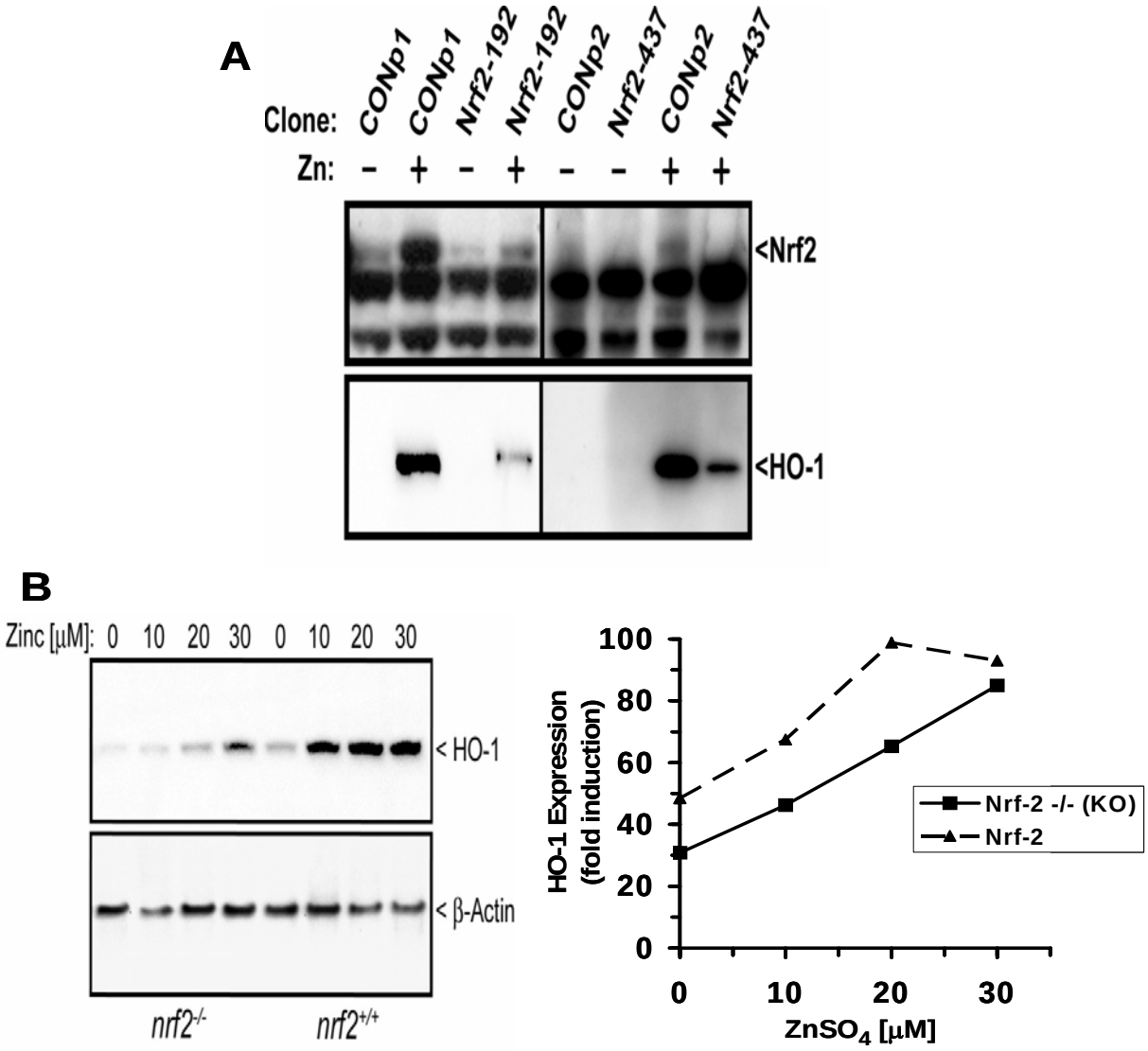

Figure 2.8 RNA I-Mediated Knockout of Nrf-2 Diminishes *hmox*-1 Induction by Zinc. A. Hepa cells stably transfected with the control (Conp 1. Conp.2)of Nrf-2 shRNA (Nrf-2-192, Nrf-437) expression plasmids were treated with vehicles(-)or 80 μM of zinc (+) for 5 h. B. Whole cell protein extracts (40 μg /lane). Nrf-2:10g /lane). Nrf-2 :10g/lane 11), were subjected to immunoblot analysis.

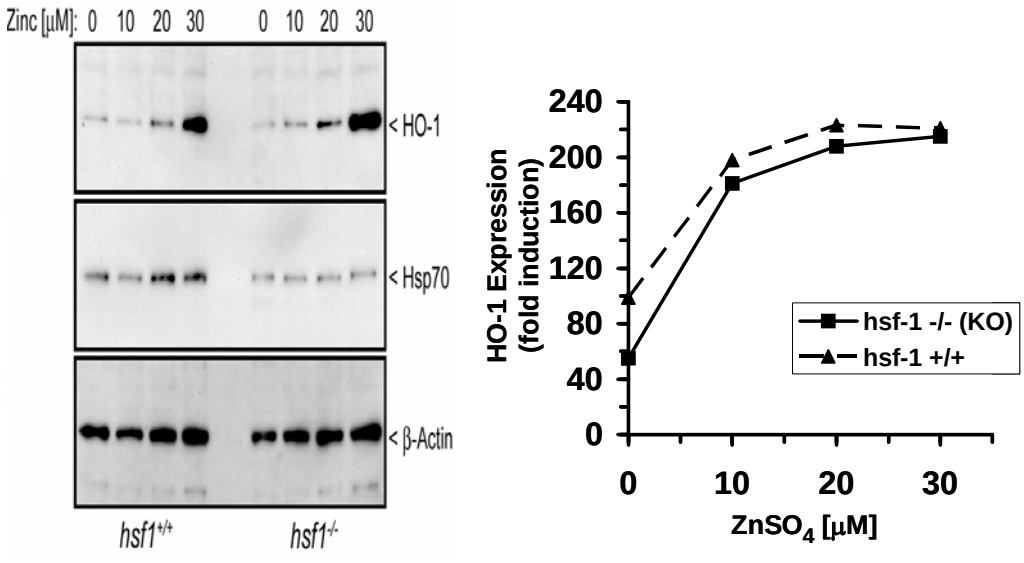

Figure 2.9 Lacking HSF-1 has no Effect on the Induction of HO-1 in Response to $ZnSO_4$ in Mouse Embryo Fibroblasts. HSF-1 is not required for optimal induction of *hmox*-1 by $ZnSO_4$ in mouse embryo fibroblasts. Embryonic fibroblasts from Nrf-2+/+(WT) and Nrf-2-/-(KO) mice were treated with 0, 10, 20, or 30μM $ZnSO_4$ cell extracts (10 μg) wild-type (WT) and -to HSF-1-/-, (KO) MEF for 5 h, and were then subjected to immunoblot analysis.

and transfected HO-1 promoter-reporter constructs (Figures 2.2); HO-1 expression is upregulated in a dose-dependent manner by exposure to zinc; and HO-1 is induced by an abundance of materials, including many heavy metals. Hepa cells were assayed for baseline and zinc-stimulated HO-1 protein and mRNA levels. Dose-dependent HO-1 production was observed after administration of increasing doses of $ZnSO_4$ (Fig. 2.2 A). HO-1 mRNA levels were also relative to the amount of zinc exposure (Fig. 2.2A). Both protein and mRNA expression were highest around the same concentrations of $ZnSO_4$, at 80 μM. Hepa cells were also transfected with pHO15luc and luciferase activity was quantified (Fig. 2.2C). Expression of the reporter construct in the transfected cells was augmented with the increasing concentrations of $ZnSO_4$. Luciferase activity was induced more than 35-fold at its peak response.

2.7.2 Identification of Sequence Elements Necessary for Gene Activation by Zinc

Previous studies have demonstrated that two 5' distal enhancer regions, E1 and E2, mediate mouse *hmox*-1 gene activation in response to multiple inducers including heme, cadmium, and 15-deoxy-$\Delta^{12,14}$–prostaglandin J_2 (Alam et al., 1999; Alam et al., 2000; He et al., 2001). The role of E1 and E2 in $ZnSO_4$ responsiveness was tested using mutant derivatives of pHO-15luc. Targeted deletion of a 521-bp fragment containing the 268-bp E1 enhancer (ΔE1) inhibited $ZnSO_4$ responsiveness by greater than 80%, whereas deletion of the 161-bp E2 fragment reduced induction by only 10%, indicating a greater importance of E1 in this response.

E1 enhancer region , however, are required for optimal induction, as deletion of E1 (Figure 2.3A), completely abolished zinc responsiveness. The specificity of the E1 and E2 in this

response is demonstrated by the fact that deletion of sequences between -1.3 kb and -3.5kb (ΔB) did not affect zinc-induced luciferase expression.

