

LAI, CHENGZHI, M.S. The Effects of Cadmium on Heme Oxygenase-1 in HCT116 Human Colon Epithelial Cells With or Without Iron Deficiency. (2009)
Directed by Dr. George Loo. 96 pp.

Metals may have contrasting biological effects. Iron, a component of a variety of iron-containing proteins, plays an important role in maintaining a healthy human body. In contrast, cadmium, a contaminant and carcinogen, has been considered one of the most toxic elements in the environment. As a defending mechanism against the exposure to cadmium, cells increase expression of cytoprotective genes such as heme oxygenase-1 (HO-1). Although both the importance of iron and the toxicity of cadmium are well known, it is not clear whether iron is required for the defending mechanism (that is, the upregulation of HO-1) against the toxicity of cadmium. In my thesis project, the effects of cadmium on HO-1 in HCT116 human colon epithelial cells with or without iron deficiency were investigated. It was found that cadmium upregulated HO-1 mRNA and protein expression and enzyme activity, but these effects were decreased by desferoxamine (DFO), an iron chelator, suggesting iron plays a critical role in cadmium-induced upregulation of HO-1. This conclusion was supported by two other observations: 1) another iron chelator, 2',2'-dipyridyl (DPD), also decreased the upregulating effects of cadmium on HO-1 mRNA and protein expression; 2) iron sulfate, but not zinc sulfate and copper sulfate, restored the upregulating effects of cadmium on HO-1 mRNA and protein expression and enzyme activity in iron-deficient cells caused by the pretreatments with iron chelators, DFO or DPD. Further experiments were conducted to help explain the observations. There

were two primary findings: 1) cadmium decreased intracellular glutathione levels, similar in the effects of glutathione inhibitors, ethacrynic acid (EA) and buthionine sulfoximine (BSO); however, only cadmium and EA, but not BSO, increased the expression of HO-1 mRNA and the nuclear expression of nuclear factor-E2-related factor-2 (Nrf-2), suggesting that cadmium may have the same effect as EA to directly react with intracellular glutathione; 2) being similar in effect to iron chelators, NADPH oxidase (NOX) inhibitors such as apocynin and diphenyleneiodonium (DPI), and superoxide scavenger, tiron, decreased the upregulating effects of cadmium on HO-1 mRNA and protein expression and enzyme activity. Compiled together, the results suggest that NOX-produced ROS play an important role in cadmium-induced HO-1 upregulation; cadmium induces intracellular accumulation of ROS by depleting intracellular glutathione, and increases nuclear Nrf-2 expression, which all lead to the upregulation of HO-1 expression. Moreover, it is possible that iron chelators, DFO and DPD, deplete iron contained in heme, a component of NOX, decreasing NOX-produced ROS, therefore decreasing the upregulating effect of cadmium on HO-1. In conclusion, the results imply that cadmium could be more toxic to iron-deficient cells than to iron-sufficient cells, suggesting that cadmium exposure could result in more severe consequences in an iron-deficient population than in a healthy population.

THE EFFECTS OF CADMIUM ON HEME OXYGENASE-1 IN HCT116 HUMAN
COLON EPITHELIAL CELLS WITH OR WITHOUT IRON DEFICIENCY

by

Chengzhi Lai

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro

2009

Approved by

Committee Chair

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

Date of Acceptance by Committee

Date of Final Oral Examination

ACKNOWLEDGEMENTS

Firstly, I would like to thank my mentor, Dr. George Loo, for directing and helping me develop and finish this project with incredible patience. Secondly, I would like to thank Dr. Keith Erikson, Dr. Ron Morrison, and Dr. Gregory Raner, for their interests in this project and servicing in my committee. Thirdly, I would like to thank Ms. Jennifer Longpre, for helping me develop research skills. Fourthly, I would like to thank the Department of Nutrition, the School of Human Environmental Sciences, and the Graduate School, for providing me the financial supports including Tuition Waiver, the Graduate Assistantship, the Esther Segner Scholarship, and Summer Assistantship, which make it possible for me to complete this project. Furthermore, I am so grateful to the help offered by all the lovely faculty and staff members and graduate students in the Department of Nutrition, all the instructors lectured me, and the staff in other supporting departments. Finally, I would like to thank my wife, Jia Min, and my family for their unconditional love, supports, and encouragements.

I love you all.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	v
CHAPTER	
I. INTRODUCTION.....	1
Review of literature.....	1
Research problem.....	13
Thesis objective and hypothesis tested	13
References.....	16
II. THE INFLUENCE OF IRON DEFICIENCY ON CADMIUM-INDUCED UPREGULATION OF HEME OXYGENASE-1 IN HCT116 CELLS.....	23
Abstract	23
Introduction.....	23
Materials and methods	25
Results.....	29
Discussion	33
References.....	38
Figures.....	42
III. ELUCIDATION OF CADMIUM-INDUCED UPREGULATION OF HEME OXYGENASE-1 IN HCT116 CELLS.....	55
Abstract	55
Introduction.....	56
Materials and methods	58
Results.....	65
Discussion	69
References.....	76
Figures.....	80
EPILOGUE.....	94

LIST OF FIGURES

	Page
Figure I-1. Heme degradation catalyzed by heme oxygenase	2
Figure I-2. The transcriptional regulation of heme oxygenase-1 gene expression	6
Figure I-3. The generation and function of reactive oxygen species	8
Figure I-4. GSH/GSSG antioxidative system	9
Figure I-5. The absorption, transport, storage, and chelation of iron	12
Figure II-1. Cadmium upregulated HO-1 mRNA and protein expression in iron-sufficient HCT116 cells	42
Figure II-2. Cadmium upregulated HO-1 enzyme activity, and this effect could be decreased by desferoxamine.....	43
Figure II-3. DFO decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression	44
Figure II-4. DPD decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression	46
Figure II-5. Iron restored the upregulating effects of cadmium on HO-1 protein and mRNA expression in the cells pretreated with DFO.....	48
Figure II-6. Iron restored the upregulating effects of cadmium on HO-1 protein and mRNA expression in the cells pretreated with DPD.....	50
Figure II-7. Iron, but not copper and zinc, restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with DFO	52
Figure II-8. Iron restored the upregulating effect of cadmium on HO-1 enzyme activity in the cells pretreated with DFO	54
Figure III-1. Cadmium, EA, and BSO decreased the intracellular glutathione levels (determined by using Ellman's reagent).....	80

Figure III-2. Cadmium, EA, and BSO decreased the intracellular glutathione levels (determined by using monochlorobimane).....	81
Figure III-3. The effects of cadmium and EA on HO-1 mRNA expression.....	82
Figure III-4. Cadmium and EA, but not BSO, increased nuclear Nrf-2 expression (by using Western immunoblotting analysis and TransAM™ kit).....	84
Figure III-5. Cadmium and EA, but not BSO, increased nuclear Nrf-2 expression (by using immunocytofluorescence microscopy).....	86
Figure III-6. HCT116 cells expressed NOX-1 protein and NOX inhibitors, apocynin and DPI, and superoxide scavenger, tiron, decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression	88
Figure III-7. NADPH oxidase inhibitors, apocynin and DPI, and superoxide scavenger, tiron, decreased the upregulating effect of cadmium on HO-1 enzyme activity	90
Figure III-8. DPI, but not apocynin and tiron, inhibited NADPH: cytochrome P450 reductase (NCPR) enzyme activity.....	92
Figure III-9. Possible elucidation of the effects of cadmium on HO-1 in the cells with or without iron deficiency	93

CHAPTER I

INTRODUCTION

Review of literature

1. Brief introduction of the function, induction, and transcriptional regulation of heme oxygenase-1

Heme oxygenase (HO) is a rate-limiting enzyme that catalyzes the degradation of heme during the process of which free iron, carbon monoxide (CO), and biliverdin are produced. Biliverdin is then converted to bilirubin rapidly by biliverdin reductase (Figure I-1, page 2). To date, three isoforms of HO have been identified: HO-1, HO-2, and HO-3 (McCoubrey 1997). Among them, HO-2 is considered as a constitutively expressed isoform which is similar to HO-3; HO-1 is the inducible isoform and is also known as a 32-kDa heat shock protein (Keyse 1989). HO-1 could be induced by a variety of extra- or intra-cellular stress including the exposure to ultraviolet (UV) radiation, oxidative stress, hypoxia, heme, endotoxin, inflammatory cytokines, and heavy metals (Keyse 1989, Applegate 1991, Rizzardina 1993). The induction of HO-1 has been believed to be a protective response, and has been reported to be a rapid protective mechanism in rhabdomyolysis-induced kidney injury in rat (Nath 1992) and to have protective effects on cardiac xenograft and cold preservation of rat liver graft (Soares 1998, Redaelli 2002). It also has been reported that HO-1 induction may reduce the ischemia/reperfusion injury and chronic allograft deterioration in kidney transplants (Tullius 2002) and may protect skin fibroblasts against UV light-induced oxidative stress (Vile 1993). In contrast, HO-1 deficiency may increase

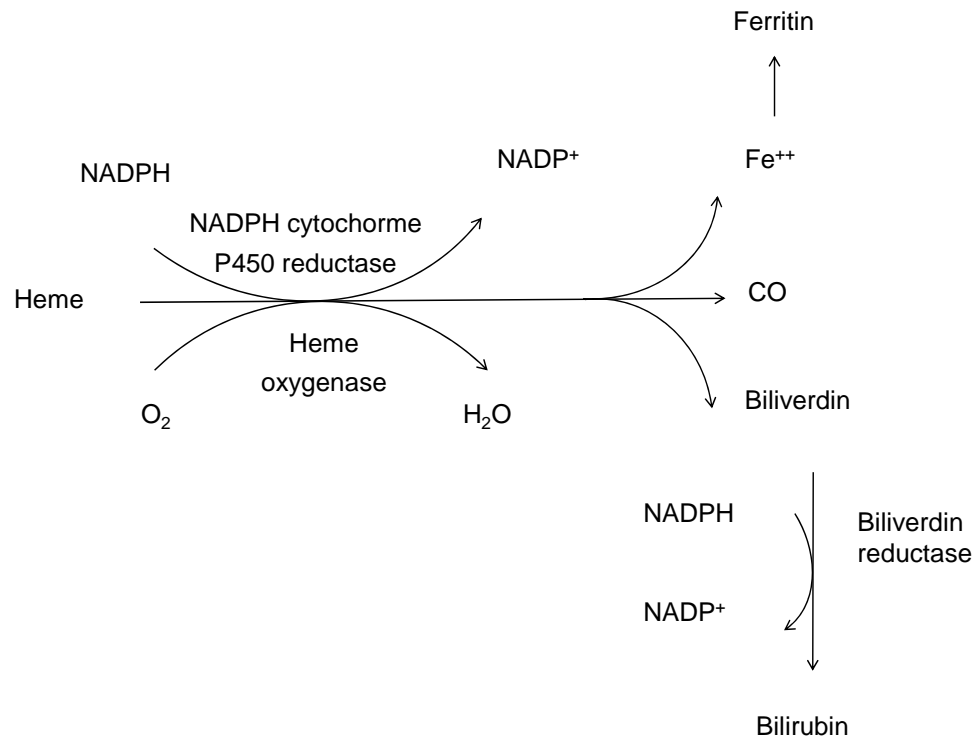


Figure I-1. Heme degradation catalyzed by heme oxygenase. Heme is degraded to free iron (Fe^{++}), carbon monoxide (CO), and biliverdin by heme oxygenase (HO). Biliverdin is then rapidly converted to bilirubin by biliverdin reductase.

iron accumulation in mice and decrease stress defense against oxidant damage in cells (Poss 1997). It was reported that a six year old human patient with HO-1 deficiency exhibited growth retardation, persistent anemia, and eventually died of intracranial hemorrhage (Yachie 1999).

The basic mechanism of the protective effects of HO-1 has not been fully demonstrated. But it has been believed that the products heme degradation, carbon monoxide (CO), biliverdin/bilirubin, and iron, of the degradation of heme by HO-1 play important roles. CO, which is well known as a toxic air pollutant, has been shown to have a variety of biological functions related to neurotransmission (Snyder

1998), protection against oxidative injury and cell death (Brouard 2000), cell proliferation (Morita 1997), and tolerance of organ transplantation (Sato 2001). CO has been reported to have the properties to inhibit platelet aggregation and smooth muscle relaxation (Ramos 1989). CO also has been shown to have an anti-inflammatory effect by regulating proinflammatory cytokines including tumor necrosis factor α (TNF- α), interleukin (IL)-1 β , and IL-6 and anti-inflammatory cytokine IL-10 via the p38 and c-Jun N-terminal kinase mitogen-activated protein kinase (MAPK) pathways (Otterbein 2000, Morse 2003).

Another product of the degradation of heme by HO-1 is biliverdin. Biliverdin is quickly converted by biliverdin reductase to bilirubin, which is known as a toxin to the central nervous system (CNS) in newborns (Kappas 2004). Biliverdin and bilirubin have been shown to have diverse protective effects in vitro and in vivo. It has been reported that bilirubin has the property to scavenge ROS in vitro, reduce cellular oxidative damage, and attenuate oxidant stress in vivo (Stocker 1987). Furthermore, bilirubin has also been shown to have a protective effect against oxidative stress caused by hydrogen peroxide (Sedlak 2004). Besides that, biliverdin has been reported to have protective effects against lung injury, lethal endotoxemia, liver ischemia/reperfusion injury, and inflammatory response in a lipopolysaccharide (LPS)-induced shock model in rat (Sarady-Andrews 2005). In addition, studies have shown that the absence of human biliverdin reductase, the enzyme converts biliverdin to bilirubin, attenuated arsenate-induced cell injury and increased apoptosis in 293 kidney cells (Miralem 2005).

The functions of ferrous iron, the last product of the degradation of heme by HO-1, are controversial. On one hand, ferrous iron is known to have the property to

increase intracellular ROS by converting hydrogen peroxide to hydroxyl radical via Fenton reaction. On the other hand, the release of iron from heme may increase the synthesis of ferritin, which in turn functions as an iron scavenger (Eisenstein 1991). Studies have shown that ferritin may protect endothelial cells against oxidative stress induced by iron and UV light (Vile 1993).

As mentioned above, HO-1 could be induced in a variety of circumstances including UV radiation, oxidative stress, hypoxia, heme, endotoxin, inflammatory cytokines, and heavy metals. This induction is regulated at a transcriptional level. Several transcription factors (TFs) have been identified to be involved in the induction of HO-1. Among them, nuclear factor (NF)- κ B, activator protein-1 (AP-1), and nuclear factor-E2-related factor-2 (Nrf-2) have been considered to be the most important ones. Although a stimulus may activate more than one TFs, it is thought that the activation of NF- κ B is relative more important in cytokine- or other mediator-induced inflammatory and immune response; AP-1 is related to cell growth or death; and Nrf-2 is activated by cellular stress caused by xenobiotics and oxidants.

Studies have shown that the activation of HO-1 expression involves interactions between a variety of basic-leucine zipper (bZIP) TFs and enhancer elements including Maf recognition element (MARE) (Kataoka 1994) and the stress responsive element (StRE) (Inamdar 1996). Nrf-2 is a bZIP protein which prominently dimerizes with another bZIP protein, small Maf protein (Itoh 1995, Marini 1997). This dimer then may bind to MARE, resulting in the activation of HO-1 gene expression. However, the small Maf protein may also bind to basic leucine zipper transcription factor 1 (Bach1), another bZIP protein, forming a heterodimer which may competitively bind to MARE, and repress HO-1 gene expression. Therefore, Bach1 is regarded as a

repressor of HO-1 gene expression (Igarashi 1998). In normal conditions, Nrf-2 is sequestered in the cytoplasm as an inactive form by Kelch-like-ECH-associated protein 1 (Keap1). Keap1 binds to Nrf-2, preventing Nrf-2 to be translocated into nucleus via increasing the degradation of Nrf-2 by ubiquitin-proteasome pathway, hence preventing HO-1 to be activated by Nrf-2 (Steward 2003). Keap1 is a protein rich in cysteine residues which make it to be sensitive to xenobiotics and oxidative stresses (Itoh 1999 and 2003, McMahon 2003). In the condition of oxidative stress, Nrf-2 is dissociated from Keap1 and is translocated into nucleus where it forms heterodimer with small Maf protein, and activate HO-1 gene expression (Figure I-2, page 6).

Cadmium has been shown to be a strong inducer of the expression of HO-1 gene (Taketani 1989). As for the mechanism, it has been reported that cadmium upregulates HO-1 gene expression by increasing the export of Bach1 from the nucleus (Suzuki 2003) and by stabilizing Nrf-2 through a mechanism of decreasing the degradation of Nrf-2 by the ubiquitin-proteasome pathway (Steward 2003).

2. Brief introduction of the exposure, absorption, and toxicity of cadmium

Cadmium, which has a long elimination half-life of 10-30 years, has been considered one of the most toxic elements in the environment (Jarup 1998). Cadmium has been classified as a contaminant by the U.S. Environmental Protection Agency (EPA) (ATSDR 1998) and a carcinogen of category 1 by the International Agency for Research on Cancer (IARC) (IARC 1993). It has been estimated that at least 512,000 U.S. employees work in an environment exposing them to cadmium (Wittman 2002) and the situation in developing countries is believed to be much more severe (Sethi 2006).

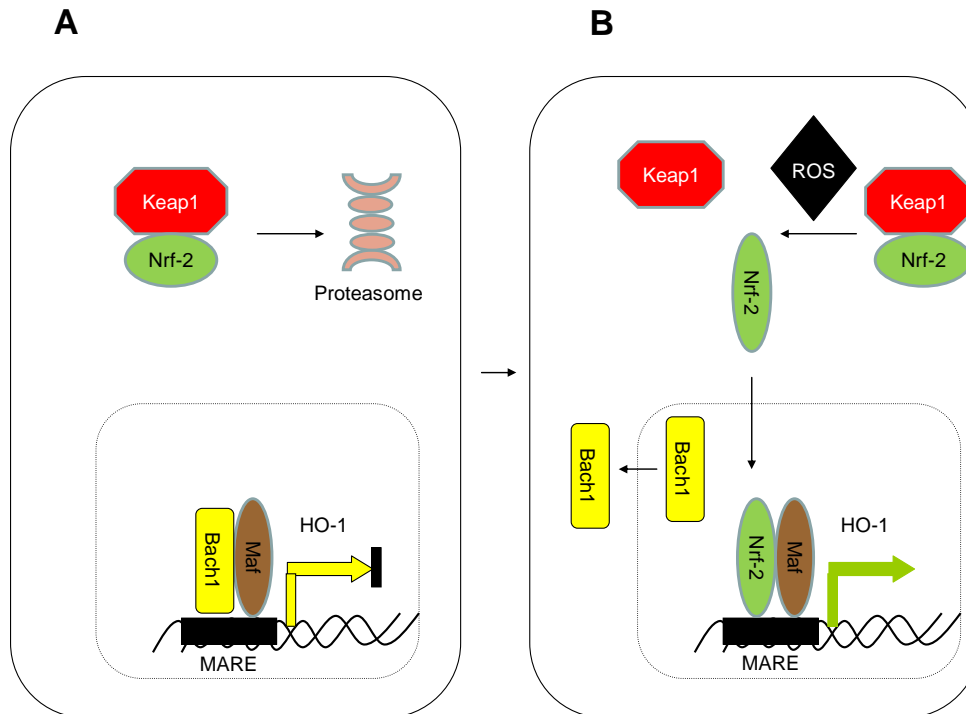


Figure I-2. The transcriptional regulation of heme oxygenase-1 gene expression. A: in normal conditions, the heterodimer of Bach1 and small Maf protein binds to MARE, and Keap1 sequesters Nrf-2 in an inactive form in cytosol, preventing Nrf-2 to be translocated into nucleus and increasing the degradation of Nrf-2 by proteasome, hence preventing HO-1 gene expression activated by Nrf-2. B: with the intracellular accumulation of reactive oxygen species (ROS), Nrf-2 is dissociated from Keap1 and is then translocated into nucleus where it forms heterodimer with small Maf protein, and activates HO-1 gene expression.

Cadmium exposure may result from mining, industrial emission, contaminated soil, pigments, plastic stabilizers, anti-corrosive products, and tobacco smoke. Plants absorb cadmium from cadmium-contaminated soil or water resulting in elevated cadmium concentrations in plant and animal products. People consuming these plant and animal products may have a higher risk of cadmium toxicity. Cadmium may be absorbed from respiratory tract and gastrointestinal tract. The absorbance from gastrointestinal tract is lower than from respiratory tract, but study has shown that low

dietary intakes of calcium, protein, zinc, iron, and copper may increase the absorption of cadmium. After being absorbed, cadmium is mostly accumulated in liver where it induces and binds to a cysteine-rich protein, metallothionein, and in kidney where it ultimately damages renal function (Jarup 1998).

Due to its long half-life, the accumulation of cadmium in human body is irreversible, causing kidney dysfunction, bone deformation, obstructive lung disease, hypertension, and cancers of lung, prostate, breast, and pancreas. The mechanism of cadmium toxicity has not been fully understood. But oxidative stress induced by cadmium seems to be involved. It has been shown that cadmium has the property to induce the production of reactive oxygen species (ROS), causing lipid peroxidation of cell membrane and DNA damage. The DNA damage has been believed to be involved in carcinogenesis caused by cadmium.

