IDENTIFICATION OF NRF1 (NFE2L1) AS A HYPOXIA-INDUCIBLE FACTOR AND ITS OXYGEN-DEPENDENT REGULATION *IN VITRO* AND *IN VIVO*

by

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Abstract

Oxygen and iron-sensing pathways are tightly interrelated. For example, a group of proteins known as hypoxia-inducible factors (HIFs) mediate the responses of mammalian cells to variations in ambient oxygen levels and are regulated in an iron- and oxygen-dependent manner. HIFs activate the expression of genes responsible for iron metabolism, transport and storage. Similarly, both iron overload and oxygen deprivation (hypoxia) conditions can stimulate the production of reactive oxygen species (ROS) that can be harmful to biomacromolecules, altering their structure and function. The cap-n-collar (CNC) subfamily of basic leucine zipper (bZIP) transcriptional factors includes Nrf (nuclear factor-erythroid 2 p45 subunit-related factor) 1 and 2 proteins that activate the expression of the genes, important to iron storage and metabolism, as well as to the cellular defense against ROS. The CNC-bZIP factors bind to a DNA sequence known as the Antioxidant-Response Element (ARE) to either activate or repress the ARE-driven gene expression under various physiological conditions. This project revealed that hypoxic conditions activate Nrf1, demonstrating further interrelatedness between the oxygen and iron metabolism pathways. In addition to describing oxygen-dependent regulation of Nrf1, it has been demonstrated that bisphenol A (BPA), a compound thought to produce ROS intracellularly and a common environmental pollutant, can also activate Nrf1 and the expression of its target genes. Finally, using mouse models, it was shown that the protein binding to an ARE sequence diminishes with age in the lung with concomitant changes in the Nrf1/Nrf2 ratio. Taken together, the work presented in this thesis reveals how Nrf1 is regulated under different, oxygen-dependent conditions. Since

Nrf1 can be very important for human health and Nrf1 is a key regulator of the cellular antioxidant defense system and proteasomal gene expression, this work can be a useful starting point for developing the strategies for pharmacological activation of Nrf1, which may be beneficial in numerous diseases where oxidative stress is a factor, such as neurodegenerative diseases, brain injury, acute lung injury and cerebral ischemia.

Preface

This thesis is written in an integrated thesis format, summarizing the work that, at the time of the thesis submission, has been published already (Chapter 1), submitted (Chapters 3 and 4) or will soon be submitted for a publication in a peer-review journal (Chapter 5).

Status of the manuscripts at the time of thesis submission

Chapter 1:

Chepelev, N.L., and Willmore, W.G. 2011. Regulation of iron pathways in response to hypoxia. *Free Radic. Biol. Med.* 50, 645-666.

Chapter 3:

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Chapter 4:

Chepelev, N.L., Enikanolaiye, M., Chepelev, L.L., Chen, Q., Scoggan, K., Coughlan, M., Jin, X., and Willmore, W.G. 2011. Bisphenol A activates Nrf1/2-antioxidant response element pathway in HEK293A cells. Submitted to Chemical Research in Toxicology on

March 31, 2011; submission number: tx-2011-00132j. The manuscript is currently undergoing the revisions suggested by the reviewers.

Chapter 5:

Chepelev, N.L., Zhang, H., Liu, H., McBride, S., Willmore, W.G., Morgan, T., Finch, C., Davies, K., and Forman, H. The Yin-yang of Nrf1 and Nrf2 in aging mouse lung. The expected date of submission is August 15, 2011.

Statement of contributions

I have contributed towards the research described in this thesis as follows:

1. Jointly with Dr. Willmore, I formulated the hypotheses,
2. I planned and executed the experiments and collected the data,
3. I analyzed the data, jointly with Dr. Willmore,
4. In collaboration with Dr. Willmore, I trained and co-supervised six undergraduate students: Joshua Bennitz, who helped with the optimization of luciferase assays and the cloning of N-terminally-tagged Nrf1, Ioana Nicolau and Julia DiLabio, who participated in some preliminary data collection, Ting Huang and Skye McBride, who participated in C-terminally-tagged Nrf1-FLAG cloning and site-directed mutagenesis and Mutiat Enikanolaiye, who participated in the glutathione and ROS assays and antibody microarrays,
5. With the help of Dr. Willmore, I prepared the manuscripts that form the basis for this thesis,

6. In collaboration with the Health Canada group (Dr. Xiaolei (Dawn) Jin) and Dr. Willmore, I designed and performed the experiments with BPA described in Chapter 4,

7. In collaboration with the University of Southern California, Los Angeles (USC LA) group (Dr. Henry Forman (PI), Dr. Hongqiao Zhang, Dr. Kelvin Davies (PI) and Dr. Caleb Finch (PI)) and Dr. Willmore, who oversaw my proposal for the SFRBM Mini-Fellowship application, I participated in the experimental design of the experiments described in Chapter 5. Together with Dr. H. Zhang, an assistant professor working in Dr. Forman's lab, I was involved in data collection and analyses using mouse lung tissue and drafted a manuscript as described in Chapter 5.

I would like to formally acknowledge the contributions of my co-authors of the manuscripts that form the basis of this thesis. As stated above, Dr. Willmore's contributions included formulating hypothesis, experimental design, data analysis and manuscript preparation for all four manuscripts included in this thesis. Leonid Chepelev calculated bond dissociation enthalpy value for BPA and Charlie Chen, Kylie Scoggan and Melanie Coughlan (scientists from Health Canada) performed the real-time qPCR experiments on the material provided from our laboratory; these workers contributed to the research described in Chapter 4. The USC LA group described above was involved in planning experiments, presented in Chapter 5 of this thesis, including animal care and treatment while my role in this was to work with the lung tissue from these animals to perform the final set of the experiments. The contribution of other co-authors, undergraduate students from the Willmore lab, is acknowledged above.

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List of Abbreviations

AA - Amino Acid

AAPH - 2,2'-azobis(2-amidinopropane) Dihydrochloride

AD - Acidic Domain

ALAD - δ-Aminolevulinate dehydratase

ALAS2 - Erythroid-specific 5-Aminolevulinate Synthase

ARE - Antioxidant Response Element

Bach - BTB (Broad-complex, Tramtrack, and Bric-a-brac) and CNC (Cap'n'Collar) Homology 1, Basic Leucine Zipper Transcription Factor

BDE - Bond Dissociation Energy

BPA - Bisphenol A

BSO - Butathione Sulfoxamine

bZIP - Basic Leucine Zipper

Cp - Ceruloplasmin

CHX - Cycloheximide

CNC-bZIP - Cap'n'Collar-Basic Leucine Zipper

Co-IP - Co-immunoprecipitation

COS - African Green Monkey-derived, SV40-transfected Cells

CPRG - Chlorophenol Red-β-D-galactopyranoside

DMEM - Dubecco's Modified Eagle's Medium

DNA - Deoxyribonucleic Acid

DMOG - Dimethyloxalylglycine

DMSO - Dimethylsulfoxide

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic Acid

EMSA - Electrophoretic Mobility Shift Assay

EPR - Electron Paramagnetic Resonance

EpRE - Electrophile Response Element

ER - Endoplasmic Reticulum

ERAD - ER-associated Protein Degradation

ETC - Electron Transport Chain

FLAG - An Octapeptide Tag C-DYKDDDDK-N

FCS - Fetal Calf Serum

FECH - Ferrochelatase

FTL - Ferritin Light Chain

FTH - Ferritin Heavy Chain

GAPDH - Glyceraldehyde-3-phosphate Dehydrogenase

Gclm - Glutamate-cysteine Ligase Modifier Subunit

Gclc - Glutamate-cysteine Ligase Catalytic Subunit

GS - Gas Chromatograph

GSH - Reduced Glutathione

GSSG - Oxidized Glutathione

H_2DCFDA - 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein Diacetate

HEK - Human Embryonic Kidney Cells

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid

HIF - Hypoxia-Inducible Factor

hFLF - Human Fetal Lung Fibroblast Cells

HMBS - Porphobilinogen Deaminase

HO-1 - Heme Oxygenase-1

HS - Horse Serum

HRP - Horseradish Peroxidase

IgG - Immunoglobulin G

IRP - Iron-response Proteins

kDa - KiloDalton

Keap1 - Kelch-like ECH (Erythroid Cell-derived Protein with Cap'n'Collar Homology)-Associated Protein1

LIP - Labile Iron Pool

LXXLAP - Oxygen-dependent Degradation Motif

MAPK – Mitogen-activated Protein Kinase

Mf - Mitoferrin

MT - Metallothionein

MTT - Methylthiazolyldiphenyl-tetrazolium Bromide

MSD - Mass Selective Detector

MW - Molecular Weight

NAC - N-acetylcysteine

NCS - Newborn Calf Serum

Neh6L - Neh (Nrf2-ECH homology) 6-like Domain

NO - Nitric Oxide

Nrf - Nuclear Factor-Erythroid 2 p45 Subunit-related Factor

NST - Asn/Ser/Thr-rich Domain

NTD - N-terminal Domain

OA - Okadaic Acid

p45NFE2 - Nuclear Factor-erythroid 2 p45 Subunit

p250, p140, p120, p95, p65, p23 - Various Forms of Nrf1 Protein

PBS - Phosphate Buffered Saline

PEST - an Amino Acid Region Rich in Pro, Glu, Thr and Ser

PHD - Prolyl Hydroxylase

PKC - Protein Kinase C

PMSF - Phenylmethanesulfonylfluoride

PM - Particulate Matter (Nanoparticles with a Diameter < 200 nm)

qPCR - Quantitative PCR

Rac - Rho Family GTPase

RNA - Ribonucleic Acid

RNAi - RNA Interference

ROOH - Peroxyl

ROO• - Peroxyl Radical

ROS - Reactive Oxygen Species

SR - Ser Repeat Domain

Src - Sarcoma Protein Kinase

tBHQ - tert-Butylhydroquinone

Tf - Transferrin

TfR - Transferrin Receptor

1 Chapter: General Introduction

1.1 Hypoxia, its Challenges and Main Regulatory Proteins

Hypoxia constitutes a stress for all mammalian cells. It is encountered at high altitudes, during prenatal development, as well as in some pathophysiological conditions such as ischemia, cardiovascular and pulmonary diseases, cancer and sleep apnea. Mammalian cells have evolved complex mechanisms to "sense" changes in ambient oxygen concentrations and mount appropriate adaptive responses to return to a state of homeostasis. It should be emphasized that different cells and tissues *in situ* are exposed to variable oxygen levels (about 2-5 % O_2 (Stiehl *et al.*, 2006) compared to 21% air oxygen concentration typically used in cell culturing). Therefore, the term "hypoxia" is relative and attempts to describe oxygen concentrations significantly lower than normal, physiological levels to which a given cell type, tissue or organ is exposed.

Mammals cope with hypoxia by: 1) increasing the oxygen-carrying capacity and rate of blood flow to hypoxic organs and tissues; and 2) switching from aerobic (i.e. oxidative phosphorylation) to anaerobic (e.g. glycolysis) metabolic pathways. The former involves activating gene and protein expression responsible for erythropoiesis (erythropoietin), angiogenesis (vascular endothelial growth factor), vasodilation (NO synthase), iron transport (transferrin (Tf), Tf receptor (TfR), ceruloplasmin (Cp) and mitoferrin (Mf)), storage (ferritin) and heme turnover (erythroid-specific 5-aminolevulinate synthase (ALAS2), ferrochelatase (FECH) and heme oxygenase-1 (HO-1)). The latter strategy relies on the up-regulation of key glycolytic enzymes, including

aldolase A, glucose-6-phosphate isomerase, phosphoglycerate kinase 1 and pyruvate kinase M (Semenza *et al.*, 2004; Naughton, 2003).

These adaptations are primarily mediated by the key regulatory proteins of iron metabolism, hypoxia-inducible factors (HIFs) and iron-response proteins 1 and 2 (IRP1 and IRP2). These regulatory proteins, in turn, appear to be the targets of oxygen-dependent modifications of their structure and function, which allows HIFs and IRPs to act as "molecular switches" for oxygen sensing, response and adaptation, regulating transcription (HIFs) or translation (IRPs) of their targets. In addition, Cap'N'Collar – basic Leu Zipper (CNC-bZIP) transcriptional factors appear to be important gene expression activators and repressors of HO-1, ferritin and β-globin as well as cellular antioxidant defense and detoxification system genes.

1.2 Iron's Role in Oxygen Sensing

Iron (Fe) is intimately associated with oxygen sensing in cells. Since Fe is indispensable to oxygen transport, its shortage can create low oxygen conditions (hypoxia) as a result of diminished O_2 delivery to cells and tissues. Both Fe and O_2 depletion may compromise ATP production by the electron transport chain (ETC) and thus both deficiencies may trigger similar signaling pathways. Given close association between O_2 and Fe homeostasis, it was proposed that the heme moiety of some heme proteins could function as an oxygen sensor (Goldberg *et al.*, 1988). According to this early hypothesis, a ligand-dependent change in the conformation of this heme moiety was responsible for oxygen

sensing based on the fact that hypoxia-inducible induction of the erythropoietin gene was abolished by CO, which binds to heme iron strongly and selectively, as well as by heme synthesis inhibitors. Soon after the discovery of hypoxia-inducible factor-1 (HIF-1), a master regulator of hypoxic gene expression (Wang and Semenza, 1993), this hypothesis was modified to explain the role of the enigmatic heme protein in the context of HIF-1 stabilization and activation during hypoxia. It was envisioned that reversible O_2 binding to the Fe atom of the heme protein resulted in the production of superoxide, $O_2^{\bullet-}$, subsequently convertible to other reactive oxygen species (ROS) using Fe^{2+} as a catalyst and that ROS were capable of oxidizing HIF-1, rendering it inactive under normoxia (Bunn and Poyton, 1996). During hypoxia, however, the levels of ROS were thought to be diminished, resulting in stabilization of HIF-1 and its activation of hypoxia-inducible gene expression. After two landmark publications on the role of prolyl hydroxylases (PHDs; iron-binding proteins) in controlling HIF levels (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001), the idea that an oxygen sensor molecule is a heme protein became less noticeable in the literature. Despite this, Fe still occupies a central role in O_2 sensing due to its direct involvement in PHD-catalyzed HIF hydroxylation. It was further suggested that hypoxic elevation of ROS (generated probably by the increased half-life of reactive semi-quinone intermediates at complex III of the electron transport chain; see Klimova and Chandel, 2008) could affect the oxidation status of iron bound to PHD, interfering with PHD function during hypoxia (Kaelin, 2005), perhaps through the Src- (Sarcoma) protein kinase Rac (Rho family GTPase) dependent activation of mitogen-activated protein kinase (MAPK, Bell *et al.*, 2005). Unlike the earlier hypothesis, which asserted that diminished ROS production during hypoxia activated HIF (Wang and Semenza, 1993),

the current view on the potential involvement of increased ROS in PHD inactivation and hypoxic HIF stabilization (Kaelin, 2005) relies on more recent evidence of increased ROS generation by complex III of the mitochondrial ETC during hypoxia (Guzy *et al.*, 2005; Brunelle *et al.*, 2005; Bell *et al.*, 2007). To date, Rieske Fe-S (iron-sulphur) proteins, PHDs, and heme-recycling enzyme heme oxygenase 2 (HO-2) have all been described as putative oxygen sensing systems ((Guzy *et al.*, 2005; Brunelle *et al.*, 2005; Bell *et al.*, 2007; Acker *et al.*, 2006; Fähling *et al.*, 2009). Remarkably, all of these proposed O_2 sensors are iron-binding or heme-binding proteins.

1.3 The Generation of Reactive Oxygen Species is Associated With Both Iron and Hypoxia

At physiological pH, ferrous (Fe^{2+}) ion spontaneously oxidizes to produce ROS including superoxide, hydrogen peroxide and the highly reactive hydroxyl radical, capable of covalently damaging proteins, lipids and DNA (Halliwell and Gutteridge, 2007). The pro-oxidant capacity of iron has long been recognized and the most famous example is so-called "Fenton reaction" in which iron reacts with hydrogen peroxide to generate the hydroxyl radical according to the following equation:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$

The propensity of iron to generate ROS, inducing oxidative damage, is thought to contribute to the development and progression of some neurodegenerative diseases such

as Alzheimer's, Parkinson's and Huntington's disease (Altamura and Muckenthaler, 2009). Iron for Fenton reactions comes from the intracellular redox-active iron pool, also known as the labile iron pool (LIP). In most organisms, LIP is kept small and does not constitute a major source of ROS (Breuer *et al.*, 1997). Iron within LIP is thought to exist mostly in ferric form, complexed with citrate or ADP, with a small fraction of LIP consisting of cytotoxic ferrous iron (Kohgo *et al.*, 2008). The level of LIP reflects the overall iron status of the cell (Galaris and Pantopoulos, 2008). Due to its toxicity, LIP is maintained only as a minor fraction (3-5%) of the total cellular iron (Kruszewski, 2003). Thus it was found that catalytic iron, available for Fenton reactions, is in the micromolar range in the plasma of diseased patients and is undetectable in healthy people (see (Halliwell, 2009) and references therein). To diminish pro-oxidant activity of iron, many organisms employ iron sequestration since protein-bound iron displays a decreased tendency to generate ROS and induce oxidative damage (Halliwell and Gutteridge, 2007). However, if the iron concentrations exceed the capacity of iron-detoxification systems (iron transport, sequestration and oxidation of toxic Fe^{2+} to less reactive Fe^{3+}), excessive production of hydroxyl and lipid radicals takes place as a direct consequence of Fe^{2+} excess (Rothman *et al.*, 1992). Ironically, in addition to iron excess, iron deficiency can also contribute to oxidative damage as a result of increased electron leakage from the faulty iron-dependent components of mitochondrial ETC (Halliwell and Gutteridge, 2007).

Similar to iron deficiency or overload, hypoxia has also been known to contribute to increased rates of ROS formation (see Klimova and Chandel, 2008) for a

comprehensive review). Approximately 1 to 5% of the oxygen used for oxidative phosphorylation is converted to superoxide as a result of incomplete reduction of oxygen to water by the ETC. There is a large body of evidence suggesting that hypoxia increases superoxide production from complex III of ETC (possibly by increasing the life-time of hydroquinone intermediate (Andreini *et al.*, 2009; Chandel *et al.*, 2000; Guzy *et al.*, 2007). Superoxide is then converted to hydrogen peroxide in the cytosol, activating oxygen-dependent signaling pathways responsible for HIFα stabilization (Klimova and Chandel, 2008; Aragonés *et al.*, 2008). HIF, in turn, activates the hypoxic gene expression of major proteins involved in iron metabolism.

1.4 The CNC-bZIP Subfamily of Transcriptional Factors Include Nrf1 and Nrf2

The CNC-bZIP factors include the Nuclear Factor-Erythroid 2 p45 Subunit-related Factors (Nrf1, Nrf2, Nrf3, NF-E2 p45), and more distant members Bach1 and Bach2. These play an important role in regulating the expression of such iron-related genes as ferritin (both light (FTL) and heavy (FTH) chains), β-globin and HO-1. By binding to an enhancer element known as the Antioxidant Response Element (ARE; consensus sequence: TGCTCAGTCAT; also referred to as the Electrophile Response Element, EpRE), Nrf1, Nrf2, Nrf3 and NF-E2 p45 activate ARE-driven gene expression under various physiological conditions (Figure 1.1). Bach1 and Bach2 act as dominant repressors of ARE-driven gene expression by heterodimerizing with the Maf proteins on the ARE (Dhakshinamoorthy *et al.*, 2005; Sun *et al.*, 2002; Igarashi *et al.*, 1998).

The ARE sequence is very similar to the Maf recognition element (MARE) sequence such that the terms "ARE" and "MARE" are often used interchangeably (Igarashi and Sun, 2006). The CNC-bZIP transcriptional factors bind to AREs as heterodimers with small Maf proteins (for review see (Motohashi *et al.*, 2002). Interestingly, small Maf-NF-E2 p45, Maf-Nrf1, Maf-Nrf2 or Maf-Nrf3 heterodimers activate while small Maf-Bach1 heterodimers repress the expression of *β-globin* and *ho-1* genes (Sun *et al.*, 2004). Thus, cells possess the ability to fine-tune the expression of iron-related genes, either by activation or repression, through the same ARE regions bound by different members of the same subfamily of proteins.

It has been extensively demonstrated that hypoxia leads to increased mitochondrial ROS production (see Klimova and Chandel (2008) for a review), although some controversy regarding whether ROS concentrations increase during hypoxia still exists. Therefore, it seems reasonable to hypothesize that hypoxic response and antioxidant response pathways are connected and some of our observations point out to the existence of an interesting link between hypoxia and the antioxidant response pathway. It will be discussed in greater detail further that using a luciferase reporter vector with three ARE elements from chicken β-globin enhancer (3 x ARE-luciferase (Igarashi *et al.*, 2004)), we noticed that the luciferase activity upon transient co-transfection of Nrf1-FLAG and 3 x ARE-luciferase was significantly higher during hypoxia treatment of COS7 and HEK293 cells (Chepelev *et al.*, submitted). Interestingly, hypoxic induction of Nrf1 was abolished by antioxidant N-acetylcysteine. Using a similar approach, we were able to show hypoxic inducibility of Nrf2 as well. This suggests that

hypoxia activates Nrf1 and Nrf2 transactivation activity through ROS signaling pathways, potentially inducing gene expression of ferritin, *ho-1* and other ARE-controlled genes.

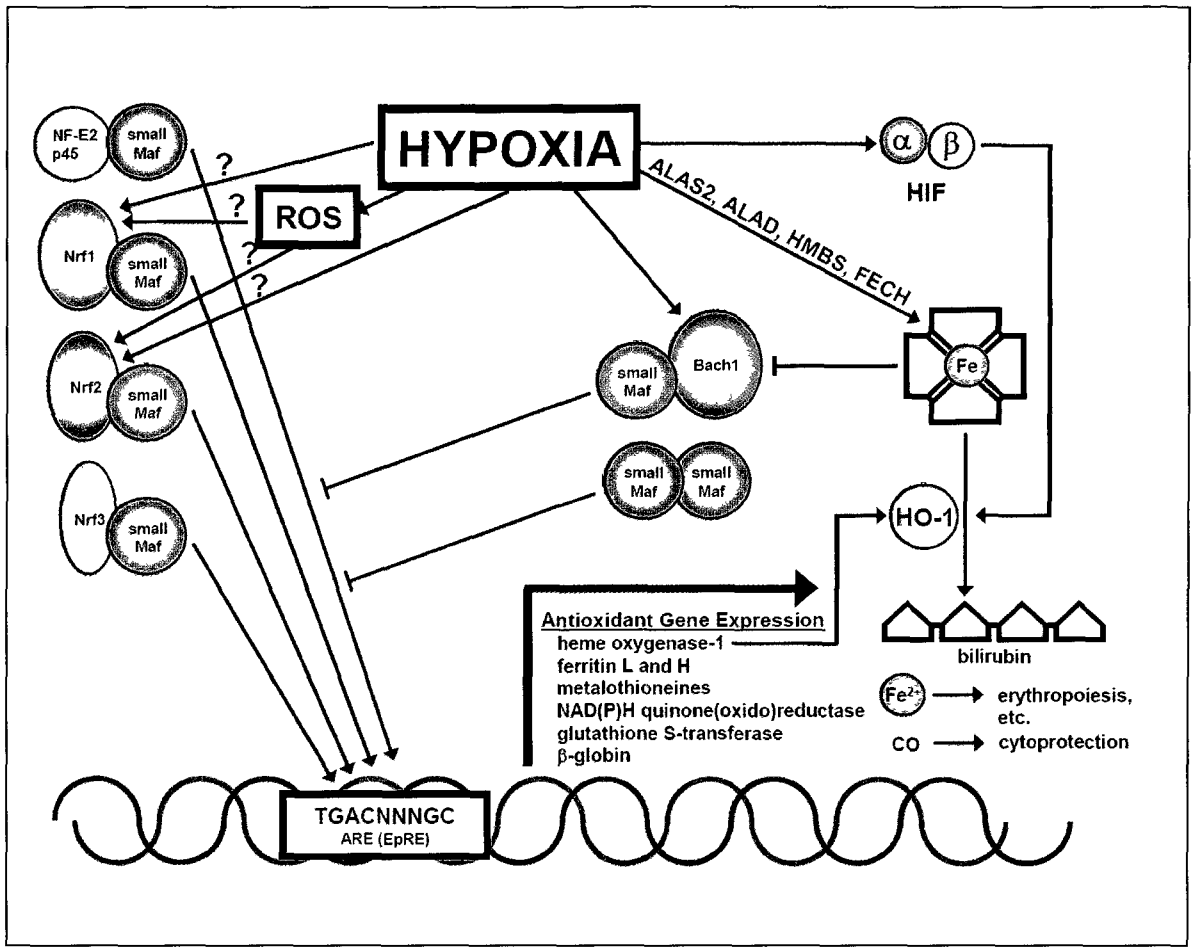

HYPOXIA
ROS
HIF
ALAS2, ALAD, HMBS, FECH
NF-E2 p45
small Maf
Nrf1
Nrf2
Nrf3
Bach1
Fe
HO-1
bilirubin
Antioxidant Gene Expression
heme oxygenase-1
ferritin L and H
metalothioneines
NAD(P)H quinone(oxido)reductase
glutathione S-transferase
β-globin
Fe2+
erythropoiesis, etc.
CO
cytoprotection
TGACNNNGC
ARE (EpRE)

Figure 1.1. The CNC-bZIP Transcription Factors Regulate Antioxidant-Response Element (ARE)/Electrophile-Response Element (EpER)-driven Gene Expression. The CNC-bZIP trancription factors are located at the integration point of the oxygen and iron metabolism regulatory pathways. CNC-bZIP factors include activators Nrf1, Nrf2, Nrf3, NE-F2 p45 and repressors Bach1 and Bach2 and bind to ARE (EpRE) as heterodimers with small Maf proteins. Hypoxia induces heme synthesis and heme dislodges Bach1 from its interaction with Maf-occupied AREs, favoring the binding of activators (Nrf1, 2 and 3) to the ARE and activating the expression of ferritin, HO-1 and β-globin genes. Hypoxia is known to stabilize the α subunit of heterodimeric HIF factors and upregulate the expression of genes and/or proteins involved in iron transport and metabolism, many of which also contain functional AREs (e.g., ferritin, heavy and light chains, β-globin and HO-1). Cell type- and tissue-specific upregulation of heme synthesis in erythroid-related cells and augmented heme degradation in other cell types can help the process of "iron redistribution" during hypoxic challenge such that more red blood cells can be produced to increase the oxygen-carrying capacity of the blood. We hypothesize that hypoxia activates Nrf1 and Nrf2 transactivation through ROS pathways. ALAS2, erythroid-specific aminolevulinate synthase; ALAD, δ-aminolevulinate dehydratase; HMBS, porphobilinogen deaminase; FECH, ferrochelatase. Taken from Chepelev and Willmore (2011).

Antioxidant and cytoprotective genes, regulated transcriptionally through their AREs, include NAD(P)H:quinone oxidoreductase 1 (*nqo1*), the glutathione-S-transferases (*gst*), ferritin light (*ftl*) and heavy (*fth*) chains, heme oxygenase-1 (*ho-1*), catalase (*cat*) and superoxide dismutase (*sod*) (Jung and Kwak, 2010). Since Nrf1 controls phase 2 detoxification enzymes that aid in metabolism and removal of potential carcinogens, and due to the fact that potent ARE-inducers such as sulforaphane are known chemopreventive agents, understanding the mechanisms of Nrf1 regulation may aid in the development of cancer therapeutics. Furthermore, due to the cytoprotective nature of phase 2 enzymes against oxidative stress-induced neurodegeneration (Satoh *et al.*, 2009), manipulation of the upstream factors controlling these enzymes (e.g., Nrf1 and Nrf2) could be useful in the search of therapeutic targets against chronic neurodegenerative diseases (Satoh *et al.*, 2009).

1.5 Heme Oxygenase-1 is a Hypoxia- and Oxidative Stress-inducible Enzyme

Heme oxygenase-1 (HO-1; E.C. 1.14.99.3) is the major enzyme involved in the recycling of heme and is controlled by multiple mechanisms. It catalyzes the first and rate-limiting step of heme breakdown by cleaving the heme ring at the alpha methane bridge to form biliverdin (heme + NAD(P)H + H^+ + $3O_2$ ↔ biliverdin + Fe^{2+} + CO + $NAD(P)^+$ + $3H_2O$). The central iron of heme substrate is kept in reduced state by NAD(P)H. Since the majority of the iron utilized for hypoxia-inducible heme synthesis comes from cellular hemoproteins (Peyssonnaux *et al.*, 2008), heme needs to be recycled at a higher rate in low oxygen conditions to satisfy the growing need for iron, required for heme synthesis

in the bone marrow, in order to stimulate the production of red blood cells; therefore, heme degradation enzymes are expected to be up-regulated in response to erythropoietin stimulation to keep up with the increased iron demand (Soe-Lin *et al.*, 2008).