The activity of the E1 region was examined in more detail because of its greater contribution to zinc responsiveness. E1, as originally defined, contains three copies of a sequence motif, termed the stress-response element (StRE), that are essential for transcription activation in response to multiple agents including heme. Arsenite, the StRE-binding protein implicated in these responses, is the transcription factor Nrf-2 (Alam et al., 2003). Additionally, a functional HSE was identified immediately downstream of the E1 enhancer (Alam et al., 2003; Stewart et al., 2003). Because Nrf-2 is subjected to proteasomal degradation (Alam et al., 2003; Stewart et al., 2003) and HSEs have been implicated in HO-1 expression by heavy metals (Alam et al., 2003), it was reasoned that one or both types of elements may be responsible for *hmox*-1 activation by zinc. To test this hypothesis, the original E1 enhancer was extended to include the HSE resulting in the 321-bp $E1^+$ fragment. (Incidentally, both the StREs and the HSE are deleted in the ΔE1 derivative of pHO-151uc). Luciferase reporter constructs under the control of WT or mutant $E1^+$ were stably transfected into Hepa cells and assayed for zinc inducibility. As shown in Figure 2.4, treatment of cells harboring the WT $E1^+$ construct with 80 μM $ZnSO_4$ increased luciferase activity by 12-fold over basal levels, whereas mutants lacking either the StREs {mutS} and {mutH}, exhibited significantly reduced induction by 80 %. While mutants lacking the {mutH} had no significant effect, no induction was observed with a mutant lacking both types of mutant response elements ({mut S},{mut H}). Since there is no StRE in E2 region, E2 plasmid construct not used. These results indicate that only the StREs are required for optimal *hmox-1* activation by $ZnSO_4$.

2.7.3 Transcription Factors Potentially Responsible for *hmox*-1 Activation by Zinc

The principal StRE-binding protein responsible for *hmox-1* activation (by multiple agents) is the transcription factor, Nrf-2, and the best characterized as StRE regulators. To better understand the mechanism of *hmox-1* activation by zinc, the effect of zinc on the expression of transcription factors in Hepa cells was examined. As shown in Figure 2.5C, zinc treatment promoted a nuclear accumulation of Nrf-2. This factor was detected as early as 30 min and continued to increase up to the last time point tested (4h). This is consistent with a role for Nrf-2 in *hmox-1* regulation, since an increase in cytoplasmic HO-1 was not detected until 3 h after initiation of zinc treatment (Figure 2.5B). Additionally, zinc-induced Nrf-2 is known to have StRE-binding activity (Stewart et al., 2003). Zinc treatment of Hepa cells did not activate HSF-1, (Figure 2.7B). It is well-known that Nrf-2 is not the sole regulator of HO-1. Other transcription factors such as Maf (Alam et al., 2003) contribute to HO-1 induction, and this explains why we have a lot of HO-1 at lower zinc concentration Fig 2.6 in In addition, HO-1 regulation by zinc in MEF cells was examined. Zinc treatment stimulated HO-1 expression in a dose-dependent manner in WT cells, but this response was not significantly altered in cells lacking HSF-1(Figure 2.8). Consistent with that observed in Hepa cells, zinc did not activate HSF-1 as judged by: 1) the absence of electrophoretic retardation, (Figure 2.7B) and 2) lack of Hsp70 (Figure 2.8)induction in MEF. These results corroborate the earlier conclusion that the HSE/HSF pathway is not involved in *hmox-1* activation by zinc.

2.7.4 Nrf-2 and StRE Mediate *hmox*-1 Gene Activation by Zinc

Previous studies from our laboratory using a dominant negative mutant (DNM) of Nrf-2 have implicated this transcription factor in *hmox-1* activation by cadmium (Alam et al., 1999; Alam et al., 2000). Given the newl findings of StRE, and its role as a target for the transcription

factor, the role of Nrf-2, and also , on the induction of the E1+-luciferase fusion gene in response to zinc was investigated. Because of the limited specificity of DNMs, it was decided to inhibit or knock down Nrf-2 expression by RNA interference (RNAi) using several individual small hairpin RNA (shRNA) constructs made against Nrf-2 . Initial experiments tested the efficacy of these constructs. Nrf-2 shRNA constructs were tested for their ability to inhibit Nrf-2 transactivation of a luciferase reporter under the control of three tandem copies of a StRE, p3xStRE-luc. In transient transfection assays using Hepa cells, co-expression of either of the shRNA/Nrf-2 constructs decreased luciferase activity by approximately 75%, with Nrf-2[192] demonstrating the most effective response (Figure 2.8A).

The basal- and zinc-induced transcription was then measured by luciferase assays in the cells containing pHO15luc. RNAi-mediated knockdown of Nrf-2 diminished HO-1 production, while HSF-1 knockdown did not (Figure 2.9). The normal Hepa cells were also stably transfected with two different Nrf-2 shRNA constructs, Nrf-2[192] and Nrf-2[437]. Whole cell protein extracts were then assayed for Nrf-2 and HO-1 protein expression. The Nrf-2[192] treated cells showed diminished Nrf-2 and HO-1 expression, while the cells transfected with Nrf-2[437] showed no Nrf-2 and significantly less HO-1(Figure 2.8A).

2.7.5 How Does Zinc Activate HO-1 Expression?

The Keap 1-dependent model of Nrf-2 degradation provides a plausible mechanism. Recently, several groups have identified critical cysteine residues in Keap1 that are required for Keap1-dependent ubiquitinoylation of Nrf-2 and the Keap1-mediated repression of Nrf-2 activation under basal conditions (Dinkova-Kostova et al., 2002; Levonen et al., 2003; Zhang and Hannink, 2003). Cysteine residues contain thiol groups that react with inducers by alkylation, oxidation, reduction, or thiol interchange ─ which results in modification of the

protein, such as the formation of disulfide bonds, and protein conformational change (Prochaska et al., 1985; Dinkova-Kostava et al., 2001). Mutation of these residues resulted in loss of repression of Nrf-2. In addition, selective cysteine residues (Cys257, Cys273, Cys288, and Cys297) were shown to interact with several inducers, including dexamethasone mesylate and 15d-PGJ2. The cysteine residues in Keap1 appear to function as redox-sensitive molecular switches that react with inducers through their cysteine-thiol groups, resulting in the disruption of the Keap1-Nrf-2 complex and release of Nrf-2. Furthermore, zinc was shown to bind to protein-sulfhydryl groups of MT and GSH molecules (Waisberg, 2003, Waalkes, 2003). Two possible mechanisms for Nrf-2 release are: 1) Zinc binds the thiol groups in the cysteine residues of Keap1, or 2) ROS generated by zinc oxidizes the thiol groups in the cysteine residues of Keap 1. Both scenarios promote conformational changes in Keap1, resulting in release of Nrf-2.

Finally, future work is required to answer questions, such as whether other antioxidants are more effective than zinc in stimulating Nrf-2 levels. This needs to be investigated, because there are less potent inducers of HO-1 mRNA. One explanation for this discrepancy is that the steady-state level of Nrf-2 is not the sole determinant of Nrf-2 activity. Such activity is likely to require post-translational modification, such as phosphorylation, which may occur optimally in response to zinc or other antioxidants. This possibility is supported by the observation that mutation of the StREs within E1 enhancer t completely abolish zinc-dependent transcription activity of the enhancer (Figure 2.4).

Additional support is provided for a non-StRE/Nrf-2 model such as: the toxicity of zinc is associated with the promotion of cellular oxidative stress, either by enhanced production of ROS or inhibition of cellular antioxidant defenses. This imbalance in redox homeostasis is expected to alter protein conformation through various mechanisms, including modification of glutathione-

protein mixed disulfide bonds and inter- and intra-protein-protein disulfide bonds (Senisterra et al., 1997; Zou et al., 1998b, Dinkova-Kostova et al., 2001). Accumulation of damaged and unfolded proteins would then activate HSF-1, as described previously.

The preliminary data presented in this study suggests that the StRE motifs play an important role in the E1+ enhancer. In luciferase gene reporter studies, mutation of StREs and demonstrated their necessity and cooperativity in the transcriptional activation of *hmox-1* gene by zinc. Additionally, RNAi knockdown studies and the use of Nrf-2 deficient cells demonstrated that Nrf-2 and StRE are both necessary for E1+ activation and HO-1 protein expression by zinc. Taken together, the data suggest that cooperativity exists between StRE and Nrf-2. The synergistic effects of StRE and Nrf-2 could arise by several different mechanisms: 1) a direct interaction between transcription factors in which one or both interact with the transcriptional machinery, 2) no interaction between transcription factors but one or both interact with the transcriptional machinery, or 3) an indirect interaction between transcription factors via coactivator(s) in which interaction with the transcriptional machinery can occur through the coactivator or through Nrf-2 and StRE alone, together. These protein interactions can result in altered DNA-binding activities or transactivation properties (Lin et al., 1990; Chinenov and Kerppola, 2001; Courey, 2001).