3. Brief introduction of the definition, production, and property of reactive oxygen species

Reactive oxygen species (ROS), including superoxide, hydroxyl radicals, and hydrogen peroxide, are small molecules and free radicals derived from oxygen. Reactive oxygen species (ROS) are constantly generated in response to a variety of stimuli. The generation of ROS starts from the production of superoxide which is rapidly converted to hydrogen peroxide by superoxide dismutase or in a low pH environment. Hydrogen peroxide then may be converted to hydroxyl radical via Fenton reaction. ROS could be generated as by-products of the usage of oxygen in mitochondrion, xanthine oxidase, peroxisome, and cytochrome P450 (Balaban 2005, Harrison 2004, Schrader 2004, Gottlieb 2003). ROS may also be produced from NADPH oxidase (NOX) and function as defending molecules against exogenous

pathogens in neutrophils (Geiszt 2003).

Studies have shown that ROS have the property to interact with a variety of molecules including carbohydrates, proteins, lipids, and nucleic acids, therefore appear to have a wide range of functions including immune defense (Geiszt 2003), inflammatory (Swindel 2007), cellular signaling (Furst 2005), gene expression (Clark 2004), cell growth regulation (Burdon 1995), and angiogenesis (Kuroki 1996) (Figure I-3, page 8). A number of studies have shown that ROS are involved in the

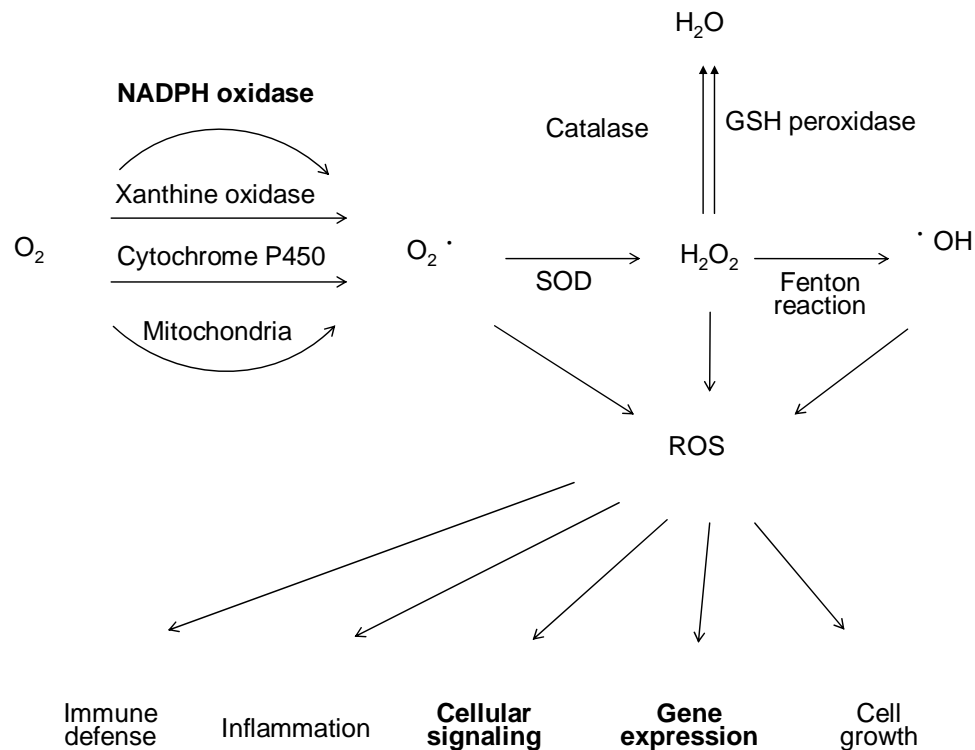


Figure I-3. The generation and function of reactive oxygen species. Reactive oxygen species (ROS) are generated in mitochondrion, xanthine oxidase, peroxisomes, NADPH oxidase, and cytochrome P450, beginning with the generation of superoxide ($O_2^{\cdot -}$), which is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) or in a low pH environment. Hydrogen peroxide (H_2O_2) then is catalyzed to hydroxyl radical ($\cdot OH$) via Fenton reaction. The functions of ROS include immune defense, inflammatory activity, cellular signaling, gene expression, cell growth regulation, and angiogenesis.

developments of a variety of health disorders, including cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion injury, and aging (Dhalla 2000, Jenner 2003, Dalle-Donne 2006). Normally, several defense mechanisms protect cells from ROS-induced damage, one of which is antioxidant defense mechanism. Antioxidant defense mechanisms include enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzyme substances such as ascorbic acid (vitamin C), α -tocopherol (vitamin E), glutathione (GSH)/glutathione disulfide (GSSG), carotenoids, and flavonoids. Among them GSH/GSSG system has been considered to play a major role in defense against ROS (Schafer 2001) (Figure I-4, page 9).

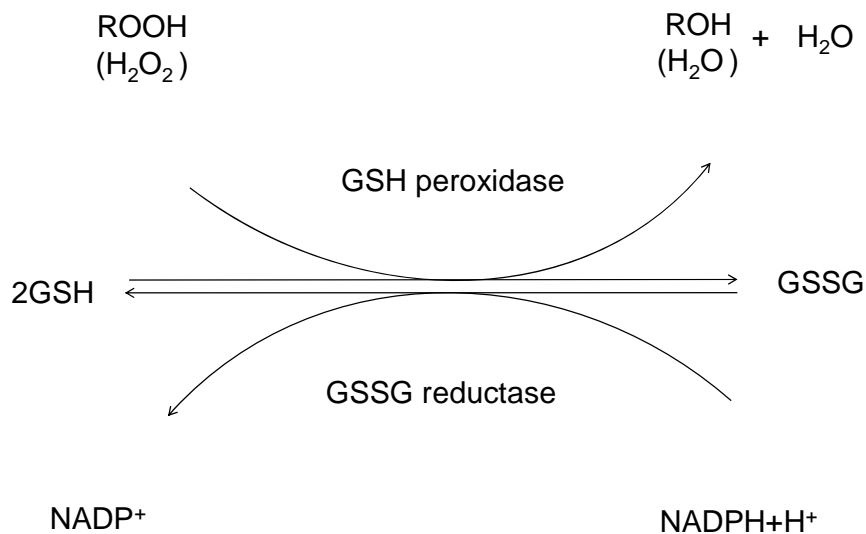


Figure I-4. GSH/GSSG antioxidative system. Organic peroxides (ROOH) or hydrogen peroxide (H₂O₂) is converted to their hydroxyl form (ROH) and water, respectively, by glutathione (GSH) peroxidase. At the same time, two molecules of GSH are converted to one molecule of glutathione disulfide (GSSG) which could be converted back to two molecules of GSH by GSSG reductase. GSH/GSSG ratio is commonly used to be an indicator of oxidative stress in cells and tissues.

4. Brief introduction of the requirement for iron in human body and the source, absorption, function, and deficiency of iron

The adult human body contains 2-4 g iron, or 38-50 mg iron/kg body weight. The majority (about 70%) of iron is contained in heme and hemoglobin (Ponka 1997). Since iron could be recycled from senescent erythrocytes, very little iron, 0.7-1.0 mg iron/kg body weight/day for men and postmenopausal women or 1.3-1.4 mg/kg body weight/day for premenopausal women, is lost (Smith 2005). The National Academy of Sciences, Institute of Medicine, and Food and Nutrition Board suggest that the Recommended Dietary Allowances (RDA) for iron are 8 mg/day for men older than 19 years and women older than 51 years and 18 mg/day for women aged from 19 to 50 years (The National Academies, 2001). General speaking, there are two major sources of iron: heme iron, which is rich in liver and organ meats, and nonheme iron, which is rich in nuts, dark green leafy vegetables, and dried fruits. Iron absorption occurs primarily in duodenum (Smith 2005).

Iron-containing heme is released from hemoglobin or myoglobin, and is rapidly absorbed into enterocyte of the small intestine. In enterocyte, ferrous iron is released from the degradation of heme by heme oxygenase-1 (HO-1). The iron released from nonheme iron-containing food is in the ferric form, which is reduced to ferrous iron in the intestinal lumen by duodenal cytochrome b (Dcytb). Ferrous iron is then transported into the enterocyte of the small intestine by natural resistance associated macrophage protein 2 (nramp2) or one of its isoforms, divalent metal transporter 1 (DMT1). Then, another transporter, ferroportin, exports ferrous iron, both from heme iron and nonheme iron, from enterocyte to circulation, followed by the oxidation of ferrous iron to ferric iron by hephaestin. Ferric iron in the circulation is attached to

transferrin which delivers iron to bone marrow or other tissues. Iron-attached transferrin in the circulation therefore binds to transferrin receptor (TfR) in the surface of tissue cells. The complex of ferric iron-binding transferrin and TfR is then imported to endosome via endocytosis. After being reduced to ferrous form in the acid environment of the endosome, iron is exported from the endosome by DMT1, and is used to synthesize iron-containing proteins. The extra iron is stored in ferritin which may bind up to 4,500 iron atoms (Pantopoulos 2004). Two iron chelators are used in my thesis: desferoxamine (DFO) and 2', 2'-dipyridyl (DPD). DFO is relatively hydrophilic and preferentially binds to ferric iron (1:1), but DPD is hydrophobic and binds to ferrous iron (3:1) (Figure I-5, page 12).

Iron functions as a component of several iron-containing proteins including hemoglobin/myoglobin which transports/stores oxygen, proteins involved in electron transport (such as cytochromes b and c, NADPH dehydrogenase, and ubiquinone-cytochrome c reductase), monooxygenases/dioxygenases (such as tyrosine monooxygenase), peroxidases (such as catalase), oxidoreductases (such as xanthine oxidase and dehydrogenase), and other iron-containing proteins (such as ribonucleotide reductase which is involved in DNA synthesis) (Smith 2005).

However, iron deficiency is still very prevalent, and has been considered an international health problem influencing an estimated two billion people worldwide, most of which are young children and women in reproductive age, especially in developing countries. It was reported that the prevalence of low iron stores was 10-19% in the U.S. females aged from 12 to 49 years in 2002 (Ramakrishnan 2002). The most common result of iron deficiency is iron deficiency anemia. Iron deficiency may also impair the performance in cognitive tasks and learning ability in children,

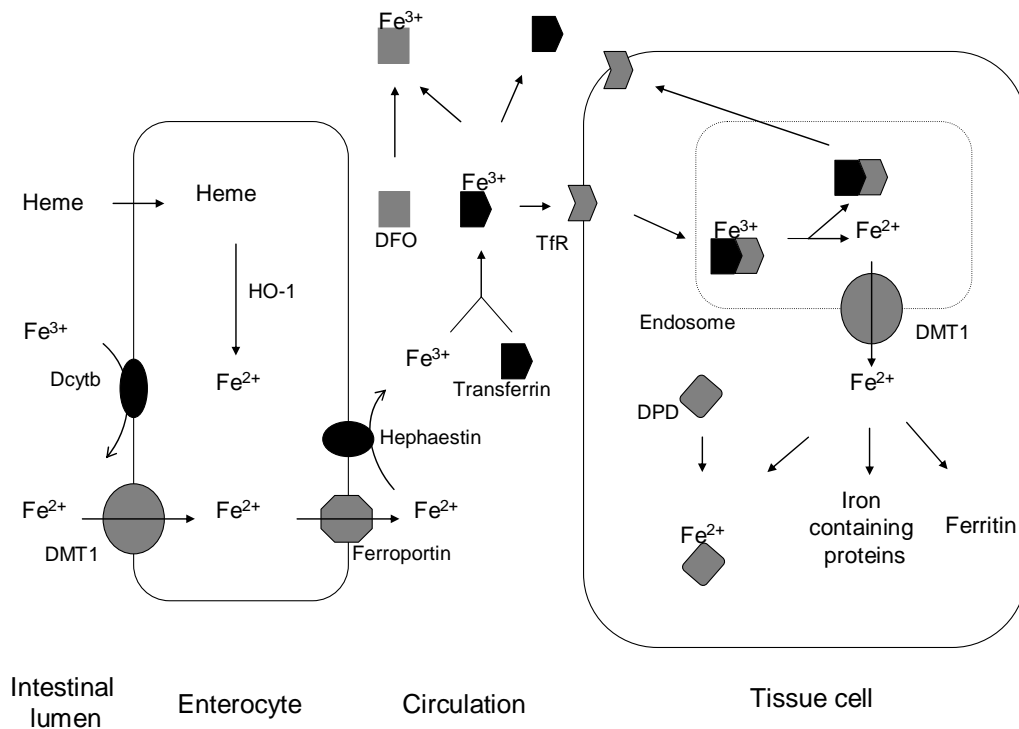


Figure I-5. The absorption, transport, storage, and chelation of iron. Iron-containing heme is released from hemoglobin or myoglobin, and is rapidly absorbed into enterocyte of the small intestine. In enterocyte, ferrous iron (Fe^{2+}) is released from the degradation of heme by heme oxygenase-1 (HO-1). The iron released from nonheme iron-containing food is in the ferric form (Fe^{3+}), which is reduced to ferrous iron (Fe^{2+}) in the intestinal lumen by duodenal cytochrome b (Dcytb). Ferrous iron (Fe^{2+}) is then transported into the enterocyte by natural resistance associated macrophage protein 2 (nramp2) or one of its isoforms, divalent metal transporter 1 (DMT1). Then, another transporter, ferroportin, exports of ferrous iron, both from heme iron and nonheme iron, from enterocyte to circulation, followed by the oxidation of ferrous form iron (Fe^{2+}) to ferric form iron (Fe^{3+}) by hephaestin. Ferric iron (Fe^{3+}) in the circulation is attached to transferrin which delivers iron to bone marrow or other tissues. Iron-attached transferrin in the circulation therefore binds to transferrin receptor (TfR) in the surface of tissue cells. The complex of ferric iron-binding transferrin and TfR is then imported to endosome via endocytosis. After being reduced to ferrous form (Fe^{2+}) in the acid environment of the endosome, iron is exported from the endosome by divalent metal transporter 1 (DMT1), and is used to synthesize iron-containing protein. The extra iron is stored in ferritin which may bind to 4,500 iron atoms. Two iron chelators are used in my thesis: desferoxamine (DFO) and 2', 2'-dipyridyl (DPD). Desferoxamine (DFO) is relatively hydrophilic and preferentially binds to ferric iron (Fe^{3+}) (1:1), whereas 2', 2'-dipyridyl (DPD) is hydrophobic and binds to ferrous iron (Fe^{2+}) (3:1).

working performance and productivity in adults, and capability to maintain body temperature (Smith 2005). It also has been shown that iron deficiency may increase cadmium absorbance 5-20% (Nordberg 1985), and that low concentration of serum ferritin ($< 12 \mu\text{g/L}$) may increase kidney lesions caused by cadmium (Jarup 1998).

Research problem

As reviewed above, metals may have contrasting biological effects. Iron and cadmium are generally regarded as an important micronutrient and toxic environmental agent. Iron is an important micronutrient which has important functions in human bodies. However, cadmium is a contaminant and carcinogen, which causes damages and cancers. When exposed to cadmium, cells increase the expression of HO-1 to protect them against cadmium toxicity.

Although both the importance of iron and the toxicity of cadmium are well known, it is not clear whether iron is required to the upregulation of HO-1 caused by cadmium, the defending mechanism against cadmium toxicity. To understand this question is important, because the answer could help to better develop an efficient strategy to defend against cadmium toxicity, especially in developing countries where both iron deficiency and cadmium exposure are prevalent.

Thesis objective and hypothesis tested

The objective of my thesis was to evaluate the influence of a metal micronutrient deficiency, iron deficiency, on cadmium-induced upregulation of HO-1 gene expression. My central hypothesis was that iron plays a critical role in cadmium-induced upregulation of HO-1 gene expression. This hypothesis was formulated based on the observation that iron chelator desferoxamine (DFO)

decreased the upregulating effect of cadmium on HO-1 activity in HCT116 human colon epithelial cells. The rationale of my thesis was that it was expected to provide some basic knowledge to better develop a strategy to protect people, especially the people with iron deficiency, against cadmium toxicity.

There were two major specific aims included in my thesis. Specific aim No. 1 was to evaluate the influence of iron deficiency on the upregulating effects of cadmium on HO-1 gene expression. The working hypothesis for this specific aim was that iron deficiency may influence the upregulating effect of cadmium on HO-1. To test the hypothesis, the following two approaches were made: 1) investigating the influence of iron deficiency caused by iron chelators on the upregulating effect of cadmium on HO-1 in HCT116 cells; 2) investigating the effects of the restoration of iron on the upregulating effect of cadmium on HO-1 in iron-deficient cells caused by iron chelators. It was expected that iron deficiency caused by iron chelators would impair, and the treatment with iron in iron-deficient cells caused by iron chelators may restore, the upregulating effect of cadmium on HO-1.

Specific aim No. 2 was to provide a possible explanation of the effects of cadmium on HO-1 gene expression in the cells with or without iron deficiency. The working hypothesis for this specific aim was that cadmium upregulates HO-1 gene expression by depleting intracellular glutathione and that NADPH oxidase (NOX)-produced ROS play an important role in cadmium-induced HO-1 upregulation. To test the hypothesis, the following two approaches were made: 1) comparing the effects of cadmium and glutathione scavengers on the intracellular glutathione and HO-1 gene expression; 2) investigating the influences of NOX inhibitors and superoxide scavenger on the upregulating effect of cadmium on HO-1. It was

expected that NOX-produced ROS would play an important role on cadmium-induced upregulation of HO-1, and that cadmium would upregulate HO-1 by depleting intracellular glutathione.

My thesis was expected to provide some basic knowledge about the influence of iron deficiency on cadmium-induced upregulation of HO-1 and a possible explanation of the effects of cadmium on HO-1 gene expression in the cells with or without iron deficiency. The outcomes of my thesis could help people understand the mechanism of cadmium toxicity and better develop an efficient strategy to defend against cadmium toxicity, especially in a population with iron deficiency.

The detailed approaches obtaining the two specific aims of my thesis are presented in the next two chapters.

References

- Agency for Toxic Substances and Disease Registry (ATSDR). 1999 Toxicological Profile for Cadmium. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service;1999:242.
- Applegate LA, Luscher P, Tyrrell RM. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res* 1992;51:974-8.
- Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, aging. *Cell* 2005;120:483-95.
- Brouard S, Otterbein LE, Anrather J, et al. Carbon monoxide generated by heme oxygenase-1 suppresses endothelial cell apoptosis. *J Exp Med* 2000;192:1015-26.
- Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radical Biol Med* 1995;18:775-94.
- Clark RA, Valente AJ. Nuclear factor kappa B activation by NADPH oxidases. *Mech Ageing Dev* 2004;125:799-810.
- Dalle-Donne I, Rossi R, Colombo R, et al. Biomarkers of oxidative damage in human disease. *Clin Chem* 2006;52:601-23.
- Dhalla NS, Temsah RM, Netticadan T. Role of oxidative stress in cardiovascular diseases. *J Hypertens* 2000;18:655-73.
- Eisenstein RS, Garcia-Mayol D, Pettingell W, et al. Regulation of ferritin and heme oxygenase synthesis in rat fibroblasts by different forms of iron. *Proc Natl Acad Sci USA* 1991;88:688-92.
- Furst R, Brueckl C, Kuebler WM, et al. Atrial natriuretic peptide induces mitogen-activated protein kinase phosphatase-1 in human endothelial cells via

- Rac1 and NAD(P)H oxidase/Nox2-activation. *Circ Res* 2005;96:43-53.
- Harrison R. Physiological roles of xanthine oxidoreductase. *Drug Metab Rev* 2004;36:363-75.
- Geiszt M, Witta J, Baffi J, et al. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J* 2003;17:1502-4.
- Gottlieb RA. Cytochrome P450: major player in reperfusion injury. *Arch Biochem Biophys* 2003;420:262-7.
- Igarashi K, Hoshino H, Muto A, et al. Multivalent DNA binding complex generated by small Maf and Bach1 as a possible biochemical basis for beta-globin locus control region complex. *J Biol Chem* 1998;273:11783-90.
- Inamdar NM, Ahn YI, Alam J. The heme-responsive element of the mouse heme oxygenase 1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. *Biochem Biophys Res Commun* 1996;221:570-6.
- International Agency for Research on Cancer. Beryllium, cadmium, mercury and exposures in the glass manufacturing industry, in: International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC Scientific Publications, Lyon 1993;58:119-237.
- Itoh K, Igarashi K, Hayashi N, et al. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Mol Cell Biol* 1995;15:4184-93.
- Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 1999;13:76-86.

- Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* 2003;8:379-91.
- Jarup L, Berglund M, Elinder CG, et al. Health effects of cadmium exposure-a review of the literature and a risk estimate. *Scand J Work Environ Health* 1998;24:1-51.
- Jenner P. Oxidative stress in Parkinson's disease. *Ann Neurol* 2003;53:S26-36.
- Kappas A. A method for interdicting the development of severe jaundice in newborns by inhibiting the production of bilirubin. *Pediatrics* 2004;113:119-23.
- Kataoka K, Noda M, Nishizawa M. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol Cell Biol* 1994;14:700-12.
- Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* 1989;86:99-103.
- Kuroki M, Voest EE, Amano S, et al. Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo. *J Clin Invest* 1996;98:1667-75.
- Marini MG, Chan K, Casula L, et al. hMAF, a small human transcription factor that heterodimerizes specifically with Nrf1 and Nrf2. *J Biol Chem* 1997;272:16490-7.
- McCoubrey WKJr, Huang TJ, Maines MD. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* 1997;247:725-32.
- McMahon M, Itoh K, Yamamoto M, et al. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant

- response element-driven gene expression. *J Biol Chem* 2003;278:21592-600.
- Miralem T, Hu Z, Torno MD, et al. Small interference RNA-mediated gene silencing of human biliverdin reductase, but not that of heme oxygenase 1, attenuates arsenite-mediated induction of the oxygenase and increases apoptosis in 293A kidney cells. *J Biol Chem* 2005;280:17084-92.
- Morita T, Mitsialis SA, Hoike H, et al. Carbon monoxide controls the proliferation of hypoxic smooth muscle cells. *J Biol Chem* 1997;272:32804-9.
- Morse D, Pischke SE, Zhou Z, et al. Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J Biol Chem* 2003;278:36993-8.
- Nath KA, Balla J, Jacob HS, et al. Induction of heme oxygenase is a rapid protective response in rhabdomyolysis in the rat. *J Clin Invest* 1992;90:267-70.
- Nordberg GF, Kjellstrom T, Nordberg M. Kinetics and metabolism, in: Friberg L, Elinder CG, Kjellstrom T, et al, eds. *Cadmium and health: a toxicological and epidemiological appraisal. Exposure Dose and Metabolism*. Boca Raton: CPR Press 1985;103-78.
- Otterbein LE, Bach FH, Alam J, et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 2000;6:422-8.
- Pantopoulos K. Iron metabolism and the IRE/IRP regulatory system An update. *Ann NY Acad Sci* 2004;1012:1-13.
- Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood* 1997;89:1-25.
- Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci USA* 1997;94:10925-30.

- Ramakrishnan U, Frith-Terhune A, Cogswell M, et al. Dietary intake does not account for differences in low iron stores among Mexican American and non-Hispanic white women: Third National Health and Nutrition Examination Survey, 1988-1994. *J Nutr* 2002;132:996-1001.
- Ramos KS, Lin H, McGrath JJ. Modulation of cyclic guanosine monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide. *Biochem Pharmacol* 1989;38:1368-70.
- Redaelli CA, Tian YH, Schaffner T, et al. Extended preservation of rat liver graft by induction of heme oxygenase 1. *Hepatology* 2002;35:1082-92.
- Rizzardina M, Terao M, Falciani, et al. Cytokine induction of haem oxygenase mRNA in mouse liver: interleukin 1 transcriptionally activates the haem oxygenase gene. *Biochem J* 1993;290:343-7.
- Sarady-Andrews JK, Liu F, Gallo D, et al. Biliverdin administration protects against endotoxin induced acute lung injury in rats. *Am J Physiol* 2005;289:L1131-7.
- Sato K, Balla J, Otterbein L, et al. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J Immunol* 2001;166:4185-94.
- Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001;30:1191-212.
- Schrader M, Fahimi HD. Mammalian peroxisomes and reactive oxygen species. *Histochem Cell Biol* 2004;122:383-93.
- Sedlak TW, Snyder SH. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics* 2004;113:1776-82.

- Sethi PK, Khandelwal DJ. Cadmium exposure: health hazards of silver cottage industry in developing countries. *Med Toxicol* 2006;2:14-5.
- Smith JL, Groff JL, Gropper SS. *Advanced nutrition and human metabolism*. 4th ed. Wadsworth, 2005.
- Soares MP, Lin Y, Anrather J, et al. Expression of heme oxygenase-1 can determine cardiac xenograft survival. *Nat Med* 1998;4:1073-7.
- Stewart D, Killeen, E, Naquin R, et al. Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *J Biol Chem* 2003;278:2396-2402.
- Stocker R, Yamamoto Y, McDonagh AF, et al. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043-6.
- Snyder SH, Jaffrey SR, Zakhary R. Nitric oxide and carbon monoxide: parallel roles as neural messengers. *Brain Res Brain Res Rev* 1998;2:167-75.
- Suzuki H, Tashiro S, Sun J, et al. Cadmium induces nuclear export of Bach1, a transcriptional repressor of heme oxygenase-1 gene. *J Biol Chem* 2003;278:49264-53.
- Swindle EJ, Metcalfe DD. The role of reactive oxygen species and nitric oxide in most cell-dependent inflammatory process. *Immunol Rev* 2007;217:186-205.
- Taketani S, Kohno H, Yoshinaga T, et al. The human 32-kDa stress protein induced by exposure to arsenite and cadmium ions is heme oxygenase. *FEBS Lett* 1989;245:173-6.
- The National Academies. *Recommended Dietary Allowances*. 2001.
- Tullius SG, Nieminen-Kelha M, Buelow R, et al. Inhibition of ischemia/reperfusion injury and chronic graft deterioration by a single-donor treatment with

cobalt-protoporphyrin for the induction of heme oxygenase-1. *Transplantation* 2002;74:591-8.

Vile GF, Tyrrell RM. Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. *J Biol Chem* 1993;268:14678-81.

Wittman R, Hu H. Cadmium exposure and nephropathy in a 28-year-old female metal worker. *Environ Health Perspect* 2002;110:1261-6.

Yachie A, Niida Y, Wada T, et al. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* 1999;103:129-35.

CHAPTER II
THE INFLUENCE OF IRON DEFICIENCY ON CADMIUM-INDUCED
UPREGULATION OF HEME OXYGENASE-1 IN HCT116 CELLS

Abstract

Metals may have contrasting biological effects. Iron and cadmium are generally regarded as an important micronutrient and a toxic environmental agent, respectively. However, it is not clear whether iron is required to help defend cells against cadmium toxicity. In the present study, the effects of cadmium on the cytoprotective gene, heme oxygenase-1 (HO-1), in HCT116 human colon epithelial cells with or without iron deficiency caused by iron chelators, deferoxamine (DFO) or 2',2'-dipyridyl (DPD) were investigated. It was found that cadmium upregulated HO-1 mRNA, protein expression and enzyme activity in iron-sufficient cells, but these effects could be decreased by DFO. Similarly, another iron chelator, DPD, decreased the upregulating effects of cadmium on HO-1 mRNA and protein expression. Furthermore, it was found that iron, but not copper and zinc, restored the upregulating effects of cadmium on HO-1 mRNA and protein expression and enzyme activity in the cells pretreated with DFO or DPD. These results suggest that iron plays an important role in the defending mechanism, HO-1 upregulation, against the toxicity of cadmium or other potential xenobiotics.

Introduction

The undesirable nature of cadmium is well known. As a by-product of the mineral industry and a component mainly used in battery production, cadmium is one of the

most toxic elements in the environment (Patrick 2003). Cadmium exposure may result from mining, industrial emission, contaminated soil, pigments, plastic stabilizers, anti-corrosive products, and tobacco smoke. Plants absorb cadmium from cadmium-contaminated soil or water resulting in elevated cadmium concentrations in plant and animal products. People consuming these plant and animal products are more likely to suffer from cadmium exposure. Cadmium toxicity has been a health concern in industrial workers and populations living in polluted areas, especially in developing countries (Sethi 2006). Cadmium may be absorbed from respiratory tract and gastrointestinal tract. Due to its long half-life, the accumulation of cadmium in human body is irreversible. Cadmium has been shown to cause kidney dysfunction, bone deformation, obstructive lung disease, hypertension, and cancers of lung, prostate, breast, and pancreas (Akesson 2005, Jin 2004, Satarug 2004, Shukla 2000, Ekman 1999, Coyle 2004, Schwartz 2000). The basic mechanisms of cadmium toxicity are not fully understood. However, cadmium has been shown to have the property to induce the production of reactive oxygen species (ROS), causing dysfunction of mitochondria, lipid peroxidation of cell membrane, and DNA damage (O'Brien 1998, Stohs 2001).

Heme oxygenase-1 (HO-1), one isoform of heme oxygenase (HO), the rate-limiting enzyme in the degradation of heme, has captured lots of interests for decades because of its protective functions including antioxidative and anti-inflammatory effects (Morse 2002). The expression of HO-1 can be upregulated by various forms of stress, one of which is the exposure to cadmium (Poss 1997, Taketani 1989, Yachie 1999).

In contrast, iron has been well known as a vital micronutrient for good health,

participating in the utilization of oxygen and being an important component of numerous iron-containing proteins which are involved in oxygen transport and storage, electron transport, and DNA synthesis. However, iron deficiency is still one of the most prevalent nutritional disorders worldwide, affecting an estimated 2 billion people, causing iron deficiency anemia, worsening fatigue, and other health problems, especially in developing countries (Smith 2005, Ramakrishnan 2002).

Both cadmium exposure and iron deficiency are prevalent in developing countries. However, it is unclear whether iron is required to help protect humans against cadmium exposure. The interaction between cadmium and iron at the cellular level is not totally understood due to inadequate research in this area. In the present study, the effects of cadmium on HO-1 in HCT116 cells with or without iron deficiency were investigated. It was found that cadmium upregulated HO-1 mRNA and protein expression and enzyme activity in iron-sufficient HCT116 cells. However, these effects could be decreased in iron-deficient cells caused by the pretreatments with iron chelators, desferoxamine (DFO) or 2',2'-dipyridyl (DPD). Interestingly, the treatment with iron sulfate, but not zinc sulfate and copper sulfate, restored the upregulating effects of cadmium on HO-1 mRNA and protein expression and enzyme activity in iron-deficient cells caused by DFO or DPD. These results suggest that iron may play a critical role in the cadmium-induced upregulation of HO-1.

Materials and methods

1. Materials

HCT116 human colon adenocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). Anti-HO-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Cadmium chloride, McCoy's 5A cell culture

media and other reagents were purchased from Sigma-Aldrich Company (St. Louis, MO).

2. Cell culture and treatments

HCT116 cells were grown in McCoy's 5A cell culture media (Sigma-Aldrich, MO) in a humidified atmosphere of 5% carbon dioxide (CO₂), 95% air at 37 °C, with the supplement of 2.2 g/L sodium bicarbonate (Sigma-Aldrich, MO), 11.5 ml/L penicillin/streptomycin solution (Lonza, MD), 4.1 ml/L amphotericin B (Mediatech, VA), and 113 ml/L fetal bovine serum (Mediatech, VA). After reaching a confluence of 80%, depending on the experiments, the cells were: 1) exposed to 0-50 μM cadmium chloride for 5 hours; 2) pretreated with 0-100 μM desferoxamine (DFO) or 2',2'-dipyridyl (DPD) for 24 hours, followed by the exposure to 0-50 μM cadmium; 3) treated with 0-50 μM desferoxamine (DFO) (Sigma-Aldrich, MO), or 2', 2'-dipyridyl (DPD) (Sigma-Aldrich, MO) for 24 hours, followed by the treatment with 0-100 μM iron sulfate (FeSO₄) (Mallinckrodt Chemical Works, NY) for 3 hours before the exposure to 0-50 μM cadmium for 5 hours; 4) treated with 0-50 μM DFO for 24 hours followed by the treatment with 0-100 μM FeSO₄, copper sulfate (CuSO₄) (Fisher Scientific, NJ), or zinc sulfate (ZnSO₄) (Baker Analyzed, NJ) for 3 hours before the exposure to 0-50 μM cadmium for 5 hours.

3. Determination of HO-1 mRNA expression

The cells were harvested and centrifuged at 500 rpm for 5 minutes. Total RNA was isolated from the cells by using a Qiagen RNeasy Mini kit (Qiagen, CA). The HO-1 mRNA expression was determined by reverse transcription polymerase chain reaction (RT-PCR) analysis using a Qiagen OneStep RT-PCR kit (Qiagen, CA) and HO-1 primers (Qiagen, CA). The HO-1 PCR upper and lower primer sequences were:

5'-TGTGGCAGCTGTCTCAAACCTCCA-3' and 5'-TTGAGGCTGAGCCAGGAACAGAGT-3'. The β -actin QuantumRNA primer/competimer sets (Ambion, TX) were used as internal standard. The RT-PCR conditions were 30 minutes at 50 °C, followed by 15 minutes at 95 °C (RT), then 0.5 minutes at 95 °C, 0.5 minutes at 60 °C, and 1 minute at 72 °C (PCR) for 25 cycles. The resulting cDNA products were separated by electrophoresis in 2% agarose gel with ethidium bromide staining. For the target gene (HO-1), the PCR product size was 175 base pairs (bp). For the internal control, β -actin, the PCR product size was 294 bp.

4. Western immunoblotting analysis of HO-1 protein

The cells were harvested, washed and centrifuged in 500 rpm for 5 minutes in phosphate buffered saline (PBS). The cells were then lysed using radioimmunoprecipitation assay (RIPA) buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and Roche Complete Protease Inhibitor Cocktail, pH 7.4) and sat on ice for 30 minutes. Then, the cell lysate was centrifuged at 15,000 rpm for 20 minutes at 4 °C, and the supernatant was saved. The protein concentration of the supernatant was determined using a BCA protein assay kit (Pierce, IL). Then the cell lysate supernatant (100 μ g protein) was electrophoresed using Novex NuPAGE (Invitrogen, CA) mini-gels (4-12% Bis-Tris) in NuPAGE MOPS SDS Running Buffer (Invitrogen, CA), followed by blotting the gel to a nitrocellulose membrane. Blotting was performed for 1 hour at 25 °C in NuPAGE Transfer Buffer (Invitrogen, CA). The membrane was incubated overnight at 4 °C with HO-1 goat monoclonal antibody (Santa Cruz, CA) in blocking buffer consisting of 5% skim milk powder in TBST (20 mM Tris-HCl, 150 mM sodium chloride,

0.05% Tween 20, pH 7.4) (1:1,000). After being washed three times with TBST for 30 minutes, the membrane was incubated for 2 hours at 25 °C with rabbit anti-goat IgG/HRP conjugate (Santa Cruz, CA) in blocking buffer (1:100,000). Finally, after being washed again, the membrane was placed in a plastic pouch for incubation with Thermo Scientific Super Signal West Femto Luminol/Enhancer Solution (Thermo Scientific, IL) for 5 minutes before analysis (Kodak digital scienceTM image station 440 CF). Then the membrane was washed with Restore Western Blot Stripping Buffer (Thermo Scientific, IL) and incubated with mouse monoclonal anti- β -actin antibody in blocking buffer (1:40,000) for 2 hours at 25 °C. After being washed three times with TBST for 30 minutes, the membrane was incubated with HRP-conjugated goat polyclonal anti-mouse IgG (Santa Cruz, CA) in blocking buffer (1:200,000) for 1 hour at 25 °C. Then, the membrane was washed in TBST and processed for analysis as before.

5. Determination of HO-1 enzyme activity

The cells were harvested, washed and centrifuged at 500 rpm for 5 minutes in 4 °C PBS. Cell pellets were resuspended in 100 mM phosphate buffer containing 2 mM magnesium chloride (MgCl₂), and were sonicated. After being spun at 15,000 rpm for 10 minutes at 4 °C, the supernatant was saved and protein in the supernatant was determined. Then 0.8 mg protein was used to quantitate HO-1 enzyme activity in a reaction volume of 1 ml, containing 0.1 ml liver cytosol, 1 mM NADPH, 0.25 mM hemin, 2 mM glucose-6-phosphate, and 0.002 U/ μ L glucose-6-phosphate dehydrogenase. The reaction was performed at 37 °C for 1 hour in the dark and stopped by adding 1 ml of chloroform. The tubes were vortexed thoroughly followed by centrifugation at 500 rpm for 5 minutes. Finally, the chloroform layers were

measured for the absorbance of a visible light at 464 nm with the background at 530 nm in a spectrophotometer. The HO-1 enzyme activity was expressed as formation of pmol bilirubin per hour per milligram of protein.

6. *Statistical analysis*

HO-1/ β -actin mRNA ratio and HO enzyme activity data were analyzed via two-tailed unpaired t test using SPSS 16.0 for Microsoft Windows XP (SPSS Inc., IL).

Results

1. *Cadmium upregulated HO-1 mRNA and protein expression in iron-sufficient HCT116 cells*

To investigate the effect of cadmium on the expression of HO-1, the cells were exposed to 0-50 μ M cadmium chloride for 5 hours. It was found that HO-1 mRNA was constitutively expressed at a relative low level in control cells (0 μ M cadmium), and the treatments with 10-50 μ M cadmium increased HO-1 mRNA expression (Figure II-1 B, page 42); the fold increase of HO-1 mRNA/ β -actin mRNA ratios in the cells treated with cadmium appeared to be dose-dependent manner ($p < 0.05$) (Figure II-1 A, page 42); similarly, the treatments with cadmium increased HO-1 protein expression (Figure II-1 C, page 42).

2. *Cadmium upregulated HO-1 enzyme activity, and this effect could be decreased by an iron chelator, desferoxamine (DFO)*

It was also found that as depicted in Figure II-2 (page 43), HO-1 enzyme activity was relative low in control cells; the treatment with cadmium dramatically increased HO-1 enzyme activity; the pretreatments with 25-50 μ M DFO significantly decreased the upregulating effect of cadmium on HO-1 enzyme activity ($p < 0.05$).

3. DFO decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression

Because the pretreatment of DFO led to a decreased upregulating effect of cadmium on HO-1 enzyme activity, we managed to determine if HO-1 protein and mRNA were also decreased. The cells were pretreated with 0-100 μM DFO for 24 hours, followed with or without the exposure to 0-50 μM cadmium for 5 hours. It was found that the treatment with 50 μM cadmium increased HO-1 protein expression, and pretreatment with 25-100 μM DFO decreased the upregulating effect of cadmium on HO-1 protein expression (Figure II-3 C, page 44). Furthermore, it was found that HO-1 mRNA was expressed at a relative low level in control cells (0 μM cadmium and 0 μM DFO), and the treatment with 50 μM cadmium increased HO-1 mRNA expression; however, the pretreatments with 25-100 μM DFO decreased the upregulating effect of cadmium on HO-1 mRNA (Figure II-3 B, page 44); the ratio of HO-1 mRNA/ β -actin mRNA in the cells treated with 50 μM cadmium was increased dramatically compared to the control cells, but the ratios of HO-1 mRNA/ β -actin mRNA in the cells treated with 25-100 μM DFO and 50 μM cadmium were significantly decreased compared to the cells treated with 50 μM cadmium ($p < 0.05$) (Figure II-3 A, page 44).

4. Another iron chelator, 2',2'-dipyridyl (DPD), decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression

To confirm that the decreased upregulating effects of cadmium on HO-1 protein and mRNA expression was caused by iron deficiency, several additional experiments were conducted. First, the effect of another iron chelator, 2',2'-dipyridyl (DPD), on the upregulating effects of cadmium on HO-1 protein and mRNA was tested. Cells

were pretreated with 0-100 μM DPD for 24 hours, followed with or without the exposure to 0-50 μM cadmium for 5 hours. It was found that HO-1 mRNA was constitutively expressed at a relative low level in control cells (0 μM cadmium and 0 μM DPD), and the treatment with 50 μM cadmium increased HO-1 mRNA expression; however, the pretreatments with 50-100 μM DPD decreased the upregulating effect of cadmium on HO-1 mRNA (Figure II-4 B, page 46); the ratio of HO-1 mRNA/ β -actin mRNA in the cells treated with 50 μM cadmium was dramatically increased compared to the control cells, but the ratios of HO-1 mRNA/ β -actin mRNA in the cells treated with 50-100 μM DPD and 50 μM cadmium were significantly decreased compared to the cells treated with 50 μM cadmium ($p < 0.05$) (Figure II-4 A, page 46); similarly, the treatment with 50 μM cadmium increased HO-1 protein expression, and the pretreatments with 10-100 μM DPD decreased the upregulating effect of cadmium on HO-1 protein expression (Figure II-4 C, page 46).

5. Iron restored the upregulating effects of cadmium on HO-1 protein and mRNA expression in the cells pretreated with DFO or DPD

Next, experiments were conducted to investigate whether iron could restore the upregulating effects of cadmium on HO-1 protein and mRNA in the cells pretreated with DFO or DPD. Cells were treated with 0-100 μM iron sulfate (FeSO_4) for 3 hours after the pretreatment with 0-50 μM DFO or DPD for 24 hours, then cells were exposed to 0-50 μM cadmium for 5 hours. It was found that the treatments with 50-100 μM FeSO_4 restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with 50 μM DFO (Figure II-5 B, page 48) or DPD (Figure II-6 B, page 50), respectively; the ratios of HO-1 mRNA/ β -actin mRNA was significantly increased in the cells treated with DFO, 50-100 μM FeSO_4 , and

cadmium compared to the cells treated with DFO and cadmium ($p < 0.05$) (Figure II-5 A, page 48); similarly, the ratios of HO-1 mRNA/ β -actin mRNA were significantly increased in the cells treated with DPD, 100 μ M FeSO₄, and cadmium compared to the cells treated with DPD and cadmium ($p < 0.05$) (Figure II-6 A, page 50); furthermore, the treatments with 50-100 μ M FeSO₄ restored the upregulating effect of cadmium on HO-1 protein expression in the cells pretreated with 50 μ M DFO (Figure II-5 C, page 48) or DPD (Figure II-6 C, page 50).