The human HO-1 protein is composed of 288 amino acids and its expression is increased by its heme substrate (Yoshida *et al.*, 1988) as well as by heavy metals, bromobenzene, endotoxins, cytokines, hypoxia, oxidative stress and UV radiation (Maines, 1992). It is located in the endoplasmic reticulum (ER) and is generally thought to exist as a homodimer in most organisms where it has been characterized; however, some recent work (Hwang *et al.*, 2009) suggests that HO-1 monomers form homodimers and even oligomers in the ER.

Hypoxia has long been known to affect HO-1 expression (for a review, see Shibahara *et al.*, 2007). The general response of HO-1, however, appears to be cell- and species-specific. Hypoxia repressed *ho-1* mRNA expression in primary cultures of human umbilical vein endothelial cells, human astrocytes, and human coronary artery endothelial cells (Nakayama *et al.*, 2000). On the other hand, hypoxia increased HO-1 expression in human lung A549 cells, which was markedly attenuated by HIF-1α knockdown (Hänze *et al.*, 2003). Hypoxia also induced HO-1 in rat liver (Bonkovsky *et al.*, 1986), heart (Katayose *et al.*, 1993), as well as in cultured animal cells, including Chinese hamster ovary cells (Murphy *et al.*, 1991), rat ventricular smooth muscle cells (Lee *et al.*, 1997; Morita *et al.*, 1995), and rat myocytes (Eyssen-Hernandez *et al.*, 1996). In mice, a 163 kilobase pair (kb.p.) hypoxia-responsive region is found approximately 9.5 kb.p. away

from the transcriptional start site of the *ho-1* gene and contains two functional HREs (Lee *et al.*, 1997). Induction of HO-1 by HIF during hypoxic conditions was found to be cardioprotective (Czibik *et al.*, 2009) and provided protective effects in retinal ischemia (Zhu *et al.*, 2007). In the human *ho-1* gene, three putative HREs are found from -121 to -126 (CACGTG) and -5,619 to -5,624 upstream (TACGTG) as well as +1,812 to +1,817 downstream (AACGTG) from the transcriptional start site (Willmore *et al.*, unpublished results). Whether these are functional HREs remains to be tested.

A potential hypoxia-inducible repression of *ho-1* gene expression by Bach1, a heme- and an ARE-binding protein, has been reported (Kitamuro *et al.*, 2003). An ARE is found in the human *ho-1* gene, which is not surprising given that HO-1 is regarded as an antioxidant enzyme (Levonen *et al.*, 2007) which removes potentially cytotoxic heme, produces an antioxidant billirubin and carbon monoxide that confers proper regulation of vascular functions. The *ho-1* ARE is required for Bach1-mediated repression of a reporter gene by hypoxia in human glioblastoma, lung cancer and umbilical vein endothelial cells (Kitamuro *et al.*, 2003). It appears that CNC-bZIP-ARE and HIF-HRE controls could have some overlapping functions in terms of HO-1 regulation as reported by Lee *et al.* (1997). These workers observed marked HIF-dependent increase of *ho-1* mRNA in whole rats and cultured rat cells treated with hypoxia. Interestingly, treatment with cobalt, which mimics the hypoxic condition, led to the accumulation of *ho-1* mRNA in both wildtype and HIF-deficient Chinese hamster ovary cells (Gong *et al.*, 2001), suggesting that HO-1 expression is controlled at least at two different levels. Again, multiple ways of HO-1 control may allow cells to fine-tune gene expression during various oxygen and

iron conditions. In addition, increased hypoxic expression of HO-1 in vascular systems produces CO, thought to confer proper regulation of vascular functions and provide negative feedback on hypoxia induction (Huang *et al.*, 1999; Ryter and Choi, 2009). I believe the work presented here describes some very interesting results regarding the HO-1 dimerization, which might be helpful in further elucidating the function of this important cytoprotective enzyme, which has been a subject of numerous studies and the manipulation of which may be beneficial in molecular therapy of diseases, involving tissue injury (Ryter and Choi, 2009).

1.6 Nrf1 Regulation Differs From That of Nrf2

Nrf2 is a well-established master regulator of oxidative stress-inducible gene expression (Pi *et al.*, 2010). Currently, the mechanisms controlling Nrf2 activity have been studied in great detail with over 2,000 articles catalogued in PubMed, while studies examining Nrf1 regulation are lacking, totaling only 47 PubMed articles to date. Some of the potential reasons why Nrf1 has received less attention compared to Nrf2 may include: i) the fact that, unlike *Nrf2* global knock-out mice, *Nrf1*-deficient animals are non-viable and die at the embryonic stage (Chan *et al.*, 1998); ii) the concept that Nrf2 is responsible for the inducible ARE-driven gene expression (Ohtsuji *et al.*, 2008), such that it can be activated and studied using multiple stimuli while Nrf1 appears to be involved in the constitutive expression of the ARE target genes (Ohtsuji *et al.*, 2008); and iii) the hypothesis that Nrf1 may play a role in regulating membrane-dependent biological processes (Zhang and Hayes, 2010), expanding the function of Nrf1 beyond that of a transcription factor and,

perhaps, providing researchers with the data that can not be conceptualized easily. Despite this apparent lack of the reports on Nrf1, it has been shown that Nrf1 may play just as an important role in human pathologies as Nrf2 does (Sykiotis and Bohmann, 2010). Nrf 1 is ubiquitously expressed and is found in all tissue and cell types. Given that Nrf2 regulation has been described in greater detail compared to Nrf1, the regulation of Nrf2 can be a starting point in understanding the molecular mechanisms of the Nrf1 control. According to the established model, Nrf2 is kept in the cytosol under homeostatic conditions by interaction with Kelch-like ECH-associated protein 1 (Keap1) (Itoh *et al.*, 1999), which acts as an E3 ubiquitin ligase adaptor and facilitates the ubiquitination of Nrf2 and its rapid degradation by the proteasome (Sekhar *et al.*, 2002). When the cell experiences oxidative stress, the Keap1-mediated proteasomal degradation of Nrf2 is compromised (Kobayashi *et al.*, 2006), allowing Nrf2 to dissociate from Keap1 and translocate to the nucleus to activate ARE-driven gene expression (see Figure 1.2).

In contrast to the canonical model of Nrf2 regulation, more recent analysis has shown that homeostatic Keap1-Nrf2 interactions are not permanent and take place in the nucleus *via* transient shuttling of Keap1 into that compartment and that Nrf2 is bound to certain ARE-containing genes even under normal homeostatic conditions (Nguyen *et al.*, 2009).

Nrf2 contains six Nrf2-erythroid-derived CNC homology protein (ECH) homology (Neh) domains that are highly conserved across species (Figure 1.3). The Neh1 domain is located within the CNC-bZIP domain and enables Nrf2 interaction with its

dimerization partners, small Maf proteins. The Neh2 is an inhibitory domain of Nrf2 as it allows Nrf2 interaction with Keap1, which targets Nrf2 for proteasomal degradation. The Neh3, Neh4 and Neh5 domains are transactivation domains that bind proteins involved in the transcription, including cAMP Response Element Binding Protein (CBP) (Katoh et al, 2001). Interestingly, CBP contains histone acetyltransferase activity (Motohashi and Yamamoto, 2004), which is expected to be helpful for transcription by remodeling chromatin structure, and opens up DNA for the binding of the basal transcription machinery. Secondly, CBP is thought to act as a bridging protein that recruits the basal transcription machinery to specific sites of transcription initiation, aiding the binding of the RNA polymerase II holoenzyme to the promoter regions (Kim *et al.*, 1998).

It is noteworthy that while Nrf2 is regulated by rapid proteasomal degradation, mediated by Keap1, Nrf1 appears to play a role in regulating the proteasome itself. Recent studies have established Nrf1 as a pivotal transcriptional regulator of the genes of subunits of the proteasome. Thus, Nrf1 activates proteasome gene expression upon proteasome inhibition treatment in human Ea.hy926 cells (Steffen *et al.*, 2010) and mouse embryonic fibroblasts (Radhakrishnan *et al.*, 2010) to compensate for the loss of proteasome activity. Similarly, the tissue-specific Nrf1 conditional knock-out in mouse brain leads to proteasomal impairment in Nrf1-, but not Nrf2-deficient cells, further confirming the importance of Nrf1 as a translational regulator of proteasomal gene expression (Lee *et al.*, 2011).

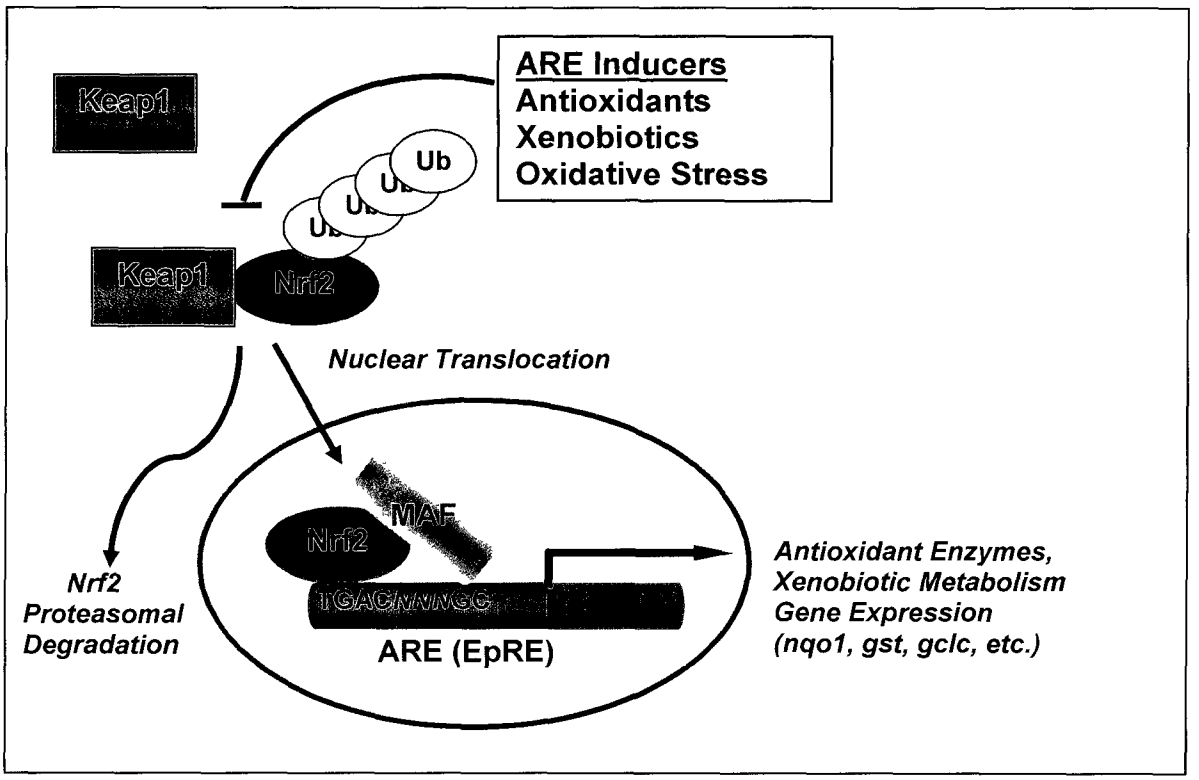


Figure 1.2. The Canonical Model for the Nrf2 Regulation by the Proteasomal Degradation. Under normal homeostatic conditions, Nrf2 is thought to be held in inactive state in the cytoplasm through its interaction with Keap1, which targets Nrf2 for ubiquitination and proteasomal degradation. Under appropriate stimuli, elicited by the ARE inducers and following oxidation of redox-sensitive Cys residues of Keap1, Nrf2 dissociates from Keap1, enters the nucleus and activates the expression of the ARE-containing genes. According to more recent data, Keap1 is capable of entering the nucleus to mediate the proteasome-dependent degradation of the Nrf2 in the nucleus (Nguyen *et al.*, 2009).

The Neh6 domain is involved in Keap1-independent degradation of Nrf2. Glycogen synthase kinase 3 (GSK-3) phosphorylates a group of Ser residues located in the Neh6 domain, allowing the S-phase kinase-associated protein 1/cullin 1/F-box (SCF) protein complex (that contains the F-box protein β-transducin repeat-containing protein (β-TRCP or SCF(β-TRCP)) to bind and catalyze the ubiquitination of Nrf2 in a manner similar to that of Keap1, leading to the proteasomal destruction of Nrf2 (Rada *et al.*, 2011). Compared to Nrf2, Nrf1 has several unique domains, highly conserved among Nrf1 homologues in other species, which are thought to confer Nrf1-specific functions. Among such domains is the endoplasmic reticulum- (ER)-targeting sequence, located in the N-terminal domain (NTD, Figure 1.3) of the protein.

The activity of Nrf1 appears to be negatively controlled by the NTD, which directs Nrf1 to the ER, where Nrf1 is in its inactive state (Zhang *et al.*, 2006; Zhang *et al.*, 2009; Wang and Chan, 2007). In addition, Nrf1 is different from Nrf2 in that it contains an acidic/polar region (residues 125-155) within its acidic domain 1 (AD1). Immediately next to this region, Nrf1 contains Neh2-lke domain (Neh2L), containing Keap1-like binding motifs present in Nrf2. Later analysis revealed that, while the Neh2L region is involved in the Keap1 binding to Nrf1, it affects neither localization nor activity of Nrf1 (Zhang *et al.*, 2006). Further, Nrf1 possesses Asn/Ser/Thr-rich domain (NST) which contains seven Asn residues, at which Nrf1 is putatively glycosylated in the ER. Finally, while both factors appear to have similar DNA-binding specificity and similar dimerization domain structure, it is the difference between the transactivation domains of Nrf1 and Nrf2 which is thought to give rise to some genes being preferentially controlled

by either one of these factors (Ohtsuji *et al.*, 2008). Such structural differences may enable Nrf1 and Nrf2 to recruit different co-activators, achieving specificity in their control of ARE-driven gene expression (Ohtsuji *et al.*, 2008).

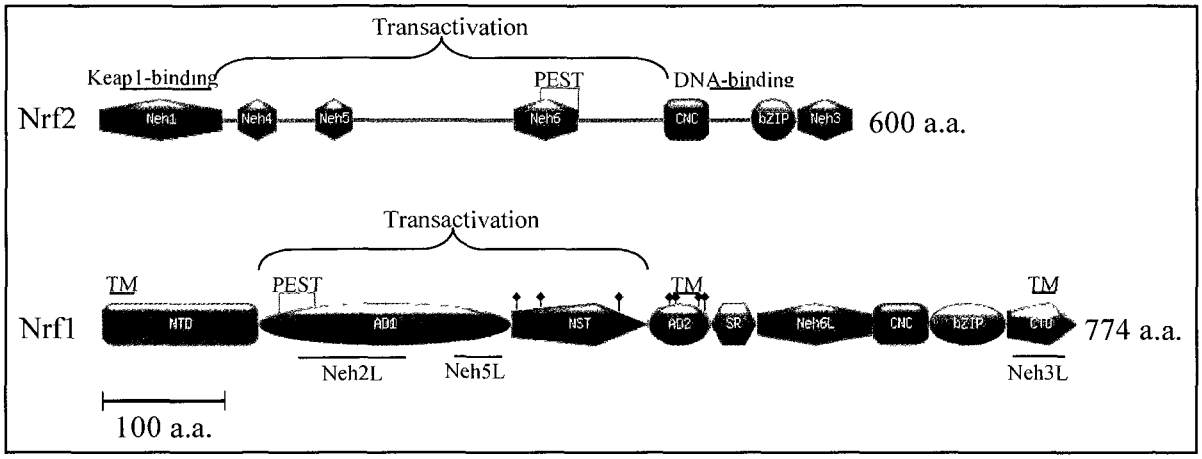


Figure 1.3. The Comparison Between the Nrf2 and Nrf1 Domains. The two factors have been named as "NF-E2-like" factors based on the presense of the the CNC domain. Nrf2-erythroid-derived CNC homology protein (ECH) homology (Neh) domains on Nrf2 and Neh-like (Neh2L, Neh2L and Neh3L) domains on Nrf1 are indicated. The Nrf1 domains are: NTD, N-terminal domain AD1, acidic domain 1, NST (Asn/Ser/Thr-rich region), AD2, acidic domain 2, SR (Ser repeat) domain and CTD, C-terminal domain. Both transcription factors contain CNC, cap'n'collar domain and bZIP, basic Leu zipper domains. TM indicates putative trans-ER membrane glycosylation sites (on NST and AD2 domain of Nrf1) are represented by sticks. Note that only Nrf1 possesses the NTD and both Nrf1 and Nrf2 contain a strong PEST sequence. Drawing of the domains and specific regions was performed to scale using the Prosite Mydomains feature of the ExPASy (http://prosite.expasy.org/mydomains). The word "domain" is used here to indicate specific amino acid regions rather than independent folding motifs.

For example, while both Nrf1 and Nrf2 appear to bind to the metallothionein 1 (*mt1*) promoter with equal affinity, Nrf2 was able to only weakly activate the *mt1* ARE-driven reporter vector in a co-transfection reporter assay. Similarly, Nrf2 stabilization by Keap1 knockdown in mice was ineffective at *mt1* induction, while other well-established Nrf2 target genes were downregulated in Keap1-deficient animals (Ohtsuji *et al.*, 2008), suggesting that, despite the fact that both Nrf1 and Nrf2 bind the *mt1* ARE, only Nrf1 can form necessary interactions with the coactivators, resulting in the transcription of this gene.

The uniqueness of Nrf1 is also manifested in that, unlike Nrf2 or Nrf3, it is essential for embryonic development; *nrf1* -/- mice die at mid-late gestation, presumably due to anemia-induced hypoxia (Chan *et al.*, 1998). Liver-specific knock out of Nrf1 resulted in the development of *nrf1*-/- mice hepatic cancer with the phenotype reminiscent to nonalcoholic steatohepatitis, presumably due to the increased overexpression of the cytochrome P450 4A enzymes, catalyzing the oxidation of fatty acids and diminished ARE-driven gene expression (Xu *et al.*, 2005). Similarly, hepatocytes isolated from the *nrf1*-/- animals contained higher number of lipid vesicles and exhibited greater proliferation of smooth ER (Ohtsuji *et al.*, 2008; Xu *et al.*, 2005). In contrast, *nrf2*-/- and *nrf3*-/- mice showed no obvious phenotype under unstressed conditions (Chan and Kan, 1999; Derjuga *et al.*, 2005). The structural features of Nrf1, most notably, the presence of the NTD with a potential cholesterol recognition sequence (-L/V-X_{1-5}-Y-X_{1-5}-K/R-, where X and Y are any different amino acids), and the observation that Nrf1 is localized to detergent-resistant lipid rafts of the ER (Zhang *et al.*,

2009), suggests that Nrf1 is anchored to the ER and intramembrane proteolysis of Nrf1 releases the active portion of the protein to travel to the nucleus and act as a transcription factor. This is similar to the regulation of another membrane-bound transcription factor, the Sterol Regulatory Element-binding Protein (SREBP). This, as well as the fact that *nrf1*-/- mice are embryonic lethal (*nrf2*-/- animals live to adult stage and are healthy under unstressed conditions), is consistent with the idea that Nrf1 has acquired distinct roles from other CNC-bZIP transcription factors during its molecular evolution.

Nrf1 localizes primarily to the ER (see Figure 1.4) as well as the nuclear envelope membrane (Zhang *et al.*, 2009). The ER membrane-resident form of Nrf1 represents a low activity, glycosylated protein with an apparent molecular weight of 120 kDa (p120), while the nuclear form (p95) is active, and unglycosylated (Zhang *et al.*, 2007). The ER location of Nrf1 is thought to be suitable for the maintenance of the ER redox homeostasis (Zhang *et al.*, 2009), perhaps by affecting the ER membrane lipid organization *via* its amphipathic, transmembrane α-helices and participating in membrane-dependent biological events (Zhang and Hayes, 2010). Nrf1 may, for example, affect the rigidity of the ER membrane, acting as a diffusion barrier to membrane lipids (Zhang and Hayes, 2011). This ER homeostasis-maintaining function could not be attributed to any other ER-resident protein because the absence of Nrf1 in mouse hepatocytes, isolated from the *Nrf1*-/- animals, contained higher number of lipid vesicles and exhibited greater proliferation of smooth ER (Xu *et al.*, 2005). This suggested that Nrf1 is required for the proper function of the ER. It has also been proposed that the localization of Nrf1 within the ER determines the activity of this CNC

factor and that the ER redox status and Nrf1 glycosylation status could cause Nrf1 to relocate from the ER to the nucleus (Zhang *et al.*, 2009). As ER-resident Nrf1 represents glycosylated Nrf1 and nuclear-localized Nrf1 is unglycosylated, it has been hypothesized that Nrf1 deglycosylation could represent the main mechanism of its regulation (Zhang *et al.*, 2009) and that Nrf1 is activated by the proteasomal cleavage of its N-terminus to remove the inhibitory NTD, producing smaller, more active forms of Nrf1 (Schultz *et al.*, 2010). In addition, there is a p65 form of Nrf1, presumably arising from Nrf1 translation initiating at an internal start codon (Chan *et al.*, 1993) although the possibility of the p65 arising as a result of a proteolytic cleavage can not be ruled out (Zhang *et al.*, 2007). The p65 form has been shown to act as a dominant negative inhibitor of Nrf2-mediated, ARE-driven luciferase activity (Wang *et al.*, 2007). Apart from antagonistic competition of Nrf1 p65 (and, potentially, full-length Nrf1) with Nrf2 for the ARE binding site, the *nrf2* promoter contains two ARE sequences (Kwak *et al.*, 2002), which may provide Nrf1 with yet another means of regulating Nrf2 expression.

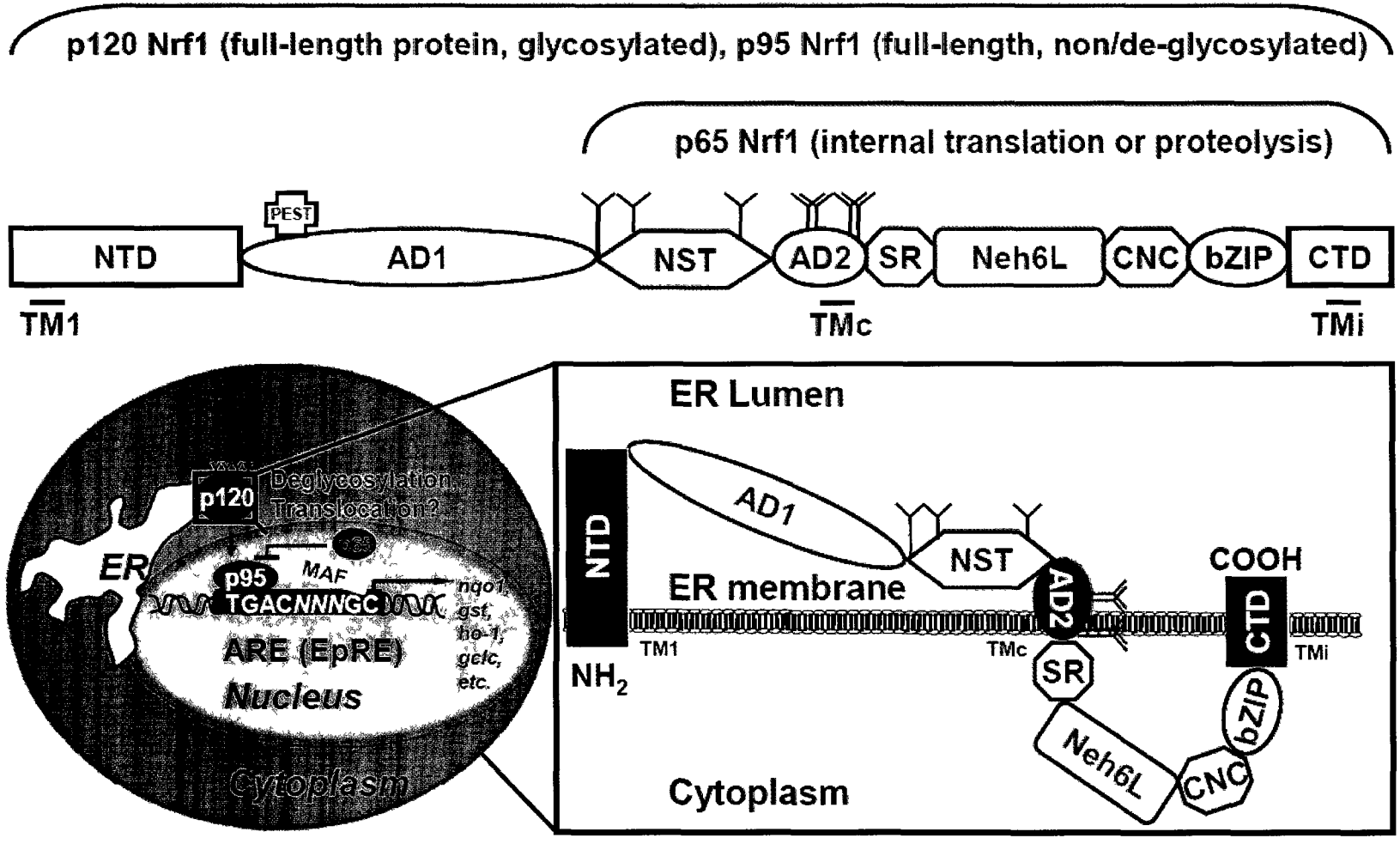

p120 Nrf1 (full-length protein, glycosylated), p95 Nrf1 (full-length, non/de-glycosylated)
p65 Nrf1 (internal translation or proteolysis)
PEST
NTD
AD1
NST
AD2
SR
Neh6L
CNC
bZIP
CTD
TM1
TMc
TMi
p120
Deglycosylation
Translocation?
ER
p95
MAF
TGACNNNGC
ARE (EpRE)
Nucleus
Cytoplasm
ngo1,
gst,
ho-1,
gclc,
etc.
ER Lumen
NTD
AD1
NST
AD2
COOH
ER membrane
CTD
TM1
TMc
TMi
NH2
SR
bZIP
Neh6L
CNC
Cytoplasm

Figure 1.4. The Structural Domains in Human Nrf1 (hNrf1) and the Predicted Topology of Nrf1 Within the ER membrane. The domains (independent structural and homology regions) were identified using a multiple amino acid (aa) alignment with mouse Nrf1 (mNrf1) aa sequence and domain designations as reported in (Zhang *et al.*, 2006). The hNrf1 domains are: NTD, N-terminal domain (aa 1-124); AD1, acidic domain 1 (aa 125-324); NST, Asn/Ser/Thr-rich region (aa 325-432); AD2, acidic domain 2 (aa 433-482); SR, Ser repeat (aa 483-519); Neh6L, Neh6-like (aa 520-611); CNC, cap'n'collar (aa 612-655); bZIP, basic Leu zipper domain (aa 656-717); CTD, C-terminal domain (aa 718-772). The topology of mNrf1 was predicted by Zhang and others (2009). TM1, TMi and TMc are putatitve trans-ER membrane regions. Nrf1 is synthesized as an ER-targeted protein and, once inserted into the ER membrane *via* TM1, TMi and TMc, is glycosylated in the ER lumen; the Nrf1 glycoprotein is referred to as p120. Following the translocation of the luminal part of the p120 into the nucleoplasm, it is unglycosylated to become active p95 Nrf1, heterodimerizes with small Maf or c-Jun proteins and binds to the ARE (EpRE) to activate the expression of genes involved the antioxidant defense and phase II detoxification metabolism. In *vitro*, Nrf1 deglycosylation is achieved by PNGase F. Internal translation or proteolysis by yet unidentified protease gives rise to p65 Nrf1, dominant negative repressor of the ARE-driven gene expression. Glycosylation sites are represented by "Y". Drawing of the domains and specific regions of Nrf1 is to scale.