In summary, this study identified one of the conserved motifs in the E1 enhancer, the StRE that is involved in zinc-induced *hmox-1* gene regulation. The data demonstrate that the StRE(s) appear to activate the *hmox-1* gene in response to zinc.

Analyses have led to three main findings: 1) the identification of a functional StRE in the 5' end of the E1 that binds to Nrf-2 in response to zinc, 2) the knowledge that both Nrf-2 and StRE are necessary and cooperate together in the transcription activation of the *hmox-1* gene by

zinc, and 3) that not all HO-1 inducers utilize the HSE/HSF-1 pathway for activation. Collectively, these findings point to the possibility of an independent cellular heat shock response pathway that is responsible for *hmox-1* gene regulation. This finding is consistent in one part with previous work done by Stewart et al. (2003) in which it was demonstrated that the conserved StREs within the *hmox-1* E1 enhancer, in conjunction with the transcription factor Nrf-2, mediate gene activation in response to cadmium in several cell types including Hepa cells (Alam et al., 2000; He et al., 2001; Stewart et al., 2003).

3. ZINC-MEDIATED INDUCTION OF THE HEME OXYGENASE-1 GENE IN MOUSE HEPA CELLS BY THE EXTRACELLULAR – REGULATED KINASE (ERK1)

3.1 Introduction

3.1.1 Zinc and Signal Transduction

The three classes of the mitogen-activated protein kinase (MAPK) family are: extra cellular signal-activated protein kinases (ERK), Jun N-terminal kinases, and p38 MAP kinases. The kinases activated by cells exposed to zinc include protein kinases C (PKC) JNK, extracellular regulated kinase 1/2 ERK1/2, p38 kinases (Zhang et al., 1998; Seo et al., 2001; Kohda et al., 2006; Kim et al., 2007), tyrosine kinases, and casein kinases II (Masuya et al., 1999). Support for the involvement of cellular protein kinases in the regulation of transcription factors comes from studies using inhibitors of various kinases. The studies showed that inhibition of PKC and MAPK/ERKs blocked zinc induced overexpression of c-Jun and c-Fos (Zhang, et al., 2001).

One of the approaches to study zinc and its relation to signal transduction pathways used glucose deficiency (GD) induced cell death, which was demonstrated through regulation of ERK1/2. It was shown by switching the GD–induced necrosis to apoptosis by decreasing the ROS production through the action of superoxide dismutase (SOD), which was brought about by the inhibition of copper /zinc degradation. This led to activation of caspase 3 and caspase 9, which has a pro-apoptotic activity (Kim et al., 2007). In another study of the rat renal cortex, cell nuclear fractions appeared after zinc treatment, which caused an increase of 4-hydroxynonena (a well-known lipid peroxidation). Zinc increased the expression of pERK and pELK- in the nuclear fraction, which later suppressed by U0126 (p-ERK1/2) inhibitor (Kohda et al., 2006).

In another study, a well known toxin, peroxynitrite, has been demonstrated to play a major role in white-matter pathogenesis by using oligodendrocytes (OLs) as a primary cell

culture. Since it oligodendrocytes expresses the myelin basic protein, it has been shown that the maintaining intracellular zinc homeostasis prevents oxynitrite progression by inhibiting p-ERK42/44 phosphorylation of ERK42/44, inhibiting activation of 12-lipoxygenase, and eliminating the accumulation of reactive oxygen species (Zhang et al., 2005). Zinc deficiency has also been proven to increase cellular proliferation: in a model for neuronal cells. When cells are incubated with low zinc media, the cell showed an increase in cell oxidants and hydrogen peroxide (H_2O_2), which increases JNK and p38 activation, high nuclear AP-1-DNA binding activity, and AP-1-dependent gene expression. However, the addition of the catalase reversed the reduction of cell proliferation (Zago et al., 2005). In another study, the oxidative role of zinc has been implicated by inhibition of the glutathione cuased by the intracellular zinc which causes an increase in the ERK1/2 activation in HT22 cells (McLaughlin et al., 2001). Another study supporting the oxidative role of zinc found that zinc released from intracellular stores by MIT triggers the activation of ERK1/2; persistent ERK1/2 activation also is observed in glutamate-induced oxidative toxicity in the HT22 neuroblastoma cell (Zhang et al., 2004).

My goal in this chapter is to show which MAPKs are activated as a response to zinc induction and which ones are responsible for the upregulation of the HO-1 expression. I will be showing all theseobservations by using Lucifirase assay, by measuring the proteins level, using cell knock outs, and by using MAPKs inhibitors.

3.1.2 Zinc-Mediated Induction of Heme Oxygenase-1 Gene in Mouse Hepa Cells by Extracellular-Regulated Kinase (ERK1)

All of the previously mentioned studies have focused on demonstrating that zinc plays an important role in the signal transduction pathway(s), which has a profound positive impact on the oxidative stress response.

Redox-sensitive transcription factor NF-E2-related factor-2 (Nrf-2) has been demonstrated as the potential regulator of the stress response elements (StrE) (Nguyen, et al., 2002). When there is no oxidative stress condition, Nrf-2 is in the cytoplasm binding to Keap-1 (Itoh et al., 1999). Under oxidative stress condition, this complex is disrupted, thereby releasing Nrf-2, which subsequently translocates to the nucleus where it binds to other transcription factors such as Maf and Jun members (Hayes and McMahon, 2001). Phosphorylation and stabilization are required to liberate Nrf-2 from Nrf-2-Keap1 (Huang et al., 2002), and since the Nrf-2 has a half-life of 30 min, it becomes degraded by the ubiquitin-proteasome pathway (Alam et al., 2002).

In this study, the effect of zinc on the activation of signal transduction, and the signaling pathways leading to HO-1 expression was investigated.

3.2 Experimental Procedures

3.2.1 Materials

The tissue culture media and gentamicin were from Invitrogen (Carlsbad, CA), and fetal bovine serum was obtained from Mediatech (Herndon, VA). Antibodies against transcription factors (Nrf-2) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), whereas anti-rat HO-1 was acquired from StressGen Biotech. Corp. (Victoria, Canada). Protease inhibitors were purchased from Calbiochem (San Diego, CA). Reagents for luciferase assays were acquired from Sigma Chemical Company (St. Louis, MO). All other chemicals were of reagent grade.

3.2.2 Immunoblotting

The 30 µg of cell lysate were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corp. Blots were analyzed with the appropriate antibodies (1:500), anti-

ERK2 (1:1000), anti-p38 (1:1000), anti-Nrf-2 (1:16,000), (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-Ser^{473} AKT1 (1:1000), anti-phospho-ERK2 (1:1000), anti-phospho-p38 (1:1000), Inc., Beverly, MA); anti-phospho-JNK (1:1000) (Upstate Biotechnology, Inc., Lake Placid, NY); and anti-HO-1 (1:1000) (Stressgen Biotech Corp., Victoria, British Columbia, Canada). Appropriate peroxidase-conjugated secondary antibodies (1:10,000) were used to detect the proteins of interest by enhanced chemiluminescence.

3.3 Results

3.3.1 Several MAPK Signaling Pathways Are Employed During Zinc-Induced *hmox*-1 Expression

MAPK signaling pathways are used during many different types of cell activity. Different transcription factors are activated by various combinations of these kinases. The action of MAPK signaling pathways, which consists of the phosphorylation cascade of several kinases and proteins, has been implicated in *hmox-1* expression by several inducers. This study explored several of the main players including ERK1/2, JNK, p38, and AKT. AKT, a serine/threonine-specific kinase, is phosphorylated by PI-3K. Cells were treated with zinc for increasing amounts of time and then measured for expression of the kinases, as well as their respective phosphorylated forms (Figure 3.1). Phosphorylation of ERK, JNK, and p38 all appear to increase with time of zinc exposure, and these phosphorylated before the induction of HO-1.

Dominant negative mutants (DNM) of several intracellular signaling molecules were created and transfected into cells along with the reporter plasmid. These cells were then treated with zinc and tested for luciferase activity (Figure 3.3A).