6. Iron, but not copper and zinc, restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with DFO

Considering DFO can also chelate other metals such as copper and zinc, the effects of the treatments iron, copper, or zinc on the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with DFO were compared. Cells were pretreated with 0-50 μ M DFO for 24 hours, followed the treatment with 0-100 μ M iron sulfate (FeSO₄), copper sulfate (CuSO₄), or zinc sulfate (ZnSO₄) for 3 hours, and then cells were exposed to 0-50 μ M cadmium for 5 hours. It was found that the treatment with 100 μ M FeSO₄ restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with 50 μ M DFO; however, no restoring effect of the treatments with CuSO₄ or ZnSO₄ on the upregulating effect of cadmium on HO-1 mRNA was observed in the cells pretreated DFO (Figure II-7 B, page 52); the ratio of HO-1 mRNA/ β -actin mRNA was significantly increased in the cells treated with DFO, FeSO₄, and cadmium compared to the cells treated with DFO and cadmium ($p < 0.05$); but the ratios of HO-1 mRNA/ β -actin mRNA in the cells treated with DFO, CuSO₄ or ZnSO₄, and cadmium were not different from in the cells treated with DFO and cadmium (Figure II-7 A, page 52).

7. Iron restored the upregulating effect of cadmium on HO-1 enzyme activity in the cells pretreated with DFO

Finally, the effect of iron on the upregulating effect of cadmium on HO-1 enzyme activity in the cells pretreated with DFO was tested. Cells were pretreated with or without 0-50 μM DFO for 24 hours, followed with the treatments with or without 0-100 μM FeSO_4 for 3 hours. Then the cells were exposed to 50 μM cadmium for 5 hours. It was found that the treatment with FeSO_4 significantly restored the upregulating effect of cadmium on HO-1 enzyme activity ($p < 0.05$) (Figure II-8, page 54).

Discussion

In the present study, the results indicate that cadmium upregulated HO-1 mRNA and protein expression and enzyme activity in iron-sufficient HCT116 cells (Figure II-1 and 2, page 42-43), which is consistent with other scientists' work (Taketani 1989, Gong 1997). In the Taketani study, they found that the exposure to cadmium or arsenite upregulated a 32-kDa HO protein expression and enzyme activity in HeLa and HL 60 cells. Similarly, the Gong group found that the exposure to cadmium upregulated the expression HO-1 as well as other genes in rat lung epithelial cells. One of the mechanisms of the toxicity of cadmium has been believed to cause the intracellular accumulation of ROS (O'Brien 1998, Stohs 2001). In fact, it is considered to be critical for cells exposed to cadmium to be able to fully express HO-1 to help defend against oxidative stress. Actually, some adverse effects of HO-1 deficiency have been shown in vivo and in vitro. In 1999, a persistent endothelial damage caused by oxidative stress was reported in a six-year old patient with HO-1 deficiency (Yachie 1999). Also, an increased production of free radicals was observed

in HO-1 deficient embryonic fibroblasts exposed to cadmium, and these cells were more sensitive to hemin- or hydrogen peroxide-caused cytotoxicity (Poss 1997). Studies have shown that the anti-oxidative property of HO-1 may come from the following facts: first, HO-1 degrades the pro-oxidant heme, resulting in a decrease of heme and an increase of iron; second, iron release from heme may induce the production of ferritin (Vile 1994), which may in turn sequester free iron and reduce the overall intracellular oxidant status; finally, biliverdin and bilirubin, two other products of heme degradation which were considered as waste products, have been shown to have potent antioxidant properties (Stocker 1987, Neuzil 1993).

Interestingly, it was observed in the present study that the iron chelator DFO decreased the upregulating effects of cadmium on HO-1 mRNA and protein expression and enzyme activity (Figure II-2 and 3, page 43-44). These results suggest that iron may be playing a critical role in the increased expression of HO-1 caused by cadmium. Similar results were observed by Fogg et al. (Fogg 1999). In their study (Fogg 1999), they found that pretreatment with DFO to human pulmonary artery endothelial cells decreased the upregulated expression of HO-1 mRNA caused by the exposure to hyperoxia, specially to heme and free iron, and concluded that iron plays a critical role in regulating HO-1 expression caused by hyperoxia. Their study focused on the effect of DFO on HO-1 expression in the cells with iron overload caused by the treatment with heme and free iron. Hence they were interested in finding out if iron toxicity could be reduced by DFO, instead of studying cellular iron deficiency caused by DFO. The present study focused on the effect of cadmium on HO-1 expression in iron-deficient cells caused by the treatment with DFO. Furthermore, the conclusion of Fogg study was not entirely persuasive because as mentioned above, besides iron,

DFO also has the property to chelate other metals such as zinc and copper. In the present study, three additional experiments were conducted to confirm the iron role in the upregulation of HO-1 caused by cadmium. First, the effect of another iron chelator, DPD, on the upregulating effects of cadmium on HO-1 mRNA and protein expression were investigated. It was observed that DPD, similar to DFO, decreased the upregulating effects of cadmium on HO-1 mRNA and protein expression (Figure II-4, page 46). Second, after the pretreatments with DFO or DPD and before the exposure to cadmium, the cells were treated with iron, and it was found that the treatment with iron restored the upregulating effects of cadmium on HO-1 mRNA and protein expression and enzyme activity in the cells pretreated with DFO or DPD (Figure II-5 and 6, page 48-50; Figure II-8, page 54). Finally, after the pretreatment with DFO, the cells were treated with iron, zinc, or copper, and it was found that only iron, but not zinc and copper, restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with DFO (Figure II-7, page 52). Put together, the results strongly suggest that iron plays a critical role in the cadmium-induced upregulation of HO-1.

As discussed before, cadmium may cause intracellular accumulation of ROS. However, the mechanism is not well demonstrated. Because cadmium is not a redox-active metal, it is not able to generate ROS itself. Some researchers proposed that cadmium increases the cellular ROS level via indirect processes, including inhibiting the electron transfer chain in mitochondrion (Latinwo 2006, Wang 2004) and decreasing cellular antioxidants (Stohs 1995). Some others have shown that cadmium may replace zinc, iron and copper from enzymes or proteins disturbing the cellular balance of these metals, hence resulting in the generation of ROS (Block 1992,

Fotakis 2006).

ROS includes superoxide, hydroxyl radical and hydrogen peroxide which are by-products of oxygen metabolism and could be generated in response to extraneous stimuli. Furthermore, ROS could be generated from several different sources including mitochondrion, xanthine oxidase, peroxisomes, and cytochrome P450. Among them, mitochondrion and NADPH oxidase have been considered two major sources of ROS (Fleury 2002, Lambeth 2004).

Iron deficiency caused by DFO and DPD decreased the upregulating effects of cadmium on the enzyme activity and the expression of protein and mRNA of HO-1, indicating a modulation at a pre-transcriptional level, very likely by interrupting the production of ROS in the two major sources, mitochondrion, or NADPH oxidase. Since NADPH oxidase (NOX) contains two hemes participating in the electron transport in the enzyme (Finegold 1996), then it is possible that iron chelators, DFO and DPD, deplete the iron in the heme, a component of NOX, affecting the electron transport in the enzyme and reducing the production of ROS, therefore decrease the upregulating effect of cadmium on HO-1.

In conclusion, the present study shows that cadmium upregulated HO-1 mRNA and protein expression and enzyme activity in iron-sufficient HCT116 cells. However, these upregulating effects could be decreased and be restored by the treatment with iron, but not copper and zinc, in iron-deficient cells caused by the pretreatments with iron chelators, DFO or DPD. These observations suggest that iron plays an important role to the upregulation of HO-1 by cadmium, which has been thought to be a defending mechanism against cadmium toxicity. These observations imply that with cadmium exposure, population with iron deficiency could have a higher risk to

develop adverse health problems than iron-sufficient population.

References

- Akesson A, Lundh T, Vahter M, et al. Tubular and glomerular kidney effects in Swedish women with low environmental cadmium exposure. *Environ Health Perspect* 2005;113:1627-31.
- Block C, Freyermuth S, Beyersmann D, Malviya AN. Role of cadmium in activating nuclear protein kinase C and the enzyme binding to nuclear protein. *J Biol Chem* 1992;267:19824-8.
- Buchet JP, Lauwerys R, Roels H, et al. Renal effects of cadmium body burden of the general population. *Lancet* 1990;336:699-702.
- Coyle YM. The effect of environment on breast cancer risk. *Breast Cancer Res Treatm* 2004;84:273-88.
- Durante W. Heme oxygenase 1 in growth control and its clinical application to vascular disease. *J Cell Physiol* 2003;195:373-82.
- Ekman P. Genetic and environmental factors in prostate cancer genesis: identifying risk cohorts. *Eur Urol* 1999;35:362-9.
- Finegold AA, Shatwell KP, Segal AW, et al. Intramembrane bis-heme motif for transmembrane electron transport conserved in a yeast iron reductase and the human NADPH oxidase. *J Biol Chem* 1996;271:31021-4.
- Fleury C, Mignotte B, Vayssiere JL. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 2002;84:131-41.
- Fogg S, Agarwal A, Nick HS, et al. Iron regulates hyperoxia-dependent human heme oxygenase 1 gene expression in pulmonary endothelial cells. *Am J Respir Cell Mol Biol* 1999;20:797-804.
- Fotakis G, Timbrell JA. Role of trace elements in cadmium chloride uptake in

- hepatoma cell lines. *Toxicol Lett* 2006;164:97-103.
- Galan A, Garcia-Bermejo L, Troyano A, et al. The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays). *Eur J Cell Biol* 2001;80:312-20.
- Goering PL, Fisher BR, Kish CL. Stress protein synthesis induced in rat liver by cadmium precedes hepatotoxicity. *Toxicol Appl Pharmacol* 1993;122:139-48.
- Gong Q, Hart BA. Effect of thiols on cadmium-induced expression of metallothionein and other oxidant stress genes in rat lung epithelial cells. *Toxicology* 1997;119:179-91.
- Jin T, Nordberg G, Ye T, et al. Osteoporosis and renal dysfunction in a general population exposed to cadmium in China. *Environ Res* 2004;90:353-9.
- Lambeth JD. Nox enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 2004;4:181-9.
- Latinwo LM, Yanardag R, Bolkent S, et al. Influence of combined antioxidants against cadmium induced testicular damage. *Environ Toxicol Pharmacol* 2006;21:235-40.
- Manca D, Richard AC, Tra HV, et al. Relation between lipid peroxidation and inflammation in the pulmonary toxicity of cadmium. *Arch Toxicol* 1994;68:364-9.
- Morse D, Choi AM. Heme oxygenase 1: the “emerging molecule” has arrived. *Am J Respir Cell Mol Biol* 2002;27:8-16.
- Neuzil J, Stocker R. Bilirubin attenuates radical mediated damage to serum albumin. *FEBS Lett* 1993;331:281-4.
- O'Brien P, Salacinski HJ. Evidence that the reactions of cadmium in the presence of

- metallothionein can produce hydroxyl radicals. *Arch Toxicol* 1998;72:690-700.
- Patrick L. Toxic metals and antioxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity. *Altern Med Rev* 2003;8:106-28.
- Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci USA* 1997;94:10925-30.
- Ramakrishnan U, Frith-Terhune A, Cogswell M, et al. Dietary intake does not account for differences in low iron stores among Mexican American and non-Hispanic white women: Third National Health and Nutrition Examination Survey, 1988-1994. *J Nutr* 2002;132:996-1001.
- Satarug S, Nishijo M, Ujtin P, et al. Evidence for concurrent effects of exposure to environmental cadmium and lead on hepatic CYP2A6 phenotype and renal function biomarkers in non-smokers. *Environ Health Perspect* 2004;112:1512-8.
- Schwartz GG, Reis IM. Is cadmium a cause of human pancreatic cancer? *Cancer Epidemiol Biomark Prev* 2000;9:139-145.
- Sethi PK, Khandelwal DJ. Cadmium exposure: health hazards of silver cottage industry in developing countries. *Med Toxicol* 2006;2:14-5.
- Shukla GS, Chiu JF, Hart BA. Cadmium-induced elevations in the gene expression of the regulatory subunit of gamma-glutamylcysteine synthetase in rat lung and alveolar epithelial cells. *Toxicology* 2000;51:45-54.
- Smith JL, Groff JL, Gropper SS. *Advanced nutrition and human metabolism*. 4th ed. Wadsworth, 2005.
- Staessen JA, Roels H, Emelianov D, et al. Environmental exposure to cadmium, forearm bone density, and risk of fracture: prospective population study. *Lancet* 1999;353:1140-4.

- Stocker R, Yamamoto Y, McDonagh AF, et al. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043-6.
- Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. *Free Rad Biol Med* 1995;18:321-36.
- Stohs SJ, Bagchi D, Hassoun E, et al. Oxidative mechanisms in the toxicity of chromium and cadmium ions. *J Environ Pathol Toxicol Oncol* 2001;20:77-88.
- Taketani S, Kohno H, Yoshinaga T, et al. The human 32-kDa stress protein induced by exposure to arsenite and cadmium ions is heme oxygenase. *FEBS Lett* 1989;245:173-6.
- Vile G.F, Basu-Modak S, Waltner C, et al. Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proc Natl Acad Sci USA* 1994;91:2607-10.
- Wang Y, Fang J, Leonard SS, et al. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radic Biol Med* 2004;36:1434-43.
- Yachie A, Niida Y, Wada T. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase 1 deficiency. *J Clin Invest* 1999;103:129-35.

Figures

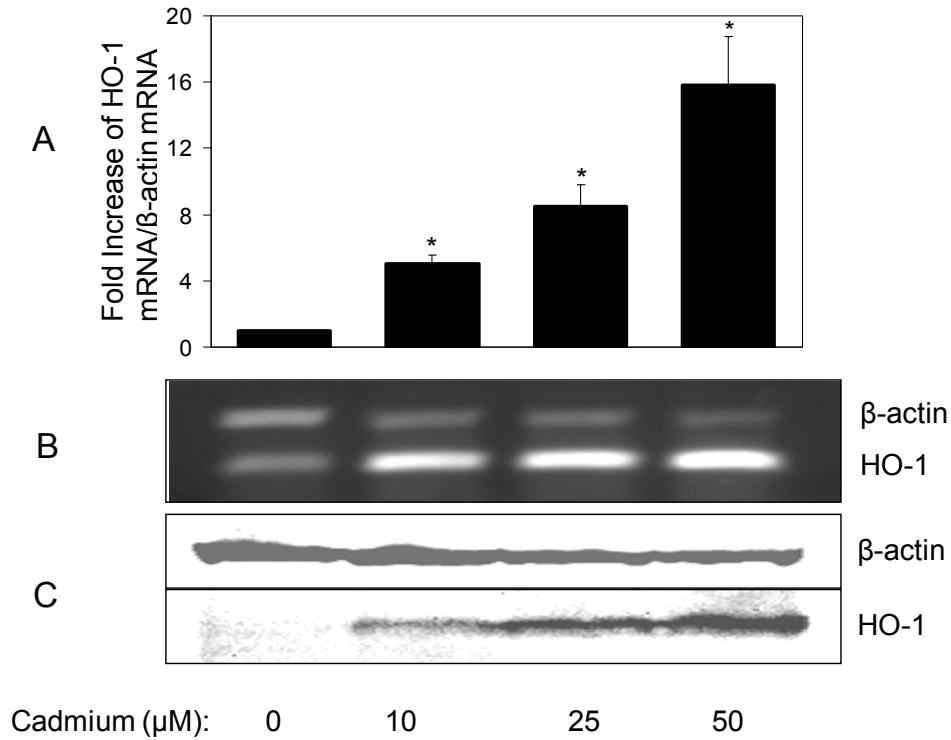


Figure II-1. Cadmium upregulated HO-1 mRNA and protein expression in iron-sufficient HCT116 cells. Cells were treated with 0-50 μM cadmium for 5 hours. B: HO-1 mRNA was constitutively expressed in control cells, and the treatments with 10-50 μM cadmium increased HO-1 mRNA expression. A: the ratios of HO-1 mRNA/ β -actin mRNA were increased in the cells treated with 10-25 μM cadmium, and this increasing effects appeared to be a dose-dependent manner ($p < 0.05$). C: similarly, the treatments with cadmium increased HO-1 protein expression. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 10, 25, or 50 μM cadmium vs. control, $p < 0.05$).

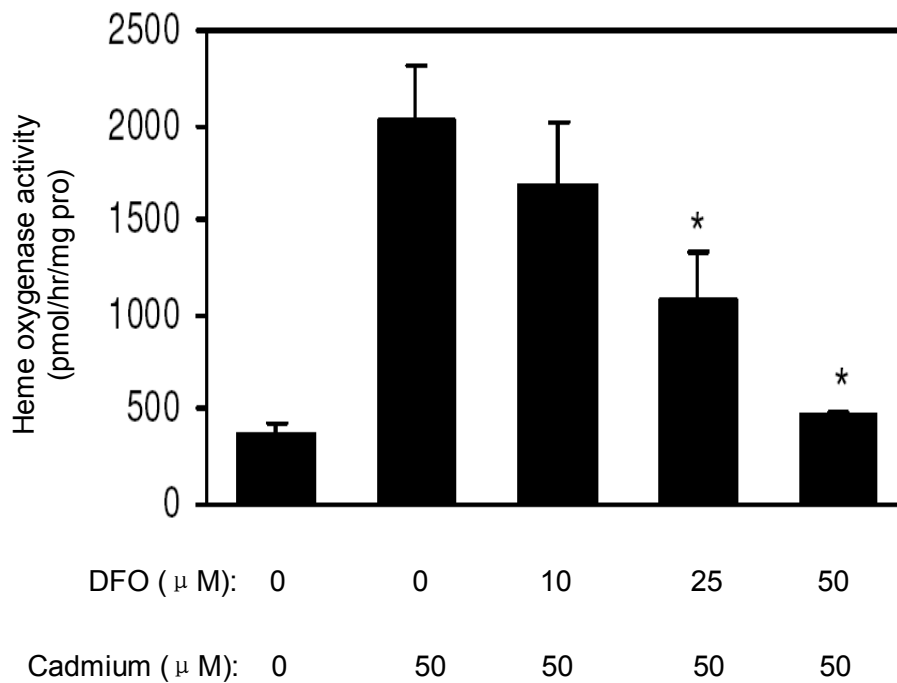


Figure II-2. Cadmium upregulated HO-1 enzyme activity, and this effect could be decreased by desferoxamine. Cells were pretreated with 0-50 μM desferoxamine (DFO) for 24 hours, followed by the exposure to 0-50 μM cadmium for 5 hours. HO-1 enzyme activity was relative low in control cells. The treatment with 50 μM cadmium increased HO-1 enzyme activity. The pretreatments with 25-50 μM DFO significantly decreased the upregulating effect of cadmium on HO-1 enzyme activity ($p < 0.05$). The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 25 or 50 μM DFO plus 50 μM cadmium vs. the treatment with 0 μM DFO plus 50 μM cadmium, $p < 0.05$).

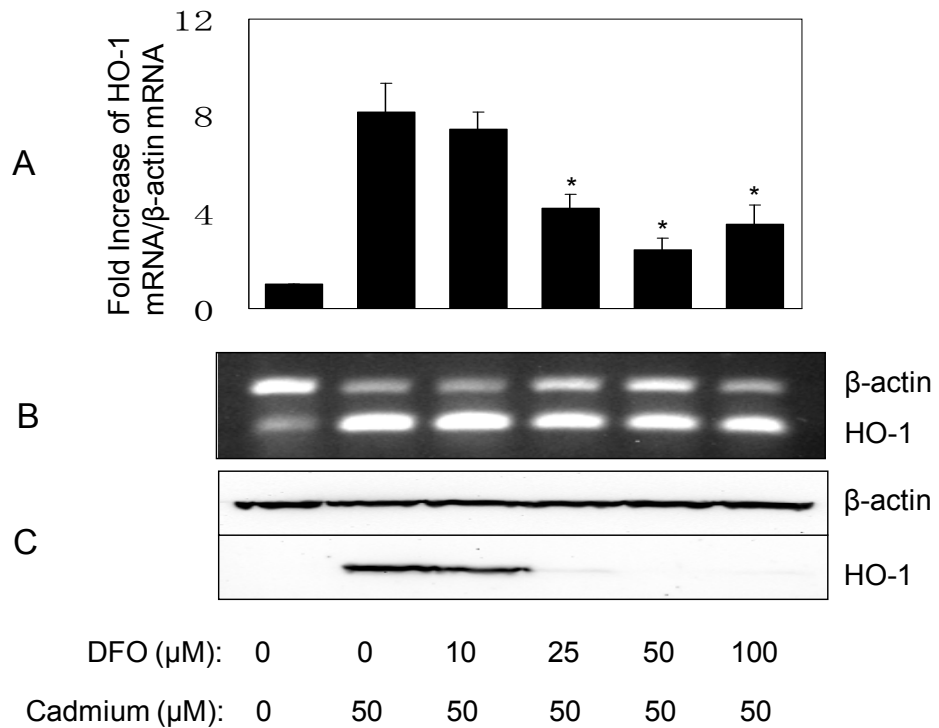


Figure II-3. DFO decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression. Cells were treated with 0-100 μM DFO for 24 hours, followed with or without the exposure to 0-50 μM cadmium for 5 hours. B: HO-1 mRNA was constitutively expressed in control cells, and the treatment with 50 μM cadmium increased HO-1 mRNA expression; however, the pretreatments with 25-100 μM DFO decreased the upregulating effect of cadmium on HO-1 mRNA expression. A: the ratio of HO-1 mRNA/β-actin mRNA in the cells treated with 50 μM cadmium was dramatically increased compared to the control cells; however, the ratios of HO-1 mRNA/β-actin mRNA in the cells treated with 25-100 μM DFO and 50 μM cadmium were significantly decreased compared to the cells treated with 50 μM cadmium (p<0.05). C: similarly, the treatment with 50 μM cadmium increased HO-1 protein

expression, and the pretreatments with 25-100 μM DFO decreased the upregulating effect of cadmium on HO-1 protein expression. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 25, 50, or 100 μM DFO plus 50 μM cadmium vs. the treatment with 0 μM DFO plus 50 μM cadmium, $p < 0.05$).