In addition to the negative regulation of Nrf2 by p65 Nrf1, reported by Wang and colleagues (2007), the conditional knock-out work with hepatocyte-specific Nrf1-knockout mice showed that Nrf1 deficiency upregulated the Nrf2-target genes, such as *gclc*, *nqo1* and *ho-1*, while this effect was abolished in Nrf2-lacking animals (Ohtsuji *et al.*, 2008). It is plausible to hypothesize that the upregulation of the Nrf2-controlling genes in the livers of the Nrf1-deficient animals is due to the failure of structurally-different transactivation domains of Nrf1 to form the necessary interactions with the co-activator proteins to activate the transcription of the Nrf2-target genes. Therefore, when the inhibitory effect of Nrf1 is removed, more Nrf2-ARE interactions can take place, resulting in increased gene expression. Another possibility is that the lack of Nrf1 can create oxidative stress conditions, which would stimulate Nrf2-driven gene expression (Ohtsuji *et al.*, 2008). Yet, another plausible scenario is the one in which a competition for the ARE binding takes place between the p65 Nrf1 and Nrf2 such that the knockout of *nrf1* removes the negative, inhibitory action of Nrf1, leading to increased transcription of certain ARE-driven genes (Yamamoto, personal communication; Forman, personal communication). Such Nrf1-Nrf2 competition could be a useful way of turning down unnecessary transcription of antioxidant genes which, if left unrestrained, could lead to promoting the proliferation of precancerous cells and their survival (Wang *et al.*, 2007). The present thesis examines, in greater detail, the functional interaction between Nrf1 and Nrf2 factors, in addition to examining the relationship between different forms of Nrf1, as well as the regulation of Nrf1

by various conditions and post-translational modifications, are described in the Research Hypotheses section in greater detail.

1.7 ROS, Nrf2 and Their Potential Involvement in Aging

According to the oxidative stress theory of aging, the current version of the free radical theory of aging, proposed nearly 60 years ago (Harman, 1956), the use of oxygen by all aerobic cells creates ROS, damaging cellular macromolecules even under normal physiological conditions (Sohal and Weindruch, 1996). The accumulation of oxidative damage increases with age and is thought to be the major cause of senescence (Sohal an Weindruch, 1996). In support of this theory, multiple studies reported that aging organisms display increased oxidative damage (Bokov *et al.*, 2004); however, the accumulated genetic evidence, aimed at altering the extent of oxidative damage in animals, suggests that oxidative damage plays a major role in aging only under suboptimal conditions and plays a very minor role in aging in unstressed environments (Salmon *et al.*, 2009). Similarly, there is some evidence, suggesting that the susceptibility of animals to pro-oxidants, compounds augmenting oxidative stress within cells, increases with age (Gordon *et al.*, 2010). At the systemic level, greater susceptibility of the elderly to environmental toxicants may be the consequence of the age-associated changes in the nervous system (Ginsberg *et al.*, 2005) while at the cellular level, aging is associated with diminished expression of the antioxidant genes, orchestrated by Nrf2 (Suh *et al.*, 2004; Shih and Yen, 2006). Interestingly, the proper function of the Nrf2-

ARE pathway seems to be crucial for long-lived animals such that Nrf2 has been regarded as the "gatekeeper of species longevity" (Lewis *et al.*, 2010). Paradoxically, aged animals display impaired Nrf2 function and reduced Nrf2 levels when its activity is most needed (Bohman and Sykiotis, 2010). Since Nrf2 is inducible by multiple pro-oxidants, nanoparticles generated by the vehicular traffic have a tendency to generate free radicals due to the presence of organic hydrocarbon and transition metals (Morgan *et al.*, 2011; Xia *et al.*, 2006), the treatment of young and aged mice with airborne particles was thought to be a suitable model for the study of the Nrf2-ARE pathway induction *in vivo*. Given the potentially negative regulation of Nrf2 by Nrf1, which has been discussed in greater detail above, we wanted to consider whether Nrf1 activity and expression is altered by age and whether such an alteration can provide a clue to Nrf2 inactivation in aged animals.

1.8 Bisphenol A, the Mechanism of Its Action in Biological Systems and Its Relation to Oxygen Metabolism and Antioxidant Enzymes

Bisphenol A (BPA) is used in the production of polycarbonate plastics and epoxy resins for baby bottles, liners of canned food, medical equipment and consumer electronics (Benachours and Aris, 2009). Unfortunately, BPA-containing products can act as a source of the human exposure to BPA, especially under high temperature, exposure to sunlight, acidic or basic conditions (Kang *et al.*, 2003). Indeed, over 90% of the U.S. population has detectable levels of BPA in urine with the chemical being most prevalent

in children samples (Calafat *et al.* 2005). Although initially thought of as a weak estrogenic compound, it is now apparent that the potency of BPA to stimulate several cellular responses is comparable to estradiol (Rubin, 2011) and BPA is thought to act as an endocrine disruptor, interfering with normal pre- and post-natal development (O'Connor and Chapin, 2003). The developing liver of embryos and neonates has relatively weak capacity to metabolize BPA (Takahashi and Oishi, 2000). The latter fact probably explains the reason Canadian government banned the use of BPA-containing plastics in the production of baby food bottles in 2008. More specifically, BPA exposure was found to lead to unusual reproductive organ development (Lang *et al.* 2008), diabetes (Alonso-Magdalena *et al.* 2010) and decreased fetal survival and fetal body weight (Rubin *et al.* 2009).

BPA has the propensity to be metabolized to a catechol and then, subsequently, to an ortho-quinone and these two species can participate in the redox cycling (Kovacic, 2010). More specifically, the tendency to generate ROS is due to the highly reactive nature of the ortho-quinones that can donate electrons to molecular oxygen, producing superoxide, $O_2^{\bullet-}$ and other potentially damaging forms of ROS. Previous studies suggested that BPA treatment of cultured mammalian cells, in the order of 50-100 μM BPA for 6-24 hours, increases the formation of ROS and the production of antioxidant enzymes, many of which are transcriptionally regulated by the ARE. However, despite increased expression, many antioxidant enzymes were found to be inactivated by BPA application. For instance, BPA treatment of cultured human Chang liver cells led to inactivation of antioxidant enzymes catalase and glutathione peroxidase (CAT and GPx,

respectively, Oh and Lim, 2008). Both CAT and GPx are thought to be controlled by the ARE pathway (Itoh *et al.*, 2005; Ohtsuji *et al.*, 2008). Similar inactivation of GPx and CAT was seen *in vivo* in mice and rats during the provision of a BPA-rich diet to mice (Kabuto *et al.*, 2003) and rats (Chitra *et al.*, 2003). I hypothesized that the stimulatory effect of the BPA on ROS generation could be due to the impairment of the ARE-Nrf1/2 pathway and declined mRNA expression of the ARE-controlled antioxidant enzymes.

1.9 Research Hypotheses

Three main themes have been considered during the completion of this thesis. These pertain to the regulation of Nrf1 by: i) the proteasome (and proteasome inhibitors) and hypoxia, ii) BPA, and iii) aging and airborne particles. The common theme in using these treatments and conditions is that they are all thought to be related to oxidative stress conditions and thus fall within the topic of "oxygen-dependent" regulation of Nrf1.

First, the proteasome regulates some rapidly-degraded transcription factors, such as Nrf2 and HIF-α. In addition, the proteasome is responsible for destruction of aged, oxidized, misfolded or unfolded proteins and the impairment of proteasomal function can lead to augmented intracellular ROS production and cell death (Grim *et al.*, 2011). Proteasome inhibition also stimulates the hypoxia- and oxidative stress-inducible gene expression through the stabilization of transcription factors HIF-α and Nrf2, respectively. *I hypothesized that Nrf1 is regulated by the proteasome through rapid proteasomal degradation. I also hypothesized that hypoxia has an effect on the Nrf1-ARE pathway. In*

addition, I hypothesized that there could be several post-translational mechanisms of Nrf1 regulation. To test these hypotheses, I used cultured mammalian cells in conjunction with the proteasomal inhibitors and hypoxic conditions and analyzed the expression of Nrf1 and the function of Nrf1, overexpressed in the cells and subjected to different conditions using luciferase reporter assay.

Next, given the reported pro-oxidant activity of BPA in cultured cells, *I hypothesized that BPA might lead to ROS generation by affecting the Nrf1/2-ARE pathway-mediated expression of antioxidant genes.* To test this hypothesis, I used BPA treatment of cultured human cells. ROS levels were measured using conventional fluorescence-based assays and the expression of the regulatory (Nrf1 and Nrf2) and target (Nqo1 and HO-1) proteins was analyzed using immunoblotting and real-time quantitative RT-PCR. The function of Nrf1 and Nrf2, overexpressed in the human cultured cells was measured upon BPA treatment using luciferase reporter assays.

Finally, I investigated the potential regulation of Nrf1 by aging and particulate exposure using *in vivo* mouse models. *I hypothesized that Nrf1 can negatively regulate the transactivation activity of Nrf2 in vitro and in vivo and that Nrf1 may be differentially expressed in mousemice as a function of age.* To test this hypothesis, I used luciferase reporter assays and overexpressed short (p65) and full-length Nrf1 forms, in addition to Nrf2, and followed the transactivation activity of Nrf2 in the presence of Nrf1. In addition, I used the mouse models of airborne particle exposure since airborne particles are reactive centres with rich chemistry stemming from the presence of organic

hydrocarbons and transition metals and analyzed and I analyzed Nrf1 and Nrf2 protein expression and DNA binding functions using lung protein extracts.

2 Chapter: Methods

2.1 Justification for the Use of Specific Cell Lines and Experimental Design

COS7 and HEK293A are immortalized cells from the kidney of African green monkey and human embryos, respectively. These cells undergo transfection very easily and are routinely used for transcription factor studies. For example, Numazawa and co-workers used COS7 to study the activation of Nrf2 by atypical protein kinase C (2003). Since Nrf1 is expressed ubiquitously (Chan *et al.*, 1993), the use of a cell line in which some basal expression of Nrf1 protein exist in addition to the expression of the FLAG-tagged Nrf1 was considered appropriate to study the function of this transcription factor, which is a common strategy, reflected in other publications (e.g., Furukawa and Xiong, 2005). Simultaneous utilization of luciferase reporters with overexpressed transcription factors usually does not provide researchers with the same degree of confidence as when utilizing other experiments, as will be discussed in Chapter 6 (Figure 6.2); however, this approach still provides important clues to a regulation of transcription factor and such an approach is often employed by researchers in the field. This approach has been utilized to reveal the transactivation regions of Nrf3 (Chénais *et al.*, 2005). Hypoxic activation of Nrf1-FLAG, seen initially in COS7 cells, was confirmed using Nrf1-FLAG overexpression in the HEK293 cells. WFF 2002 cells are whole fetal fibroblasts derived from human, female, and non-cancerous fetuses. This cell line was used to confirm the data, obtained with proteasomal inhibition of Nrf1, as seen in COS7 cells, in order to reveal if proteasomal regulation of Nrf1 is a universal mechanism of Nrf1 regulation, seen in different species. For cycloheximide (CHX) experiments, Nrf1 p120 was first stabilized by proteasomal inhibition in COS7 cells and then the proteasome inhibitor-

containing medium was removed and CHX was added to inhibit protein synthesis. The assumption here was that the increase in the proteasome-degraded proteins, achieved by proteasomal inhibition did not overwhelm the catalytic activities of the proteasome and that the rate of Nrf1 degradation was not affected by pre-treatment with proteasomal inhibitor MG-132, which is known to inhibit the proteasome reversibly (Pajonk *et al.*, 2005). Such as strategy for the determination of a half-life was employed for HIF-1α in which HIF-1α was first stabilized by pre-treatment with 10 μM MG-132 for 15 min, followed by CHX exposure (Chachami *et al.*, 2004).

Another important consideration taken into account was the toxicity of certain treatments, dependent upon the time and concentration of a condition or a compound exposure, provided to the cells. This has been addressed by using the time and concentrations previously reported in the literature as being non-cytotoxic (e.g., Nouhi *et al.*, 2007) and the lack of cytotoxicity upon treatment with proteasome inhibitors has been confirmed using cell viability assays, including the methylthiazolyldiphenyl-tetrazolium bromide (MTT), trypan blue and cell sorting/GFP assays (data not shown).

The cellular context of the experiments was also considered in the interpretation of the data. For example, since the phosphatase and kinase inhibitors used were not Nrf1-specific and were expected to affect other proteins, regulated by phosphorylation, it has been explicitly discussed (section 3.7) that the experimental outcomes could not provide any information, suggesting whether or not the phosphorylation status of Nrf1 itself has been affected by the treatment and the possibility of the existence of other proteins,

acting on Nrf1 in a phosphorylation-dependent manner, has been mentioned. AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride), a known peroxyl radical generator, is usually used at high millimolar (10-100 mM) concentrations to achieve a steady, continuous rate of the radical generation (Niki, 1990) and has been used to study the oxidative inactivation of several enzymes, including catalase, alcohol dehydrogenase and horseradish peroxidase (reviewed in Chepelev *et al.*, 2009) at these concentrations.

For the work presented in Chapter 4, the cell viability with BPA treatment was performed. The 24-hour time interval for treatment with several concentrations of BPA is common methodology in many toxicological studies that examine effects of specific chemicals published to date. For example, Li *et al.* (2005) used a single concentration of tert-butylhydroquinone (tBHQ) and a single time point (24 hours) to discover genes, whose expression was altered by the tert-hydroquinone treatment. Likewise, in this work, a known inducer of Nrf2, tBHQ, (Li et al, 2005), was used as a positive control for Nrf2 transactivation activity.

For Chapter 5, human bronchial epithelial (HBE1) cells were used as they retain the phenotype of normal bronchial epithelial cells and could, therefore, be a useful model of human lung cells (Yankaskas *et al.*, 1993). Finally, mice, treated with airborne particles, were used for Nrf1/2 studies as the airborne particles used for the experiment were found to contain stable, carbon-centred radicals (Morgan *et al.*, 2011). Lung was used as the tissue of choice as it was in direct contact with the inhaled particles.

2.2 Cell Culture and Treatments

COS7 cells were grown in DMEM supplemented with 10% NCS and 3% P/S/A (300 units/mL penicillin G, sodium salt, 300 μg/mL streptomycin sulfate and 0.75 μg/mL Fungizone ® in 0.85% saline) in a humidified atmosphere with 5% CO_2 at 37°C. HEK293A and WFF2002 cells were grown under the same conditions, but with different sera (10% HS and 10% FCS, respectively). Hypoxic conditions were achieved by setting O_2 at 1%, CO_2 at 5% and the balance N_2 in triple-gas incubators (Thermo Forma, Rockford, IL) or by incubating the cells with chemicals at the concentrations and times indicated in the figure legends.

2.3 Immunoblotting and Co-immunoprecipitation

Harvested cells were lysed in 50 to 100 μL of cell lysis buffer (20 mM HEPES pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% glycerol, 1 mM DTT and 0.5 mM PMSF) and an equal amount of total protein (determined using BioRad protein assay and diluted 1:1 with 2 X Laemmli loading buffer) was loaded on a 10% SDS-PAGE, run at 120 V for 1.5 to 2 hours and transferred onto Immobilon PVDF membrane (Millipore, Bedford, MA) overnight at 4°C. Membranes were probed with 5% milk in Tris-Buffered Saline, Tween-20 (TBST) for 1 hour. Western blot analysis for Nrf1 was performed using mouse anti-human Nrf1, HO-1 and Nqo1 antibody (1:1,000 dilution) and rabbit anti-human Nrf2 (Santa Cruz Biotechnology and horseradish peroxidase-labelled goat anti-mouse or goat anti-rabbit IgG secondary antibody (1:2,000 dilution; DAKOCytomation,

Mississauga, ON). FLAG- tagged Nrf1 was visualized using horseradish peroxidase (HRP)-conjugated mouse anti-FLAG M2 antibodies (1:4,000 dilution; Sigma-Aldrich). As a loading control, membranes were probed with mouse anti-human β-tubulin (1:4,000 dilution, Developmental Studies Hybridoma Bank, Iowa City, IA) or stained with Ponceau S red stain. Blots were developed by enhanced chemiluminescence substrate (Millipore, Bedford, MA) and Kodak X-Omat blue film (Perkin-Elmer, Waltham, MA). Film was scanned using a CanoScan LIDE 80 scanner (Canon, Lake Success, NY) and band densitometry was measured using AlphaEaseFC software, version 3.1.2 (Alpha Innotech/Cell Biosciences, Santa Clara, CA). For co-IP, the protein complexes were immunoprecipitated from 125 μg total lysate protein with 20 μL Protein A/G-Sepharose beads and 2 μg anti-Nrf1 antibody. The immunoprecipitated complexes were subjected immunobloting using anti-ubiquitin antibody.

2.4 Cloning of Nrf1-FLAG

Human Nrf1 gene from mammalian gene collection (accession number BC010623) was inserted into a modified pCR3.1 mammalian expression vector using NdeI and EcoRI and then introduced into CMV-5a-FLAG vector (Sigma-Aldrich) using EcoRI and KpnI restriction enzymes to create N- and C-terminally FLAG-tagged Nrf1, respectively.

2.5 Transient Transfections and Luciferase Reporter Assays

A luciferase reporter plasmid containing three AREs from chicken β-globin enhancer (3 x ARE-luciferase) was a kind gift from Dr. Masayuki Yamamoto (Tohuku University) and has been described elsewhere (Igarashi *et al.*, 1994). N-terminally myc-tagged (pCDNA3-myc3-Nrf2) and N-terminally FLAG-tagged Nrf2 (pPROEX-HTc-Flag3-Nrf2) were obtained from Addgene (Addgene plasmids 21555 and 21553 Cambridge, MA) and has been described previously (Furukawa and Xiong, 2005). Cells were seeded in 6-cm or 6-well plates at a density of 140,000 cells/mL and approximately 24 hours later were transiently transfected with Lipofectamine 2000 as per the manufacturer's protocol (upon becoming at least 70% confluent). For a 6-cm plate, 0.2 μg of β-galactosidase, 8.0 μg of 3 x ARE-luciferase and 2.4 μg of Nrf1-FLAG, myc-Nrf2 or pCR3.1 (empty vector) plasmid DNA, were used. Twenty-four or forty-two hours later, cells were treated for 24 or 6 hours, respectively, and harvested such that the total amount of time after transfection was 48 h. A firefly luciferase reporter gene assay was performed to measure ARE-driven transcriptional activity. Cells were lysed in 25 to 50 μL of lysis buffer (25 mM glycylglycine (pH 7.8), 1% Triton X-100, 15 mM KH_2PO_4, 15 mM $MgSO_4$, 4 mM EDTA, 1 mM DTT and a complete protease inhibitor cocktail tablet (Roche , Mannheim, Germany)). Approximately 4 to 12 μL of supernatant was added to 76 μL of luciferase assay buffer (2 mM ATP in lysis buffer) and luciferase activity was assayed on a FLUOstar OPTIMA (BMG LABTECH, Offenburg, Germany) luminescence microplate reader. The reaction was initiated by the injection of 50 μL of 200 μM luciferin solution. Luciferase assay values were normalized to β-galactosidase assay results (a measure of transfection efficiency). For β-galactosidase assay, cell lysates were incubated in 85 μL of 0.2 mg/mL of CPRG in 60 mM Na_2HPO_4, (pH 8.0), 10 mM

KCl, 1 mM $MgCl_2$, and 1 mM DTT, for 5 to 10 minutes, and monitored at 580 nm. Transfection experiments were reproduced at least three times and are presented as means ± S.E.M.

2.6 Electrophoretic Mobility Gel Shift Assays

Cells were lysed as described above and 7.5 - 20 μg nuclear or total protein was reacted with a biotinylated dsDNA ARE probe of the human *gclm* promoter (Kwong *et al.*, 1998) and was subjected to non-denaturing electrophoresis according to the manufacturer's instructions (Panomics, Fremont, CA). Protein-DNA complexes were transferred onto an Amersham Hybond-N^+ membrane (GE Healthcare, Buckinghamshire, UK) and visualized using streptavidin-HRP and chemiluminescence as described above for Western blots. The identity of the ARE-bound complex was investigated using 2 μg non-specific (pre-immune serum with immunoglobulin G (IgG) or actin) antibodies per reaction for immunodepletion (Dickinson *et al.*, 2003). In this case, the protein extract is incubated with a given antibody for 2 hours at room temperature prior to the addition of the probe. To control for specific binding to the ARE probe, an excess of cold (unlabelled probe) provided by the manufacturer at an unspecified concentration (Panomics) was employed.

2.7 Computational and Bioinformatics Analyses

To compute the gas-phase bond dissociation enthalpies of the O-H bonds in BPA, the medium level method 2 (MLM2; Wright *et al.*, 2001) was used. Briefly, the molecular geometries were first approximated using lowest energy conformer generation procedure at the Austin Model 1 (AM1) level. The lowest energy conformer geometry was then used as a starting point for a geometry optimization procedure at the density functional theory (DFT) level with a Becke, Three-Parameter, Lee-Yang-Parr (B3LYP) functional and a 6-31G(d) basis set. The optimized geometry was used to compute the frequencies, at the same level of theory, with a correction factor of 0.9806, at 298.15°K temperature and 1 atm. pressure, in order to obtain the enthalpic correction factors for each compound. The final single-point energy of each compound was computed using the B3LYP functional and a 6-311+G(2d,2p) basis set. The overall gas-phase enthalpy of formation was computed as a sum of the enthalpic correction factor computed in the frequency calculation step and the final single-point energy. The procedure was identical for the closed-shell and open-shell (e.g. radicals) compounds, with the exception that the latter had a single-point energy computed with the open-shell (RO)B3LYP functional. The enthalpy of formation of the hydrogen atom was -0.5 hartree. In order to compute the Bond Dissociation Energy (BDE) for the transformation RO-H -> RO• + H•, the enthalpies of formation of reactants were subtracted from those of products.

ClustalW tool (Larking *et al.*, 2007) was used for multiple amino acid alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Nrf1 PEST domain was identified using PESTfind algorithm that was previously located at https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm. This tool

identifies the amino acid regions rich in Pro, Glu, Ser and Thr, compares them to known, short-lived proteins with PEST sequences and provides an index, indicative of the strength of a given putative PEST sequence. The prediction of phosphorylation sites was performed using NetPhos 2.0 server available at http://www.cbs.dtu.dk/services/NetPhos/ (Bloom *et al.*, 1999). This tool uses neural networks and defined sequence logos of phosphorylation sites of Tyr, Ser and Thr, derived from the experimentally-confirmed phosphorylation sites. The prediction of kinase-specific phorphorylation sites (Bloom *et al.*, 2004) was performed using http://www.cbs.dtu.dk/services/NetPhosK/. The underlying principles of this method are very similar to NetPhos 2.0 and rely on neural network-assisted analysis of a given amino acid sequence by comparing it to the experimentally-derived, site-specific preferences for the phosphorylation of several well-studied kinases, including protein kinase C (PKC). The phosphorylation prediction tools were used to predict the potential phosphorylation sites and the effect of the intracellular phosphorylation status on the Nrf1 activity was assessed by further experiments.

2.8 Cell Viability (MTT) Assay

The principle behind this assay is the oxidation of yellow methylthiazolyldiphenyl-tetrazolium bromide (MTT) by mitochondrial NADH dehydrogenase to produce a purple formazan product, whose formation can be followed spectrophotometrically. Cells were seeded at a density of 50,000 cells/mL and grown for 48 h and treated with the indicated BPA concentrations for 24 h. MTT was then added (0.5 mg/mL) for 2 h and the absorbance was read at 570 nm with correction at 630 nm.

2.9 ROS, Glutathione and Carbonyl Assays

Intracellular ROS were detected using the Image-iT LIVE Green Reactive Oxygen Species Kit (Molecular Probes). Cells were grown for 48 h as described above, washed in PBS, incubated in 10 μM of 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA; an intracellular ROS probe) for 30 min, washed in PBS and treated with BPA for 24 h using phenol red-free medium. AAPH, a generator of peroxyl and alkoxyl radicals (Niki, 1990), served as a positive control. Free and total reduced glutathione (GSH) was measured using the DetectX Glutathione Fluorescent Detection Kit (Arbor Assays). Butathione sulfoxamine (BSO), an inhibitor of GSH synthesis (Yim *et al.,* 1994), served as a positive control. The generation of carbonyl groups, as a marker of oxidative damage to proteins (Chevion *et al.*, 2000), was assessed using the OxyBlot Protein Oxidation Detection Kit (Millipore) according to the manufacturer's protocol using 7.5 μg of total cellular protein per sample.

2.10 Total RNA Isolation and Real-time Quantitative PCR (real-time qPCR)

Total RNA from frozen cell pellets was extracted using the RNeasy Mini Kit (Qiagen). Procedures for cDNA synthesis and real-time qPCR were as previously described (Chen *et al.*, 2009). Briefly, 1 μg total RNA was reverse-transcribed to synthesize cDNA with the Retroscript Kit (Applied Biosystems/Ambion). Real-time qPCR was performed on Mx4000 Multiplex Quantitative PCR System using TaqMan

Gene Expression Assays (Applied Biosystems; see **Table 4.1** for assay information). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was unaffected by BPA treatments (data not shown) and was considered a valid housekeeping gene. The fold change in expression for the gene-of-interest relative to GAPDH (gene-of-interest/GAPDH) was calculated and compared to the control group (set as 1.0).

2.11 Nanoparticle Production, Mouse Exposure and Tissue Processing

Nanoparticle (also referred to as "particulate matter" or PM) collection and mouse treatment have been described previously (Morgan *et al.*, 2011). Briefly, nanoparticles were collected with a High-Volume Ultrafine Particle (HVUP) Sampler (Misra *et al.*, 2002) at 400 L/min in Los Angeles City near the CA-110 Freeway. This is considered to be the mix of fresh ambient particles mostly from vehicular traffic nearby this freeway (Ning *et al.* 2007). The nanoparticles with diameter less than 200 nm were collected on pre-treated Teflon filters (20 x 25.4 cm, PTFE, 2 μm pore; Pall Life Sciences, Covina, CA). The collected nanoparticles were transferred into aqueous suspension by 30 min soaking of nanoparticle-loaded filters in Milli-Q deionized water (resistivity 18.2 megaΩ; total organic compounds <10 ppb; particle-free; bacteria levels <1 CFU/ml; endotoxin-free glass vials), followed by 5 min. vortexing and 30 min. sonication. Aqueous nanoparticle suspensions were pooled and frozen as a stock at -20°C, which is chemicaly stable for at least 3 months (Li *et al.* 2003). The nanoparticle suspensions were re-aerosolized by a VORTRAN nebulizer using compressed particle-free filtered air as previously described (Morgan *et al.*, 2011).

C57BL/6 male mice (3 month- and 18 month-old) were maintained under standard conditions with *ad libitum* Purina Lab Chow (Rancho Cucamonga, CA) and sterile water. Just before the exposure to particles, mice were transferred from home cages to exposure chambers, which provided free movement. Temperature and airflow parameters were controlled for adequate ventilation and minimization of the build-up of animal-generated contaminants (skin dander; CO_2, NH_3). Re-aerosolized nanoparticles or ambient air (control) was delivered to the sealed exposure chambers for 5 hr/day, 3 days/week, for 10 weeks. Mice had normal weight and showed no signs of respiratory distress. After isoflurane anesthesia, mice were euthanized and the tissue was collected and stored at -80°C until processing. All rodents were treated humanely, with regard for alleviation of suffering and following the procedures approved by the USC Institutional Animal Care and Use Committee.

For protein extraction, approximately 30 mg lung tissue was ground in glass homogenizer using 100 μL of ice-cold CER I reagent from NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Rockford, IL), supplemented with protease and phosphatase inhibitors to prepare cytosolic fraction according to the manufacturer's protocol. For nuclear extraction, 50 μL of NER reagent from the same kit, supplemented with phosphatase and protease inhibitors, was used, using the manufacturer's instructions. Protein determination was carried out using Bradford assay (BioRad, Hercules, CA).

2.12 Oxygen Radical Absorbance Capacity (ORAC) Assay

The antioxidant activity of lung cytoplasmic extracts was measured using the ORAC assay as descrubed previously (Cao *et al.*, 1993). Three micrograms of cytoplasmic protein was used per well and the assay was run in triplicate. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble vitamin E analogue), was used to establish the reference range. Peroxyl and alkoxyl radicals were generated using AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride; final concentration 0.158 M), prepared fresh for each run. Fluorescein (final concentration 0.114 μM) was used as a fluorescence probe and rutin (final concentration 5.0 μM) was used as a positive control. Fluorescence was measured at an excitation and emission wavelengths of 360 and 520 nm, respectively, using an automated plate reader (FLx800 Fluorescence Microplate Reader, BioTek) with 96-well plates. Analyses were conducted in 70 mM potassium phosphate buffer pH 7.4 at 37°C. ORAC values were reported as μmol of Trolox equivalents (μmol

TE) that produces the same area under the curve as one microgram of total protein (μmol TE/μg total protein).

2.13 Statistical Analyses

Data are presented as means ± S.E.M. of at least three independent experiments. The results were considered statistically significant at $p < 0.05$ for the Student's paired t-test unless otherwise indicated.