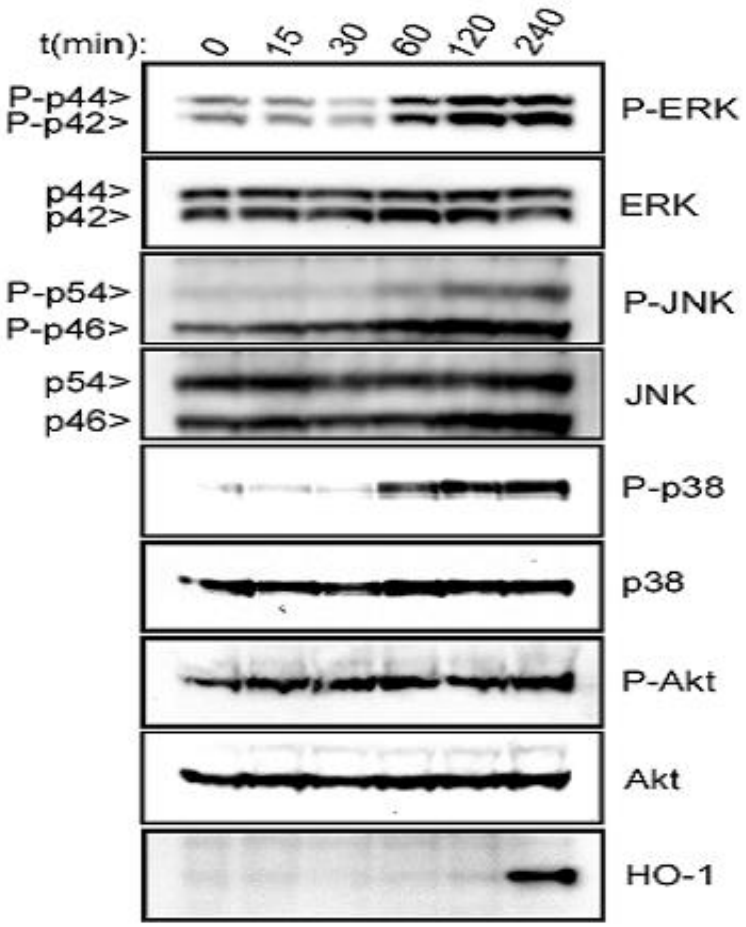

Figure 3.1 Effect of Zinc on phosphrylation of protein kinases Induction of HO-1 by Zinc. Map kinases beside P1-3K are activated in Hepa cells. Hepa cells were incubated for 24 h. ERK, phosphorylated ERK(P-ERK),p38 and phosphorylated o-p-38 (p-38) ,c-Jun ,N-terminal kinase (JNK) and phosphorylated c-Jun N-terminal kinase (p-JNK) proteins were detected by western blotting. MAPKs are activated by dual phosphorylation of threonine and tyrosine residues located in the 'activation lip'of the conserved core kinase sequence, the activated species (p-p44, p-p42 (ERK1/2), p-p54, p-p46(JNK), p-p38(P-38), P44, P42, P54, P46, and p38 are controls respectively.

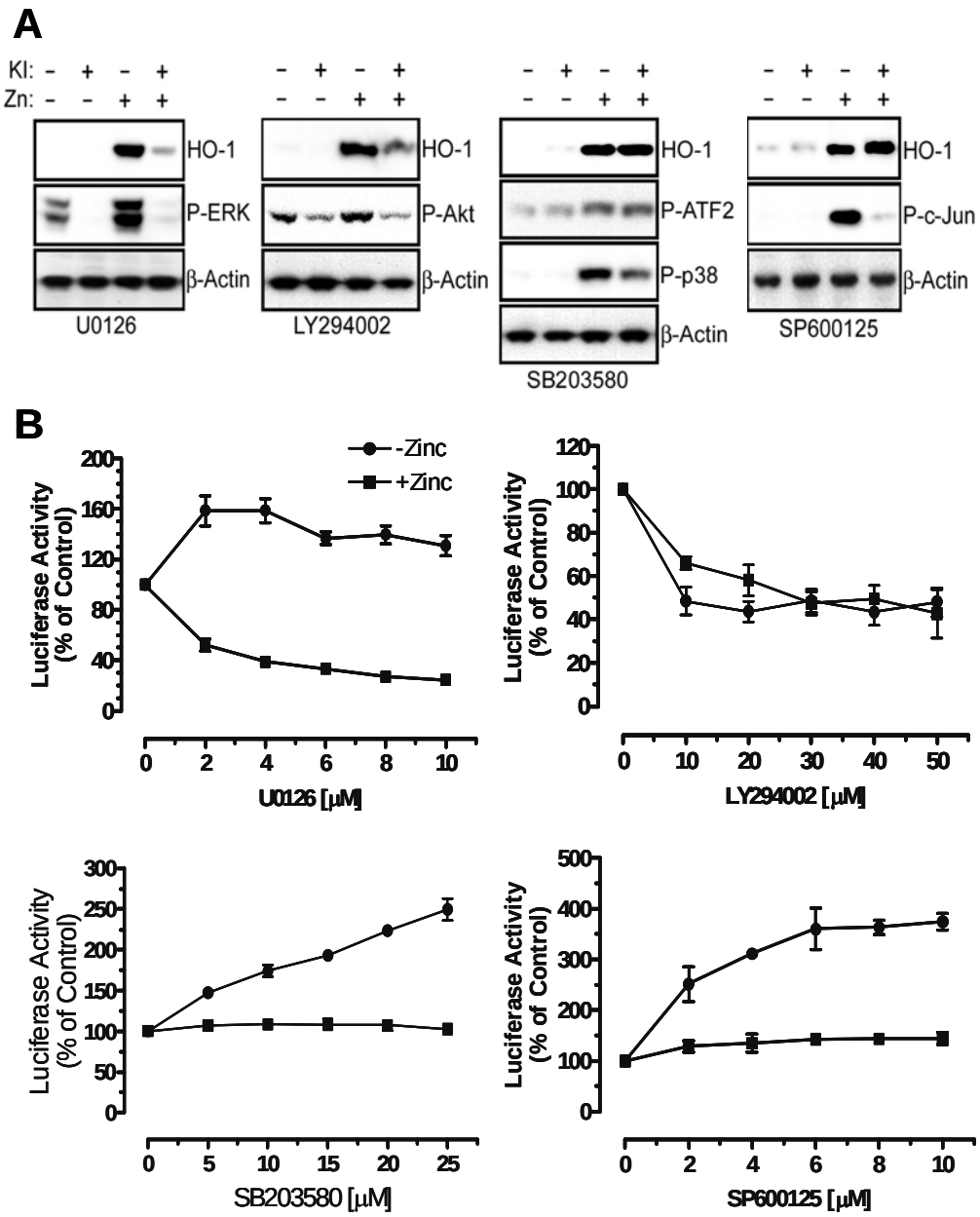

Figure 3.2. Effect of MAPK and PI-3K Inhibitors on Induction of HO-1 by Zinc**.** (A) Hepa cells were maintained in a low serum medium for 24 h and then were pre-incubated with UO126 (ERK1/2 inhibitor), 40 μM LY294002 (p38-specific inhibitor), 5 μM SB203580 (AKT-specific inhibitor), or 10 μM SP600125 (a C-junk inhibitor) for 15 min. Map kinases beside PI3K activated in Hepa cells. Hepa cells were incubated for 24 h and ERK, phosphorylated ERK(P-ERK), p38 and phosphorylated o-p-38 (p-38), c-Jun, N-terminal kinase (JNK) and phosphorylated c-Jun N-terminal kinase (p-JNK) proteins were detected by western blotting. (B) Hepa cells were maintained in low serum medium and submitted to 80 μM $ZnSO_4$ for 3 h. $ZnSO_4$ treatment induced luciferase activity in the mouse HO-1 promoter in the presence of the MAPK and PI-3K inhibitors.