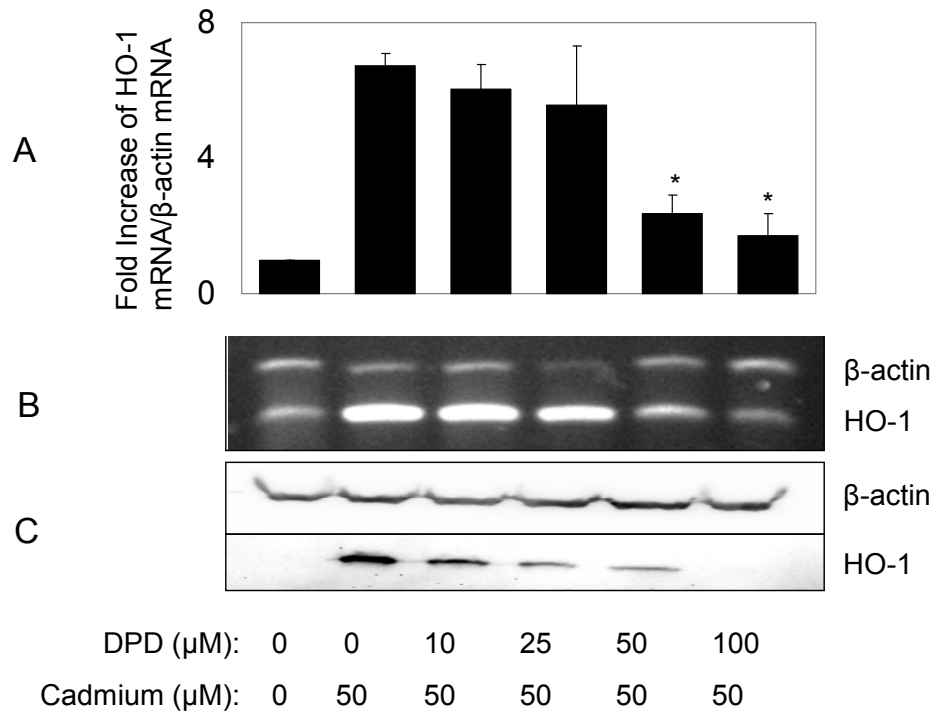


Figure II-4. DPD decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression. Cells were treated with 0-100 μM DPD for 24 hours, followed with or without the exposure to 0-50 μM cadmium for 5 hours. B: HO-1 mRNA was constitutively expressed in control cells, and the treatment with 50 μM cadmium increased HO-1 mRNA expression; however, the pretreatments with 50-100 μM DPD decreased the upregulating effect of cadmium on HO-1 mRNA expression. A: the ratio of HO-1 mRNA/β-actin mRNA in the cells treated with 50 μM cadmium was increased dramatically compared to the control cells; however, the ratios of HO-1 mRNA/β-actin mRNA in the cells treated with 50-100 μM DPD and 50 μM cadmium were significantly decreased compared to the cells treated with 50 μM cadmium ($p < 0.05$). C: similarly, the treatment with 50 μM cadmium increased HO-1 protein expression, and the pretreatments with 10-100 μM DPD decreased the upregulating

effect of cadmium on HO-1 protein expression. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 50 or 100 μ M DPD plus 50 μ M cadmium vs. the treatment with 0 μ M DPD plus 50 μ M cadmium, $p < 0.05$).

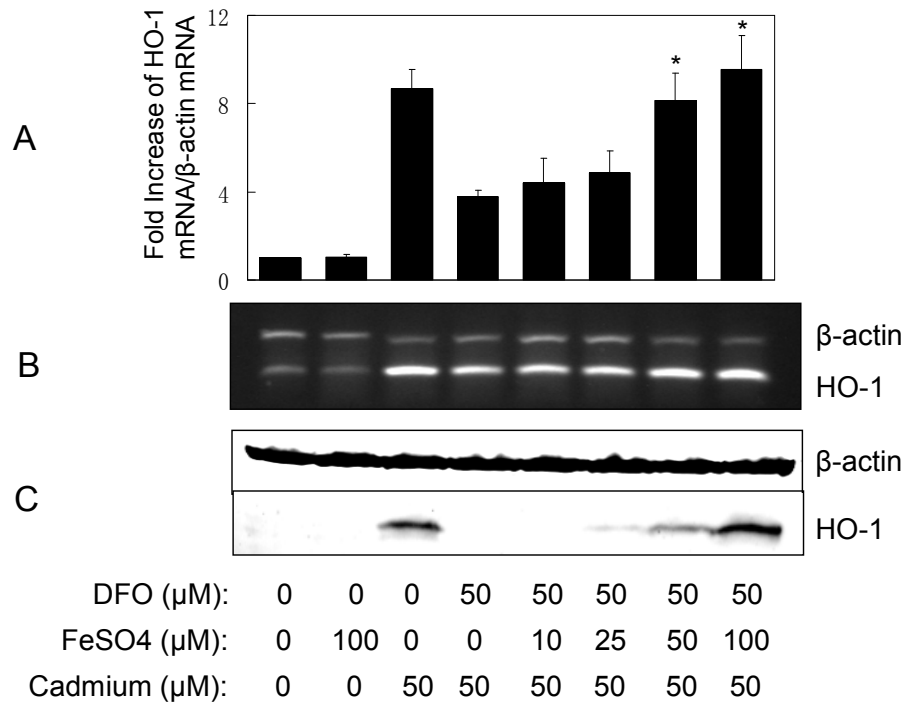


Figure II-5. Iron restored the upregulating effects of cadmium on HO-1 protein and mRNA expression in the cells pretreated with DFO. Cells were treated with 0-100 μM iron sulfate (FeSO₄) for 3 hours after the pretreatment with 0-50 μM DFO for 24 hours, then cells were exposed to 0-50 μM cadmium for 5 hours. B: the treatments with 50-100 μM FeSO₄ restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with 50 μM DFO. A: the ratios of HO-1 mRNA/ β -actin mRNA was significantly increased in the cells treated with DFO, 50-100 μM FeSO₄, and cadmium compared to the cells treated with DFO and cadmium ($p < 0.05$). C: the treatment with 50-100 μM FeSO₄ restored the upregulating effect of cadmium on HO-1 protein expression in the cells pretreated with 50 μM DFO. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 50 μM DFO, 50 or 100 μM FeSO₄, and 50 μM cadmium vs. the

treatments with 50 μM DFO, 0 μM FeSO_4 , and 50 μM cadmium, $p < 0.05$).

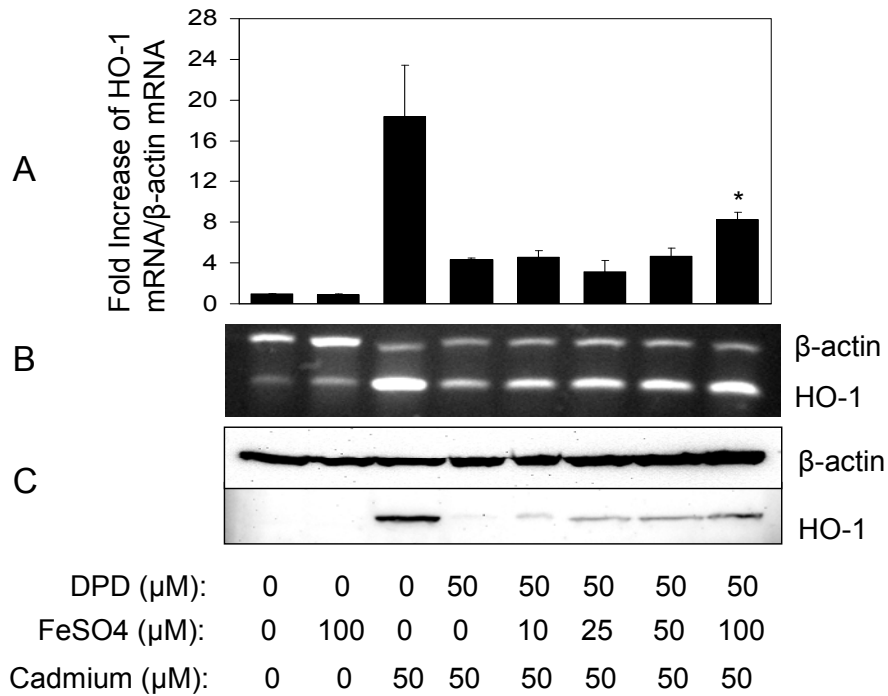


Figure II-6. Iron restored the upregulating effects of cadmium on HO-1 protein and mRNA expression in the cells pretreated with DPD. Cells were treated with 0-100 μM iron sulfate (FeSO₄) for 3 hours after the pretreatment with 0-50 μM DPD for 24 hours, and then cells were exposed to 0-50 μM cadmium for 5 hours. B: the treatment with 100 μM FeSO₄ restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with 50 μM DPD. A: the ratios of HO-1 mRNA/β-actin mRNA was significantly increased in the cells treated with DPD, 100 μM FeSO₄, and cadmium compared to the cells treated with DPD and cadmium (p<0.05). C: the treatment with 100 μM FeSO₄ restored the upregulating effect of cadmium on HO-1 protein expression in the cells pretreated with 50 μM DPD. The values given are means ± S.E.M. from three independent experiments (*, the treatments with 50 μM DPD, 100 μM FeSO₄, and 50 μM cadmium vs. the treatments

with 50 μM DPD, 0 μM FeSO_4 , and 50 μM cadmium, $p < 0.05$).

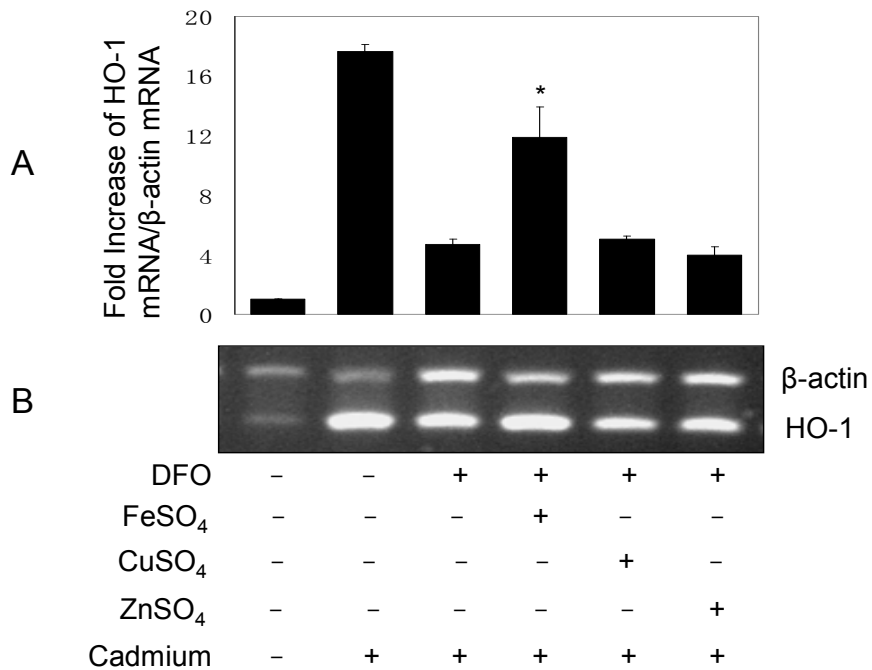


Figure II-7. Iron, but not copper and zinc, restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with DFO. Cells were pretreated with 0-50 μ M DFO for 24 hours, followed the treatment with 0-100 μ M FeSO₄, copper sulfate (CuSO₄), or zinc sulfate (ZnSO₄) for 3 hours, and then cells were exposed to 0-50 μ M cadmium for 5 hours. B: the treatment with 100 μ M FeSO₄ restored the upregulating effect of cadmium on HO-1 mRNA expression in the cells pretreated with 50 μ M DFO; however, no restoring effect of the treatments with CuSO₄ or ZnSO₄ on the upregulating effect of cadmium on HO-1 mRNA was observed in the cells pretreated DFO. A: the ratio of HO-1 mRNA/ β -actin mRNA was significantly increased in the cells treated with DFO, FeSO₄ and cadmium compared to the cells treated with DFO and cadmium ($p < 0.05$); but the ratios in the cells treated with DFO, CuSO₄ or ZnSO₄, and cadmium were similar to the cells treated with DFO

and cadmium. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 50 μ M DFO, 100 μ M FeSO₄, 0 μ M CuSO₄, 0 μ M ZnSO₄, and 50 μ M cadmium vs. the treatments with 50 μ M DFO, 0 μ M FeSO₄, 0 μ M CuSO₄, 0 μ M ZnSO₄, and 50 μ M cadmium, $p < 0.05$).

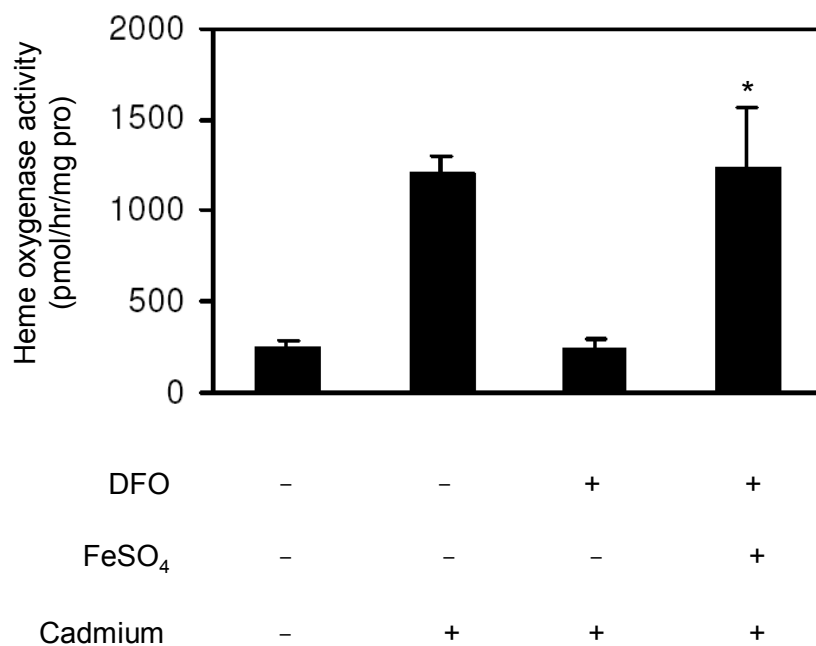


Figure II-8. Iron restored the upregulating effect of cadmium on HO-1 enzyme activity in the cells pretreated with DFO. Cells were pretreated with without 0-50 μM DFO for 24 hours, followed by treatments with or without 0-100 μM FeSO_4 for 3 hours. Then the cells were exposed to 0-50 μM cadmium for 5 hours. The treatment with FeSO_4 significantly restored the upregulating effect of cadmium on HO-1 enzyme activity ($p < 0.05$). The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 50 μM DFO, 100 μM FeSO_4 , and 50 μM cadmium vs. the treatments with 50 μM DFO, 0 μM FeSO_4 , and 50 μM cadmium, $p < 0.05$).

CHAPTER III
ELUCIDATION OF CADMIUM-INDUCED UPREGULATION OF HEME
OXYGENASE-1 IN HCT116 CELLS

Abstract

As reported previously, cadmium upregulated mRNA and protein expression and enzyme activity of the cytoprotective gene, heme oxygenase-1 (HO-1), in iron-sufficient HCT116 cells, but these upregulating effects could be weakened and then be restored by the treatments with iron in iron-deficient cells caused by iron chelators, desferoxamine (DFO) or 2', 2'-dipyridyl (DPD), indicating that iron plays a critical role in cadmium-induced HO-1 upregulation. In the present study, experiments were conducted to help explain these observations. It was found that cadmium, ethacrynic acid (EA), and buthionine sulfoximine (BSO) decreased intracellular glutathione levels. Cadmium and EA, but not BSO, increased HO-1 mRNA expression. EA also increased HO-1 protein expression. However, the upregulation of HO-1 protein and mRNA caused by cadmium or EA could be decreased by adding glutathione (GSH) or N-acetyl-cysteine (NAC) into the cell culture media before the treatments with cadmium or EA. Cadmium and EA, but not BSO, increased nuclear expression of nuclear factor erythroid-2 related factor 2 (Nrf-2). Furthermore, it was found that HCT116 cells express NADPH oxidase (NOX)-1, and NOX inhibitors, apocynin and diphenyleneiodonium (DPI), or superoxide anion scavenger, tiron, decreased cadmium-induced upregulation of HO-1 protein and mRNA expression and enzyme activity. Finally, it was found that DPI, but not apocynin and tiron inhibited

NADPH:cytochrome P450 reductase (NCPR) enzyme activity. Put together, these results suggest that cadmium may deplete intracellular glutathione resulting in the intracellular accumulation of NOX-produced ROS, which may cause the translocation of Nrf-2 into nucleus, therefore upregulate HO-1 expression; iron chelators DFO and DPD may deplete the iron in the hemes, the components of NOX, therefore decrease the production of NOX-produced ROS and finally attenuate the upregulating effects of cadmium on HO-1.

Introduction

Cadmium is widely used in several industrial settings, causes a variety of adverse health effects including carcinogenesis (Patrick 2003). As for the mechanism, oxidative stress induced by cadmium seems to be involved. Studies have shown that cadmium has the property to induce the intracellular accumulation of reactive oxygen species (ROS) which may cause lipid peroxidation of cell membrane and DNA damage (O'Brien 1998, Stohs 2001). Cadmium has also been shown to have the ability to upregulate the expression of a variety of stress-response genes including heme oxygenase-1 (HO-1), which has been believed to be an important defending mechanism against cadmium toxicity.

It has been shown that HO-1, the rate-limiting enzyme in heme degradation (Taketani 1989), could be induced by oxidative stress (Applegate 1992) and that cadmium has the property to induce the intracellular accumulation of reactive oxygen species (ROS) by interrupting intracellular anti-oxidative system (Stohs 2001). Studies also have shown that ROS could be generated from several different sources including mitochondrion, NADPH oxidase, cytochrome P450, and other enzymes. Among them, mitochondrion and NADPH oxidase have been considered two major

sources of ROS (Fleury 2002, Lambeth 2004). It is important to note that iron is commonly required by these ROS-generating systems for the function of electron transport.

Previously, we observed that cadmium upregulated HO-1 protein and mRNA expression and enzyme activity in HCT116 human colon epithelial cells, but all of these effects could be decreased and then be restored by the treatment with iron sulfate, in iron-deficient cells caused by iron chelators, desferoxamine (DFO) or 2',2'-dipyridyl (DPD), indicating that iron plays an important role in the cadmium-induced upregulation of HO-1. Experiments were conducted to help explain the observations.

It was found that cadmium, ethacrynic acid (EA), and buthionine sulfoximine (BSO) decreased intracellular glutathione. However, only cadmium and EA, but not BSO, upregulated the expression of HO-1 mRNA and the nuclear expression of nuclear transcription factor erythroid-2 related factor 2 (Nrf-2). The upregulation of HO-1 mRNA by cadmium and EA could be decreased by adding GSH and NAC into cell culture media before the treatments with cadmium and EA. HCT116 cells expressed NADPH oxidase (NOX)-1 and NOX inhibitors, apocynin and diphenyleneiodonium (DPI), and superoxide scavenger, tiron, decreased the upregulating effects of cadmium on HO-1 mRNA and protein expression and enzyme activity. DPI, but not apocynin and tiron, also inhibited NADPH:cytochrome P450 reductase (NCPR) enzyme activity which may further explain its potent inhibiting effect on HO-1 enzyme activity. These results suggest that cadmium may deplete intracellular glutathione, resulting in the intracellular accumulation of NOX-produced ROS which may cause the translocation of Nrf-2 into nucleus, therefore upregulate

HO-1 expression; iron chelators, DFO and DPD, may deplete the iron in the hemes, the components of NOX, therefore decrease the production of NOX-produced ROS and finally decreased cadmium-induced upregulation of HO-1.

Materials and methods

1. Materials

HCT116 human colon adenocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). Anti-HO-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Cadmium chloride, McCOY's 5A cell culture media and other reagents were purchased from Sigma-Aldrich Company (St. Louis, MO).