3 Chapter: Oxygen-dependent and Proteasomal Regulation of Nrf1

3.1 Proteasomal Inhibition Stabilizes p120 Nrf1 and Ubiquitinated Nrf1

The regulation of the protein abundance through the degradation of misfolded and oxidatively damaged proteins is an important function of multi-subunit protease known as the proteasome. The 26S proteasome is the most common of these. It consists of: a) the 20S core with trypsin-, chymotrypsin- and peptidyl-glutamyl peptide-hydrolyzing proteolytic activities and b) the 19S regulatory particle, containing the ATPase activity required for unfolding of the protein substrates prior to their degradation by the 20S core. The attachement of ubiquitin, a short 76 amino acid protein, serves as the tag recognized by the 19S regulatory particle. It is estimated that about 80% of cellular proteins are degraded by the ubiquitin-proteasome system (Crawford *et al.*, 2011). It appears that not only misfolded or damaged proteins are the substrates of the proteasome, but also a number or regulatory proteins, including transcription factors. For instance, the key transcription factors, involved in hypoxia, inflammation and oxidative stress signalling cascades, including HIF-α, IκB and Nrf2 respectively, are rapidly degraded by the proteasome (Taguchi *et al.*, 2011). In other words, under unstressed conditions, the function of these factors is not required and, therefore, is repressed by continuous proteasomal degradation. Under an appropriate stimulus, however, the proteasomal inhibition of these factors is impaired and this de-repression from rapid proteasomal degradation through increased stability allows the cell to mediate appropriate responses through altered gene expression. Closely-related to Nrf1 CNC-bZIP factors Nrf2 and Nrf3 have been shown to be controlled by keeping their intracellular levels low under homeostatic conditions through proteasomal degradation (Nguyen *et al.*, 2003; Nouhi *et*

al., 2007). *I hypothesized that Nrf1 is also regulated by the proteasome through rapid proteasomal degradation.* To test if Nrf1 is also regulated *via* proteasomal degradation, I analyzed its protein expression in the presence of proteasomal inhibitors clasto-lactacystin-β-lactone (lactacystin) and MG-132. Proteasomal inhibition with both inhibitors greatly enhanced the expression of the inactive, ER-bound glycosylated form of Nrf1, p120 (Figure 3.1). Interestingly, while the density of the p120 band increased significantly (about 140% compared to controls) during MG-132 treatment, the intensity of the band, corresponding to the active, nuclear nonglycosylated p95 Nrf1 form decreased (data not shown). This could imply that higher levels of p120 could arise, at least in part, due to increased glycosylation of p95, in response to proteasomal inhibition. However, the decrease in the p95 Nrf1 seen during the MG-132 treatment was very minimal (approximately, 20%) and, while such a decrease may be statistically-significant, this could be biologically-irrelevant. Also, this was not seen for lactacystin as the p95 expression during this treatment was unaltered, indicating that the stabilization of p120 may proceed independently of p95 Nrf1. This, apparently independent regulation of p95 and p120 was also confirmed by the treatment of COS7 cells with the oxidative stressor AAPH, which acts by generating steady-state levels of peroxyl and alkoxyl radicals and is employed at high millimolar concentrations (Niki, 1990). The danger of using such a high concentration of a xenobiotic compound is that it can lead to cell death through apoptosis, providing confounding results. During the AAPH treatment, it was assumed that, in the case of cell death, lowered tubulin signal will be observed, which was not seen. In addition, no detrimental effect of the AAPH treatment was observed using cells overexpressing β-galactosidase as judged by the β-galactosidase assay (data

not presented). Upon establishing that AAPH, a known oxidative stressor, increased the Nrf1 binding to the ARE (Figure 3.2A and B), we observed the accumulation of p95 form without any apparent effect on the p120, illustrating the possibility of p95 "*de novo*" accumulation (see Figure 3.2C). Alternatively, there may be some time lag between the accumulation of p120 and p95 forms, given the time required for p120 deglycosylation conversion to p95.

Figure 3.1. Nrf1 is Stablized by Proteasomal Inhibition in COS7 cells. Cells were treated with 10 μM lactacystin (**A**) or 10 μM MG-132 (**B**) for six hours, lysed, resolved by SDS-PAGE and Western blots were performed with anti-Nrf1 antibody as described in the Methods section (Chapter 2). DMSO (the solvent used for lactacystin and MG-132) was used for controls. The positions of p95 and p120 Nrf1, as well as the loading control β-tubulin, is shown with arrows and the presense or absence of a proteasomal inhibitor treatment is indicated by + or - , respectively.

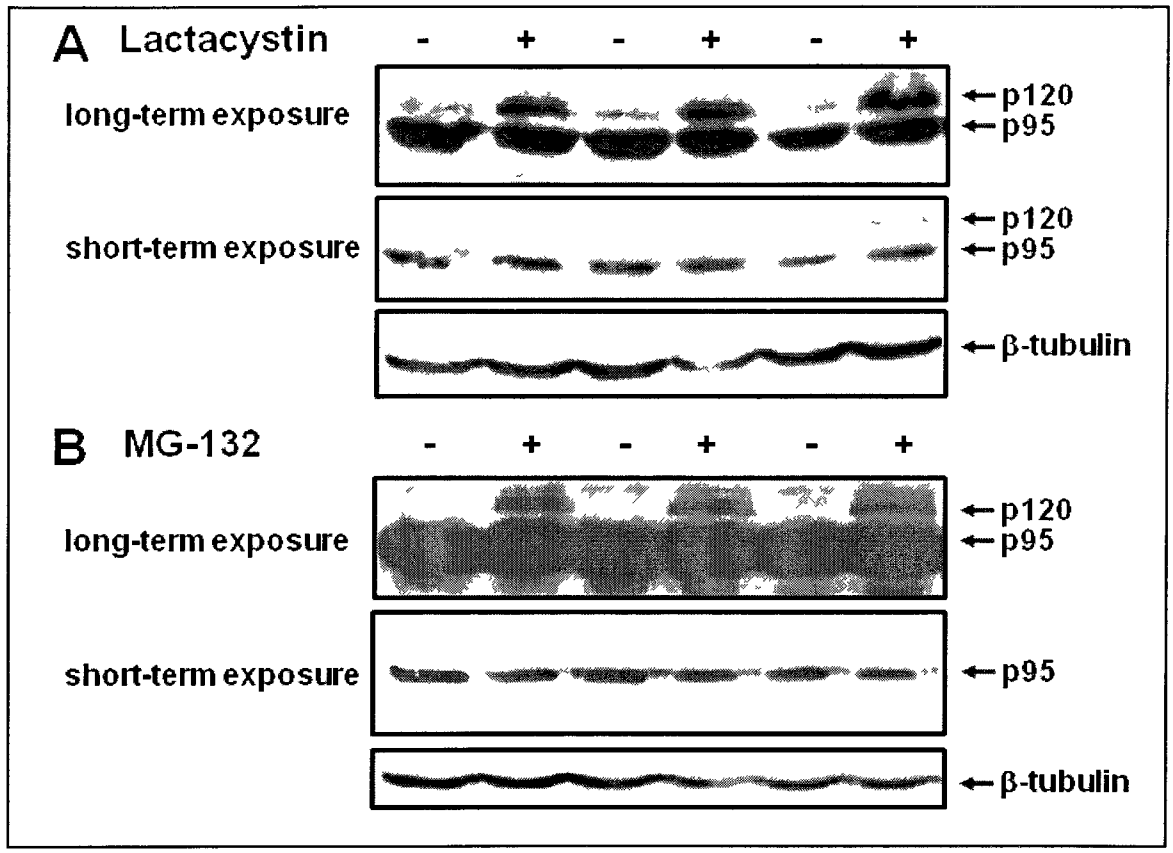
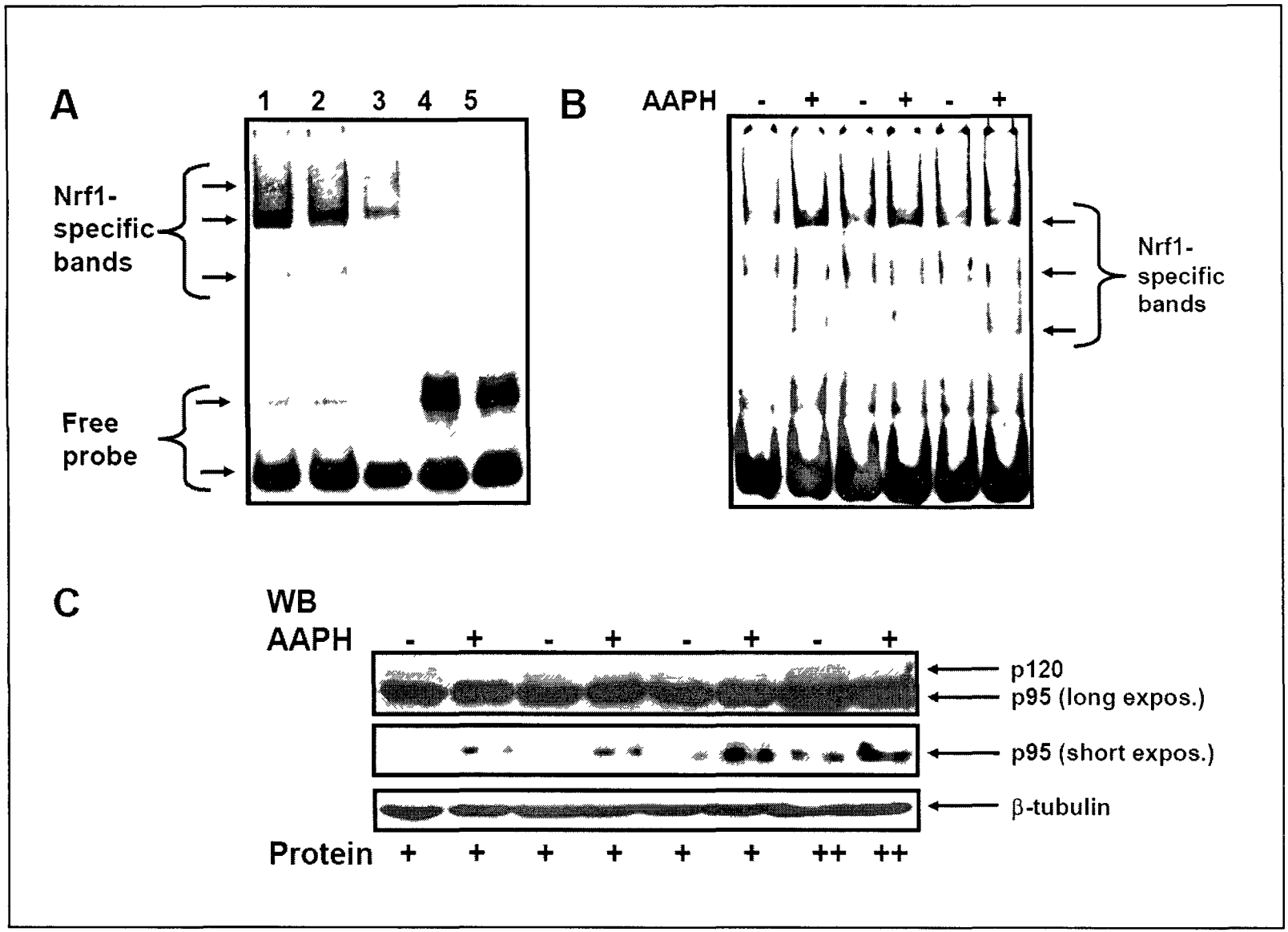

A Lactacystin
- + - + - +
long-term exposure
←p120
←p95
short-term exposure
←p120
←p95
←β-tubulin
B MG-132
- + - + - +
long-term exposure
←p120
←p95
short-term exposure
←p95
←β-tubulin

Figure 3.2. Oxidative Stressor AAPH Induces Nrf1 DNA Binding and Stabilizes p95 Nrf1 Form Independently of p120. (**A**) The Nrf1-specific band on an EMSA format was determined by including 20 μg of the COS7 cell lysate with: 1) no antibody; 2) anti-actin antibody; 3) anti-Nrf1 antibody; 4) anti-Nrf1 antibody, no lysate; and 5) neither lysate nor antibody (probe only). The experiment was performed twice with the same outcome. (**B**) COS7 cells were treated with 80 mM AAPH for 6 hours (Niki, 1990; Terao and Niki, 1986) and the lysates were subjected to EMSA and Western blotting (**C**). The positions of AAPH-inducible Nrf1-specific bands are shown with arrows in (**A**) and (**B**). In (**C**), both short- and long-time exposure of bands are shown for better clarity.

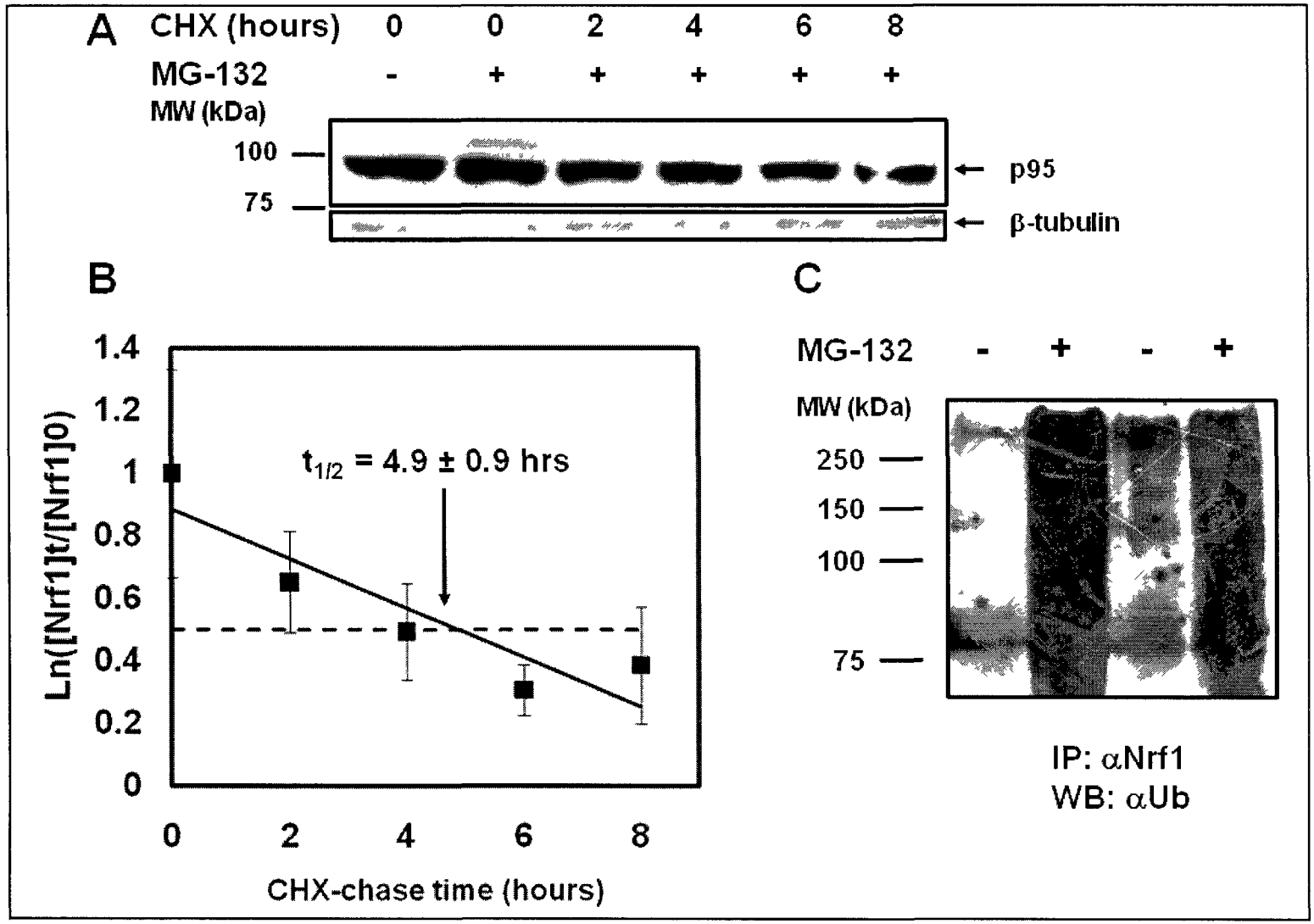

A
1 2 3 4 5
Nrf1-specific bands
Free probe
B
AAPH - + - + - +
Nrf1-specific bands
C
WB
AAPH - + - + - + - +
p120
p95 (long expos.)
p95 (short expos.)
β-tubulin
Protein + + + + + + ++ ++

3.2 Nrf1 Contains a PEST Motif and is Degraded by the Proteasome

Nrf2 and Nrf3 have half-lives ($t_{1/2}$) of approximately 15 and 30 minutes, respectively (Nguyen *et al.*, 2003; Nouhi *et al.*, 2007) and contain two strong putative PEST sequences (Nouhi *et al.*, 2007; Steward *et al.*, 2003). PEST sequences are rich in Pro, Glu, Thr and Ser and are found frequently in rapidly degraded proteins; the Ser and Thr residues are potential phosphorylation sites (Li *et al.*, 2005). The PESTfind analysis tool (http://www.at.embnet.org/toolbox/pestfind/) was used to search for any putative PEST sequence in Nrf1 and found that Nrf1 has a strong PEST motif (amino acids 141-169) in its NTD (see Figure 1.4 for Nrf1 domains). To test whether Nrf1 is indeed subject to rapid degradation, as suggested by the presence of strong PEST motif, we applied CHX and analyzed Nrf1 protein degradation by immunoblotting when protein synthesis was inhibited (Figure 3.3). A plot of band density versus time (Figure 3.3B) showed that the $t_{1/2}$ of p95 is approximately 5 ± 1 hours in COS7 cells. Compared to transcription factors Nrf2 and Nrf3, Nrf1 is more than 5 to 10 times more stable, but the Nrf proteins are less stable than other proteins that have $t_{1/2}$ between 16 (lysozyme) and 210 (phosphoglycerate kinase) hours (Rogers *et al.*, 1986). Compared to these proteins, Nrf1 can be regarded as a relatively short-lived protein; however, amongst the transcription factors, it has a relatively long half-life, suggesting that Nrf1 activity may be regulated by other mechanisms, such as post-translational modifications. Next, we found that anti-Nrf1 antibody was able to capture significantly higher levels of ubiquitinated Nrf1, under MG-132 treatment, compared to the DMSO control, in co-immunoprecipitation experiments,

as revealed by immunoprecipitation using anti-Nrf1 antibody followed by immunoblotting with anti-ubiquitin antibody (Figure 3.3C).

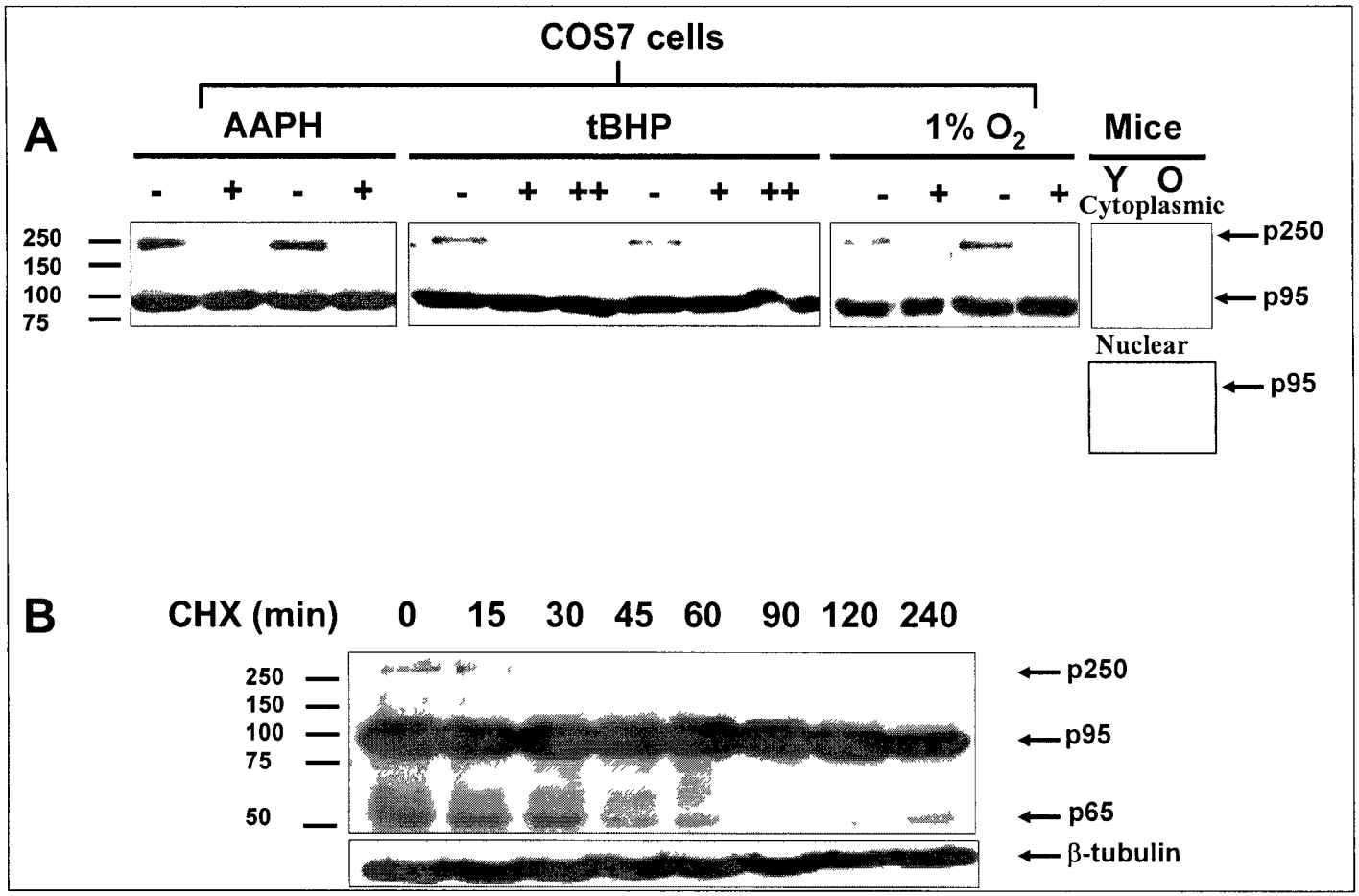
Figure 3.3 Nrf1 Undergoes Ubiquitination and Proteasomal Degradation. **(A)** COS7 cells were treated with 12 μM MG-132, a reversible proteasome inhibitor (Pajonk *et al.*, 2005), for five hours to stabilize p120, the medium was replaced by fresh medium containing 100 μg/mL of CHX and the cells were harvested at different time intervals (0-8 hours) and subjected to immunoblotting. **(B)** The graphical determination of Nrf1 p95 half-life ($t_{1/2}$) using at least three independent experiments ± S.E.M. is shown. **(C)** Immunoprecipitation was performed using anti-Nrf1 antibody as described above in the Methods section on the lysates of COS7 cells, pre-treated with 10 μM MG-132 or DMSO for six hours and the ubiquitinated Nrf1 was visualized using immunoblotting with anti-ubiquitin antibody. The results of two independent experiments are shown.


A
CHX (hours) 0 0 2 4 6 8
MG-132 - + + + + +
MW (kDa)
100
75
p95
β-tubulin
B
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ln([Nrf1]t/[Nrf1]0)
t1/2 = 4.9 ± 0.9 hrs
0 2 4 6 8
CHX-chase time (hours)
C
MG-132 - + - +
MW (kDa)
250
150
100
75
IP: αNrf1
WB: αUb

Thus, Nrf1 appears to be precisely controlled by keeping its levels low through its continuous ubiquitination and degradation by the proteasome. Given its relatively long half-life, compared to Nrf2 and Nrf3, Nrf1 may have additional modifications that allow for ubiquitination, similar to the large subunit of RNA polymerase II (RPB1), which requires phosphorylation prior to ubiquitination in order to be degraded by the proteasome (Kuznetsova *et al.*, 2003). Ubiquitination of the target protein would be dependent upon conditions required for the primary post-translational modification to occur. Thus, the turnover of Nrf1 may be higher under conditions that favor other post-translational modifications.

In addition to ubiquitination, we noticed the disappearance of a high molecular weight form of Nrf1 with an approximate molecular weight of 250 kDa (p250, see Figure 3.4) under stimulated conditions as a result of CHX, oxidative stress (AAPH or tBHQ) and hypoxia treatments. Furthermore, a similar decrease in the p250 content in aged mice tissues was observed using a different antibody, recognizing the C-terminus region of Nrf1 (C-19, Santa Cruz, see Figure 3.4). The identity of this stress-responsive band merits further investigations and could represent post-translational modification of Nrf1 (e.g., ubiquitination), Nrf1 homodimer, ER membrane-bound Nrf1 or Nrf1, covalently bound to some unknown protein. That such a complex could be observed under reducing and denaturing gel electrophoresis discussion is explained in Section 4.5 of the thesis using previously-published data.

Figure 3.4. Oxidative Stressors AAPH and tBHQ, Hypoxia, CHX and Aging Destabilize the p250 Form of Nrf1. COS7 cells were treated for six hours with (**A**) 80 mM AAPH, 100 (+) or 200 (++) μM tBHP or hypoxia for twenty-four hours or with (**B**) 100 μg/mL CHX (Schneider-Poetsch *et al.*, 2010) for the times indicated, after which cells were harvested and total cell lysates were subjected to immunoblotting with anti-Nrf1 or anti-β-tubulin antibodies. Molecular masses are indicated in kDa. The results of two independent experiments (**A**) or a representative result of three independent experiments (**B**) are shown. Nrf1 forms (p65, p95 and p250) are indicated with arrows In (**A**), Y and O refers to mouse liver cytoplasm extract samples from young (6 month) or old (21 month), respectively. Even though the expression of p95 was not seen in the cytoplasmic fraction ("Cytoplasmic"), it was clearly discernable in the nuclear fraction ("Nuclear") and the position of p250 in mouse liver sample was verified using appropriate molecular weight standards.


COS7 cells
A
AAPH
tBHP
1% O2
Mice
Y O
Cytoplasmic
Nuclear
250
150
100
75
p250
p95
p95
B
CHX (min) 0 15 30 45 60 90 120 240
250
150
100
75
50
p250
p95
p65
β-tubulin

3.3 Nrf1 Protein Expression under Proteasomal Inhibition and Hypoxia

With the observation that MG-132 and lactacystin stabilized p120, we wanted to determine if the protein expression of Nrf1 is also affected by hypoxia. To this end, we subjected COS7 and WFF2002 cells to hypoxic conditions (1% O_2). Hypoxia is known to induce the expression of genes involved in iron metabolism, many of which are transcriptionally regulated *via* the CNC-bZIP factors to which Nrf1 belongs (Chepelev and Willmore, 2011). Similarly, hypoxia activates the expression of metallothioneins (MT) 1 and 2 (Yamasaki *et al.*, 2007); these genes contain the ARE sequence and are known targets of Nrf1, but not Nrf2 (Ohtsuji *et al.*, 2008). Intracellularly, hypoxia treatment has many similarities with proteasomal inhibition; for instance, hypoxia-inducible factor alpha subunits (HIFαs) are stabilized when the proteasome is inhibited, which leads to HIFα-mediated activation of hypoxia-inducible gene expression. In addition, multiple studies have reported an increased rate of ROS production under hypoxia (Klimova and Chandel, 2008) and ROS are known activators of the ARE-Nrf1 pathway. Given that hypoxia results in the generation of ROS, we wanted to study the effects of hypoxia on Nrf1 function in order to elucidate the mechanisms of Nrf1 regulation in greater detail.