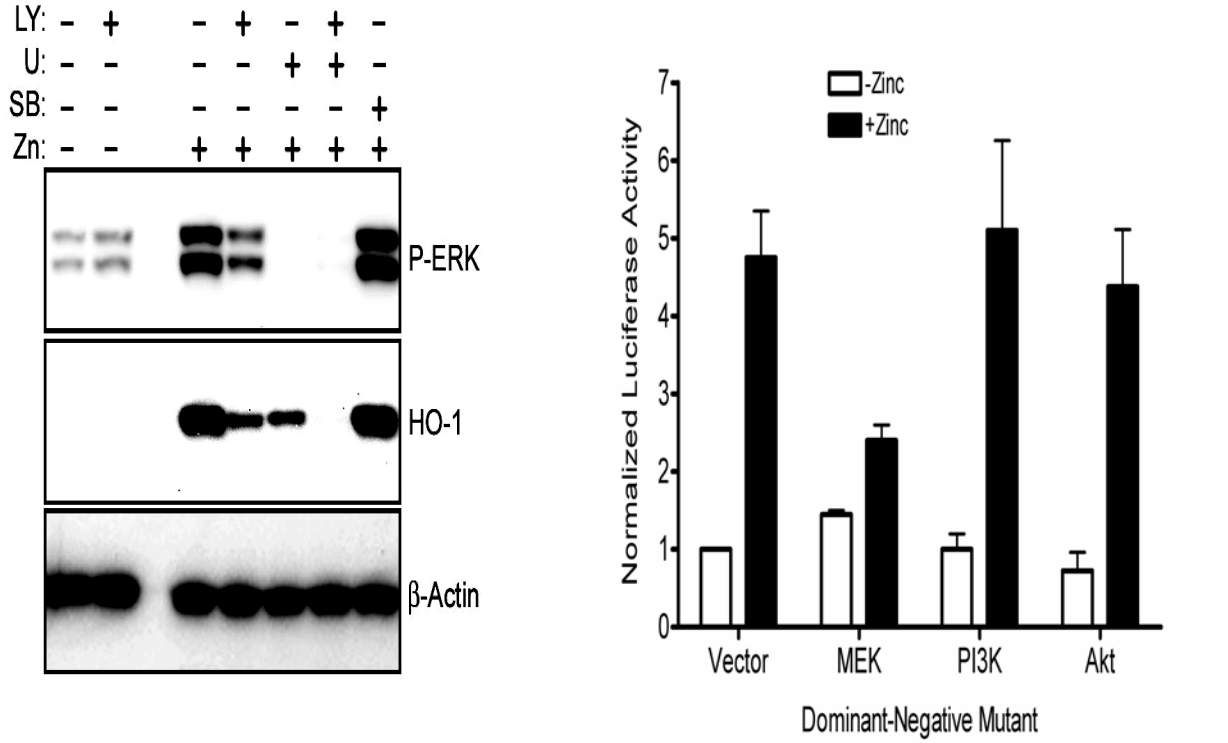

Figure 3.3 The Involvement of ERK1 Rather than AKT orPI-3K in Zinc-Mediated –*hmox*-1 Induction. In serum-free medium, cells were exposed to vehicle (DMSO) or the indicated kinase inhibitor in for 1h prior to addition of 0 or 80 uM $ZnSO_4$. Cells were incubated for an additional 4 h and then processed for immunoblot analysis. Twenty or 80ug (P-AKT) of total cell extract were used for SDS-PAGE .A representative blot from 2-3 experiments is shown in.B. The induction of HO-1 expression of the unattenuated by the dominant-negative PI-3K and AKT dominant-negative mutants (DNM) of several intracellular signaling AKT and PI-3K caused molecules to be created and transfected into cells along with the reporter plasmid. These cells were then treated with zinc and tested for luciferase activity. (mean ± SE, n=5, $p<0.01$).

3.3.2 ERK1 Alone Is Enough for *hmox*-1 Induction

A requirement for active ERK1/2, but not protein PI-3K and AKT has been shown in Figure 3.3

A and B. Moreover, the long-term oxidant effect of zinc, partially blocked by ERK1 or HO-1

inhibitors Fig 3.3A, further demonstrated that zinc attenuates oxidative stress through a pathway that involves ERK1 and HO-1 (Figure 2.5A)

Another western analysis was performed in which antibodies against P-ERK1/2 alone completely diminished the HO-1 induction (Figure 3.3A). This was done to clear the previous result, which had shown some kind of non-specific binding of the anti-p-PI-3K and AKT.

3.3.3 Involvement of the ERK Signaling Cascade in Zinc Induction of *hmox*-1

To investigate the role of ERK1/2 in zinc induction of HO-1, several ERK pathway components (activated, ERK1 activated negative,) were co-transfected with pHO-15Luc, then left untreated or treated with zinc. If the ERK was important for zinc signaling, an activated component of the pathway would increase luciferase gene activity in the absence of zinc, while the negative would block the ability of zinc to induce luciferase activity from the reporter gene. As shown in Figure 3.4A, activated ERK1 increased luciferase gene activity, indicating each component led to induction of gene expression.

3.3.4 HO-1 Expression Caused by Zinc Exposure Is Dependent upon the Transcription Factor Nrf-2 and the Activation of ERK1

Since other studies have revealed that Nrf-2 and HSF-1 play important roles in HO-1 induction by heavy metals (Alam et al., 1999; Alam et al., 2000; He et al., 2001; Stewart et al., 2003), it was investigated as to how these transcription factors may be regulating this mechanism. Treatment of Hepa cells with increasing doses of $ZnSO_4$ caused greater expression of Nrf-2 (Figure 2.6A). As HO-1 production is primarily controlled by transcriptional regulation, it was examined as to how Nrf-2 adjusts HO-1 expression in Hepa cells. EMSAs were performed using oligo 5'GATCTTTTATGCTGTGTCATGGTTT3', which showed the formation of specific DNA-protein complexes. The movement of these structures was slowed by the addition of anti-Nrf-2 antibody; meaning Nrf-2 is binding X regions on *hmox-1* (Figure 2.6B). Direct

binding of *hmox-1* cis-regulatory elements by transcription factors showed that Nrf-2 definitely contributes to the regulation of HO-1.

Next, the nuclear levels of zinc-induced Nrf-2 protein were analyzed. As shown in Figure 3.4B, zinc and ERK1 showed a strong accumulation of Nrf-2 in the nucleus (Figure 3.4B,C) where the level increase is ERK1-dependent, since it has been not blocked by the specifice inhibitorr,this makes think that ERK1 is not the only regulater of the Nrf-2. to verify the functional relation of the Nrf-2 binding to the HO-1 StRE, Hepa cells were contransfected with pHO-15luc, and expression vector for dominant negative mutant (DNM) AKT, (DNM) PI-3K and ERK-1 *hmox-1* was significantly decreased in the dominant-negative ERK1. These findings suggest that the major role of ERK1, in the zinc–induced HO-1 (Figure 3.3A, B).

3.4 Discussion

3.4.1 Induction of Transfected *hmox*-1 Promoter Luciferase Reporter Constructs by Zinc

In this study, the pathways that contribute to the increase in *hmox-1* regulation by zinc were investigated. The search for the specific MAP kinase that is responsible for the upregulation of *hmox-1* was also. First, it was found that zinc upregulated *hmox-1* by increasing the Nrf-2 level in nucleus and increasing the ERK1, which targets the Keap-Nrf-2 complex in the cytoplasm. This triggers the release of Nrf-2 and increases its concentration in the nucleus, where it binds to its target, the StREs in the E1 region located in the mouse. *hmox-1* gene promoter, at least one of the two factors needed for the mediation, increases the Nrf-2 protein levels in coordination with the increase in the ERK1. This is the first time that a specific and detailed indication of which MAP kinase upregulates *hmox-1* by zinc, even though it is well-known that zinc induces *hmox-1*.

3.4.2 Extracellular Signal Regulated Protein Kinase1 (ERK1) Is Required for *hmox*-1 Induction

It is well-known that ERK1/2 activation plays a major role in cell survival, proliferation, and differentiation (Xia et al., 1995) during cell injury. For instance, Cheng et al. (2006) show that, under oxidative stress in neural cells, ERK1/2 is the major player in inducing apoptosis after using a biocide methylisothiazolinone (MIT), which increases the zinc concentration via the oxidation of cellular thiols (Du et al., 2002). In another study, it has been demonstrated that ERK1/2 activation can be caused by another pathway; that is, glutathione depletion has been mediated by intracellular zinc release in the HT22 cells. These present some models of cell death by ERK1/2 (McLaughlin et al., 2004; Zhang, 2004).

These results show that, despite the fact that ERK1/2, p38, PI-3K, and JNK are activated by increasing the zinc concentration, they are involved to some extent in the induction of HO-1 in Hepa cells. For example, ERK1/2 is very important for HO-1 upregulation (Figure 3.2A and B), whereas, inhibition of JNK, p38, and PI-3K have minimal effect on zinc-induced HO-1 (Figure 3.2A).

These findings are similar to those of others who have searched for the specific down stream kinase that controls the increase in HO-1 induction. When a dominant-negative version of the PI-13K was used, AKT and ERK1, only a slight decrease in the HO-1 promoter was observed in the PI-3K and AKT dominant negative mutant (DNM) (Figure 3.3A). This finding agrees with other findings, where they used food phytophenols (carnosol) in PC12 cells (Numazawa et al., 2003). Other findings that did not agree used (DNM) JNK in HO-1 (Alam et al., 2000). .