2. Cell culture and treatments

HCT 116 cells are grown in McCOY's 5A cell culture media (Sigma-Aldrich, MO) in a humidified atmosphere of 5% CO₂, 95% air at 37 °C, with the supplement of 2.2 g/L sodium bicarbonate (Sigma-Aldrich, MO), 11.5 ml/L penicillin/streptomycin Solution (Lonza, MD), 4.1 ml/L amphotericine B (Mediatech, VA), and 113 ml/L fetal bovine serum (Mediatech, VA). After reaching a confluence of 80%, depending on the experiments: 1) cells were treated with 0-50 μM cadmium chloride, 0-50 μM ethacrynic acid (EA) (Sigma-Aldrich, MO), or 0-100 μM buthionine sulfoximine (BSO) (Sigma-Aldrich, MO) for 5 hours; 2) cells were pretreated with 0-10 mM glutathione (GSH) (Sigma-Aldrich, MO) or 0-10 mM N-acetyl-cysteine (NAC) (Sigma-Aldrich, MO) for 2 hours, then with or without GSH or NAC staying in the media, cells were treated with 0-50 μM cadmium chloride, 0-50 μM ethacrynic acid (EA) (Sigma-Aldrich, MO), or 0-100 μM buthionine sulfoximine (BSO) (Sigma-Aldrich, MO) for 5 hours; 3) cells were pretreated with 0-4 mM apocynin,

0-20 μ M diphenyleneiodonium (DPI) (Sigma-Aldrich, MO), or 0-20 mM tiron (Sigma-Aldrich, MO) for 2 hours, followed with the exposure to 0-50 μ M cadmium for 5 hours.

3. Determination of HO-1 mRNA expression

The cells were harvested and centrifuged in 500 rpm for 5 minutes. Total RNA was isolated from the cells by using a Qiagen RNeasy Mini kit (Qiagen, CA). The HO-1 mRNA expression was determined by reverse transcription polymerase chain reaction (RT-PCR) analysis using a Qiagen OneStep RT-PCR kit (Qiagen, CA) and HO-1 primers (Qiagen, CA). The HO-1 PCR upper and lower primer sequences were: 5'-TGTGGCAGCTGTCTCAAACCTCCA-3' and 5'-TTGAGGCTGAGCCAGGAACAGAGT-3'. The β -actin QuantumRNA primer/competimer sets (Ambion, TX) were used as internal standard. The RT-PCR conditions were 30 minutes at 50 °C, followed by 15 minutes at 95 °C (RT), then 0.5 minutes at 95 °C, 0.5 minutes at 60 °C, and 1 minute at 72 °C (PCR) for 25 cycles. The resulting cDNA products were separated by electrophoresis in 2% agarose gel with ethidium bromide staining. For the target gene (HO-1), the PCR product size was 175 base pairs (bp). For the internal control, β -actin, the PCR product size was 294 bp.

4. Western immunoblotting analysis of HO-1 protein

The cells were harvested, washed and centrifuged in 500 rpm for 5 minutes in phosphate buffered saline (PBS). The cells then lysed using radioimmunoprecipitation assay (RIPA) buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and Roche Complete Protease Inhibitor Cocktail, pH 7.4) and sit on ice for 30 minutes. Then, the cell lysate was centrifuged at 15,000 rpm for 20

minutes at 4 °C, and the supernatant was saved. The protein concentration of the supernatant was measured using a BCA protein assay kit (Pierce, IL). Then cell lysate supernatant (100 µg protein) was electrophoresed using Novex NuPAGE (Invitrogen, CA) mini-gels (4-12% Bis-Tris) in NuPAGE MOPS SDS Running Buffer (Invitrogen, CA), followed by blotting the gel to nitrocellulose membrane. Blotting was performed for 1 hour at 25 °C in NuPAGE Transfer Buffer (Invitrogen, CA). The membrane was incubated overnight at 4 °C with HO-1 goat monoclonal antibody (Santa Cruz, CA) in blocking buffer consisting 5% skim milk powder in TBST (20 mM Tris-HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4) (1:1,000). After being washed three times with TBST for 30 minutes, the membrane was incubated for 2 hours at 25 °C with rabbit anti-goat IgG/HRP conjugate (Santa Cruz, CA) in blocking buffer (1:100,000). Finally, after being washed again, the membrane was placed in a plastic pouch for incubation with Thermo Scientific Super Signal West Femto Luminol/Enhancer Solution (Thermo Scientific, IL) for 5 minutes before analysis (Kodak digital science™ image station 440 CF). Then the membrane was washed with Restore Western Blot Stripping Buffer (Thermo Scientific, IL) and incubated with mouse monoclonal anti-β-actin antibody in blocking buffer (1:40,000) for 2 hours at 25 °C. After being washed three times with TBST for 30 minutes, the membrane was incubated with HRP-conjugated goat polyclonal anti-mouse IgG (Santa Cruz, CA) in blocking buffer (1:200,000) for 1 hour at 25 °C. Then, the membrane was washed in TBST and processed for analysis as before.

5. Determination of HO-1 enzyme activity

The cells were harvested, washed and centrifuged in 500 rpm for 5 minutes in 4 °C PBS. Cell pellets were resuspended in 100 mM phosphate buffer containing 2

mM magnesium chloride (MgCl_2), and were sonicated. After being spinned at 15,000 rpm for 10 minutes at 4 °C, the supernatant was saved and protein in the supernatant was determined. Then 0.8 mg protein was used to quantitate HO-1 enzyme activity in a reaction volume of 1 ml, containing 0.1 ml liver cytosol, 1 mM NADPH, 0.25 mM hemin, 2 mM glucose-6-phosphate, and 0.002 U/ μL glucose-6-phosphate dehydrogenase. The reaction was performed at 37 °C for 1 hour in the dark and stopped by adding 1 ml of chloroform. The tubes were vortexed thoroughly followed by centrifugation at 500 rpm for 5 minutes. Finally the chloroform layers were scanned on a spectrophotometer at 464 nm with the background at 530 nm. The HO-1 enzyme activity was expressed as formation of pmol bilirubin per hour per milligram of protein.

6. Intracellular glutathione determination by using Ellman's reagent

Cells were harvested, washed with cold PBS, and centrifuged in 3,000 rpm for 3 minutes at 4 °C. Cell pellets were resuspended with lyse buffer containing 50 mM tris and 1 mM EDTA (pH 6.8), and were sonicated, followed by being spun at 15,000 rpm for 10 minutes at 4 °C. Supernatant was diluted to the concentration of 125 μg protein/100 μl , and 10 μl of 30% trichloroacetic acid (TCA) was added to 300 μl supernatant. After sitting on ice for 15 minutes, the homogenate was centrifuged at 3,000 rpm for 10 minutes at 4 °C. Finally, the absorbance of the visible light at 412 nm of the homogenate containing 100 μl supernatant, 880 μl of TE buffer containing 0.25 M tris, 20 mM EDTA (pH 8.2), and 20 μl of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was measured by using a spectrophotometer.

7. Intracellular glutathione determination by using fluorescence microscopy

Cells were washed with Hank's buffered saline solution (HBSS), and incubated

with 20 μ M monochlorobimane (Invitrogen, CA) for 30 minutes at room temperature. Then the cells were washed with HBSS again, and were examined by using fluorescence microscope (UV filter).

8. Nuclear extraction preparation

Nuclear extracts were prepared by using Active Motif Nuclear Extract Kit (Active motif, CA). Cells were washed with and harvested in 10 ml ice-cold phosphate buffered saline/phosphatase inhibitor buffer (PBS/PIB), followed by spinning at 300 rpm for 5 minutes at 4 °C. The cell pellets were resuspended with 1 ml ice-cold hypotonic buffer (HB) buffer and sit on ice in 1.5 ml tubes for 15 minutes. After adding 50 μ l 10% Nonidet P-40, the tubes were vortexed vigorously for 10 seconds, followed by spinning for 30 seconds at 4 °C. The nuclear pellets were resuspended in 50 μ l complete lyse buffer and agitated on ice for 30 minutes on an agitating platform. The homogenate was centrifuged for 10 minutes at 14,000 rpm at 4°C and the supernatant was saved.

9. Western immunoblotting analysis of nuclear Nrf-2

Nuclear extract (60 μ g protein) was electrophoresed using Novex NuPAGE (Invitrogen, CA) mini-gels (4-12% Bis-Tris) in NuPAGE MOPS SDS Running Buffer (Invitrogen, CA), followed by blotting the gel to nitrocellulose membrane. Blotting was performed for 1 hour at 25 °C in NuPAGE Transfer Buffer (Invitrogen, CA). The membrane was incubated overnight at 4 °C with Nrf-2 rabbit polyclonal antibody (1:1,000) (Santa Cruz, CA) in blocking buffer consisting 5% skim milk powder in TBST (20 mM Tris-HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4). After being washed three times with TBST for 30 minutes, the membrane was incubated for 2 hours at 25 °C with goat anti-rabbit IgG/HRP conjugate (Santa Cruz, CA) in

blocking buffer (1:20,000). Finally, after being washed again, the membrane was placed in a plastic pouch for incubation with Thermo Scientific Super Signal West Femto Luminol/Enhancer Solution (Thermo Scientific, IL) for 5 minutes before analysis (Kodak digital science™ image station 440 CF). Then the membrane was washed with Restore Western Blot Stripping Buffer (Thermo Scientific, IL) and incubated with goat polyclonal anti-lamin B antibody in blocking buffer (1:1,000) for 2 hours at 25 °C. After being washed three times with TBST for 30 minutes, the membrane was incubated with HRP-conjugated rabbit anti-goat IgG (Santa Cruz, CA) in blocking buffer (1:200,000) for 1 hour at 25 °C. Then, the membrane was washed in TBST and processed for analysis as before.

10. Determination of nuclear expression of Nrf-2 by using TransAM™ Nrf-2 kit

Nuclear Nrf-2 expression was determined by using TransAM™ Nrf-2 kit (Active Motif, CA). 10 µl (60 µg protein) nuclear extracts were added to wells of TransAM™ plate immobilized with antioxidant responsive element (ARE)-containing oligonucleotide with 40 µl complete binding buffer, and were incubated for 1 hour at room temperature with mild agitation. The wells then were washed 3 times with 200 µl wash buffer, followed by the addition of 100 µl Nrf-2 primary antibody (1:1,000 diluted in antibody binding buffer). After incubating for 1 hour at room temperature and washed 3 times with 200 µl wash buffer, 100 µl horseradish peroxidase (HRP)-conjugated second antibody (1:1,000 diluted in antibody binding buffer) was added and incubated for 1 hour at room temperature. After washing 4 times with 200 µl wash buffer, 100 µl developing solution (room temperature) was added and incubated for 2-15 minutes until a dark blue color developed. Then, 100 µl stop solution was added and the absorbance of a visible light at 450 nm with a reference

wavelength of 655 nm was measured using a spectrophotometer.

11. Determination of nuclear expression of Nrf-2 by using immunocytofluorescence microscopy

Cells were grown in LabTek chamber slides, and were fixed with formaldehyde (4% in PBS) at room temperature for 15 minutes. After being incubated with Triton X-100 (0.2% in PBS) for 5 minutes at 4 °C, the slides were blocked with bovine serum albumin (1% in PBS) for 3 hours at room temperature. Then rabbit Nrf-2 polyclonal antibody (1:500) was added and incubated overnight at 4 °C. After washing 3 times for 30 minutes, goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Porbes Inc., Eugene OR) (1:1,000) was added and incubated for 2 hours at room temperature. After washing, the slides were examined under FITC filter and UV filter (4',6-diamidino-2-phenylindole (DAPI) was used as a counterstain to visualize nuclei).

12. Examination of NADPH:cytochrome P450 reductase (NCPR) activity

The cells were harvested, washed, centrifuged in 500 rpm for 5 minutes at 4 °C PBS. Cell pellets were resuspended in 100 mM phosphate buffer containing 0.3 M potassium (pH 7.6) and were sonicated. After spinning at 15,000 rpm for 10 minutes at 4 °C, the supernatant was saved. Then, supernatant (50 µg), NADPH (final concentration, 50 µM) and cytochrome c (final concentration, 50 µM) were added to reductase assay buffer containing 50 mM potassium phosphate, 0.1 mM tetrasodium dihydrate, and 0.3 M potassium chloride (pH 7.4) (final volume, 1 ml). The initial rate of cytochrome c reduction was monitored for 10 minutes by using the absorbance at 550 nm (using an extinction coefficient of 21 mM⁻¹ cm⁻¹). NCPR enzyme activity was expressed as µmol per minute per mg protein.

13. Statistical analysis

HO-1/ β -actin mRNA ratio, HO enzyme activity, glutathione concentration, nuclear Nrf-2 expression, and NCPR enzyme activity data were analyzed via two-tailed unpaired t test using SPSS 16.0 for Microsoft Windows XP (SPSS Inc., IL).

Results

1. Cadmium, EA, and BSO decreased intracellular glutathione

To investigate the effect of cadmium on the intracellular glutathione, experiments were conducted to compare the effects of cadmium and two glutathione inhibitors, EA and BSO on intracellular glutathione levels. Cells were treated with 0-50 μ M cadmium chloride, 0-50 μ M EA, or 0-100 μ M BSO for 5 hours. The intracellular glutathione concentration was first determined by using Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). DTNB may react with thiol groups in GSH, and release TNB^- to form TNB^{2-} which has a yellow color and could be quantified in a spectrophotometer by measuring the absorbance of visible light at 412 nm. It was found that cadmium, EA, and BSO decreased the light absorbance, indicating decreased intracellular GSH levels caused by cadmium, EA, and BSO (Figure III-1, page 80). Then the result was confirmed by using monochlorobimane. When incubated with the cells, monochlorobimane enters the cells and is quickly converted to a fluorescent GSH conjugate (GS-monochlorobimane) which appears a blue color in a fluorescence microscope (UV filter). It was found that, after being incubated with monochlorobimane, the cells treated with cadmium, EA, or BSO, had less densities of blue color than the control cells, indicating decreased intracellular GSH levels caused by cadmium, EA, and BSO (Figure III-2, page 81).

2. The effects of Cadmium, EA, and BSO, on HO-1 mRNA expression

Then, experiments were conducted to compare the effects of cadmium, EA, and BSO on the expression of HO-1. Cells were pretreated with 0-10 mM GSH or 0-10 mM NAC for 2 hours, then with or without GSH or NAC remaining in the media, cells were exposed to 0-50 μ M cadmium chloride, 0-50 μ M EA, or 0-100 μ M BSO for 5 hours. It was found that cadmium upregulated HO-1 mRNA expression. However, in the cells treated with GSH and NAC, which were left in the cell culture media until the five-hour cadmium treatment finished, the upregulating effect of cadmium on HO-1 mRNA expression was decreased (Figure III-3 A, page 82). But in the cells treated with GSH and NAC which were removed from cell culture media before cadmium treatment, the upregulating effect of cadmium on HO-1 mRNA expression was not affected (Figure III-3 B, page 82). Similar effects were observed in the cells treated with EA. The treatment with EA upregulated HO-1 mRNA expression. However, in the cells treated with GSH and NAC which were remained in the cell culture media until five-hour EA treatment finished, the upregulating effect of EA on HO-1 mRNA expression was decreased (Figure III-3 C, lane 6 and 8, page 82). But in the cells treated with GSH and NAC, which were removed from cell culture media before EA treatment, the upregulating effect of EA on HO-1 mRNA expression was not affected (Figure III-3 C, lane 5 and 7, page 82). Interestingly, the treatment of BSO has not influence on HO-1 mRNA expression (data is not shown).

3. Cadmium and EA, but not BSO, increased the nuclear expression of Nrf-2

The oxidative stress-responding transcription factor nuclear factor erythroid-2 related factor 2 (Nrf-2) has been thought to play a critical role in xenobiotic-induced HO-1 upregulation. Therefore, experiments were conducted to compare the effects of

cadmium, EA, and BSO on nuclear Nrf-2 expression. Cells were treated with 0-50 μM cadmium chloride, 0-50 μM EA, or 0-100 μM BSO for 5 hours. By using Western immunoblotting analysis, it was found that cadmium and EA, but not BSO, upregulated nuclear Nrf-2 protein expression (Figure III-4 B, page 84). The result was confirmed by using TransAMTM Nrf-2 kit and Fluorescence Microscopic Analysis. When Nrf-2 containing nuclear extract added to the wells of TransAMTM plate coated with antioxidant responsive element (ARE)-containing oligonucleotide, Nrf-2 binds to ARE. Then after being incubated with a primary Nrf-2 antibody and a horseradish peroxidase (HRP)-conjugated second antibody, a dark blue color could be developed which could be quantified by measuring the absorbance of a visible light at 450 nm with a reference wave length of 655 nm using spectrophotometer. Similar to the results of Western immunoblotting analysis, the nuclear extract of the cells treated with cadmium and EA had a significantly higher light absorbance than the nuclear extract of control cells and cells treated with BSO, indicating cadmium and EA, but not BSO, upregulated nuclear Nrf-2 protein expression (Figure III-4 A, page 84). Finally, cells were grown in LabTek chamber slides and were fixed with formaldehyde. After being incubated with Triton X-100, the slides were incubated with a Nrf-2 primary antibody and a second antibody conjugated with Alexa Fluor 488 which appears as green color under a FITC filter. Then, the slides were incubated with 4',6-diamidino-2-phenylindole (DAPI) which binds strongly to DNA and appears as blue color under a UV filter. By examining under fluorescence microscope, it was found that the cells treated with cadmium and EA had higher densities of green color than the control cells and cells treated with BSO under a FITC filter. The nuclei of the cells, which had been bound to DAPI, were visualized with a blue color under a

UV filter. The images with green color under the FITC filter and images with blue color under the UV filter could be overlapped (Figure III-5, page 86). These observations suggest that cells treated with cadmium and EA had higher nuclear Nrf-2 expression than control cells and cells treated with BSO.

4. HCT116 cells expressed NOX-1 protein and NOX inhibitors, apocynin and DPI, and superoxide scavenger, tiron, decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression

To investigate whether HCT116 cells express NOX, and the role of NOX-produced ROS in HO-1 protein and mRNA expression, experiments were conducted to see if NOX-1 is expressed in the cells and the influence of NOX inhibitors, apocynin and DPI, and superoxide scavenger, tiron, on the upregulating effects of cadmium on HO-1 mRNA and protein expression. It was found that NOX-1 protein was constitutively expressed in HCT116 cells, and this expression was not influenced by the treatment with DFO (Figure III-6 A, page 88). The treatment with cadmium increased HO-1 mRNA expression; however, the inhibition of NOX and superoxide decreased the upregulating effect of cadmium on HO-1 mRNA (Figure III-6 C, page 88); the ratio of HO-1 mRNA/ β -actin mRNA in the cells treated cadmium was increased dramatically compared to the control cells; however, the ratios of HO-1 mRNA/ β -actin mRNA in the cells treated with apocynin, DPI, and tiron were significantly decreased compared to the cells treated with cadmium ($p < 0.05$) (Figure III-6 B, page 88). Similarly, the treatment with cadmium increased HO-1 protein expression, and the inhibition of NOX and superoxide decreased the upregulating effect of cadmium on HO-1 protein expression (Figure III-6 D, page 88).

5. NADPH oxidase inhibitors, apocynin and DPI, and superoxide scavenger, tiron,

decreased the upregulating effect of cadmium on HO-1 enzyme activity

Then experiments were conducted to investigate the effects of NOX inhibitors, apocynin and DPI, and superoxide scavenger, tiron, on the upregulating effect of cadmium on HO-1 enzyme activity. It was found that the inhibition of NOX and superoxide decreased the upregulating effect of cadmium on HO-1 enzyme activity ($p < 0.05$) (Figure III-7, page 90).

6. DPI, but not apocynin and tiron, inhibited NADPH:cytochrome P450 reductase (NCPR) enzyme activity

Finally, experiments were conducted to investigate the influences of DPI, apocynin, and tiron on NCPR activity. It was found that DPI (Figure III-8 A, page 92), but not apocynin and tiron (Figure III-8 B, page 92), decreased NCPR enzyme activity.

Discussion

It was observed in the previous study that iron plays a critical role in cadmium-induced HO-1 upregulation, but no mechanism was identified. In the present study, experiments were conducted to help explain the effects of cadmium on HO-1 gene expression in HCT116 cells with or without iron deficiency.

Although the mechanisms of the influences of cadmium on gene expression are not fully understood, some cellular effects of cadmium have been demonstrated. Studies have shown that cadmium may influence gene expression by increasing intracellular accumulation of ROS via interaction with mitochondrion, antioxidant enzymes including catalase and superoxide dismutase (SOD), and intracellular glutathione (Patrick 2003).

Glutathione (GSH) is the major and the most abundant intracellular antioxidant.

Studies have shown that intracellular GSH concentrations could be reduced dramatically in response to oxidative stress (Ariigo 1999). Furthermore, the GSH/GSSG ratio has been used to indicate the intracellular redox state. The decrease of intracellular GSH concentration may result in a decrease of GSH/GSSG ratio, indicating an abnormal intracellular redox state, therefore, influences the expression of variety of redox-sensitive genes by modulating the redox-sensitive transcription factors such as NF- κ B, AP-1, and Nrf-2.

Studies have shown that the thiol groups have the ability to bind to metals (Giles 2003). Therefore, the effect of cadmium on intracellular GSH was investigated by comparing with two GSH inhibitors, EA and BSO. Studies have shown that EA and BSO inhibit intracellular GSH through different mechanisms. EA depletes GSH through conjugating to GSH via glutathione-S-transferases (Shen 1995), but BSO inhibits GSH synthesis (Griffith 1979). This means that EA may have a more immediate and direct effect on the depletion of intracellular glutathione.