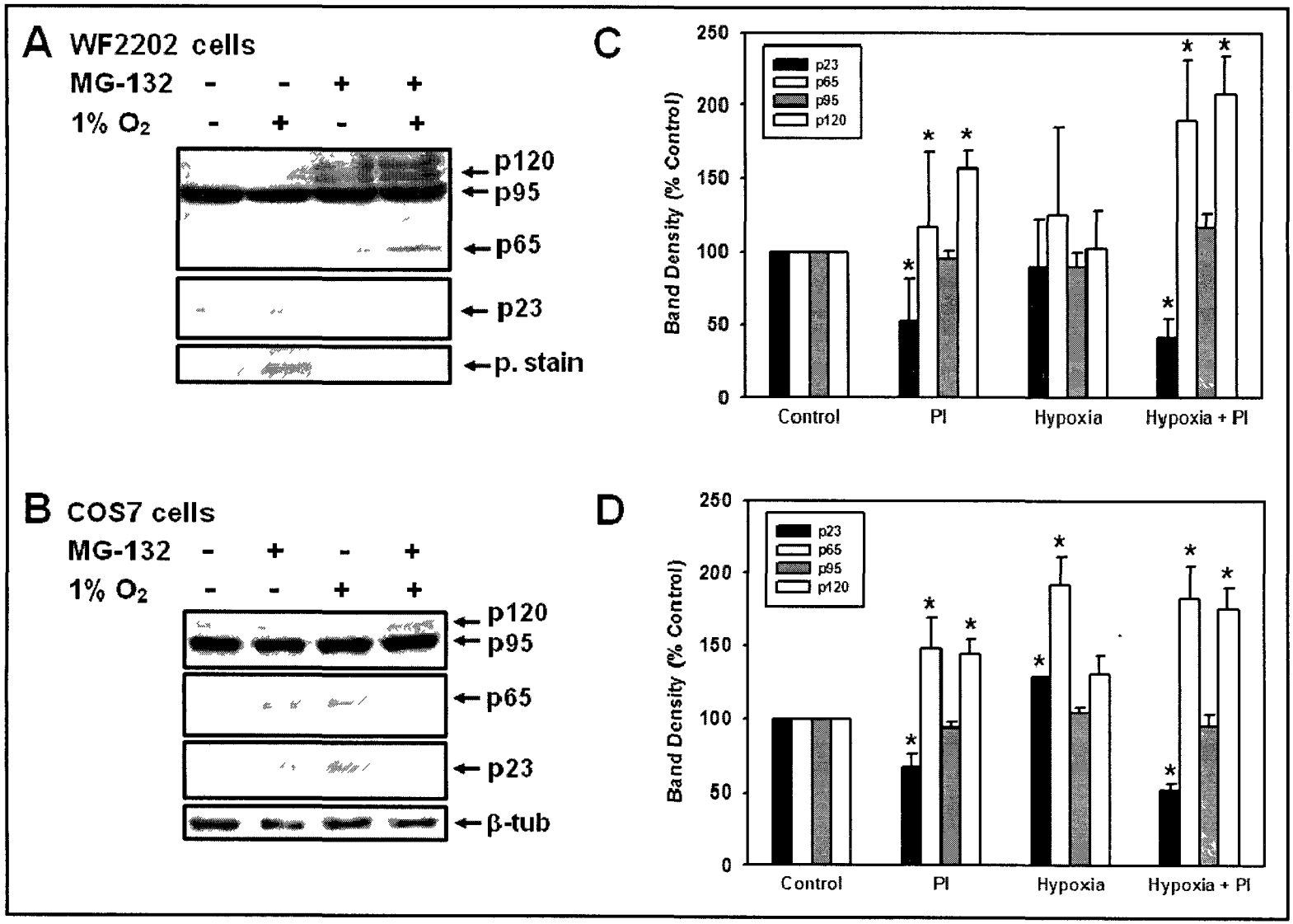
Unlike MG-132 treatment, no stabilization of the p120 form was observed in hypoxic treatments for both cell lines (Figures 3.5A and B), suggesting that the effect of combining these two treatments is not different from applying each treatment individually. Since the accumulation of the p120 form was also noticeable in WFF2002

cells (Figure 3.5A), the mechanism of Nrf1 control through proteasomal degradation is not likely to be cell-specific. In addition to increased expression of p120, another form of Nrf1 that migrated as a 140 kDa protein was detectable upon immunoblotting (Figure 3.5A). This band was seen in other instances using longer film exposure times and greater amount of protein loaded per gel lane (data not shown). Given the results (described in Figure 3.3C), indicative of Nrf1 ubiquitination, it is plausible to suggest that this higher molecular form of Nrf1 represents ubiquitinated form of p120. As ubiquitin is a small protein, comprised of 76 amino acids, and its addition shifts the molecular weight of a protein by approximately 10 kDa (Seyfried *et al.*, 2008), the Nrf1 band migrating as an approximately 140-kDa protein could represent mono- or di-ubiquitinated Nrf1.The band density corresponding to p65 Nrf1, a dominant negative inhibitor of ARE-driven gene expression (Wang *et al.*, 2007), was markedly increased after 6 hours of MG-132 treatment in WFF2002 cells (Figure 3.5A) and in both MG-132- and hypoxia-treated COS7 cells (Figure 3.5B). Very little is currently known about p65 and its role in ARE-mediated gene expression, apart from its negative effect on the ARE pathway and cell type-specific accumulation of p65 might provide some hints towards its function and regulation. We observed no changes at the mRNA levels for all four conditions tested (data not shown), supporting the involvement of post-translational modifications in Nrf1 regulation by the proteasome and hypoxia.

Interestingly, in both cell lines, the intensity of the band migrating as a 23 kDa protein ("p23" Nrf1) was diminished during proteasomal inhibition (see Figures 3.5A and B). Since our anti-Nrf1 antibody recognizes the N-terminal fragment of Nrf1 (amino

acids 191-475) and p23 is always highly expressed, this suggests that this is a product of N-terminal cleavage, in addition to the ubiquitin-dependent proteasomal degradation discussed above, p23 likely represents either protease- and/or a stable, proteasome-generated N-terminal fragment of Nrf1. A band of approximately the same molecular weight as p23 is also observed using another antibody, H-285 (Santa-Cruz, http://www.scbt.com/datasheet-13031-nrf1-h-285-antibody.html), which confirms that p23 is a fragment of Nrf1 and not some non-specific protein, cross-reacted with the antibody used. That Nrf1 is processed at its N-terminus is in line with the previous studies, suggesting that p120 Nrf1 must be processed into smaller forms in order to remove the inhibitory NTD and allow the protein to act as a transcription factor (see (Schultz *et al.*, 2010) and references therein). The N-terminal cleavage of Nrf1 might be an important way of regulating this factor as that would allow it to escape the ER and to translocate to the nucleus, where it could activate the expression of the ARE-controlled genes. The conclusion that p23 is indeed a Nrf1 fragment was based on the following: i) the N-terminal location of the Nrf1 region, recognized by the antibody employed; ii) the observation that p23 signal was diminished following proteasomal inhibition in both COS7 and WFF2002 cells (Figure 3.5), with concomitant increase in the p120 Nrf1; and iii) the fact that the overexpression of the Nrf1, FLAG-tagged at its N-terminus was able to transactivate the ARE-luciferase reporter, but could not be seen following FLAG immunoblotting (data not shown), unlike the Nrf1, FLAG-tagged at its C-terminus that can be visualized by using anti-FLAG antibody as will be shown.

Figure 3.5. Comparison of the Nrf1 Expression under Hypoxia and Proteasomal Inhibition. WFF2002 (**A**) and COS7 (**B**) cells were treated with normoxia (21% O_2), hypoxia (1% O_2), 10 μM MG-132 or hypoxia and MG-132 combined for six hours and subjected to Western blotting. The band density was normalized with respect to β-tubulin or Ponceau S red and is presented as means ± S.E.M. of at least three independent experiments for WFF2002 (**C**) and COS7 (**D**) cells. Asterisks (*) indicate significant difference ($p < 0.05$) compared to controls using Student's t-test.

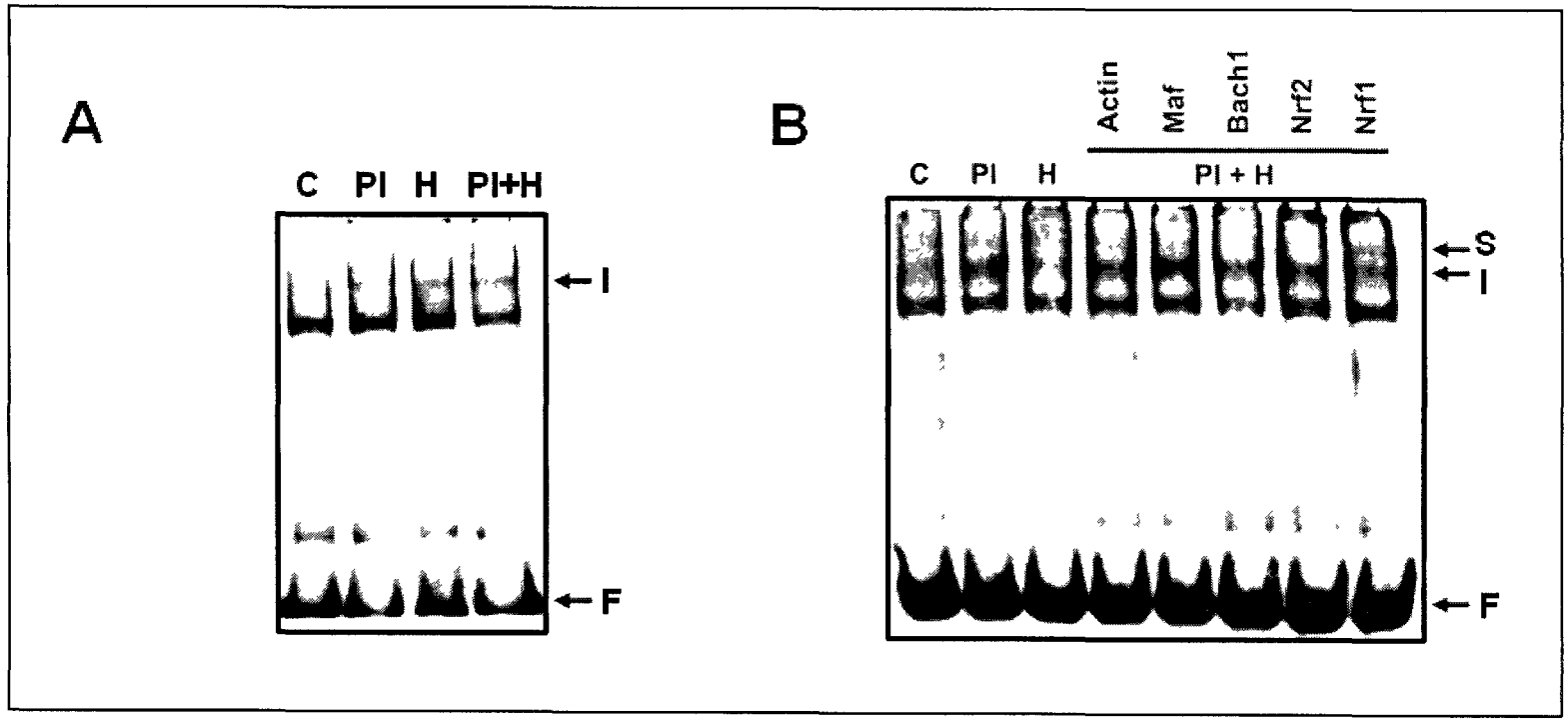

A WF2202 cells
MG-132 - - + +
1% O2 - + - +
p120
p95
p65
p23
p. stain
B COS7 cells
MG-132 - + - +
1% O2 - - + +
p120
p95
p65
p23
β-tub
C
Band Density (% Control)
250
200
150
100
50
0
p23
p65
p95
p120
Control
PI
Hypoxia
Hypoxia + PI
D
Band Density (% Control)
250
200
150
100
50
0
p23
p65
p95
p120
Control
PI
Hypoxia
Hypoxia + PI

Other ER-bound transcriptional factors are similarly regulated through intermembrane proteolysis and include the sterol-regulatory-element-binding proteins SREBP1 and SREBP2, as well as activating transcription factor 6 (ATF6), and this regulation has been well-characterized (see Wang and Chan, 2006), and references therein). Previous studies (Zhang *et al.*, 2007; Zhang and Hayes, 2010) failed to map any proteolytic cleavage site within the first 170 amino acids at the N-terminus; however, the studies were performed at normal homeostatic conditions and the possibility of Nrf1 regulation by intramembrane proteolysis under stimulated conditions cannot be ruled out (Zhang and Hayes, 2010). In contrast, a more recent study (Steffen *et al.*, 2010) provided some, albeit indirect, support for the intramembrane proteolysis of Nrf1 prior to its translocation to the nucleus from the ER.

3.4 MG-132 and Hypoxia Enhances Protein Binding to the ARE

Once we showed that proteasomal inhibition stabilizes Nrf1, we investigated whether MG-132 and/or hypoxia have any effects on Nrf1 DNA-binding to the ARE. Using electrophoretic mobility shift assays (EMSAs), we observed the appearance of MG-132- and hypoxia-inducible bands (Figure 3.6A). These results are consistent with the previously reported study of Waleh and co-workers (1998), showing hypoxia-inducible DNA-binding to AREs in human HepG2 and mouse Hepa cells.

Figure 3.6. Hypoxia and Proteasomal Inhibition Increase Protein Binding to the ARE. Biotin-labelled ARE probe from the *gclm* promoter was applied to 20 μg of total COS7 cell lysate protein in an EMSA format as described in the Methods Section, Chapter 2. (**A**) Depicts the appearance of the hypoxia- and MG-132-inducible band. C, PI, H and PI+H designate control (DMSO), proteasomal inhibition (MG-132), hypoxia (1% O_2 and DMSO) and the combination of hypoxia and MG-132 treatments, respectively. (**B**) The identity of the ARE-bound complex was probed using antibodies against the proteins indicated in the immunodepletion format. F, free probe; I, inducible band; S, supershift. Maf refers to an antibody, recognizing all three forms of small Maf proteins (MafF, G and K).

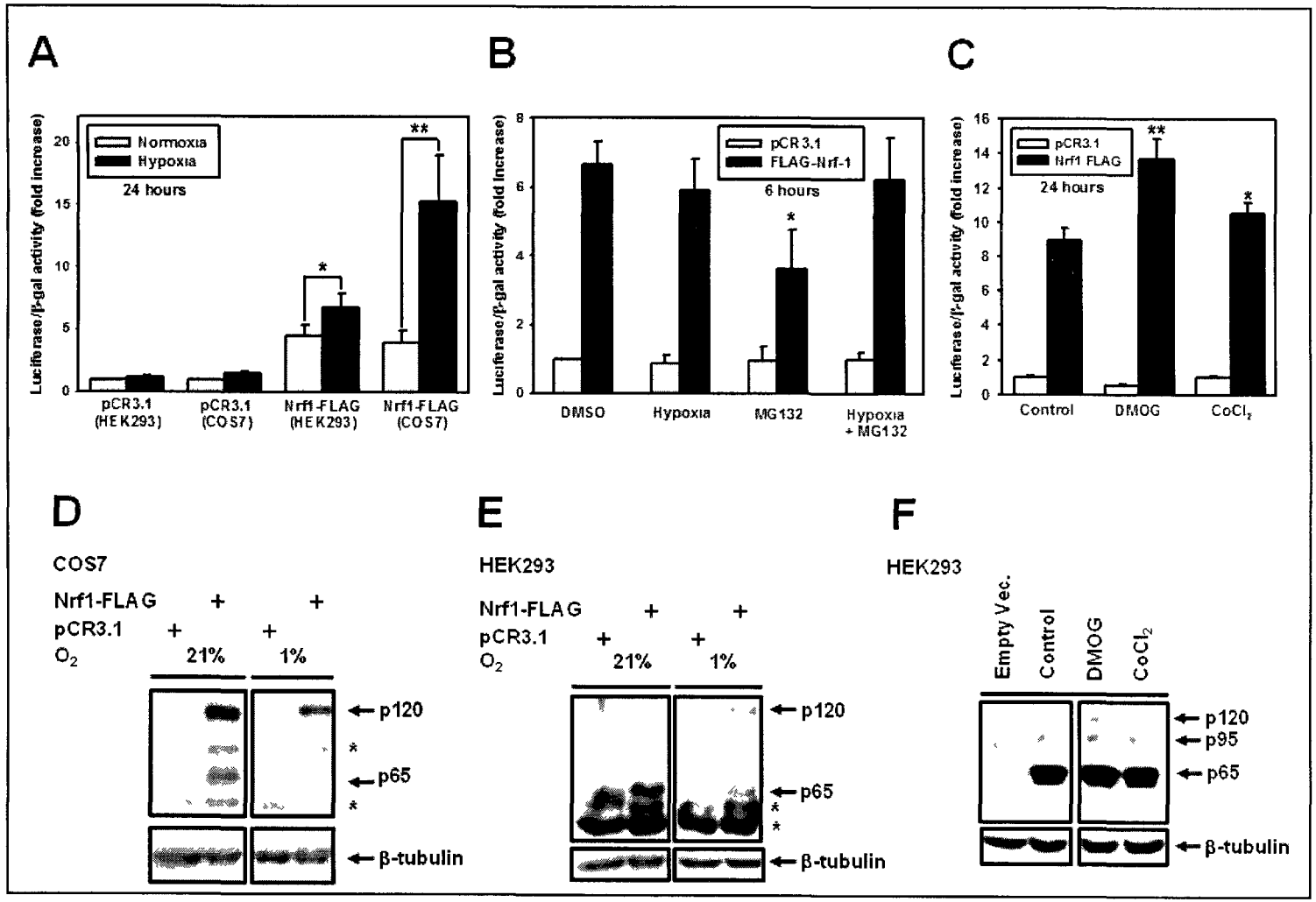

A
C
PI
H
PI+H
I
F
B
Actin
Maf
Bach1
Nrf2
Nrf1
C
PI
H
PI + H
S
I
F

To identitfy the proteins responsible for the hypoxia- and MG-132-inducible ARE-binding, we used an immunodepletion approach (Dickinson *et al.*, 2003). In this method, an antibody raised against a DNA-binding protein diminishes the intensity of an EMSA band if the DNA-binding protein is, indeed, bound to the target DNA sequence under investigation (Dickinson *et al.*, 2003). Apart from Nrf1, the obvious candidate factors that could be involved in the MG-132- and hypoxia-inducible ARE complex formation could be Nrf2 as it is stabilized by proteasomal inhibition and Bach1, whose activation by hypoxia has been previously described (Kitamuro *et al.*, 2003). As can be seen from Figure 3.6B, anti-Nrf1 antibody resulted in the appearance of an "upshift" or "supershift", not clearly discernable when other antibodies were used. This suggests potential involvement of Nrf1 in the hypoxia- and MG-132-inducible binding to an ARE probe. However, the MG-132- and hypoxia-inducible band was still robust following the anti-Nrf1 antibody addition, suggesting that other proteins may be involved in the increased ARE binding observed here. These other proteins could be some unknown ARE-binding proteins, small Mafs or other CNC-bZIP factors, known to bind to the ARE, whose binding was not seen in this experiment probably because an antibody, bound to a DNA-binding protein can block the protein-DNA interaction. Since enhanced ARE binding could result in either activation or repression of the ARE target genes, depending on the nature of the binding factor involved (activator or repressor), we used a luciferase reporter assay, in combination with Nrf1 overexpression, to gain a better understanding of the Nrf1 transactivation function in response to hypoxia and proteasomal inhibition treatments.

3.5 Hypoxia Activates and Proteasomal Inhibitors Inhibit Exogenous Nrf1 Activity

We tested if proteasomal inhibition and hypoxia have any effect on the transactivation activity of Nrf1. To this end, we used a luciferase reporter vector under the control of three AREs from chicken β-globin enhancer (3xARE-luciferase (Igarashi *et al.*, 1994)). Transient co-transfection of 3xARE-luciferase with Nrf1-FLAG in COS7 cells showed that 24-hour hypoxia treatments greatly increased the activity of Nrf1-FLAG (Figure 3.7A). Furthermore, hypoxic activation of Nrf1 is probably not cell type-specific as the same results were seen in HEK293A cells (Figure 3.7A). Neither does Nrf1 hypoxic inducibility seem to be an intrinsic property of this particular CNC-bZIP family member as exogenous Nrf2, overexpressed in COS7 cells, also responded to 1% O_2 in a manner, similar to Nrf1 (Figure 3.7A). In addition to low oxygen, Nrf1-FLAG activation is achievable by hypoxia mimetics cobalt chloride and dimethyloxalylglycine (DMOG, Figure 3.7C). In contrast, MG-132 markedly decreased the activity of Nrf1-FLAG while hypoxia had no effect on the Nrf1 activity during six-hour treatments (Figure 3.7B). Again, the action of MG-132 on exogenous Nrf1 activity can be understood in light of the current hypothesis that the processing of Nrf1 by the 26S proteasome could remove its inhibitory NTD (see Figure 1.4 for Nrf1 domains), targeting Nrf1 to the ER. Blocking Nrf1 processing into p23 and other active forms of Nrf1 (such as p95) by proteasome inhibition would interfere with Nrf1 function, which is in accord with the observed repression of the Nrf1 activity by MG-132 treatment (Figure 3.7B). Therefore, proteasomal processing seems to be a prerequisite for Nrf1 activation and function.

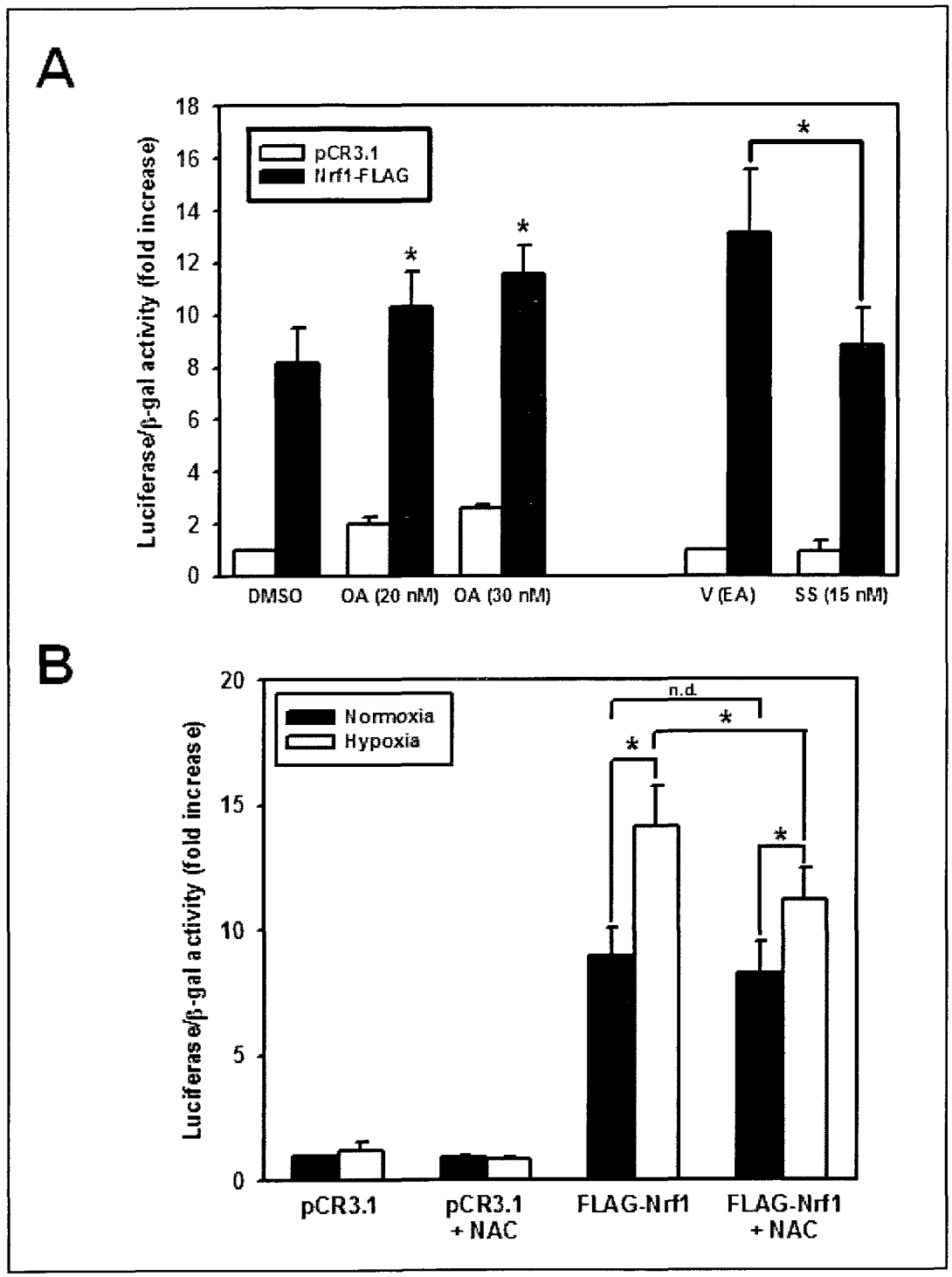
Figure 3.7. Hypoxia and Hypoxic Mimetics Activate Nrf1, while MG-132 Inhibits its Transactivation Activity. COS7 and HEK293A cells were co-transfected with 3XARE-luc, β-galactosidase and Nrf1-FLAG or Nrf2-myc plasmids, and treated with hypoxia, 10 μM MG-132, 2.5 mM DMOG or 200 μM $CoCl_2$ for twenty-four ((**A**) and (**C**)) or six (**B**) hours as indicated and the luciferase activity in the lysates was measured. The pCR3.1 plasmid, lacking Nrf1 and Nrf2, was used as a negative control and β-galactosidase activity was used to determine transfection efficiency. The means of at least three independent experiments ± S.E.M., normalized to the β-galactosidase activity and pCR3.1, are presented. Asterisks (*,**) indicate significant difference ($p < 0.05$ or $p < 0.001$, respectively) compared to controls. The lysates of hypoxia-treated (1% O_2, 24 hours) COS7 (**D**) and HEK293A (**E**) and (**F**) cells were also subjected to immunoblotting using anti-FLAG antibody. In **(D)** and **(E)**, asterisks (*) denote the non-specific, cross-reacting bands. Representative blots of three independent experiments are shown.


A
Luciferase/β-gal activity (fold increase)
Normoxia
Hypoxia
24 hours
pCR3.1 (HEK293)
pCR3.1 (COS7)
Nrf1-FLAG (HEK293)
Nrf1-FLAG (COS7)
B
pCR3.1
FLAG-Nrf-1
6 hours
DMSO
Hypoxia
MG132
Hypoxia + MG132
C
pCR3.1
Nrf1 FLAG
24 hours
Control
DMOG
CoCl2
D
COS7
Nrf1-FLAG
pCR3.1
O2
21%
1%
p120
p65
β-tubulin
E
HEK293
F
HEK293
Empty Vec.
Control
DMOG
CoCl2
p120
p95
p65
β-tubulin

3.6 Potential Effect of p65 Nrf1 on the Hypoxic Inducibility of Nrf1

The p65 form of Nrf1 is thought to be produced by either internal proteolysis (Zhang *et al.*, 2007) or as a result of translation initiation from an alternative Met codon (Chan *et al.*, 1993). The p65 Nrf1 form is thought to act as a dominant negative inhibitor of the ARE-driven gene expression (Wang *et al.*, 2007) as it contains a DNA-binding domain, but lacks a transactivation domain, such that p65 Nrf1 competes with Nrf2 for the ARE binding site. As seen in Figures 3.7D and E, the expression of the p65 form of Nrf1 markedly decreased during hypoxia treatment in both COS7 and HEK293A cell lines, while no other common change was noticeable during hypoxia. Our data presented here is in agreement with the model in which the ARE-mediated gene expression is activated as a result of down-regulation of the inhibitory p65 form of Nrf1. The two internal Met residues that are thought to give rise to p65 are M321 and M326 (Chan *et al.*, 1993) which contain consensus Kozak sequences, not present in the M1 codon. Mutating M321 and M326 to M321L and M326L, respectively, resulted in significant loss of basal Nrf1-FLAG activity and hypoxia inducibility; however, unexpectedly, mutants were not expressed at detectable levels (data not shown). This may be due to aggregation of mutant forms of the protein. Alternatively, this apparent lack of expression and any transactivation activity by the mutants could be explained by their increased instability. No significant effect of the transfection with the mutant-bearing plasmid on the cell viability was seen using the MTT assay or β-galactosidase activity (data not shown).

Figure 3.8. Phosphorylation Activates Nrf1 and Antioxidant Treatment Only Partially Abolishes the Hypoxic Inducibility of Nrf1. The luciferase assays were performed on the COS7 cell lysates, co-transfected with 3XARE-luciferase, and Nrf1-FLAG plasmids treated with (**A**) okadaic acid (OA) and staurosporine (SS), to inhibit protein dephosphorylation and phosphorylation, respectively, or with a combination of an antioxidant NAC and hypoxia (**B**) for twenty-four hours. DMSO or ethyl acetate (EA) was used as vehicle controls (V) in and the concentration of each compound used is indicated in (**A**). The means of at least three independent experiments ± S.E.M. are shown. Asterisks (*) indicate significant differences ($p < 0.05$) between indicated pairs or controls as calculated using paired Student's t-test.