A p38 inhibitor (SB 203580*)* (SB*),* and JNK-c inhibitor (SP600125) were able unable to block most of the zinc-mediated induction of HO-1at the same time they didn't decrease the HO-1 level of expression . In contrast, for the ERK1/2 pathway inhibitor, (UO126) ,activated

components were able to induce HO-1 gene expression Fig 3.2A and dominant negative AKT components were unable to block zinc induction.but mutated ERK is able to abolish HO-1 (Fig 3.2B). These experiments implicate a role for the ERK and at least an element

3.4.3 Transcriptional Regulation of *hmox*-1 Expression by Zinc in Cytoplasm and Nucleus Cytoplasmic and Nuclear Nrf-2 Protein

To investigate the potential Nrf-2 translocation through MAPK pathways, ERK1 KO MEF cells(Fig 2.5A) ,but ERK-2 is detected since the cells are ERK1.KO.MEF),In the next step I was able to detect Nrf-2 in both cytoplasm and nuclear extract in cells that were exposed to the indicated concentration of $ZnSO_4$ for 4 h. Hepa cells were treated by zinc for 5 h in the presence/absence of inhibitor (UO126) (Nrf-2 translocation is an upstream event for HO-1 expression), in whole cells and nuclear fractions were prepared for western blot analysis. As shown in Figure 2.5B and C , the Nrf-2 protein level in fractions remained unchanged following the incubation with zinc, MAPK signaling pathway inhibitor(UO126) . However, zinc evidently promoted Nrf-2 translocation into nuclei through the action of ERK1 (Fig 2.5A).

3.4.4 ERK1 KO and UO126

Zinc evidently promoted Nrf-2 translocation into nuclei, as demonstrated by the relatively no nuclear Nrf-2 expression in both the ERK1 knockout KO MEF cells and in the zinc-treated Hepa cells.(Fig 2.5 B,C) This expression was higher than in untreated Hepa cells. Zinc-induced Nrf2 translocation was not blocked by the UO126.

These results have shown the profound role that ERK1 plays in the activation of *hmox-1* zinc, since the inhibitors of P38 (SB203580), AKT specific inhibitor (LY200496), and a C-JNK inhibitor (SP600125) have no effect on HO-1 activation (Figure 3.3), while the ERK1 inhibitor UO126 completely diminishes *hmox-1* activation (Figure 3.2). Moreover, confirmation about the

role of the ERK1/2 results indicate that ERK1 is the major MAPK responsible for zinc-induced HO-1 (Figure 2.5A) is due to cross reactivity of the AKT inhibitor; (LY200496) a, similar finding on arsenite-induced HO-1 in rat hepatoma cells (Kietzmann et al., 2003). p38 has been shown to have a major role in the arsenite induction of *hmox-1* using the LMH chicken hepatoma cell line (Elbirt et al., 1998).

The extracellular signals coupled through the conserved proline–directed serine threonine kinases MAPKs by serial phosphorylation cascade (Torres and Forman, 2003). As mentioned previously, Nrf-2 is a member of the Cap'n'Collar family of basic leucine transcription factors, and plays an essential role in the StREs phase II detoxifying enzymes and stress-inducible genes (Alam et al., 2000). Previous work on oxidant research has suggested that MAPK is a central pathway involved in Nrf-2 activation and translocation for highly-specific protein synthesis, including most readily inducible *hmox-1* (Calabrese et al., 2005). These results showed that the ERK1 signal plays an important role, which is involved in Nrf-2 translocation from the cytoplasm into the nucleus and HO-1 induction by zinc. This is, to some extent, is similar to the Diallyl sulfide- treated –HepG2 (Gong et al., 2004) and ethanol-treated HMC-1 cells (Jeong et al., 2005). To clarify, it was demonstrated that the ERK1 signal is the major factor responsible for *hmox-1* upregulation, even though other signals downstream also have a role in HO-1 induction. It is a well-known idea that MAPK can be differentially regulated by the same stimuli in diverse cell types. The ERK pathway is thought to mediate cellular responses to growth and differentiation factors, whereas JNK and p38 enzymes are activated by distinct and overlapping sets of stress-related stimuli (Alam, et al., 2000). The small expression (artifact) indicated that other signaling pathway(s), tyrosine kinase, and phosphatidylinositol 3-kinase pathways are justified by the nonspecific binding of the p-PI-3K/akt to the ERK1/2.(Fig 3.3).

Finally, it is concluded that the incubation of mouse Hepa cells with zinc induce oxidative damage, which increases the ROS, thereby activating the ERK1/Nrf-2. An assay to determine this kind of protection (for example, in measuring the liver enzyme and/or the HO-1 activity caused by the high zinc content in the seafood) will be helpful.

In this study, the ability of zinc to increase transcription of endogenous HO-1, and the activity of transfected luciferase reporter gene constructs under control of the HO-1 promoter was investigated. Transient transfection assays were used to investigate the mechanism of zinc-mediated HO-1 gene expression. In mouse Hepa cells, the activities of MAP kinases, ERK, JNK, and p38 were increased by treatment with zinc. Activation of MAP kinases correlated with zinc-mediated induction of endogenous HO-1 mRNA expression. Activated components of the ERK1 kinase signaling pathway increased gene expression from an HO-1 promoter-driven luciferase reporter gene construct. An ERK1 inhibitor, a MEK inhibitor, and the ERK kinase pathways blocked most of the zinc-mediated induction of HO-1.

In contrast, for the JNK pathway, p38, and PI-3K activated components were unable to induce HO-1 gene expression but their inhibition did decrease HO-1 expression were JNK E p38 and PI-3K inhibitors unable to block zinc induction. HO-1 reporter. These experiments implicate a role for the ERK1, and at least one Nrf-2, in the zinc-mediated induction of *hmox-1* genes (Fig2.5).

These results indicate that zinc activates HO-1 expression, probably by translocation of the Nrf-2 protein in an ERK1-dependent manner (Figure 2.5A, B). Therefore, in addition to its oxidant nature, zinc activates the ERK1 survival pathway and upregulates expression of HO-1.

4. GENERAL DISCUSSION

4.1 Heme Oxygenase-1

HO-1 catalyzes the initial and rate-limiting step in heme catabolism, thereby generating equimolar quantities of iron, carbon monoxide, and biliverdin, which is metabolized further to bilirubin by biliverdin reductase (Choi and Alam, 1996; Elbirt et al., 1999; Montellano, 2000; Otterbein and Choi, 2000;Stewart et al., 2003). HO-1 is an important inducible enzyme with antioxidant and cytoprotective activities that works against oxidative stress in response to heme and also to various stress stimuli, such as arsenite, carnosol, and cadmium (Alam and Cook, 2003, Stewart et al., 2003). The cytoprotective functions of HO-1 depend on the activities of the reaction products (antioxidants biliverdin and bilirubin) and on the inducibility of HO- 1, which is regulated at the gene transcription level by two distal enhancers, E1 and E2 (Stewart et al., 2003). Identification and analysis of the enhancers resulted in the discovery of multiple StREs, which are essential and sufficient for *hmox-1* gene regulation by most inducers, including heme, cadmium and arsenite (Choi and Alam, 1996; Alam et al., 1999; Alam et al., 2000; He et al., 2001; Alam and Cook, 2003; Stewart et al., 2003; Chou, 2005). In this study, zinc activated the E1 enhancer to a greater extent and exhibited higher inducible transcription activity than E2. This suggests that E1 is crucial in *hmox-1* gene activation.

Additionally, studies identified Nrf-2, a CNC-bZIP transcription factor, as a dominant regulator that mediates inducer-dependent regulation of the *hmox-1* gene via the StRE motifs in response to several inducers including cadmium (Alam and Cook, 2003). However, the mechanism of how Nrf-2 is regulated was not completely understood.

4.2 Regulation of Nrf-2 Activity Before this study, accumulating evidence indicated that Nrf-2 was regulated, in part, by

subcellular compartmentalization in the cytoplasm, and that it was transported to the nucleus after stimulation by stress agents to activate target genes (Itoh et al., 1999; Kobayashi et al., 2002; Sekhar et al., 2002; Zipper and Mulcahy, 2002; Stewart et al., 2003). In unstimulated cells, Nrf-2 is inactive and sequestered in the cytoplasm in part, or in total, as the result of binding to a cytoskeleton-associated protein Keap1 (Itoh et al., 1999; Dhakshinamoorthy and Jaiswal, 2001; Kobayashi et al., 2002; Sekhar et al., 2002; Zipper et al., 2002; Stewart et al., 2003). Keap1 contains an N-terminal region (NTR), a broad complex, Tramtrack, Bric-a-Brac/ poxvirus and zinc finger (BTB/POZ) domain, intervening region (IVR), a double glycine region (DGR) or Keich repeats, and the C-terminal region (CTR) (Figure 4.1) (Stewart et al., 2003; Itoh et al., 2004). As Stewart (2005) further stated:

> Nrf-2 and Keap1 interact through the N-terminal Neh2 domain of Nrf-2 and several regions of Keap1 including the IVR, DGR, and CTR. Once stimulated by oxidative stress promoting agents, like zinc, Nrf-2 is liberated from Keap1 suppression, which allows Nrf-2 to translocate to the nucleus and activate target genes, like *hmox-1*. It is well known that important transcription factors are bound and repressed by inhibitory proteins as observed, for example, with the NF-kB/lkBa system (Weissman, 1997; Destero et al., 2000). (p. 122)

However, initially it was discovered that the steady-state level of Nrf-2 protein increases upon exposure to zinc as a result of ERK1 activation. Furthermore, it was observed that Nrf2 translocation was in the nucleus, not the cytoplasm, in response to zinc. This suggested an alternative mechanism during oxidative stress as presented in this model. In fact, the most important finding was that Nrf-2 regulation by zinc,.