In the present study, it was observed that cadmium decreased intracellular glutathione level. This result was similar to the effects of EA and BSO on intracellular glutathione levels (Figure III-1 and 2, page 80-81). It was also observed that cadmium and EA, but not BSO, increased HO-1 mRNA (Figure III-3, page 82) and nuclear Nrf-2 protein expression (Figure III-4 and 5, page 84-86). Therefore, it is very likely that similar to EA, cadmium decreases intracellular GSH level by directly conjugating to GSH.

To test this speculation, another experiment was conducted. The cells were treated with GSH or NAC. Then, with or without GSH and NAC left in the cell culture media, the cells were exposed to cadmium or EA. It was found that adding

GSH and NAC into the cell culture media decreased the upregulating effects of cadmium and EA on HO-1 mRNA expression, suggesting cadmium has similar conjugating effects on GSH and NAC as EA does. The decreased intracellular GSH level caused by cadmium may result in the intracellular accumulation of ROS which activates transcription factors of HO-1, therefore inducing HO-1 expression. This result is consistent with other researchers' work (Lopez 2006, Figueredo-Pereira 1998, Taketani 1989). Both in Lopez and Figueredo-Pereira studies reported a decreased intracellular GSH level in neurons after exposing to cadmium. Furthermore, Taketani group observed that cadmium have the property to upregulate HO-1 gene expression.

Several transcription factors, including heat-shock factor (HSF), nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1), and nuclear transcription factor NF-E2-related factor-2 (Nrf-2), have been shown to be related to the regulation of the expression of HO-1. Among them, Nrf-2 was thought to be particular important and related the upregulation of HO-1 by a variety of xenobiotics (Otterbin 2002, Alam 1999). In normal conditions, Nrf-2 is sequestered in the cytoplasm as an inactive form by Keap1. Keap1 binds to Nrf-2, preventing Nrf-2 translocation into the nucleus and increasing the degradation of Nrf-2 by ubiquitin-proteasome pathway, therefore preventing HO-1 upregulation activated by Nrf-2 (Steward 2003). Keap1 is a protein rich in cysteine residues which makes it to be sensitive to xenobiotics and oxidative stress (Itoh 1999 and 2003, McMahon 2003). With the appearance of ROS, Nrf-2 is dissociated from Keap1 and is translocated into the nucleus where it binds to HO-1 enhancers, including the stress responsive element (StRE) (Inamdar 1996) or Maf recognition element (MARE) (Kataoka 1994). Studies have shown that MARE/StRE,

small Maf proteins, Nrf-2, and Bach1 were involved in the regulation of HO-1 gene expression. The small Maf proteins form heterodimers with Nrf-2 to activate (Ishii 2000, Kataoka 2001), or with Bach1 to repress MARE-dependent HO-1 gene expression (Igarashi 1998). It has been reported that cadmium upregulates HO-1 expression by increasing the export of Bach1 from nucleus (Suzuki 2003) and by stabilizing Nrf-2 through a mechanism of decreasing the degradation of Nrf-2 by the ubiquitin-proteasome pathway (Steward 2003). In the present study, it was observed that cadmium upregulated HO-1 expression by increasing nuclear Nrf-2 protein expression (Figure III-4 and 5, page 84-86). Putting the results together, it is possible that cadmium may increase intracellular accumulation of ROS by depleting intracellular GSH. The accumulation of ROS then may cause the dissociation of Nrf-2 from Keap1 which decreases the degradation of Nrf-2 by ubiquitin-proteasome pathway and increases translocation of Nrf-2 from cytosol into nucleus. The increased nuclear Nrf-2 then activates HO-1 gene expression.

Studies have demonstrated that ROS could be produced in several potential sources including mitochondrion, NOX, cytochrome P450, and other enzymes. Among them, NOX and mitochondrion are believed to be two major sources of ROS. In the previous study, it was observed that DFO and DPD decreased the upregulating effect of cadmium on HO-1 gene expression, and these modulations of DFO or DPD appeared to be at the pre-transcriptional level. So, it is possible that DFO and DPD decreased the upregulating effect of cadmium on HO-1 by interrupting the production of ROS in the two major sources, mitochondrion or NOX. Since NOX contains two hemes anticipating the electron transport in the enzyme (Finogold 1996), it was speculated that DFO and DPD decrease the upregulating effect of cadmium on HO-1

by depleting the iron in the hemes, the components of NOX, hence affect the electron transport and reduce NOX-produced ROS. Therefore, in the condition of cadmium exposure in iron-deficient cells, no intracellular accumulation of ROS occurs because of possible impaired NOX function, even though the intracellular glutathione level is decreased by cadmium. This speculation was supported by the following observation: NOX inhibitors, apocynin and DPI, and superoxide scavenger, tiron, decreased the upregulating effects of cadmium on HO-1 mRNA, protein expression (Figure III-6, page 88) and enzyme activity (Figure III-7, page 90). Despite the fact that DPI (0.05-20 μ M) had a much more potent inhibiting effect on HO-1 activity than apocynin (0.5-4 mM) and tiron (5-20 mM), these effects were very similar to the effects of DFO and DPD on HO-1 mRNA and protein expression and enzyme activity, suggesting the important role of NOX-induced ROS in the cadmium-induced upregulation of HO-1.

The degradation of heme begins with the formation of ferrous iron from ferric iron in heme-HO-1 complex which needs an electron donated from NADPH:cytochrome P450 reductase (NCPR) (Yoshida 1978). This means that HO-1 activity could be influenced by the activity of NCPR. Since both NOX-1 and NCPR are flavoprotein, and DPI is a flavoprotein inhibitor (Chakraborty 2002), so it was speculated that when the cells were treated with DPI, both NOX-1 and NCPR could be inhibited by DPI; but when the cells were treated with apocynin or tiron, only the intracellular ROS level, but not NCPR enzyme activity, was decreased. To test this hypothesis, NCPR enzyme activities of the cells with the treatments with DPI, apocynin, and tiron were determined. It was found that DPI inhibited NCPR enzyme activity from a very low concentration (0.05-5 μ M) (Figure III-8 A, page 92), but no

inhibiting effect of apocynin or tiron on NCPR enzyme activity was observed (Figure III-8 B, page 92). The results may explain that why DPI was so potent on the inhibition of NCPR enzyme activity compared to apocynin and tiron, and on the other hand, support the notion that the function of HO-1 requires the participation of NCPR.

In conclusion, as depicted in Figure III-9 (page 93), the results of the present study suggest that NOX-induced ROS plays an important role on the cadmium-induced upregulation of HO-1. Cadmium depletes the intracellular glutathione which may result in the intracellular accumulation ROS. It is likely that upon the presence of ROS, Nrf-2 may be dissociated from Keap1. The dissociation of Nrf-2 from Keap1, therefore, decreases the degradation of Nrf-2 via ubiquitin-proteasome pathway and increases the translocation of Nrf-2 from cytosol into nucleus where Nrf-2 activates the expression of HO-1. Iron chelators, DFO and DPD, deplete iron in the hemes, the components of NOX, interrupting the production of ROS from NOX, therefore decrease the upregulating effects of cadmium on HO-1. The results also support the notion that the function of HO-1 requires the anticipation of NCPR.

In closing, my thesis suggests that as a contaminant and carcinogen, cadmium causes cellular damages via depleting intracellular GSH, therefore increase intracellular accumulation of NOX-produced ROS. As a defending mechanism for cytoprotection against cadmium, HO-1 mRNA and protein and enzyme activity in the cells are upregulated, but this upregulation could be weakened in iron-deficient cells caused by DFO and DPD, which implies that iron-deficient population could be more likely to develop adverse health problems than iron-sufficient population in the condition of cadmium exposure. However, there are still lots of research questions

should be answered some of which are stated in the epilogue.

References

- Alam J, Stewart D, Touchard C, et al. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase 1 gene. *J Biol Chem* 1999;274:26071-8.
- Arrigo AP. Gene expression and the thiol redox state. *Free Radic Biol Med* 1999;27:936-44.
- Applegate LA, Luscher P, and Tyrrell RM. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res* 1992;51:974-8.
- Coyle YM. The effect of environment on breast cancer risk. *Breast Cancer Res Treatm* 2004;84:273-88.
- Chakraborty S, Massey V. Reaction of reduced flavins and flavoproteins with diphenyliodonium chloride. *J Biol Chem* 2002;277:41507-16.
- Figueredo-Pereira ME, Ykushin S, Cohen G. Disruption of the intracellular sulfhydryl homeostasis by cadmium-induced oxidative stress leads to protein thiolation and ubiquitination on neuronal cells. *J Biol Chem* 1998;273:12703-9.
- Finegold AA, Shatwell KP, Segal AW, et al. Intramembrane bis-heme motif for transmembrane electron transport conserved in a yeast iron reductase and the human NADPH oxidase. *J Biol Chem* 1996;271:31021-4.
- Fleury C, Mignotte B, Vayssiere JL. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 2002;84:131-41.
- Giles NM, Watts AB, Giles GI, et al. Metal and redox modulation of cysteine protein function. *Chem Biol* 2003;10:677-93.
- Griffith OW, Anderson ME, Meister A. Inhibition of glutathione biosynthesis by

- prothionine sulfoximine (S-n-propyl homocysteine sulfoximine), a selective inhibitor of gamma-glutamylcysteine synthetase. *J Biol Chem* 1979;254:1205-10.
- Igarashi K, Hoshino H, Muto A, et al. Multivalent DNA binding complex generated by small Maf and Bach1 as a possible biochemical basis for beta-globin locus control region complex. *J Biol Chem* 1998;273:11783-90.
- Inamdar NM, Ahn YI, Alam J. The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. *Biochem Biophys Res Commun* 1996;221:570-6.
- Ishii T, Itoh K, Takahashi S, et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 2000;275:16023-9.
- Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 1999;13:76-86.
- Itoh K, Wakabayashi N, Katoh Y, et al. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* 2003;8:379-91.
- Kataoka K, Noda M, Nishizawa M. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol Cell Biol* 1994;14:700-12.
- Kataoka K, Handa H, Nishizawa M. Induction of cellular antioxidative stress genes through heterodimeric transcription factor Nrf2/small Maf by antirheumatic gold(I) compounds. *J Biol Chem* 2001;276:34074-81.

- Lambeth JD. Nox enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 2004;4:181-9.
- Lopez E, Arce C, Oset-Gasque MJ, et al. Cadmium induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture. *Free Radic Biol Med* 2006;40:940-51.
- McMahon M, Itoh K, Yamamoto M, et al. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* 2003;278:21592-600.
- O'Brien P, Salacinski HJ. Evidence that the reactions of cadmium in the presence of metallothionein can produce hydroxyl radicals. *Arch Toxicol* 1998;72:690-700.
- Otterbein LE, Choi AMK. The saga of leucine zippers continues in response to oxidative stress. *Am J Respir Cell Mol Biol* 2002;26:161-3.
- Patrick L. Toxic metals and antioxidants: part II. The role of antioxidants in arsenic and cadmium toxicity. *Altern Med Rev* 2003; 8:106-28.
- Schwartz GG, Reis IM. Is cadmium a cause of human pancreatic cancer? *Cancer Epidemiol Biomark Prev* 2000;9:139-145.
- Shen H, Ranganathan S, Kuzmich S, et al. Influence of ethacrynic acid on glutathione S-transferase transcript and protein half-lives in human colon cancer cells. *Biochem Pharmacol* 1995;50:1233-8.
- Stewart D, Killeen E, Naquin R, et al. Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *J Biol Chem* 2003;278:2396-2402.
- Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. *Free Rad Biol Med* 1995;18:321-36.

- Stohs SJ, Bagchi D, Hassoun E, et al. Oxidative mechanisms in the toxicity of chromium and cadmium ions. *J Environ Pathol Toxicol Oncol* 2001;20:77-88.
- Suzuki H, Tashiro S, Sun J, et al. Cadmium induces nuclear export of Bach1, a transcriptional repressor of heme oxygenase-1 gene. *J Biol Chem* 2003;278:49264-53.
- Taketani S, Kohno H, Yoshinaga T, et al. The human 32-kDa stress protein induced by exposure to arsenite and cadmium ions is heme oxygenase. *FEBS Lett* 1989;245:173-6.
- Yoshida T, Kikuchi G. Features of the reaction of heme degradation catalyzed by the reconstituted microsomal heme oxygenase system. *J Biol Chem* 1978;252:4230-6.

Figures

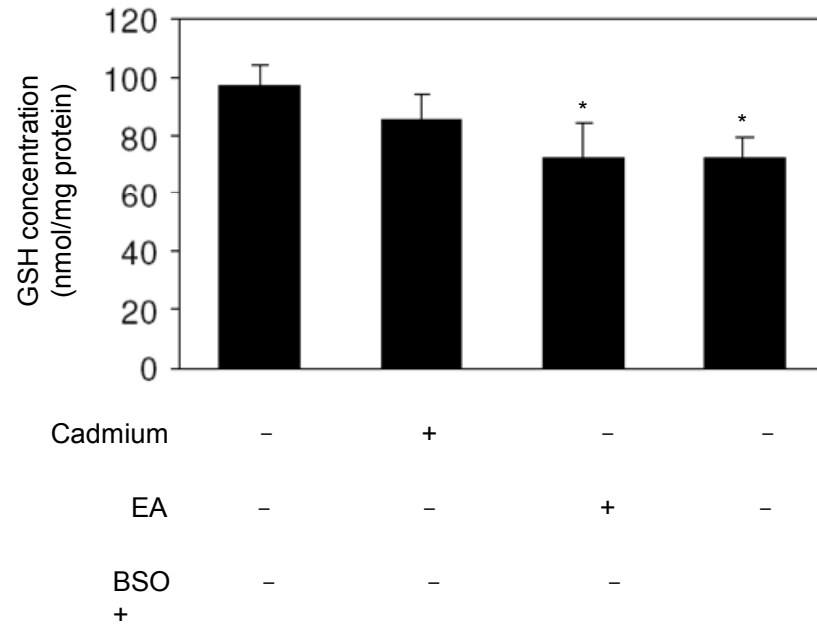


Figure III-1. Cadmium, EA, and BSO decreased the intracellular glutathione levels (determined by using Ellman's reagent). Cells were treated with 0-50 μM cadmium chloride, 0-50 μM EA, or 0-100 μM BSO for 5 hours. The intracellular glutathione concentration was determined using Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). DTNB may react with thiol groups in GSH, and release TNB^- to form TNB^{2-} which has a yellow color and could be quantified in a spectrophotometer by measuring the absorbance of a visible light at 412 nm. Cadmium, EA, and BSO decreased the light absorbance, indicating decreased intracellular GSH levels caused by cadmium, EA, and BSO. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with EA or BSO vs. control, $p < 0.05$).

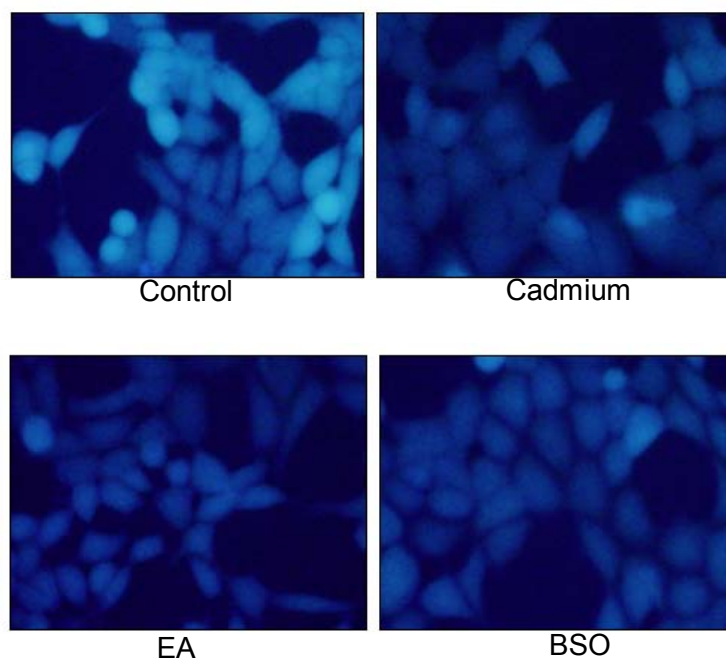


Figure III-2. Cadmium, EA, and BSO decreased the intracellular glutathione levels (determined by using monochlorobimane). Cells were treated with 0-50 μM cadmium chloride, 0-50 μM EA, or 0-100 μM BSO for 5 hours. The intracellular glutathione concentration was determined by using monochlorobimane. By incubation with the cells, monochlorobimane enter the cells and is quickly converted to a fluorescent GSH conjugate (GS-monochlorobimane) which appears a blue color under fluorescence microscope (UV filter). After being incubated with monochlorobimane, the cells treated with cadmium, EA, or BSO, have less density of blue color than the control cells, indicating decreased intracellular GSH levels caused by cadmium, EA, and BSO. The images given represent three independent experiments.

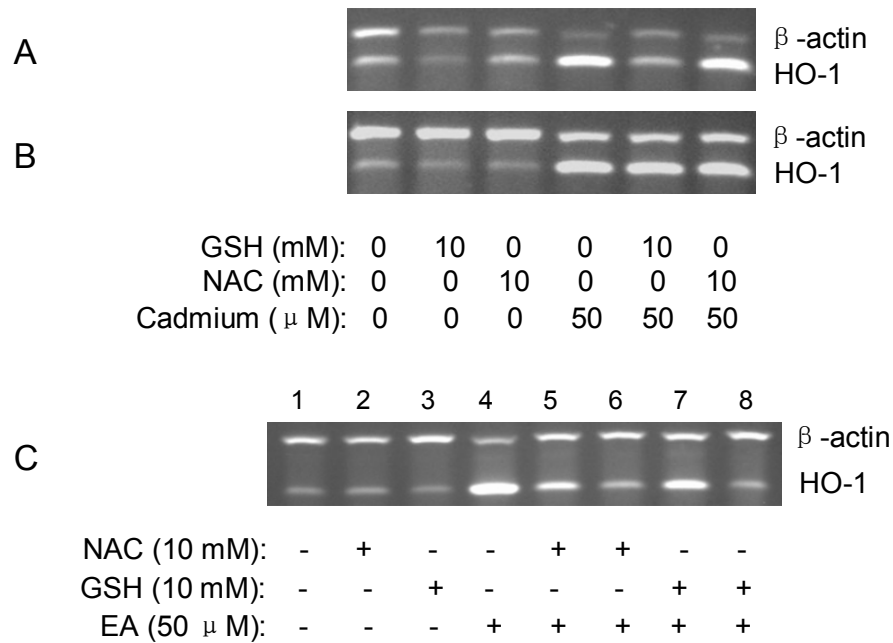


Figure III-3. The effects of cadmium and EA on HO-1 mRNA expression. Cells were pretreated with 0-10 mM GSH or 0-10 mM NAC for 2 hours, then with or without GSH or NAC remaining in the cell culture media, cells were exposed to 0-50 μ M cadmium chloride or 0-50 μ M EA 5 hours. A: cadmium upregulated HO-1 mRNA expression. In the cells treated with GSH and NAC which were left in the cell culture media until five-hour cadmium treatment finished, the upregulating effect of cadmium on HO-1 mRNA expression was decreased. B: in the cells treated with GSH and NAC which were removed from cell culture media before cadmium treatment, the upregulating effect of cadmium on HO-1 mRNA expression was not affected. C: similar effects of the treatment with EA were observed. EA upregulated HO-1 mRNA expression. In the cells treated with GSH and NAC which were left in the cell culture media until five-hour EA treatment finished, the upregulating effect of EA on HO-1

mRNA expression was decreased (lane 6 and 8); in the cells treated with GSH and NAC which were removed from cell culture media before EA treatment, the upregulating effect of EA on HO-1 mRNA expression was not affected (lane 5 and 7). The images given represent three independent experiments.