A
Luciferase/β-gal activity (fold increase)
pCR3.1
Nrf1-FLAG
DMSO
OA (20 nM)
OA (30 nM)
V (EA)
SS (15 nM)
B
Luciferase/β-gal activity (fold increase)
Normoxia
Hypoxia
n.d.
pCR3.1
pCR3.1 + NAC
FLAG-Nrf1
FLAG-Nrf1 + NAC

3.7 Nrf1 is Activated by Phosphorylation

Our quick computational analyses predicted a high probability of Nrf1 phosphorylation by cdc2 and protein kinase C (PKC) members, especially at the Ser-rich region (NST), facing the ER lumen. Zhang and others (2009) provided some evidence that phosphorylation of Nrf1 at its NTD (which contains two potential Tyr phosphorylation motifs, amino acids 62-70 in human Nrf1), may weaken the Nrf1 association with the ER, stimulating the Nrf1 trafficking to the nucleus. Given that phosphorylation by atypical PKC is known to activate Nrf2 (Numazawa *et al.*, 2003), we thought that Nrf1 could also be a subject to this modification. To test that, we performed luciferase assays on the COS7 cells co-transfected with Nrf1-FLAG and 3xARE-luciferase and subjected to protein phosphatase inhibitor okadaic acid (OA) (Nguyen *et al.*, 2003; Zhang and Forman, 2008) or pan-PKC inhibitor staurosporine (SS) (Zhang *et al.*, 2008). Figure 3.8A illustrates that OA activated Nrf1, while SS repressed Nrf1 activity, suggesting that Nrf1 phosphorylation plays a role in Nrf1 transactivation. Notably, the OA treatment resulted in about 2-fold increase of the empty vector (pCR3.1)-mediated ARE-luciferase activation, changing the ARE-driven luciferase activity from 1.0- to 2.0-fold with respect to the control vector. This suggests that there is about 1-fold increase in the "noise" signal due to other, transcriptional factors, such as Nrf2, being activated through phosphorylation. However, treatment with OA changed the ARE-luciferase reporter activity due to Nrf1-FLAG from 8.15 ± 0.7- to 10.3 ± 0.6- and 11.6 ± 0.6-fold for 0, 10- and 20-nM treatments with OA, respectively, compared to empty vector-transfected controls (pCR3.1). Therefore, the net increase in Nrf1-FLAG activity was 2.15-fold

compared to 1-fold increase due to "noise". These results, as well as a similar study on the effect of OA on Nrf2 (where an approximate 0.5-fold increase in the Nrf2 activity due to OA treatment above background was interpreted as a stimulatory effect of OA on Nrf2 activity (see Figure 6 in Nguyen *et al.*, 2003), suggests that Nrf1 positively responds to phosphorylation stimulus. Decreased transactivation activity of Nrf1, as a result of SS treatment, strengthens the claim that the phosphorylation status of Nrf1 is important for Nrf1 activity. It is possible that some upstream factors in the signaling cascade that control Nrf1 function responds to phosphorylation/dephosphorylation rather than Nrf1 itself and further studies are required to confirm the target of phosphorylation in the Nrf1 pathway.

3.8 The Potential Involvement of ROS in Hypoxic Inducibility of Nrf1

Low oxygen conditions are known to increase ROS production from mitochondrial electron-transport chain (reviewed in Klimova and Chandel, 2008). To test if increased ROS levels could be responsible for Nrf1 activation, we treated Nrf1-FLAG- and 3xARE-luciferase-transfected COS7 cells with hypoxia in combination with the antioxidant N-acetylcysteine (NAC). As seen from Figure 3.8B, induction of luciferase activity by hypoxia was only partially abolished with NAC. The data suggest that ROS may, at least in part, contribute to hypoxic activation of exogenous Nrf1. The existence of other mechanisms responsible for Nrf1 activation, however, cannot be ruled out.

3.9 The Two Proposed Levels of the Proteasomal Control of Nrf1

The levels of key inducible transcription factors are normally kept low under homeostatic conditions, through their ubiquitination and continuous degradation by the proteasome, to avoid aberrant gene expression. This is the case for transcription factors such as HIFα and the CNC-bZIP factors Nrf2 and Nrf3. As expected, we found that Nrf1 was also negatively regulated by the proteasome, being subject to ubiquitination and proteasomal degradation in agreement with Zhang and co-workers, who suggested that Nrf1 abundance may be controlled by the proteasome (Zhang *et al.*, 2009). However, given the rather high half-life of Nrf1, compared to Nrf2 and Nrf3, the existence of other regulatory mechanisms for Nrf1 is very likely. One might wonder how the proteasome, which is typically localized to the cytoplasm and nucleus, can come into contact with membrane-bound Nrf1. Perhaps, Nrf1 could be partially processed by the proteasome once p95 has been glycosylated to become p120 and prior to its integration in the ER membrane as has been suggested to be the case for Nrf3 (Chevillard and Blank, 2011). We observed high expression of the p23 fragment of Nrf1 under untreated conditions, which diminished following MG-132 treatment and provides further evidence that the p23 is derived from Nrf1 and does not represent nonspecific binding of the antibody to some other protein. Since p23 would be expected to contain about 210 amino acids, and the recognition region of our antibody is between amino acids 191-475, we believe that p23 represents an N-terminal fragment of the Nrf1, cleaved twice in the amino acids 191-475 region, with the distance between the two cleavage sites being approximately 210 amino acids. Further support of this hypothesis has been shown in previous experiments, where Site-1 or Site-2 protease cleavage sites within the first 170 amino acids of Nrf1 could not be

identified (Zhang *et al.*, 2007; Zhang and Hayes, 2010). Unlike the multicatalytic proteasome complexes, the membrane-bound Ser peptidases Site-1 and Site-2 are metalloproteases, which recognize the general motifs R-(R/K/H)-L-(A/L/S/T/F, Site-1) and GPxxN/S/G or NxxPxxxxDG (Site-2, where x stands for any amino acid) on their protein substrates, (Rojek et al., 2008; Kinch et al., 2006) and participate in two-step cleavage of their substrates, such as SREBP1 (Zhang et al., 2006). In addition to Site-1 and Site-2 proteases, the proteasome can also act as a site-specific protease that induces only partial as opposed to global protein cleavage of proteins, leading to their complete degradation, which is a more common function of the proteasome. An example of site-specific, partial proteolysis carried out by the proteasome is he proteasomal cleavage of NF-κB as described in greater detail further in this section. Our results support the hypothesis of Zhang and co-workers (Zhang *et al.*, 2007) that Nrf1 is proteolytically cleaved at regions other than the NTD. Cleavage by Site-1 and Site-2 intramembrane proteases is a well-known mechanism, responsible for the release of ER-bound transcriptional factors, but the possibility that Nrf1 is cleaved at the AD1, NST and AD2 domains merits further investigation. This possibility is also supported by Steffen and co-workers (2010), who noticed that the molecular weight of the unglycosylated Nrf1 is still higher than that of the nuclear form of Nrf1 and concluded that intramembrane proteolysis is a feasible mechanism for Nrf1 release to the nucleus from the ER.

The second or "positive" level of Nrf1 regulation by the proteasome, in addition to complete ubiquitin-mediated proteasomal degradation, appears to be partial proteolytic processing by the proteasome. In accord with the current hypothesis of the Nrf1

regulation by removal of the NTD domain from full-length Nrf1 (Schultz *et al.*, 2010), proteolytic cleavage of Nrf1 allows it to bypass insertion into the ER and travel to the nucleus. This hypothesis was supported by the fact that proteasomal inhibition with MG-132 not only stabilized p120 Nrf1 and decreased p23 levels, but also repressed Nrf1 activity. A very similar mode of activation, through the processing of p105 to the DNA-binding, p50 form of NF-κB by the 26S proteasome, has been described (Magnani *et al.*, 2001; Tanaka *et al.*, 2000). A recent study has demonstrated the involvement of Nrf1 in the "bounce-back" response, an elevated proteasomal subunit synthesis which is observed upon proteasome inhibition (Radhakrishnan *et al.*, 2010). According to the study, Nrf1 directly activates the proteasome recovery pathway upon proteasome inhibition. For that to happen, Nrf1 itself must be activated by 1 μM MG-132 treatment, which was shown by the investigators (Radhakrishnan *et al.*, 2010). This is in contrast to our results, as well as the results of others (see Figure 2 of Zhang *et al.*, 2009), demonstrating that MG-132 and another proteasome inhibitor, ALLN, applied at 10 or 13 μM concentrations respectively, represses exogenous Nrf1 activity. While both 1 and 10-13 μM concentrations of the proteasome inhibitors were able to stabilize p120 Nrf1, abolishing the proteasomal degradation of Nrf1 completely, it is conceivable that higher concentrations of proteasomal inhibitors are required for the inhibition of the Nrf1 partial processing/activation by the proteasome (which is known to possess several catalytic activities). Thus, it can be envisioned that the stimulatory effect of a proteasomal inhibitor on Nrf1 activity, resulting from its blockade of the proteasomal degradation of Nrf1, can be outweighed by the repression of the proteasome-mediated activation of Nrf1 through partial proteolytic processing at higher (≥10 μM) proteasome inhibitor

concentrations. Another possible explanation for this discrepancy is the fact that reporter plasmids with AREs from different genes (utilizing different promoters) were used in these different studies and while one set of ARE-controlled genes can be turned on by a given conditions, other ARE-driven genes can be turned off due to the complex interplay between co-activator and co-repressor proteins (Zhou and Fahl, 2001). Testing a wider range of MG-132 concentrations could have provided useful insights with respect to Nrf1 and mechanisms of negative and positive regulation by the proteasome

3.10 Potential Significance of the Stress-inducible Nrf1 Forms

Our findings have emphasized the role of multiple forms of Nrf1. Indeed, Nrf1 is known to exist in several forms, including p120 (glycosylated Nrf1), p95 (non- or unglycosylated Nrf1), inhibitory p65 form as well as 46 and 30 kDa forms (Zhang *et al.*, 2009). In addition to these, we report the existence of the p23 Nrf1 form, that seems to be a fragment of proteasomal processing of the full-length Nrf1 and another, high molecular weight p250 Nrf1 form, which is destabilized by oxidative stressors, inhibition of protein synthesis and hypoxia. Since the p250 form responds to multiple stimuli, its characterization will be useful in further attempts to uncover the mechanisms responsible for Nrf1 activation. It is plausible that the p250 form of Nrf1 represents a covalently linked protein-protein or protein-membrane interaction with Nrf1, or a Nrf1 dimer. That it is possible to observe dimer formation on the SDS-PAGE will be discussed in greater

detail in Section 4.5. Another possibility is that p250 is a currently uncharacterized, post-translationally modified, form of Nrf1 such as polyubiquitinated Nrf1.

3.11 Hypoxic Activation of Nrf1 is Accompanied by p65 Nrf1 Down-regulation

This is the first study to report Nrf1 activation by hypoxia. As it has been speculated that the location of Nrf1 in the ER membrane is suitable for the maintenance of the homeostatic redox status of the ER (Zhang *et al.*, 2009), the finding that Nrf1 is a hypoxia-inducible factor is not surprising, given that hypoxia is known to cause the ER stress due to the accumulation of the unfolded proteins in the ER lumen (Mera *et al.*, 2010). Among the genes controlled exclusively by Nrf1 (and not Nrf2) are the metallothioneins (MT 1 and 2) (Ohtsuji *et al.*, 2008). It was found that MT expression is hypoxia-inducible in PCa cells (Yamasaki *et al.*, 2007) and, according to our study, we think that Nrf1 hypoxic inducibility could be responsible for MT upregulation in response to hypoxia. Nrf1 processing must be highly regulated as its full-length p95 form can act as an activator of the ARE-driven gene expression while its shorter form, p65, acts as an apparent inhibitor of the ARE pathway. Using transient overexpression of Nrf1-FLAG, we noticed that the levels of the p65 Nrf1 were significantly lowered by hypoxia, suggesting that the cells possess the ability to control Nrf1 activity by removal of the inhibitory p65 form. The involvement of other mechanisms such as phosphorylation in the hypoxic inducibility of Nrf1 can further augment activation of this CNC-bZIP factor.

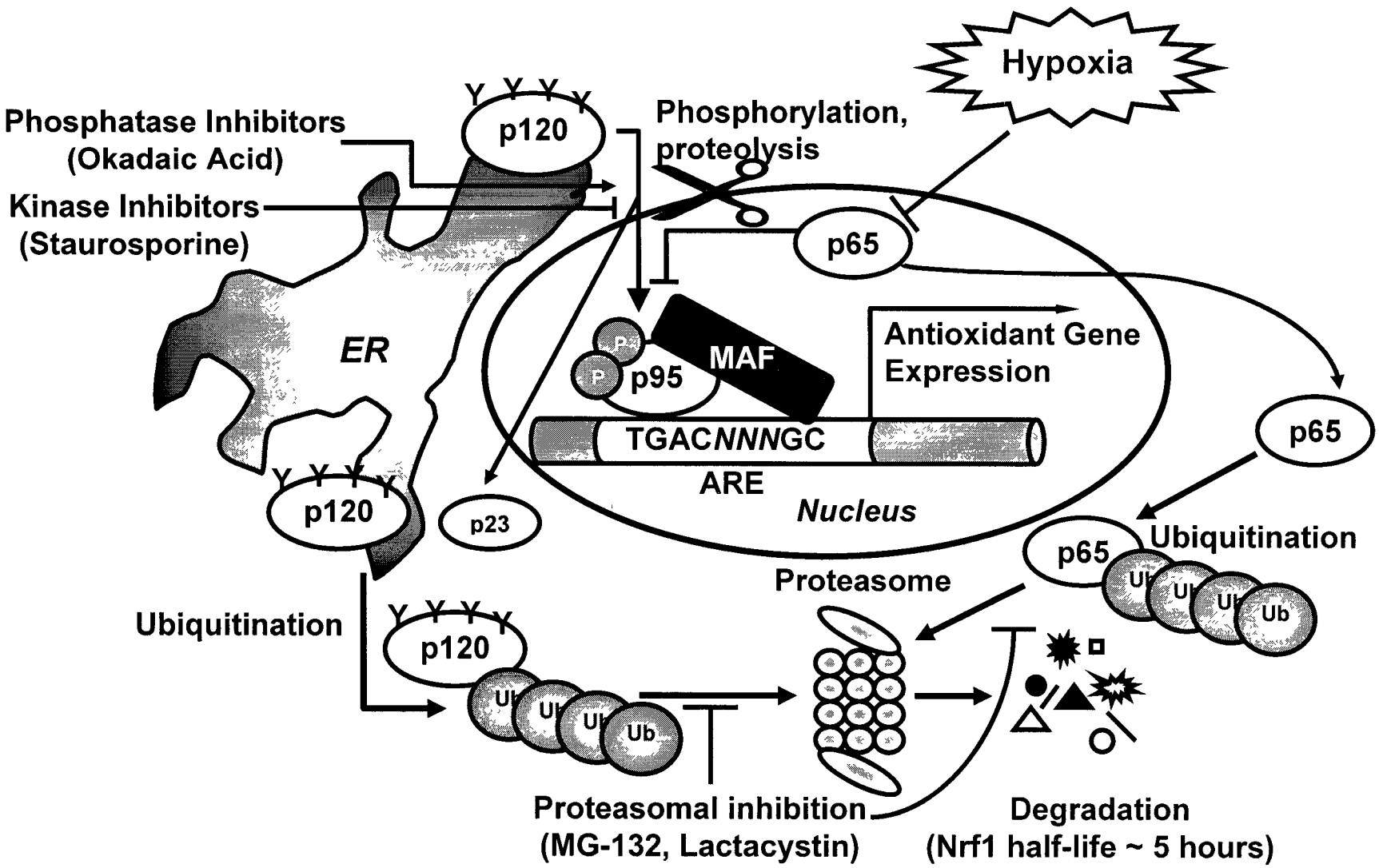
3.12 The Proposed Model for the Nrf1 Regulation by Hypoxia and the Proteasome

This study has opened up new avenues of research regarding the regulation of Nrf1. What is currently unknown is the identity of the kinase(s) and the Nrf1 residue(s) involved in Nrf1 phosphorylation. Zhang and co-workers (2009) speculated that Nrf1 phosphorylation can take place at Tyr65 and Tyr77, which could weaken Nrf1 affinity for the ER membrane and stimulate its nuclear import. As well, the proteolytic processing of Nrf1 requires further investigation to identify the protease responsible for that as well as the site of the cleavage. Furthermore, it is currently unknown how phosphorylation and processing of Nrf are controlled and if the two processes are interdependent. Given that the p250 form of Nrf1 responds to various stimuli, it would be extremely informative to determine whether this form represents a hyperglycosylated or otherwise modified form of Nrf1, why this form is destabilized under stimulated conditions and what is the significance of the destabilization.

In conclusion, our current working model of Nrf1 regulation is summarized in Figure 3.9. According to this model, Nrf1 is a subject to several regulatory events, including: i) negative regulation by the proteasome through ubiquitin-mediated degradation; ii) positive regulation by the proteasome through partial proteolytic processing to generate the p23 fragment; iii) phosphorylation and iv) derepression of its transactivation activity by the removal of the inhibitory p65 Nrf1 form. These putative mechanisms can be investigated 1) by site-directed mutagenesis to reveal the location of

the cleavage, 2) by affinity purification followed by mass spectrometry to reveal the sites of ubiquitin attachment and phosphorylation, as well as the sites of proteolytic cleavage and 3) by site-directed mutagenesis in an attempt to prevent the hypothesized translation of the *Nrf1* gene from an internal Met codon.

Figure 3.9. The Proposed Working Model for the Nrf1 Regulation by the Proteasome and Oxygen. Nrf1 is controlled at the level of protein degradation by ubiquitin (Ub)-mediated proteasomal degradation such that the half-life of Nrf1 is approximately five hours. In addition, Nrf1 is also a subject of proteasome-mediated proteolysis during which its N-terminus, containing the inhibitory NTD, is cleaved to convert Nrf1 from inactive, ER-bound p120 form to the active, nuclear p95 form, releasing the N-terminal Nrf1 fragment, p23. Nrf1 can be also activated through its phosphorylation or the phosphorylation of some other protein, which may affect Nrf1 activity indirectly. Through a currently unknown mechanism, hypoxia acts on p65 Nrf1 to diminish its expression. This relieves the Nrf1 p65-mediated repression (by the removal of p65) on the p95 transactivation activity which, in combination with p95 activation by phosphorylation, may greatly affect the expression of Nrf1-ARE target genes.


Hypoxia
Phosphatase Inhibitors
(Okadaic Acid)
Kinase Inhibitors
(Staurosporine)
p120
Phosphorylation,
proteolysis
p65
ER
P
P
p95
MAF
Antioxidant Gene
Expression
TGACNNNGC
ARE
Nucleus
p65
p120
p23
p65
Ubiquitination
Ub
Proteasome
Ubiquitination
p120
Ub
Proteasomal inhibition
(MG-132, Lactacystin)
Degradation
(Nrf1 half-life ~ 5 hours)

4 Chapter: The Effect of BPA on the Nrf1/2-ARE Pathway

4.1 BPA and its Relation to the ROS Generation and Antioxidant Enzymes

Bisphenol A (BPA) is used in the production of polycarbonate plastics and epoxy resins for baby bottles, liners of canned food and many other consumer products. Previously, BPA has been shown to reduce the activity of several antioxidant enzymes, which may contribute to oxidative stress. However, the underlying mechanism of the BPA-mediated effect upon antioxidant enzyme activity is unknown. BPA has been reported to reduce the activities of catalase and glutathione peroxidase in cultured cells (Oh and Lim, 2008), mice (Kabuto *et al.*, 2003) and rats (Chitra *et al.*, 2003), resulting in oxidative stress conditions. Other pro-oxidant effects of BPA include DNA breakage in MCF-7 cells (Iso *et al.*, 2006). Given the reported relationship between BPA, ROS generation and antioxidant enzymes, we wanted to investigate the effects of BPA on the ARE/EpRE pathway in human embryonic kidney (HEK 293) cells.

Despite previous reports on the pro-oxidant properties of BPA, we predicted that BPA would have antioxidant properties, given that it is a polyphenolic compound and polyphenols are potent antioxidants. Thus, a theoretical prediction of the antioxidant properties of BPA was attempted. A good antioxidant is expected to sacrifice an electron, usually from a weak R-H bond to reduce the attacking species, after which an antioxidant would either be regenerated by compounds such as ascorbate, or undergo a disproportionation reaction to form unreactive species, terminating the proliferation of oxidative damage. The strength of the sacrificial R-H bond has to be sufficiently low to allow for the quenching of peroxyl radicals (ROO•) through the regeneration of the

ROO-H bond, the BDE of which is approximately 88 kcal/mol. For the regeneration reaction to be favorable, the sacrificial R-H BDE has to be below that of the ROO-H bond. It is also desirable for the antioxidant in question to be quenched by cellular antioxidants, such as ascorbate; the BDE for which is 68.5 kcal/mol. A compound would exhibit antioxidant capacity if the BDE of its sacrificial R-H bond falls within the lower portion of this window (Hussain *et al.*, 2003). The weakest bond in BPA is the O-H bond, with a BDE of 83.9 kcal/mol ((calculated using the MLM2 method (Wright *et al.*, 2001)), indicating that BPA may be considered a weak antioxidant. It has been noted (Kovacic, 2010) that, as a minor route of xenobiotic metabolism, BPA can be hydroxylated at the ortho position to form a catechol, susceptible to redox cycling and ROS production (Figure 4.1). These factors make BPA unfavorable as a direct antioxidant, suggesting that the antioxidant capacity of BPA may come due to some indirect effect, such as the activation of the Nrf1/2-ARE pathway, as described in this study. Given the reported pro-oxidant activity of BPA in cultured cells, *I hypothesized that BPA might lead to ROS generation by affecting the Nrf1/2-ARE pathway-mediated expression of antioxidant genes.* The data, presented here, suggests that BPA activates the Nrf1/2-ARE pathway, as reflected by increased Nrf1 transactivation activity and gene expression of ARE target genes *ho-1* and *nqo1*.

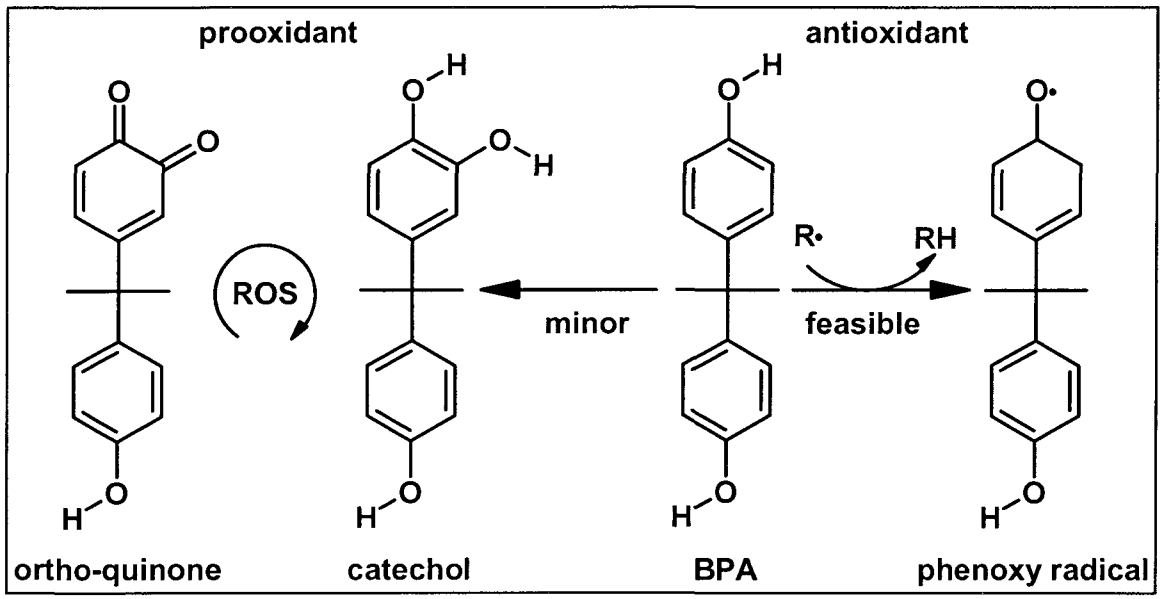


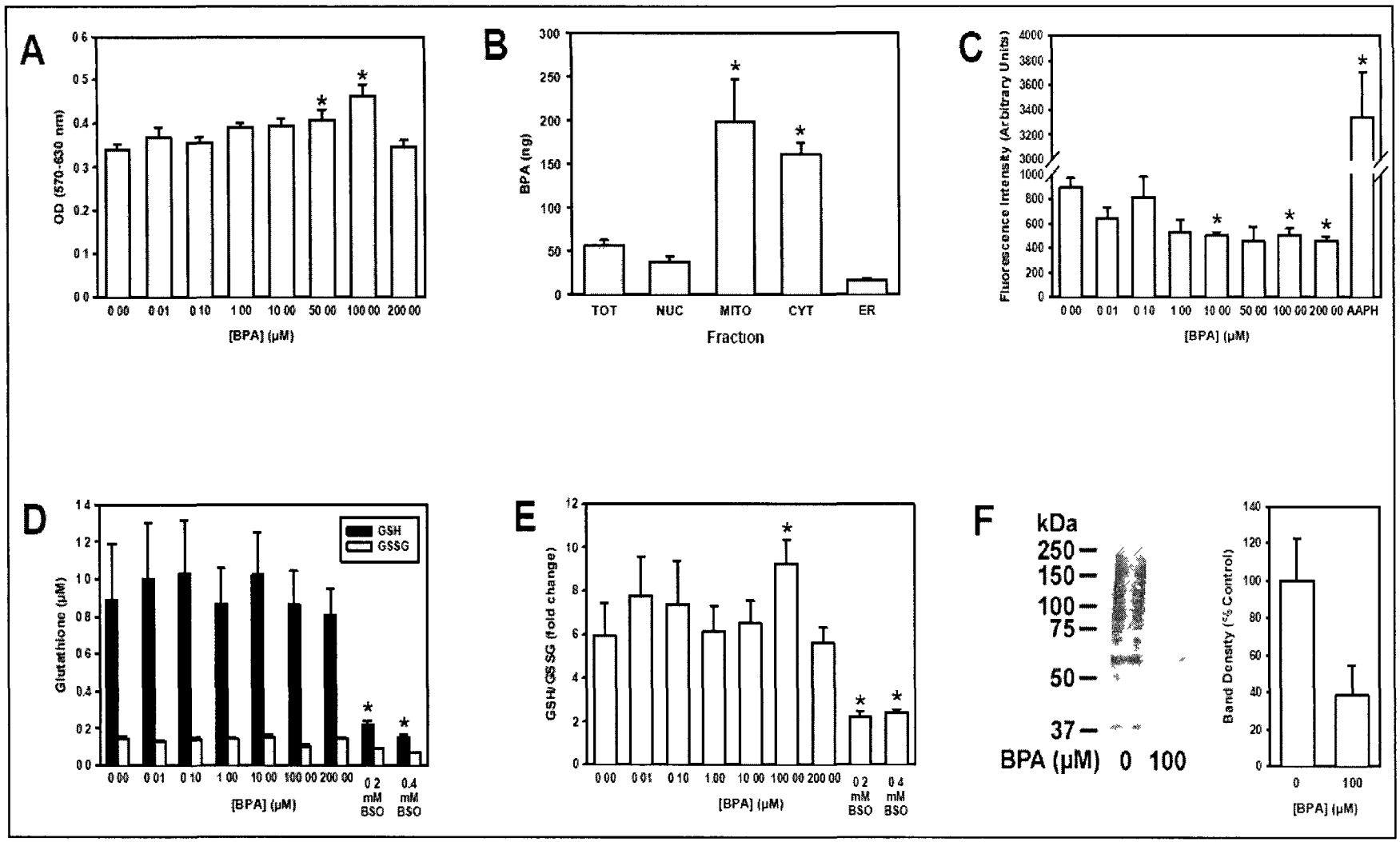
Figure 4.1. Theoretical Pro-oxidant and Antioxidant Properties of BPA. BPA has both pro-oxidant (catechol/quinone redox cycling) and antioxidant (H atom donation) potential with the antioxidant potential (predicted O-H BDE of 83.9 kcal/mol) being considerably distant from the BDE range of an ideal antioxidant (the lower part of the 68.5-88 kcal/mol window (Hussain *et al.*, 2003)).

4.2 BPA Promotes Cell Proliferation and Reduces Basal ROS Levels

First, the effect of BPA on cell viability was examined. The MTT assay results indicated that BPA is not cytotoxic and, instead, promotes cell proliferation at high (50 to 100 μM for 24 h) concentrations (Figure 4.2A). Furthermore, BPA preferentially accumulated in cytoplasmic and mitochondrial fractions (Figure 4.2B), in accordance with the previous hypothesis that BPA targets mitochondrial membranes (Ooe *et al.*, 2005). BPA treatment reduced the basal rate of ROS production by almost two-fold (Figure 4.2C). This effect

was already evident after 10 h (data not shown) and a similar result was obtained in the BPA-treated cultured human fetal lung fibroblasts (hFLF, Chen *et al.*, in preparation). We considered a possibility that the antioxidant-rich serum use in cell culture could mask the production of ROS by BPA. However, the same trend (lowered ROS signal) was noticed even in the cells cultured without serum (data not shown). Similar results were obtained for the GSH/GSSG ratio, an index of intracellular redox balance. Thus, at 100 μM BPA, the ratio was about 30% higher compared to controls (Figure 4.2D and 4.2E). A comparable trend was seen in our protein carbonyl assay, although the difference did not reach statistical significance (Figure 4.2F). These results strongly suggest that BPA induced an adaptive (or compensatory) response leading to more reduced conditions within the cell.