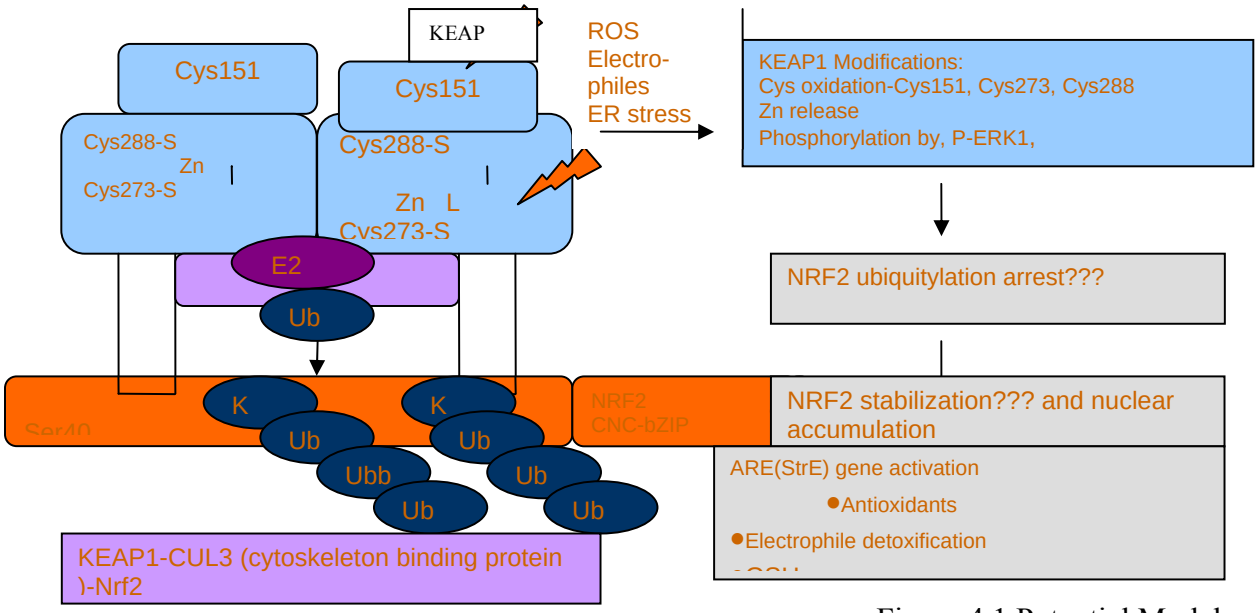

Figure 4.1 Potential Model for Zinc-Induced *hmox*-1- in Mouse Hepa Cells.

Enhanced oxidation of zinc in the hepa cells with physiological concentrations of afforded protection against glutathione depletion in response to moderately oxidized, electrophilic agents and hypochlorous acid. I subsequently established that moderately oxidized zinc rapidly stimulates phosphorylation of primarily ERK1/2, p38, MAPK, and c-jun-N-terminal kinase (JNK), leading to transcriptional activation of HO-1 expression via the Nrf-2/StRE signaling pathway.

This needs to be investigated first by decreasing the rate of Nrf-2 degradation by the ubiquitin-proteasome pathway. The second potential pathway is that, in response to oxidants like zinc, Nrf2 stability occurs by escaping recognition and degradation via the ubiquitin-proteasome pathway These are just my assumptions and further work needs to be done regarding the separation the two potential pathways .

Stewart (2005) states,

> Interestingly, the mechanism that regulates Nrf-2 degradation appears to be linked to the Keap 1-dependent subcellular compartmentalization pathway. Support for this role comes from studies showing that Keap1 functions not only to sequester Nrf-2 in the cytoplasm, but also to actively increase the rate of proteasomal degradation of Nrf-2 by directly interacting with an ETGE motif within the N-terminal Neh2 domain of Nrf-2 (Figure 3.1.) (McMahon et al., 2003; Itoh et al., 2003). Oxidative stress antagonizes this interaction resulting in the release of Nrf-2 protein and its translocation into the nucleus. (p. 124)

As described in Chapter 2, research by McMahon et al. (2003) identified the Neh2 domain of Nrf-2 as the degron sensitive to oxidative stress. Degrons are binding sites for ubiquitin ligases. The Neh2 degron is located in the N-terminus of Nrf-2 within the transcriptional activation domain and near the Keap1 binding site. Recently, several groups showed that Keap1 functions under basal conditions to negatively regulate Nrf-2 by acting as an adaptor protein for the Cullin3-Roc1 E3 ubiquitin ligase though its BTB and IVR domains (Stewart et al., 2003; Cullinan et al., 2004; Kobayashi et at., 2004; Zhang et al., 2004; Furukawa and Xiong, 2005). The Keap1-Cullin-Roc1 complex promoted ubiquitinylation of Nrf-2 and the subsequent degradation by the 26S proteasome in the cytoplasm. Furthermore, Zhang et al. (2004) demonstrated that seven lysines in the Neh2 domain of Nrf-2 are the primary determinants of Keap1-Cul2-Roc1-dependent ubiquitinylation of Nrf-2. The ubiquitin-conjugating enzymes involved in the ubiquitinylation process during basal conditions are known;

however, the Nrf-2 binding site remains unknown (Stewart et al., 2003). McMahon et al. (2004) identified a DIDLID element in the Neh2 domain of Nrf-2 that appears to be necessary for recruiting a ubiquitin ligase. In addition to the Neh2 degron required for degradation of Nrf-2 under basal conditions, a redox-sensitive degron, Neh6, was identified during oxidative stress (Stewart et al., 2003; McMahon et al., 2004). Although the Neh6 degron has been identified, the ubiquitin-conjugating enzymes involved in the ubiquitinylation process remain unknown. Furthermore, Nrf-2 appears to harbor other degrons because inhibition of both the Neh2 and Neh6 domains left a fairly short protein with a half-life of 2 h. According to Stewart et al. (2003), in the original Nrf-2-Keap1 model, cytoskeletal protein Keap1 was thought to sequester Nrf2 in the cytoplasm by binding to the actin skeleton, where it targets Nrf-2 for ubiquitin-proteasome mediated degradation. Recent work by Velichikova and Hasson (2005), however, identified a nuclear export sequence (NES) in Keap1 and demonstrated that Keap1 sequesters Nrf-2 in the cytoplasm, not by binding to the actin cytoskeleton, but via an active Crm1/exportin-dependent nuclear export pathway (Figure 4.1) (Stewart et al., 2003).

Velichikova and Hasson (2005) suggested that because Nrf-2 has a nuclear localization sequence (NLS), the Keap1 NES exports Nrf-2 to the cytoplasm for ubiquitin-mediated degradation and prevents accumulation of Nrf-2 in the nucleus and activation of target genes under basal conditions (Stewart et al., 2003). Support for this role is derived from recent research showing that Nrf2 contains both an NLS and an NES (Stewart et al., 2003; Jain et al., 2005; Li et al., 2005;). Stewart (2005) also stated,

> Finally, Zhang and colleagues (2005) demonstrated that, during oxidative stress, Keap1 is ubiquitinylated by the same Cul3 complex that ubiquitinylates Nrf-2, but that it is degraded independently from the proteasome. The mechanism that targets Keap 1 for proteasome-independent degradation needs to be studied in the future. (p. 125)

4.3 Important Findings of Current Work

This dissertation research involved a detailed investigation on the mechanism in which ERK1/Nrf-2 transduction pathway in Hepa cells stimulated by zinc, as well as the identification of a particular MAPK ERK1 and StRE in the E1 enhancer involved in zinc-dependent activation of the *hmox-1* gene.

Here, I summarize the important findings are summarized. During the investigation designed to characterize Nrf-2 activation, it was noted that various HO-1 inducers increased the steady-state level of the Nrf-2 protein. In an effort to understand the increase in Nrf-2 protein expression, it was shown that Nrf-2 translocation to the nucleus is dependent on ERK1, and the key function of the ERK1 is to precisely control the activity of the Nrf-2 in order to upregulate *hmox-1*. Furthermore, inhibition of Nrf-2 induction using ERK1 KO, MEF cells, abolished zinc-stimulated activation of Nrf-2 and, subsequently, its binding to a StRE oligonucleotide. Inhibition of Nrf-2 did not completely inhibit zinc-induced HO-1 mRNA accumulation, which demonstrates that *hmox-1* gene activation is only dependent on increased Nrf-2 protein expression. However, studies from our laboratory have shown that mutation of all three StREs in the E1 region does not completely abolish induction of a luciferase reporter construct by cadmium treatment (Alam et al., 2000; Stewart et al., 2005, unpublished data). Additionally, ectopic expression of an Nrf-2DNM does not completely inhibit activation of the *hmox-1* gene or the E1-controlled luciferase gene by cadmium and zinc in several cell types, including Hepa cells (Alam et al., 1999; Alam et al., 2000; He et al., 2001, unpublished data). These studies suggest that Nrf-2 may not be the sole regulator of zinc-dependent *hmox-1* gene activation and that other transcription factors and *cis*-acting DNA elements may be involved in *hmox-1* gene regulation.