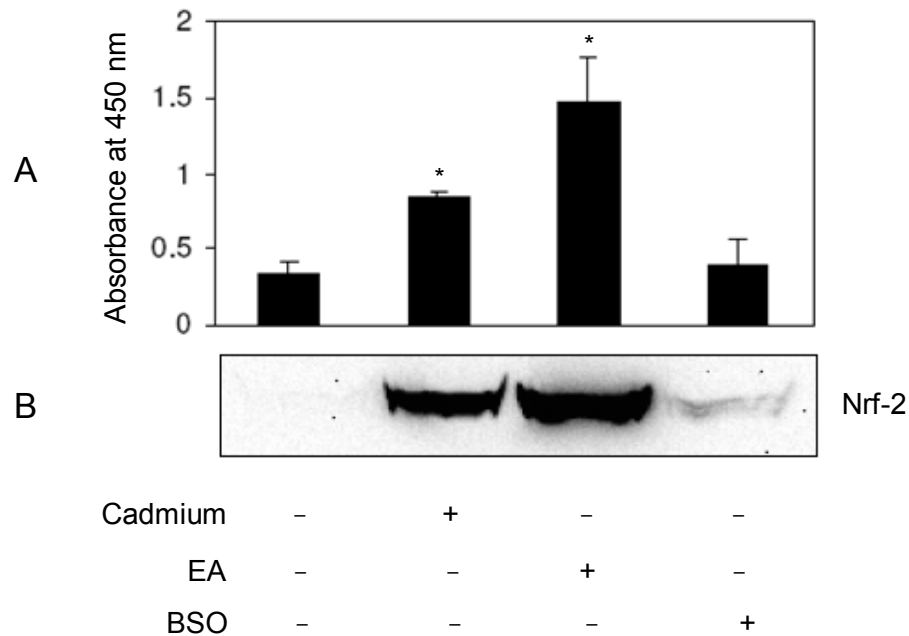


Figure III-4. Cadmium and EA, but not BSO, increased nuclear Nrf-2 expression (by using Western immunoblotting analysis and TransAMTM kit). Cells were treated with 0-50 μ M cadmium chloride, 0-50 μ M EA, or 0-100 μ M BSO for 5 hours. B: Western immunoblotting analysis indicated that cadmium and EA, but not BSO, upregulated nuclear Nrf-2 expression. A: when Nrf-2 containing nuclear extract added to the wells of TransAMTM plate which are coated with antioxidant responsive element (ARE)-containing oligonucleotide, Nrf-2 binds to ARE. After being incubated with a primary Nrf-2 antibody and a horseradish peroxidase (HRP)-conjugated second antibody, a dark blue color could be developed which could be quantified by measuring the absorbance of a visible light at 450 nm with a reference wave length of 655 nm using spectrophotometer. Similar to the result of Western immunoblotting, the nuclear extract of the cells treated with cadmium and

EA had a significantly higher light absorbance than the nuclear extract of control cells and cells treated with BSO, indicating cadmium and EA, but not BSO, upregulated nuclear Nrf-2 protein expression. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with cadmium or EA vs. control, $p < 0.05$).

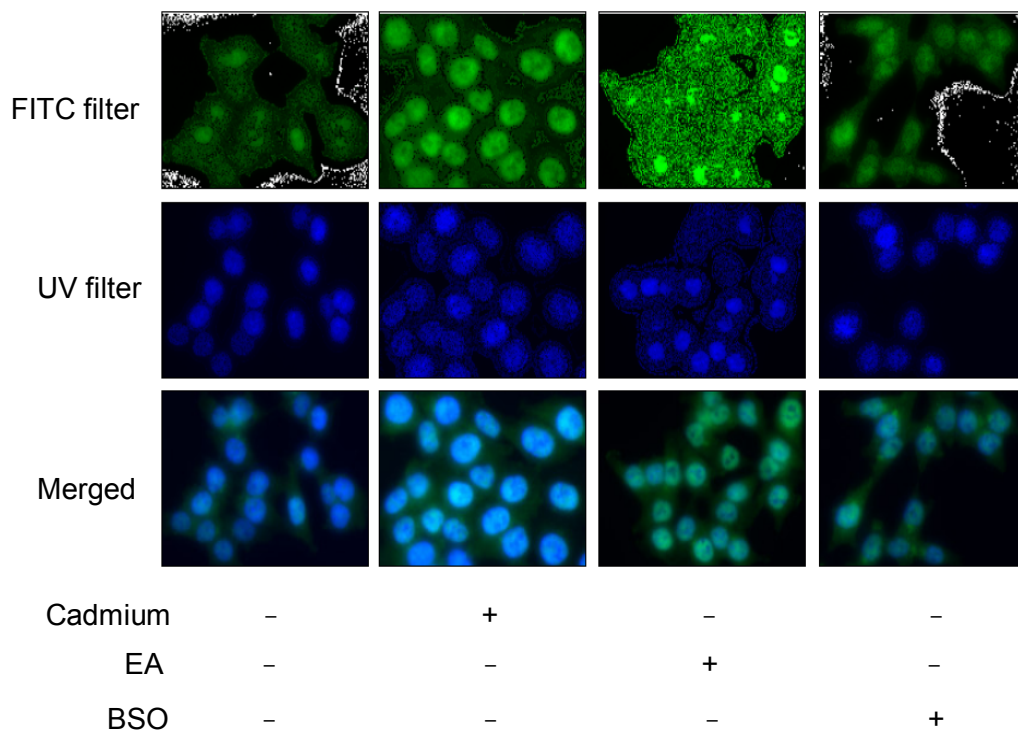


Figure III-5. Cadmium and EA, but not BSO, increased nuclear Nrf-2 expression (by using immunocytofluorescence microscopy). Cells were grown in LabTek chamber slides and were fixed with formaldehyde. After being incubated with Triton X-100, the slides were incubated with Nrf-2 primary antibody and second antibody conjugated with Alexa Fluor 488 which appears a green color under a FITC filter. Then the slides were incubated with 4',6-diamidino-2-phenylindole (DAPI) which binds strongly to DNA and appears a blue color under an UV filter. Cells treated with cadmium and EA has a higher density of green color than the control cells and cells treated with BSO under a FITC filter. The nuclei of the cells, which have been bound to DAPI, were visualized with a blue color under an UV filter. The images with green color under the FITC filter and images with blue color under the UV filter could be overlapped indicating nuclear Nrf-2 expression. These observations indicate that a

higher nuclear Nrf-2 protein expression in cells treated with cadmium and EA than in control cells and cells treated with BSO. The images give represent three independent experiments.

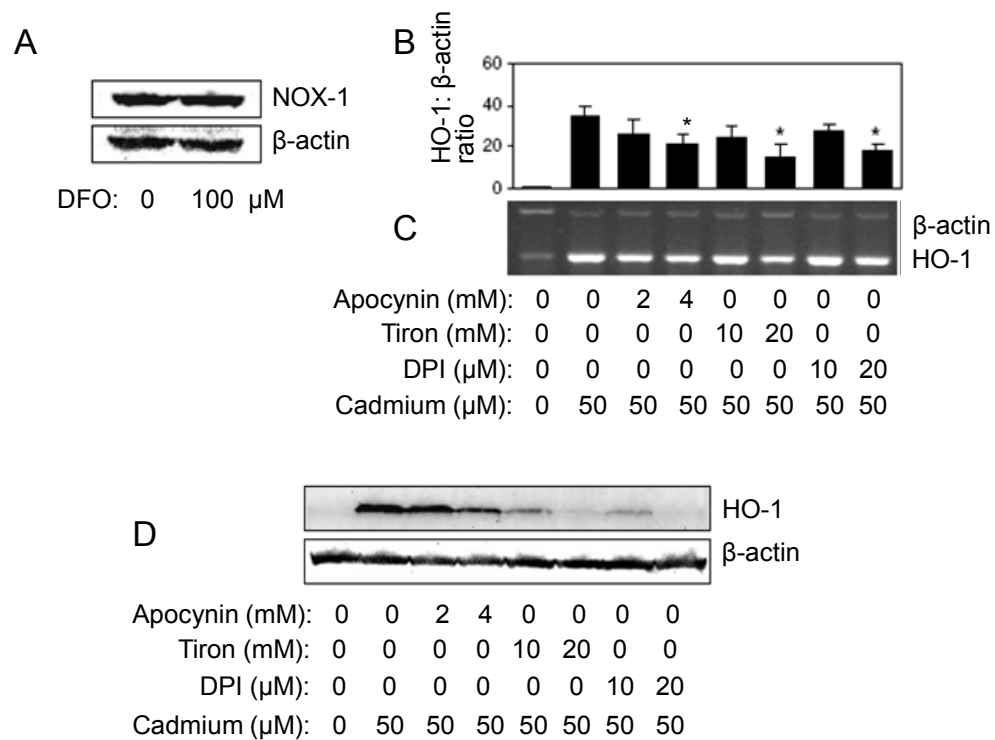


Figure III-6. HCT116 cells expressed NOX-1 protein and NOX inhibitors, apocynin and DPI, and superoxide scavenger, tiron, decreased the upregulating effects of cadmium on HO-1 protein and mRNA expression. Firstly, cells were treated with or without 0-100 μM DFO for 24 hours. Then, cells were pretreated with 0-4 mM apocynin, 0-20 μM DPI, or 0-20 mM tiron for 2 hours, followed with the exposure to 0-50 μM cadmium for 5 hours. A: NOX-1 protein was constitutively expressed in HCT116 cells, and this expression was not influenced by the treatment with DFO. C: the treatment with 50 μM cadmium increased HO-1 mRNA expression; however, the pretreatments with 4 mM apocynin, 20 μM DPI, or 20 mM tiron decreased the upregulating effect of cadmium on HO-1 mRNA. B: the ratio of HO-1 mRNA/β-actin mRNA in the cells treated with 50 μM cadmium was dramatically increased compared to the control cells; however, the ratios of HO-1 mRNA/β-actin mRNA in the cells

treated with 4 mM apocynin, 20 μ M DPI, or 20 mM tiron were significantly decreased compared to the cells treated with 50 μ M cadmium ($p < 0.05$). D: Similarly, the treatments with 50 μ M cadmium increased HO-1 protein expression, and pretreatment with 4 mM apocynin, 20 μ M DPI, or 20 mM tiron decreased the upregulating effect of cadmium on HO-1 protein expression. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 4 mM apocynin, 20 mM tiron, or 20 μ M DPI plus 50 μ M cadmium vs. the treatment with 50 μ M cadmium, $p < 0.05$).

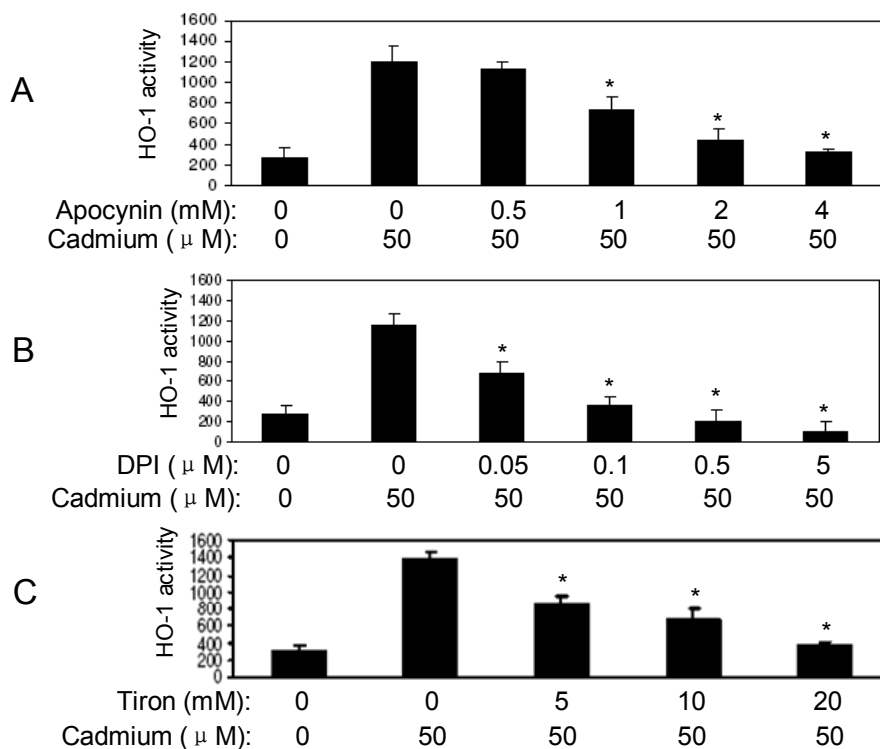


Figure III-7. NADPH oxidase inhibitors, apocynin and DPI, and superoxide scavenger, tiron, decreased the upregulating effect of cadmium on HO-1 enzyme activity. Cells were pretreated with 0-4 mM apocynin, 0-5 μM DPI, or 0-20 mM tiron for 2 hours, followed with the exposure to 0-50 μM cadmium for 5 hours. A: the treatments with 1-4 mM apocynin significantly decreased the upregulating effect of cadmium on HO-1 enzyme activity. B: the treatments with 0.05-5 μM DPI significantly decreased the upregulating effect of cadmium on HO-1 enzyme activity. C: the treatments with 5-20 mM tiron significantly decreased the upregulating effect of cadmium on HO-1 enzyme activity. The values given are means ± S.E.M. from three independent experiments (*, the treatments with 1-4 mM apocynin, 5-20 mM tiron, or 0.05-5 μM

DPI plus 50 μ M cadmium vs. the treatment with 50 μ M cadmium, $p<0.05$).

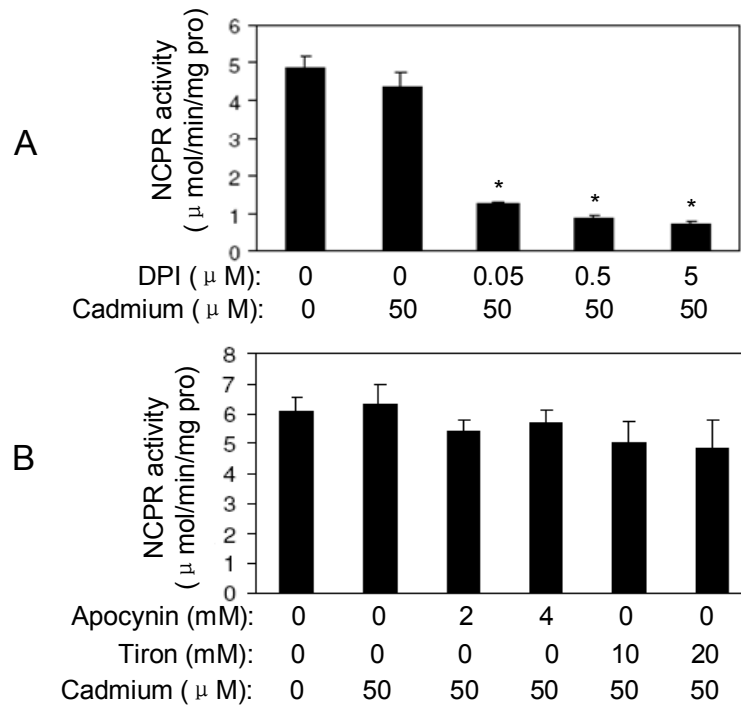


Figure III-8. DPI, but not apocynin and tiron, inhibited NADPH: cytochrome P450 reductase (NCPR) enzyme activity. Cells were pretreated with 0-4 mM apocynin, 0-5 μM DPI, or 0-20 mM tiron for 2 hours, followed with the exposure to 0-50 μM cadmium for 5 hours. A: compared to control cells and cells treated with cadmium, the treatment with 0.05-5 μM DPI significantly inhibited NADPH:cytochrome P450 reductase (NCPR) enzyme activity ($p < 0.05$). B: no inhibiting effect of apocynin and tiron on NCPR enzyme activity was observed. The values given are means \pm S.E.M. from three independent experiments (*, the treatments with 0.05-5 μM DPI plus 50 μM cadmium vs. the treatment with 50 μM cadmium, $p < 0.05$).

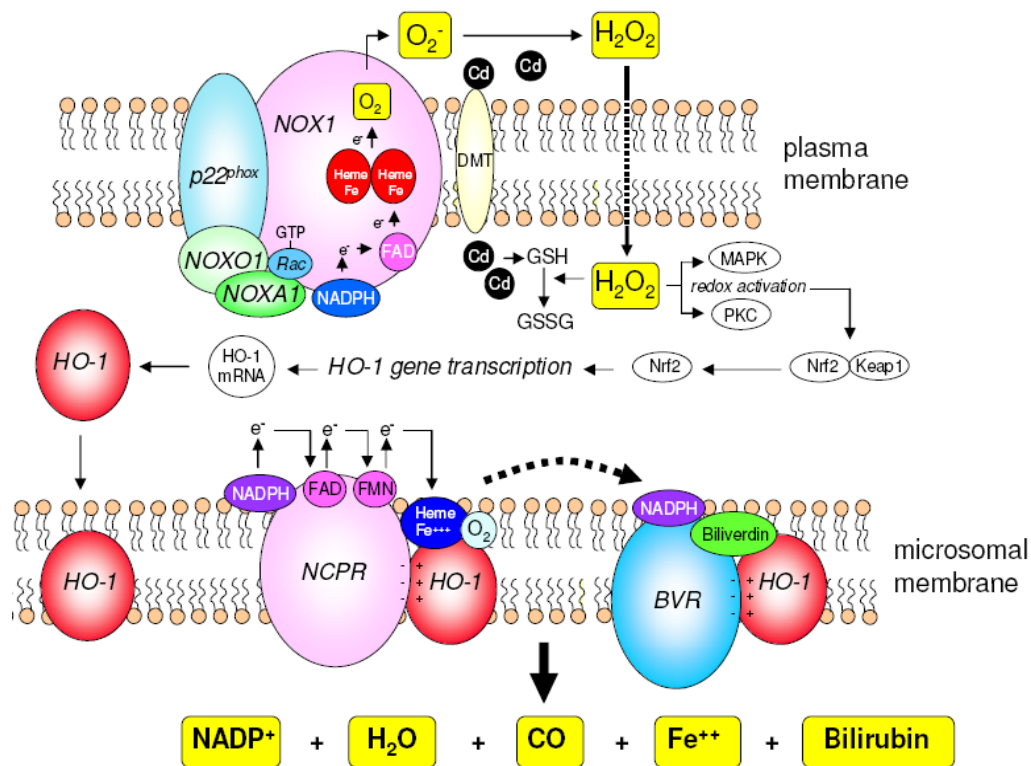


Figure III-9. Possible elucidation of the effects of cadmium on HO-1 in the cells with or without iron deficiency. NOX-induced ROS play an important role on the cadmium-induced upregulation of HO-1. Cadmium depletes the intracellular glutathione resulting in the intracellular accumulation ROS. It is likely that upon the intracellular accumulation of ROS, Nrf-2 may be released from Keap1 decreasing Nrf-2 degradation via ubiquitin-proteasome pathway and increasing the translocation of Nrf-2 from cytosol into nucleus where the expression of HO-1 is initiated. Iron chelators, DFO and DPD, deplete iron in the hemes, the components of NOX, interrupting the production of ROS from NOX, therefore decrease the upregulating effect of cadmium on HO-1. Furthermore, the function of HO-1 requires the participation of NCPR.

EPILOGUE

In Chapter II and III, the results suggest that NOX-induced ROS play an important role in the cadmium-induced upregulation of HO-1. However, studies have shown that in addition to NOX, there are several other sources of intracellular ROS including mitochondrion. The mitochondrion has been shown to be another important and major source of ROS. But the role of mitochondrion-induced ROS on cadmium-upregulated HO-1 expression is not clear. Studies have shown that the respiratory chain in mitochondrion, where ROS are produced, contains iron-containing FeS clusters and cytochromes. So, if mitochondrion produced-ROS play a role on cadmium-induced HO-1 upregulation, it is possible that these irons could also be depleted by DFO or DPD, resulting in decreased production of ROS and decreased upregulating effect of cadmium on HO-1. So future studies may be designed to investigate the effects of mitochondrion-induced ROS on cadmium upregulated HO-1 expression. To do that, the effects of mitochondrial inhibitors, such as oligomycin, on the upregulating effect of cadmium on HO-1 could be investigated.

The results in chapter III suggest that cadmium may induce cellular accumulation of ROS by depleting intracellular glutathione. With the increased ROS level, Nrf-2 could be released from Keap1 and be translocated into nucleus where it activates the expression of HO-1. Studies have shown that Keap1 is cysteine-rich protein which may be sensitive to oxidative stress, but my thesis studies did not demonstrate upstream pathways of the

activation of Nrf-2. Is there any protein kinase involved, such as protein kinase C (PKC) or MAPKs including extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38? To answer the question, the influences of the inhibitors of these protein kinases, such as H7 (for PKC), PD98059 (for ERK), AS601245 (for JNK) and SB202190 (for p38), on the upregulating effect of cadmium on HO-1 should be investigated in the future.

Studies have shown cadmium may cause intracellular accumulation of ROS which results in lipid peroxidation of cell membrane and DNA damage, and the upregulation of HO-1 by cadmium has been believed as defending mechanism against cadmium toxicity. However, what kind protective role of HO-1 on cadmium toxicity is not clear. Does cadmium cause lipid peroxidation of cell membrane or DNA damage in HCT116 cells? If it does, what are the protective effects of HO-1 against these cadmium toxicities? To answer the questions, thiobarbituric acid reactive substances (TBARS) assay for lipid peroxidation and the single cell gel electrophoresis (SCGE or comet) assay for DNA damage could be conducted in the cells with or without HO-1 gene knock out.

In my thesis, HCT116 colon cancer cell line was used. Studies have shown that cancer cells are different from normal cells in some aspects, one of which is that cancer cells may have different requirement and tolerance to ROS to maintain their cellular signaling and proliferation. In fact, it is inconclusive whether the upregulation of HO-1 is a beneficial mechanism for cancer cell proliferation or is a defending mechanism against cancer development. This means that it is not clear whether the results and conclusions obtained in my thesis could apply to normal cells, such as normal NCM460 (a normal

human colon mucosal epithelial cell line). So, similar studies should be conducted in normal cells to make a clear conclusion.

Finally, my thesis investigated the influence of iron deficiency on the defending mechanism, HO-1 upregulation, against only one of xenobiotics, cadmium. So, future studies could be done to investigate the role of iron on the defending mechanism against other xenobiotics such drugs, especially highly toxic drug used in cancer therapeutic purpose.