Figure 4.2. The Effect of BPA on Cell Viability and Intracellular Redox Status. HEK 293A cells were treated with the indicated BPA concentrations for 24 h and cell viability (**A**), subcellular BPA content (**B**), ROS generation (**C**), total glutathione content (**D**), the GSH/GSSG ratio (**E**) and protein carbonylation (**F**) were assessed as described in the Methods section. Results are presented as means ± S.E.M. ($n \geq 3$). An asterisk (*) represents a significant difference from controls (Student's paired t-test, $p < 0.05$). TOT, total cell lysate; NUC, nuclear fraction; MITO, mitochondrial fraction; CYT, cytoplasmic fraction; ER, endoplasmic reticulum fraction.


A
OD (570-630 nm)
0 0
0 1
0 2
0 3
0 4
0 5
0 6
0 00
0 01
0 10
1 00
10 00
50 00
100 00
200 00
[BPA] (μM)
B
BPA (ng)
0
50
100
150
200
250
300
TOT
NUC
MITO
CYT
ER
Fraction
C
Fluorescence Intensity (Arbitrary Units)
0
200
400
600
800
1000
3000
3200
3400
3600
3800
4000
0 00
0 01
0 10
1 00
10 00
50 00
100 00
200 00
AAPH
[BPA] (μM)
D
Glutathione (μM)
GSH
GSSG
0 0
0 2
0 4
0 6
0 8
1 0
1 2
1 4
0 00
0 01
0 10
1 00
10 00
100 00
200 00
0 2 mM BSO
0.4 mM BSO
[BPA] (μM)
E
GSH/GSSG (fold change)
0
2
4
6
8
10
12
0 00
0 01
0 10
1 00
10 00
100 00
200 00
0 2 mM BSO
0 4 mM BSO
[BPA] (μM)
F
kDa
250
150
100
75
50
37
BPA (μM) 0 100
Band Density (% Control)
0
20
40
60
80
100
120
140
0
100
[BPA] (μM)

4.3 BPA Activates Nrf1 and Nrf2 and Alters Protein Binding to the ARE

To examine protein binding to a typical ARE sequence taken from the *gclm* promoter. EMSA analysis was employed. The BPA concentrations of 0, 100 and 200 μM were selected for the EMSA analysis based on previous results of the ARE-dependent gene expression, which was significantly affected at high concentrations of BPA only as will be discussed in greater detail below. According to Figure 4.3 and the densitometric results not shown here, BPA treatment resulted in essentially no significant change in the protein binding to the labeled ARE probe. The two top bands observed in Figure 4.3 were formed as a result of specific interactions between the ARE and the ARE-binding proteins as confirmed by the addition of excess unlabelled (cold) probe. In this format, the band, whose density is affected by the inclusion of unlabelled probe is considered to be specific to a given DNA sequence because of the competition between unlabelled (cold) and labeled (hot) probes for the binding of ARE-specific proteins, with the later being present in shortage (limiting). While EMSAs are informative in terms of the overall understanding of factors binding to a given DNA sequence, such a binding event can either have a stimulatory or an inhibitory effect on the transcription of downstream genes, depending on the nature of a protein (repressor or activator) bound. In addition, the slight decrease of ARE protein binding by approximately 20% observed only with 200 μM BPA (data not shown), while statistically significant, may be biologically irrelevant in the context of the EMSA approach and therefore a more quantitative assay was required to determine Nrf1/2-ARE function in the presence of BPA. To gain a more thorough

and unambigous understanding of the effects of BPA on the Nrf1/2-ARE pathway, we employed a firefly luciferase reporter harboring three AREs from β-globin enhancer upstream of the luciferase gene (3x ARE-luciferase) or an ARE from the *gclc* gene, which was co-transfected with pCR3.1 (empty vector), Nrf1-FLAG or Nrf2-FLAG, and the transfected cells were treated with 100 μM BPA (Figure 4.4). Nrf1-FLAG activity was significantly higher with 100 μM BPA treatment, compared to controls, while Nrf2-FLAG was significantly activated by 200 μM BPA treatment. A known Nrf2 inducer, tBHQ (Li *et al.*, 2005), was used as a positive control for Nrf2 induction. The ability of tBHQ to activatet the Nrf2-mediated reporter activity, seen in Figure 4.4B, was used as a positive control for the reporter activity. However, overexpression of a transcription factor in the cells along with a reporter may produce some artefacts as will is discussed in Section 6.2 of this thesis, I wanted to examine the effect of BPA on the expression of the ARE-controlled genes using RT-qPCR.

Figure 4.3. BPA Alters Protein Binding to the ARE Consensus Sequence Probe. EMSAs were run on the nuclear lysates from the cells treated with 0, 100 and 200 μM BPA for 24 hours as described in the Methods section. A lane without protein extract (probe only) and a lane with an excess of unlabelled (cold probe) were used to determine the mobility of the free probe and the specific ARE-protein complexes, respectively. Two representative results of four independent experiments are shown.

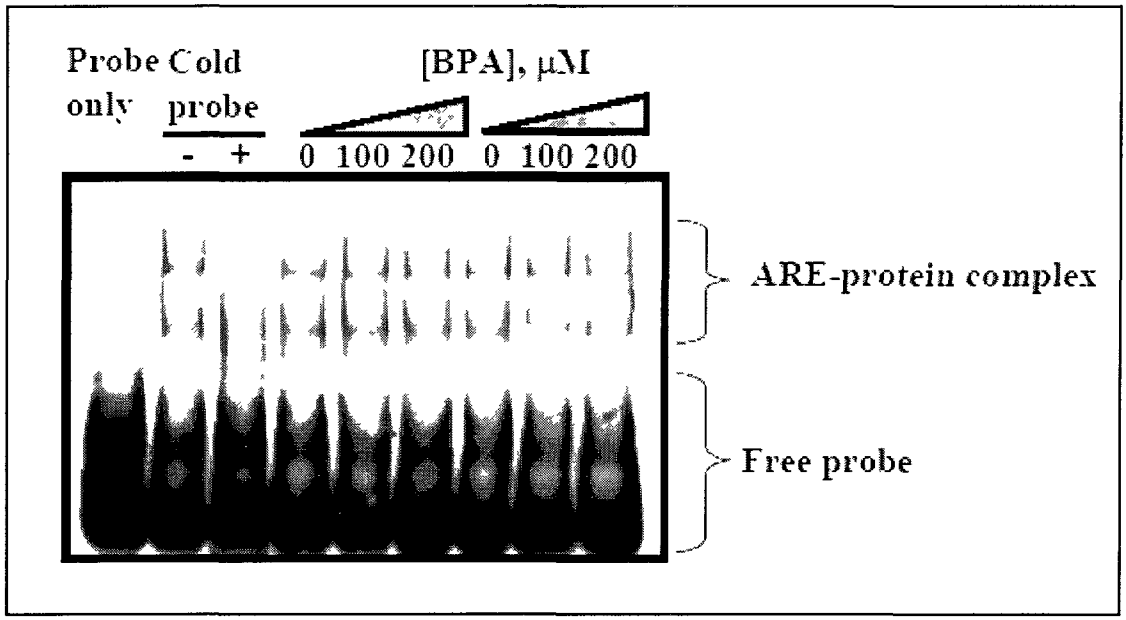
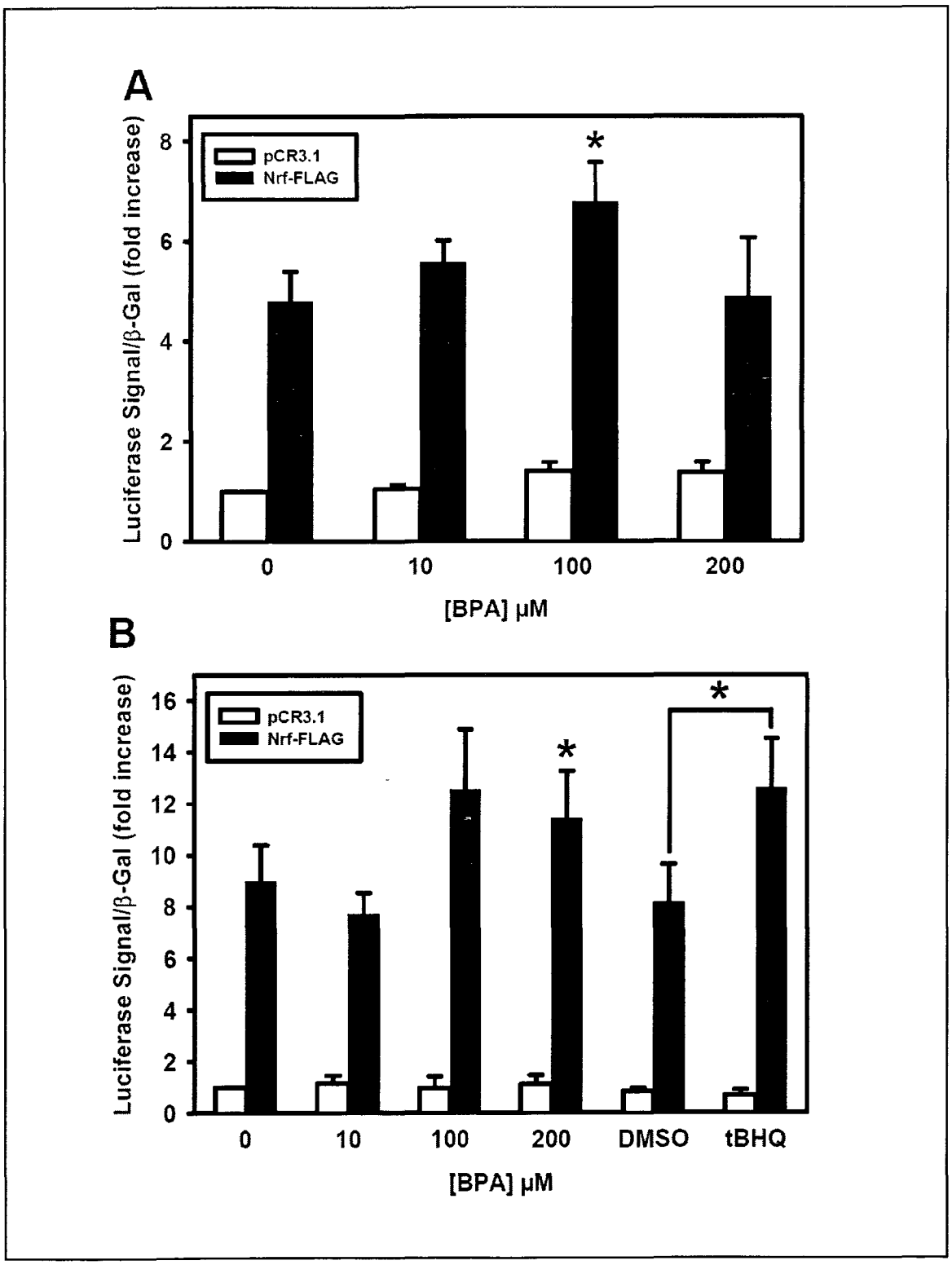

Probe only
Cold probe
- +
[BPA], μM
0 100 200 0 100 200
ARE-protein complex
Free probe

Figure 4.4. BPA Activates Exogenous Nrf1 and Nrf2 in HEK 293 Cells. The results of ARE luciferase reporter assays performed on cells co-transfected with Nrf1-FLAG (**A**), Nrf2-FLAG (**B**) or pCR3.1 (transfection control) along with β-globin 3X ARE-luciferase (**A**) or *gclc* ARE-luciferase (**B**) reporter plasmids and β-galactosidase (transfection efficiency control) and treated with 0, 10, 100 or 200 μM BPA doses for 24 h. As a positive control for Nrf2 induction, cells transfected with Nrf2-FLAG or pCR3.1 were treated with dimethylsulfoxide (DMSO, vehicle control) or 100 μM tBHQ, a known inducer of the Nrf2-mediated ARE-driven gene expression (Li *et al.*, 2005; Wang and Jaiswal, 2006). The means ± S.E.M. ($n \geq 3$) are shown. Asterisks (*) indicate significant difference from controls (containing dimethylsulfoxide (DMSO); $p < 0.05$, Student's paired t-test).


A
Luciferase Signal/β-Gal (fold increase)
pCR3.1
Nrf-FLAG
0
2
4
6
8
*
0
10
100
200
[BPA] μM
B
Luciferase Signal/β-Gal (fold increase)
pCR3.1
Nrf-FLAG
0
2
4
6
8
10
12
14
16
*
*
0
10
100
200
DMSO
tBHQ
[BPA] μM

4.4 BPA Upregulates the Nrf1/2-ARE Pathway-controlled Genes

Next, the expression of typical ARE-Nrf1/2 target genes upon BPA treatment was analyzed by RT-qPCR. Table 4.1 shows the mRNA expression of *Nrf1*, *Nrf2*, *Nqo1* and *HO-1* in response to different BPA treatments. The single p-value in Table 4.1 indicates that there was a significant difference between at least 2 doses for a specific gene (by one-way ANOVA). The p-value, however, does not indicate which groups are significantly different from one another. Therefore, a post-hoc Duncan's test was performed to determine which groups differed from one another. Significant differences between all the groups (p-value $\leq$ 0.05) have been indicated with superscript letters. Different superscript letters between rows indicated fold changes that were significantly different. One hundred μM BPA increased mRNA levels of *ho-1* 1.39-fold ($p < 0.05$, Table 4.1) and 200 μM BPA treatment increased mRNA levels of both *ho-1* and *nqo1* 1.88 ± 0.48- and 2.23 ± 0.44-fold, respectively, ($p < 0.05$, Table 4.1) when compared to all other doses. Of note is that *nrf1* mRNA levels were unaltered by 100 μM BPA treatment, suggesting that Nrf1 activation by BPA was probably mediated through post-translational modification of the protein such as ubiquitination or phosphorylation. In fact, the same BPA concentration decreased mRNA level of ubiquitin-conjugating enzyme E2T in cultured hFLF cells (Chen *et al.*, in preparation). Consistent with this is the fact that less ubiquitinated Nrf2 is immunodetectable upon BPA treatment (Figure 4.4A). At 200 μM BPA, the expression of both *nrf1* and *nrf2* was upregulated (Table 4.1). Very little is currently known about the transcriptional regulation of *nrf1* and *nrf2* genes, other than that,

under certain circumstances, their expression is regulated in the opposite fashion as is the case for the whole mouse treatment with butylated hydroxytoluene, a known antioxidant and food additive (Chevillard *et al.*, 2010).

4.5 BPA Downregulates the Protein Expression of Nrf1, Nrf2 and HO-1

To confirm the possibility that BPA activates the ARE pathway through the alteration of Nrf1 and Nrf2 protein expression, acting on these CNC-bZIP factors directly, immunoblotting was employed to determine the expression levels of the ARE-pathway regulatory (Nrf1 and Nrf2) and downstream target (Nqo1 and HO-1) proteins. The expression of the inactive, glycosylated and ER-bound Nrf1 form (p120) was significantly diminished at 100 μM BPA treatment without any apparent effect on the active nuclear form of Nrf1 (p95) (Figure 4.5). On the other hand, the active and oxidative stress-inducible form of Nrf2, migrating at approximately 100 kDa and detectable by our antibody and thought to represent ubiquitinated Nrf2 (Li *et al.*, 2005), was about four-fold reduced during BPA treatment. Similarly, the protein expression of HO-1 was about seven-fold reduced by BPA, while the expression of Nqo1 was unchanged. The fact that HO-1 protein expression was down- rather than up-regulated was puzzling and in direct contrast with our PCR results, revealing more than two-fold increase of the *ho-1* mRNA level (Table 4.1) and our experiments in hFLF cells, where the HO-1 gene and protein expression levels were increased more than 5- and 18-fold by 100 μM BPA treatment, respectively (Chen *et al.*, in -

Table 4.1 Enhanced Expression of the Nrf1/2-ARE Pathway Regulatory and Target Genes at Different BPA Concentrations.

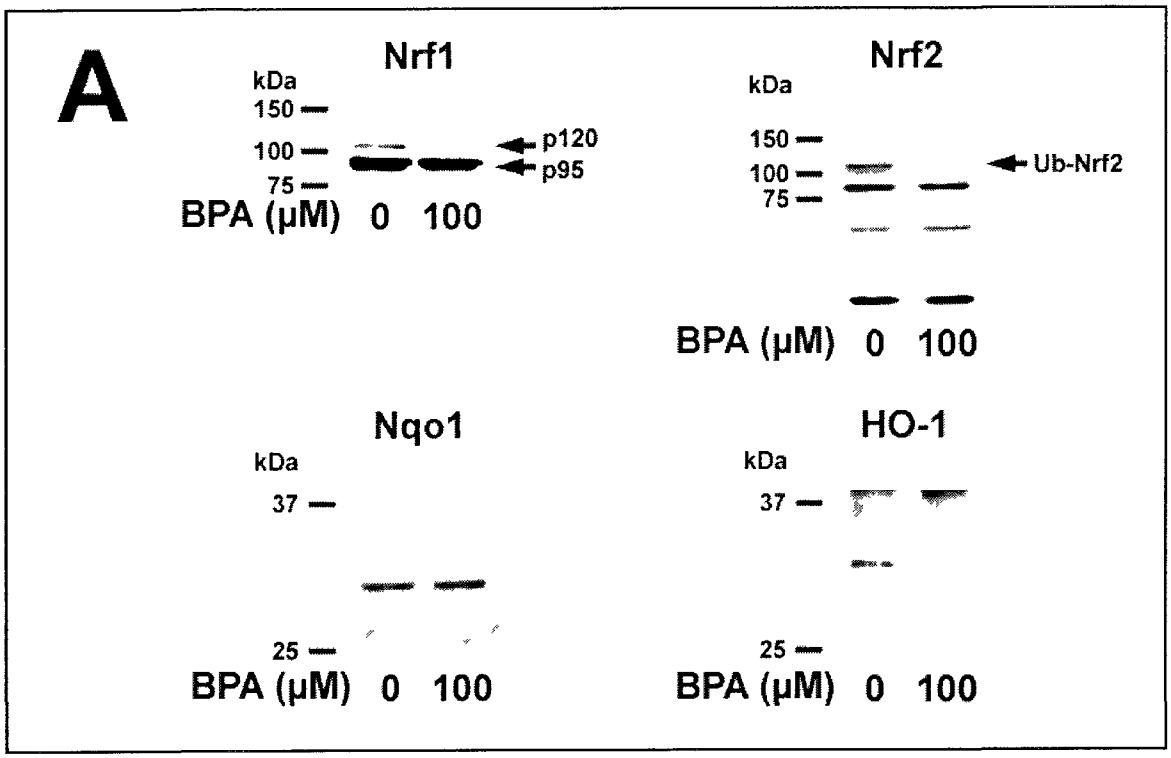
Gene		BPA Dose				p-value
Name	**TaqMan assay**	**0 µM**	**10 µM**	**100 µM**	**200 µM**	**(One-way ANOVA)**
nqo1	Hs00168547_m1	1.00 ± 0.19 [a]	1.07 ± 0.16 [a]	1.20 ± 0.20 [a]	**1.88 ± 0.48 [b]**	0.0001
ho-1	Hs00157965_m1	1.00 ± 0.12 [a]	1.04 ± 0.25 [ab]	**1.39 ± 0.28 [b]**	**2.23 ± 0.44 [c]**	< 0.0001
nrf1	Hs00231457_m1	1.00 ± 0.16 [a]	1.08 ± 0.35 [a]	1.18 ± 0.32 [a]	**1.69 ± 0.23 [b]**	0.0015
nrf2	Hs00975960_m1	1.00 ± 0.07 [a]	**1.14 ± 0.08 [b]**	**1.21 ± 0.18 [b]**	**2.04 ± 0.22 [c]**	< 0.0001*

Relative expression levels of the genes were measured by RT-qPCR analysis and normalized to GAPDH (TaqMan primer Hs 99999905_m1) expression. Values are presented as relative expression means ± SD (n = 6). The single p-value in Table 4.1 indicates that there was a significant difference between at least 2 doses for a specific gene (by one-way ANOVA). A post-hoc Duncan's test was performed to determine which groups differed from one another. Significant differences between all the groups (p-value ≤ 0.05) have been indicated with superscript letters (not indicated in the table). Means in a row not sharing a superscript letter are significantly different, $p < 0.05$ (Duncan's Test, one-way ANOVA). * Logarithmically transformed prior to statistical analysis.

preparation). We thought that the disappearance of the HO-1 monomer could be explained by protein aggregation or dimer formation through covalent bond linkage between the two HO-1 monomers as a result of increased gene and protein expression. To gain a clearer understanding of the behavior of HO-1 protein, we repeated our experiments in HEK 293 cells, paying closer attention to a potential HO-1 oligomer formation. As shown in Figure 4.6, we could demonstrate putative HO-1 dimer formation as a result of BPA treatment. Since the reducing and denaturing conditions (β-mercaptoethanol, SDS) were used for SDS-PAGE, one would expect that non-covalent associations between HO-1 monomers (aggregates) would be broken. Dimers or oligomers of HO-1 would be only seen in the case of convalent bond formation between two or more subunits. Such a formation of dimers has been observed for a heat-shock protein 25 upon reducing and denaturing SDS-PAGE (HSP25, Ho *et al.*, 2006). More importantly, the HO-1 dimerization and oligomerization has been recently reported (Hwang *et al.*, 2009) under reducing and denaturing SDS-PAGE as well. Whether HO-1 dimerization is driven by partial protein unfolding or by an increase in HO-1 concentration can be tested in a cell-free system utilizing recombinant HO-1, treated with BPA, as has been done previously for citrate synthase that undergoes protein unfolding, aggregation and SDS-PAGE-resistant oligomerization in response to oxidative stress *in vitro* (Chepelev *et al.*, 2009). Dimerization of HO-1 could be important for its activity, which is now thought to be catalytically active as a dimer/oligomer (Hwang *et al.*, 2009). Thus the BPA-mediated dimerization of HO-1 is worth further investigation.

Figure 4.5. Protein Expression of the Nrf1/2-ARE Pathway Regulatory (Nrf1 and Nrf2) and the Target Proteins (HO-1 and Nqo1) as a Result of BPA Treatment.

HEK 293 cells were treated and with 0 and 100 μM BPA for 24 hours and Western blots (**A**) were performed as described in the Methods Section. For semi-quantitative densitometric analysis (**B**), band density (the means ± S.E.M. ($n \geq 3$) are presented) was normalized to Ponceau S red-stained bands (loading control). Asterisks (*) indicate significant differences ($p < 0.05$, Student's paired t-test) from controls.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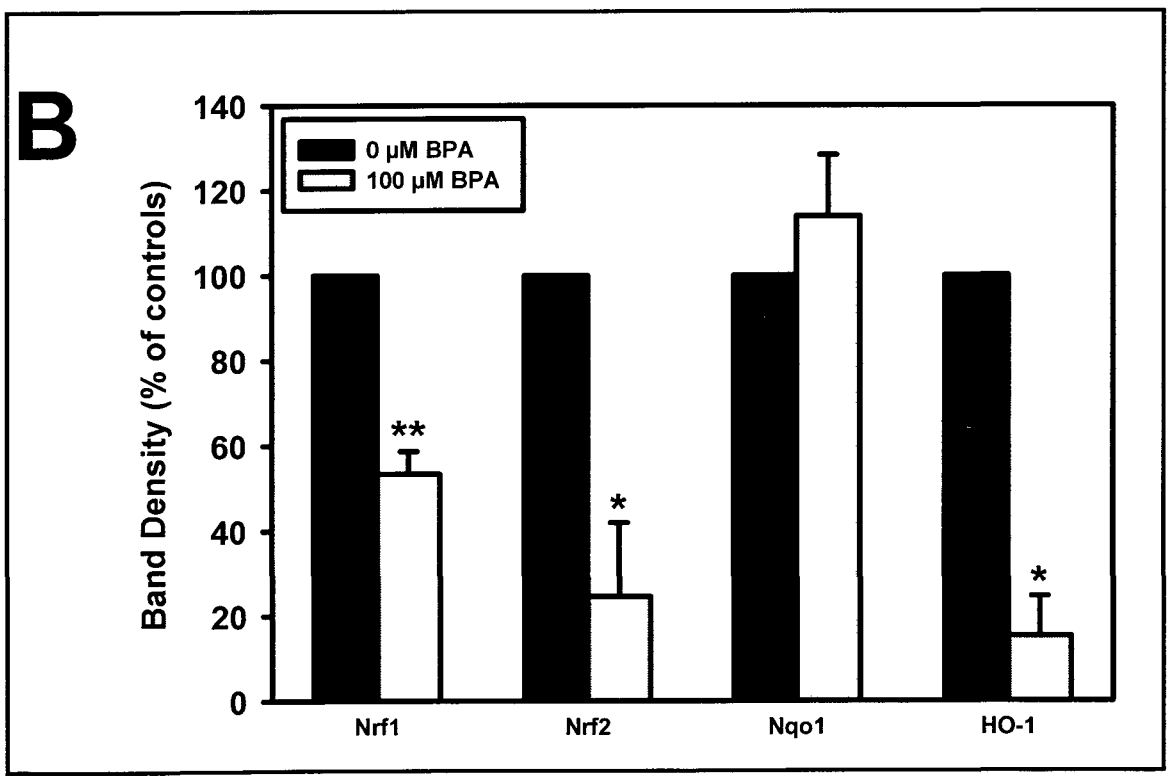
Nrf1
kDa
150
100
75
p120
p95
BPA (μM) 0 100
Nrf2
kDa
150
100
75
Ub-Nrf2
BPA (μM) 0 100
Nqo1
kDa
37
25
BPA (μM) 0 100
HO-1
kDa
37
25
BPA (μM) 0 100


B
Band Density (% of controls)
140
120
100
80
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40
20
0
0 μM BPA
100 μM BPA
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*
*
Nrf1
Nrf2
Nqo1
HO-1

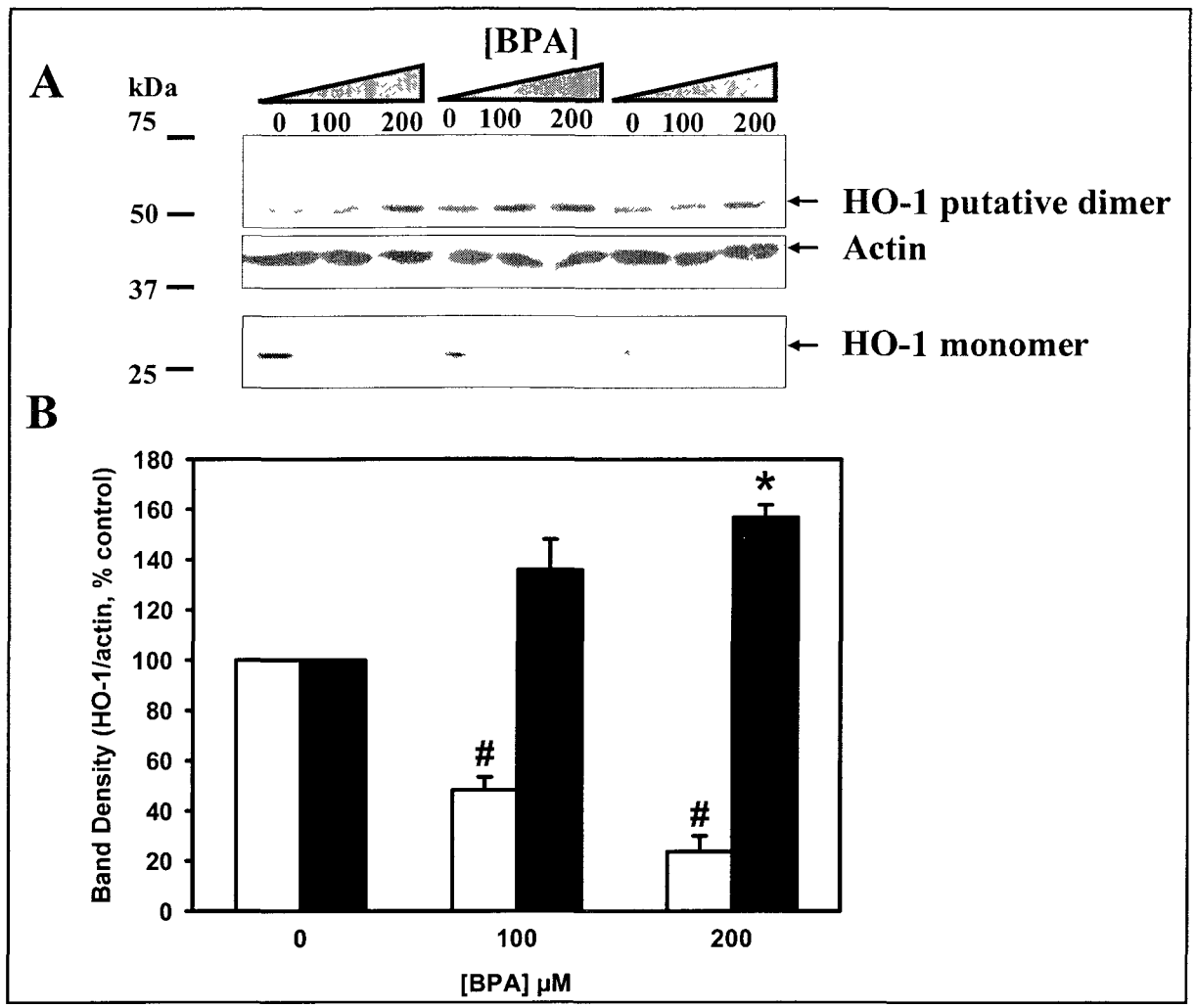


Figure 4.6. HO-1 Putative Dimer Formation as a Result of BPA Treatment. (A) HEK293A cells were subjected to BPA treatment (0, 100 or 200 μM for 24 hours) and the total cellular extract was subjected to immunoblotting as described in the Methods Section. The position of the HO-1 monomer, actin loading control and putative HO-1 dimers are indicated by arrows. **(B)** Graphical representation of the band densities compared to control (0 μM BPA, means ± S.E.M. ($n \geq 3$)) is shown. Asterisk (*) and the number signs (#) indicate statistically significant differences from controls ($p < 0.05$ and 0.01, respectively, as calculated using paired Student's t-test).