4 Implication the Role of MAPK ERK1 and its Impact on Nrf-2

HO-1 induction by zinc. Furthermore, the ERK pathway is mainly responsible for zinc-derived HO-1 induction, while another MAPK (S) is mainly responsible for zinc upregulation of HO-1 induction. In addition, it was found that UO126 ERK1 inhibitor showed higher HO-1 inhibiting effects in zinc-treated Hepa cells than untreated Hepa cells. Hence, findings suggest that HO-1 is induced mainly through the ERK pathway under normal conditions and zinc incubation, rather than through the PI-3K pathway under zinc incubation or multi-pathways. Interestingly, UO126 showed almost the same inhibitive effect on zinc-induced Nrf-2 translocation. This apparent discrepancy is due to inhibitors nonspecific binding that other signaling pathways, such as protein kinase C, tyrosine kinase, and PI-3K pathways, which might also mediate zinc-derived Nrf-2 translocation to induce HO-1 expression for regulation of Nrf-2.

4.5 Implication of HO-1 Induction by Zinc and the Role of ERK1

Previous work done on antioxidants has shown that there is an activation of MAPK in response to an antioxidant. For example, Cheng et al. (2006) demonstrated that HO-1 induction in response to Alpha-lipoic acid (ALA) is a natural oxidant that phosphorylates P44/42 MAPK pathways and increases HO-1 expression in A10 cells. In another study (An et al., 2004), ERK1/2 and other MAPKs accumulated in SH-SY5Y cells of Alzheimer's disease (An, Bjorkdahl et al., 2005). Furthermore, Yao et al. (2007) demonstrated that flavonoids, including quercetin, protected human hepatocytes from ethanol-induced oxidative stress by ERK1/2, which translocates the Nrf-2 into the nucleus and induces HO-1 (Yao et al., 2007).

In the events immediately following ERK1 activation by zinc, which acts on the Nrf-1-Kelch complex, it was oberved that the initial activation of ERK1 played an important role in the increase—most likely through phosphorylation (Pearson et al., 2001). This needs to be

investigated later. It was also observed that Nrf-2 played an essential role in zinc–mediated HO-1 induction. Other factors need to be identified, and questions need to be answered

The magnitude of the ERK1 signal and its transient nature have been well known about the extracellular signal-regulated kinases (ERKs), which are characterized by the TEY consensus sequence in the activation loop. The signal is activated by many factors during cell proliferation and differentiation (Pearson et al., 2001; Murphy et al., 2006). Furthermore, MAPKs signals are very heterogeneous, since ERK1 has a specific function─ phosphorylation/dephosphorylation of transcription factors. In these studies, ERK1,Nrf-2 has a critical characteristic. For example, Nrf-2 is translocated to the nucleus, findings demonstrate the common function of ERKS after being in the nucleus during phosphorylation of the Thr and Tyr residues at the T*X*Y motif (Pearson et al., 2001). After this, ERK1 can phosphorylate many transcription factors and, subsequently, these factors regulate the expression of many genes (Shen et al., 2006).

These findings agree wih my findings; when I used the ERK1 KO, there was a significant reduction in the HO-1 expression ,compared to the wild type Fig 2.5A. Previous investigations of ERK signals have shown that the signals are transient, which implies that the down-regulation of the ERKs is controlled by dephosphorylation (Farooq et al., 2004).

Taking all these facts into account, it was concluded that the findings are real. Other factors contributing to Nrf-2 activation should be studied further to determine if other factors contribute to ERK1 signals in terms of phosphorylation and dephosphorylation.

In summary it can be concluded, in comparison to the findings of others, that incubation of mouse Hepa cells with zinc increases HO-1 induction and mediates the zinc effect via ERK1 via ERK1/Nrf-2 transduction.

.4.6 Implication of Cooperativity between Two Pathways in hmox-1 Regulation

The preliminary data presented have suggested cooperation between Nrf-2-StRE and Nrf-2-ERK1 in the motifs of the E1+ enhancer and the MAPK ERK1 in response to zinc. Having these two major factors activated pathways can be beneficial in *hmox-1* regulation. The StREs serve as the binding sites for several transcription factors, of which Nrf-2 seems to be dominant. Likewise, the *hmox-1* gene is activated by a variety of stress-associated agents; however, both systems are not responsive to the same inducer. This flexibility in promoter binding and activation by various inducers allows regulation under more varied cellular conditions and via several mechanisms, thus increasing the combinatorial possibilities that determine the level of transcription. Additionally, having two pathways allows for fine-tuning of the transcription response. Cooperation between the ERK1 and StRE can amplify the response, depending on the inducers and transcription factors involved. Also, these two pathways can serve as back-up systems so that if either of the Nrf-2 StRE pathways is inhibited, one of the transcription factors will still be able to respond and up-regulate partial HO-1 expression. Finally, under nonstress conditions, there are more options to use these transcription factors, for instance in cellular growth and development.

In conclusion, these studies addressed the regulation of *hmox-1* gene by two element transcription factors, Nrf-2 and StRE, in response to zinc. Nrf-2 was shown to be a highly labile protein in response to oxidants such as zinc Evidences come from my results which show the cooperative role of the Nrf-2 and StRE, and as one more piece of evidence, whenever we mutated the StRE and or the ERK1, this had a profound negative impact on Nrf-2 induction.

How it escapes recognition and degradation by the ubiquitin-proteasome machinery and activates the *hmox-1* gene needs to be investigated.

Analyses of the conserved motifs in the E1 enhancer led to the discovery of the well-characterized StRE as an additional element (besides the Nrf-2) involved in zinc-induced *hmox-1* gene regulation. Furthermore, the data presented suggests that both Nrf-2 and StRE function in a cooperative manner to activate the *hmox-1* gene in response to zinc. Establishing the cooperative role of both Nrf-2 and StRE in *hmox-1* regulation will require further analysis, including examination of StRE and Nrf-2 deficient mice, as well as the interaction that occurs between these two transcription factors and the second pathway that involves the ERK1, which is activated in the presence of ROS. This leads, first, to its translocation to the nucleus where phosphorylation of the Nrf-2 occurs, and then to HO-1 gene induction. The identification of other factors involved in this mechanism is needed.

Finally, a better understanding of why certain inducers use the StRE/Nrf-2 HSE/HSF-1and ERK1-Nrf-2 pathway will help to establish the different mechanisms involved in *hmox-1* regulation and to provide important information on new avenues of research and strategies for therapeutic intervention and treatment in diseases involving oxidative stress and HO-1 expression.

On the basis of the data obtained in this study, the literature, previous studies with Nrf-2 (Alam et al., 1999; Alam et al., 2000; He et al., 2001; Stewart et al., 2003), and speculation of plausible mechanisms of HO-1 activation by zinc, a model for *hmox-1* gene activation by zinc in Hepa cells is proposed (Figure 4.1). In unstimulated cells, Nrf-2 is a highly labile protein that binds to Keap1, which is rapidly and specifically translocated by the action of the ROS either in the cytoplasm or in the nucleus (Figure 3.5A), and by the action of the MAPKs (ERK1-1), Nrf-2 is phosphorylated. The *hmox-1* gene is either repressed or exhibits low levels of gene activity by StRE-binding proteins (StRE-BP). Likely candidates for repression include heterodimers

between StRE and Nrf-2. Recent studies show that Bach1 and MafK each bind the *hmox-1* StREs, and targeted deletion of the *bach1* gene results in increased constitutive expression of HO-1 mRNA and protein (Ogawa et al., 2001; Sun et al., 2002). Whether there are repressor proteins at the HSE site is unknown. Upon cellular stimulation, between Nrf-2 and Keap1, Nrf-2 protein is released and translocates into the nucleus (Figure 4.1). In the nucleus, Nrf-2, after phosphorylation, displaces StRE-BP repressors. Likewise, zinc toxicity generates ROS, which results in protein damage.

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VITA

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Salim then returned to Jordan, where he was employed by Jordan University as a clinical immunology instructor. In 2001, Salim was awarded a scholarship from Jordan University to study in the USA. Salim was accepted, in 2001, into the graduate program in the Department of Pathobiological Sciences at Louisiana State University, and completed the requirements for the degree in the spring of 2008. Salim is married to Nisrin -aburaida, and they have son, Jasper, and beautiful daughter, Dana.