4.6 The Proposed Model for the Effect of BPA on the Nrf1/2-ARE Pathway

BPA-induced proliferation of HEK 293 cells, observed in this study, could be explained by the activation of the Nrf1/2-ARE pathway. For instance, constitutive expression of Nrf2 in human lung carcinoma cells may equip the cells with higher levels of the antioxidant enzymes HO-1 and Nqo1, favoring their unrestrained proliferation (Padmanabhan *et al.*, 2006). Given the evidence supporting the activation of the Nrf1/2-ARE pathway presented here, it is plausible to suggest that BPA acted on the pathway to activate the expression of genes involved in cell growth and proliferation. A similar result was reported by Oh and Lim in Chang liver cells (2008), but the exact mechanisms of BPA-induced cell proliferation and the contributions of Nrf1 and Nrf2 to the process, await further investigation.

The fact that BPA decreased the basal rate of ROS production is in contrast to previous publications (Oh and Lim, 2008; Kabuto *et al.*, 2003; Chtira *et al.*, 2003; Ooe *et al.*, 2005), but in agreement with a similar study in hFLF cells (Chen *et al.*, in preparation). This effect of BPA is in line with the reported antioxidative effects of estrogens, phytoestrogens and polyphenols (Mitchell *et al.*, 1998). It has been shown that these compounds can activate the antioxidant cellular defense system through the MAPK-Nrf2-Keap1 pathway. Similar to our RT-qPCR data, microarray analysis of gene expression in hFLF cells suggested an upregulation of the Nrf1/2-mediated stress response pathways by 100 μM BPA (Chen *et al.*, in preparation). Further

studies of the activities of Nrf1/2 downstream targets will reveal if they are involved in BPA-specific responses as well as responses to similar phenolics in general.

Halliwell reported some potential artifacts of cell culture studies (Halliwell, 2009). Among these is culturing cells at oxygen concentration about 10-15 times greater than *in vivo* oxygen levels to which cells are exposed, which could increase the rate of ROS generation. This might explain the fact that HO-1, which is typically stress-inducible, was expressed even under control conditions. If the expression of HO-1, and perhaps other antioxidant enzymes, were increased even prior to BPA application, cells may have been well-buffered against a potential pro-oxidant BPA attack. In addition, BPA acting as antioxidant could counteract elevated ROS levels, creating a more reduced intracellular environment and returning the HO-1 expression back to normal. This scenario, however, is not likely due to the poor predicted antioxidant properties of BPA (see the Section 4.1). Some antioxidant potential of BPA has been previously described, including the inhibition of methacrylate polymerization (Kadoma and Fujisawa, 2000) and prevention of iron-stimulated lipid peroxidation in rats (Kabuto *et al.*, 2003). Given poor predicted antioxidant properties of BPA and the evidence that BPA may act on the Nrf1/2-ARE pathway, the scenario in which BPA could act on the components of the Nrf1/2-ARE pathway to increase the expression of antioxidant enzymes (e.g., glutathione synthesis enzymes, such as *gclc* and *gclm*) to produce more reduced conditions and relieve hyperoxia-mediated oxidative stress, seems more probable. Another interesting possibility is that BPA could reduce the rate of mitochondrial ROS production, since it has been suggested

that chemicals with a structure, similar to that of BPA have a tendency to accumulate in the mitochondrial membrane (which is in agreement with Figure 4.2 B) because it contains interior hydrophobic proteins and was shown to reduce complex I activity of the mitochondrial electron transport chain (ETC, Ooe *et al.*, 2005). Decreasing the flux through the ETC by lower substrate availability or reduced expression of ETC complex I components is thought to reduce the rate of mitochondrial ROS production (Semenza *et al.*, 2010).

To explain the BPA-provoked activation of Nrf1 (Figure 4.4), it would be tempting to speculate that this occurs *via* deglycosylation of the ER-resident p120 form (since its expression was about 2-fold lower compared to controls) to increase the pool of active p95 form in the nucleus. However, it has not been shown that Nrf1 deglycosylation occurs *in vivo*. As no change in p95 Nrf1 protein expression was observed, BPA could potentially activate Nrf1 by a currently uncharacterized post-translational modification of Nrf1. This could potentially be phosphorylation since a closely-related family member, Nrf2, is activated by this modification (Numazawa *et al.*, 2003). Similarly, Zhang and co-workers (Zhang *et al.*, 2007) suggested that Nrf1 phosphorylation may weaken the association of Nrf1 with the ER membrane, stimulating its nuclear translocation. Furthermore, we observed some evidence, supporting that Nrf1-FLAG activity is dependant upon the phosphorylation of Nrf1 (Chepelev *et al.*, submitted) and further studies will reveal the significance and the mechanisms by which Nrf1 activity can be regulated through its phosphorylation.

Despite the increase in *ho-1* gene expression (Table 1), the protein levels of the HO-1 were approximately seven times lower at high (100 μM) BPA doses (Figure 4.5). The discrepancy between mRNA and protein expression of HO-1 during BPA treatment may be explained by HO-1 dimerization, observed clearly as the appearance of a band with an approximate molecular weight of 55 kDa (Figure 4.6; the molecular weight of the HO-1 monomer is approximately 33 kDa (Yoshiki *et al.*, 2001)) during cell treatment with BPA. This would result in lowered HO-1 monomer protein expression, despite its increased gene expression. Indeed, a recent study has shown that HO-1 exists as dimers and oligomers in the ER, which form through the hydrophobic interactions of the transmembrane segments (TMS, aa 266-285) of HO-1 monomers, of which Trp270 plays a major role (Hwang *et al.*, 2009). Without the TMS-TMS interaction, HO-1 is not anchored to the ER membrane and is a subject of ubiquitination and proteasomal degradation (Hwang *et al.*, 2009; Lin *et al.*, 2008). To the author's best knowledge, this is the first report of a condition, which induced dimerization of HO-1. Given that HO-1 is an enzyme implicated in numoerous clinical conditions and induced by multiple stimuli, the BPA-induced dimerization of HO-1 merits further investigation to disentangle the mechanism(s), responsible for the dimer formation.

In conclusion, it can be envisioned that BPA stimulates the Nrf1/2-ARE pathway as shown by increased mRNA levels of *ho-1* and *nqo1* (Table 1) and increased transactivation activity of Nrf1-FLAG and Nrf2-FLAG (Figure 4.4). It is conceivable that BPA acted on the mitochondrial ETC to reduce the flow of

metabolites through the ETC, reducing the rate of mitochondrial ROS generation and altering the intracellular redox status. Figure 4.7 summarizes the current working model for the effect of BPA on the Nrf1/2-ARE pathway. Finally, our study suggests that HEK 293 cells can withstand even high micromolar BPA concentrations, at least within the timeframe studied (0-24 hours), without any significant cytotoxic effects.

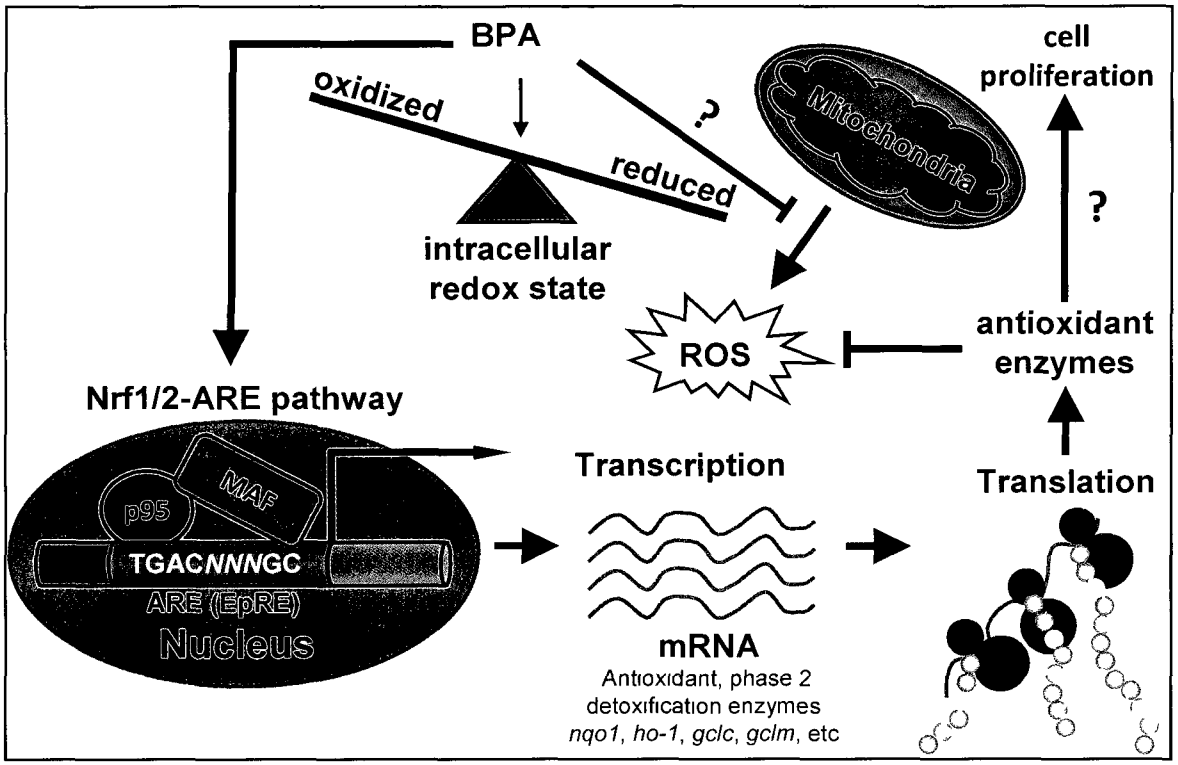


Figure 4.7. The Proposed Model for the BPA Effect on the ARE-Nrf1/2 pathway. BPA treatment results in reduced intracellular conditions in HEK 293 cells (Figure 4.2C-E). This could be due to the activation of the ARE-Nrf1/2 pathway (Figure 4.4) such that increased expression of antioxidant enzymes (Table 4.1) could lead to increased cell proliferation (Figure 4.2A). Cytoplasm and mitochondria appear to the main compartments of BPA accumulation (Figure 4.2B) and BPA could also reduce the steady-state ROS levels arising from various intracellular sources such as mitochondria.

5 Chapter: Nrf1 Regulation in Aging Mice

5.1 Aging, Air Particulate Exposure and their Relation to the ROS Generation, Nrf1 and Nrf2

As has been discussed in the Chapter 1, oxidative stress and ROS contribute to the age-related complications in humans under stress-related conditions and play a minor role under optimal environmental conditions. Indeed, in laboratory animals in which the expression of the ARE-controlled antioxidant enzymes, such as superoxide 1(SOD1), CAT and GPx, has been abrogated through knockout, significantly higher mortality rates and succeptibility to age-accociated diseases has been shown (Salmon *et al.*, 2010). This concept of increased succeptibility of mammals to environmental toxicants and stress, in general, has led us to consider a model in which both young and old animals were subjected to an environmental stress in order to evaluate the responsiveness of both group of animals towards inducing the expression of the antioxidant genes through the Nrf1/2-ARE pathway.

Airborne ultrafine particles, generated by vehicular traffic, are just one example of many widespread environmental stressors. Airborne particles, with a mean diameter of 60 nm, display a pronounced free radical signal as measured by electron paramagnetic resonance (EPR) spectroscopy, revealing the presence of the carbon-centered free radicals, stable for at least 30 days after collection (Morgan *et al.*, 2011). The inhalation of ambient ultrafine particles, with diameters less than 100 nm, can induce ROS formation and augment oxidative stress in the lung (Donaldson *et al.*, 2005; Nel, 2005; Oberdörster *et al.*, 2005). In the murine phagocyte model of the lung susceptibility to the

particles, the nanosize group of airborne particles (< 200 nm) induced ROS-mediated damage of the subcellular organelles and increased intracellular ROS production (Xia *et al.*, 2006). Interestingly, nanoparticles are capable of generating ROS even under abiotic conditions (Morgan *et al.*, 2011; Xia *et al.*, 2006). Similarly, the treatment of human lung epithelial cells with combustion-derived fly ash nanoparticles induced ROS generation and augmented the production of glutathione (GSH), a major cellular antioxidant (Diabaté *et al.*, 2011).

Concomitantly with the elevation of basal oxidative stress in aged animals, the expression and activity of antioxidant enzymes, such as glutamylcysteine ligase catalytic (CGLC) and regulatory (GCLM) subunits, is lowered in many aged tissues (see Suh *et al.*, 2004 and references therein). GCLC and GCLM are rate-limiting enzymes of the GSH biosynthesis. It was shown that decreased expression and activity of Nrf2, the master regulator of the inducible antioxidant gene expression, may be responsible for the weakened cellular antioxidant defense in the aged rats (Suh *et al.*, 2004; Shih and Yen, 2006) In contrast, long-lived Ames dwarf mice exhibit elevated expression of antioxidant enzymes, including HO-1 NQO1. More importantly, these animals possess greater inducibility of Nrf2 and the Nrf2-target genes when challenged with the pro-oxidant diquat (Sun *et al.*, 2011), indicative of their greater capacity to counteract disturbances in their homeostatic intracellular redox environment. It has been noted that long-lived rodents naked mole-rats and white-footed mice have elevated basal Nrf2 expression and GSH content and that Nrf2 homologue SKN-1 in worms and CncC in flies confer these organisms increased longevity (Lewis *et al.*, 2010). Therefore, it has been hypothesized

that augmented Nrf2 activity is a common feature of the long-lived animals and that Nrf2 (or its homologues) act as the "gatekeeper of species longevity" (Lewis *et al.*, 2010).

Despite its emerging pro-longevity function, the reasons for declined presence and performance of Nrf2 have not been addressed adequately. As Nrf2 is a tightly regulated protein, multiple mechanisms can be responsible for its age-dependent loss of expression and activity, including somatic loss-of-function mutation of *Nrf2*, increased proteolysis of the factor and altered expression and activities of kinases, phosphatases or DNA-binding proteins, leading to reduced transcription of the ARE-target genes, including *Nrf2* itself (Sykiotis and Bohmann, 2010). An emerging mechanism of Nrf2 regulation is through the closely-related family member, Nrf1. A short form of Nrf1 (p65Nrf1) is a dominant negative inhibitor of the Nrf2/ARE-mediated gene expression (Wang *et al.*, 2007). In addition, the *Nrf1* deficiency resulted in the paradoxical increase of the expression of the Nrf2-target genes in mice liver, which was abolished in *Nrf2/Nrf1* double knockout animals (Ohtsuji *et al.*, 2008).

In the current study, the relationship between Nrf1 and Nrf2 has been explored using Nrf1 RNA interference and Nrf1/Nrf2 overexpression in cultured cells as well as in mice models of aging and airborne particle exposure. *I hypothesized that Nrf1 can negatively regulate the transactivation activity of Nrf2 in vitro and in vivo and that Nrf1 may be differentially expressed in mousemice as a function of age.* The data presented here supports a model in which Nrf1 negatively affects the expression of the Nrf2-controlled genes. In addition, in the lungs of mice exposed to airborne particles, aging

diminished Nrf2 expression, and thus its DNA-binding function, as well as the inducibility of the ARE-controlled genes and the increase in the cellular antioxidant capacity. In contrast, the expression of the full-length and short forms of Nrf1 was elevated in the lung of the aged- and particle-treated animals. Taken together, these data support a model in which age-dependent change of the homeostatic Nrf1/Nrf2 ratio may impair cellular capacity to counteract deleterious effects of oxidative stressors.

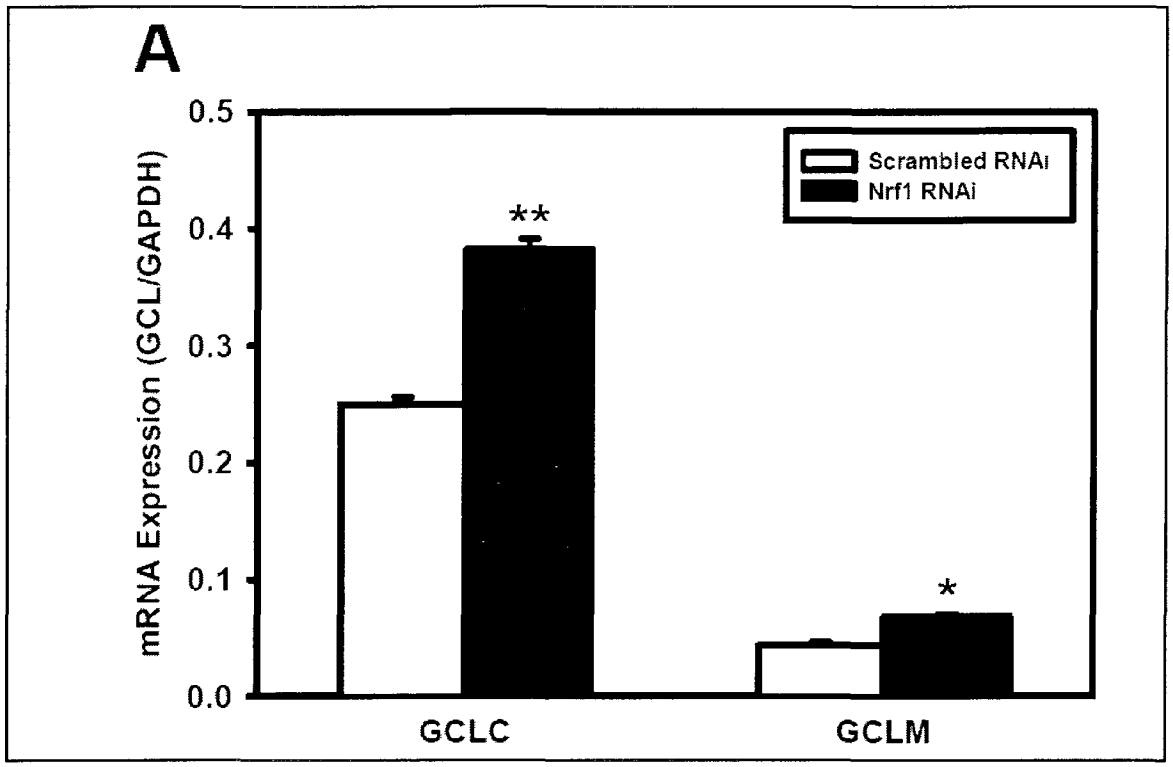
5.2 *Nrf1* Silencing Activates the Expression of the ARE-target Genes

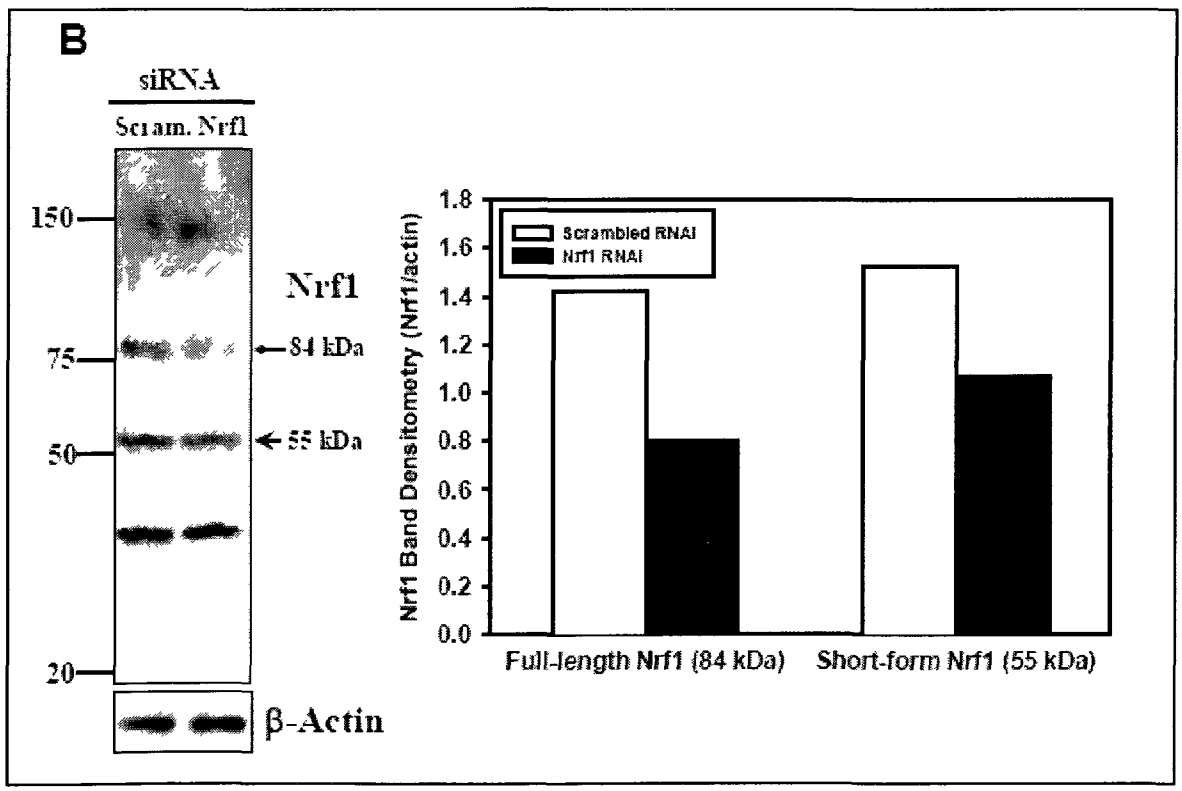
To investigate potential contribution of Nrf1 to the expression of the well-established targets of the Nrf2/ARE-driven gene expression, Nrf1 silencing using interfering RNA oligos (RNAi) was performed in the HBE1 cells. The effect of Nrf1 silencing was examined on the gene expression of *GCLC* and *GCLM* using real-time quantitative PCR (real-time qPCR). These are known Nrf2-ARE pathway targets, whose transcription is induced by pro-oxidants 4-hydroxynonenanl and acrolein (Zhang and Forman, 2009). Counter-intuitively, *Nrf1* silencing resulted in approximately 1.5-fold increase in the *GCLC* and *GCLM* basal expression (Figure 5.1A).

The effect of the Nrf1 silencing on the Nrf1 protein expression itself was confirmed with Western blotting which revealed that the intensity of Nrf1 bands, migrating with apparent molecular weights of 84 and 55 kDa, were reduced to about 60 and 70 % of the controls, respectively (Figure 5.1B). Typically, RNAi does not abolish the protein expression of its target mRNA completely and silencing efficiency close to

50% is considered to be acceptable in the literature as was the case with *Nrf1* silencing performed by Zhang and co-workers with about 50% efficiency (2009). It is noteworthy that this banding pattern is different from that obtained using the lysates of HEK293 and WFF2002 cells, which showed distinct bands of 120, 95 and 65 kDa. The difference is probably due to the use of a different cell line, in which different isoforms of Nrf1 may be expressed, in accordance with previous reports on some cell-specific Nrf1 isoforms (as reviewed by Biswass and Chan, 2010). Typically, Nrf1 immunoblotting produces bands with apparent molecular weights of 120 and 95 kDa, that are thought to represent glycosylated and unglycosylated Nrf1 respectively (Zhang *et al.*, 2007). However, Nrf1 can be seen as an 85-kDa polypeptide using other antibodies such as those provided by R&D Systems (http://www.rndsystems.com/pdf/MAB5306.pdf). Our data is also consistent with previous reports of several Nrf1-specific bands, seen upon Nrf1 overexpression, and Nrf1 detection by Western blotting including an unstable, presumably, unglycosylated 85-kDa band (Zhang and Hayes, 2010) and a shorter form of endogenous Nrf1, migrating with an apparent molecular weight of 55 kDa and thought to be the Nrf1, proteolytically-cleaved around the Asn/Ser/Thr- (NST)-rich region (Zhang *et al.*, 2009). Both Zhang and co-workers (2009) and the current study utilized the anti-Nrf1 antibodies raised against the C-terminal portions of Nrf1; therefore, the 55-kDa Nrf1 lacks the N-terminal (NTD) domain, keeping the factor in the endoplasmic reticulum-(ER)-bound, inactive state. This short Nrf1 form can still act as transcriptional activator as it contains at least two of the three Nrf1 transactivation domains (acidic domain (AD)1, NST and AD2 (Zhang *et al.*, 2009; residues 125-324, 325-432 and 433-482 in hNrf1, respectively)). The decrease of the basal *GCLC* and *GCLM* expression, observed

Figure 5.1. *Nrf1* Silencing Activates the Expression of the ARE-target Genes. (A) HBE1 cells were treated with 50 nM scrambled (control) or *Nrf1*-specific siRNA (Santa Cruz) for 72 hours and the mRNA expression of *GCLC* and *GCLM* was analyzed with the RT-qPCR. The data was normalized to the *GAPDH* expression and is presented as means ± S.E.M. (n = 3). Single and double asterisks (* and **) indicate statistical significance from the controls with $p < 0.05$ and $p < 0.001$, respectively. **(B)** The change of Nrf1 protein expression upon silencing with either scrambled or *Nrf1*-specific siRNA and the relative Nrf1/actin levels was accessed by immunoblotting. The relative density of the Nrf1-specific bands, shown with arrows, is graphed as a representative result of three independent experiments with similar outcome.


A
mRNA Expression (GCL/GAPDH)
0.5
0.4
0.3
0.2
0.1
0.0
Scrambled RNAi
Nrf1 RNAi
**
*
GCLC
GCLM


B
siRNA
Scram. Nrf1
150
75
50
20
Nrf1
84 kDa
55 kDa
β-Actin
Nrf1 Band Densitometry (Nrf1/actin)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Scrambled RNAi
Nrf1 RNAi
Full-length Nrf1 (84 kDa)
Short-form Nrf1 (55 kDa)

here, is in accord with previous findings (Ohtsuji et al., 2008) in the hepatocytes of Nrf1 knockout mice.

5.3 Exogenous Nrf1 Suppresses Nrf2 Induction by tert-Butylhydroquinone (tBHQ)

Next, to determine the effect of full-length (p120Nrf1) or short form (p65Nrf1) of Nrf1, ARE-luciferase reporter assays were performed. To this end, HEK293 cells were transfected with GCLC- and GCLM-ARE upstream of the firefly luciferase gene along with p120Nrf1, p65Nrf1 and Nrf2. After co-transfection, the cells were treated with a pro-oxidant inducer of the Nrf2-ARE pathway, tBHQ (Wang and Jaiswal, 2006). When both GCLC- and GCLM-ARE luciferase reporters were used, the transactivation activity of p65Nrf1 was approximately 2-3 times lower than that of the full-length Nrf1 (Figure 5.2). This is consistent with the findings of Zhang and co-workers (2009) who showed that the transactivation activity of Nrf1 comes mainly from the AD1 and NST domains of the protein, with a minor contribution from the AD2 domain. Naturally, the activity of p65Nrf1 is lower than that of p120Nrf1 as the former lacks the AD1. As expected, Nrf2 transactivation activity was increased by tBHQ treatment with both GCLC- and GCLM-ARE reporters. With GCLC-ARE, both inducible and basal Nrf2/ARE-driven reporter activity was markedly inhibited by both p65Nrf1 (in accord with Wang and co-workers (2007)) and by p120Nrf1 (Figure 5.2A). Although the activity of Nrf2 was not as pronounced in GCLM-ARE reporter activation, both forms of Nrf1 were efficient in

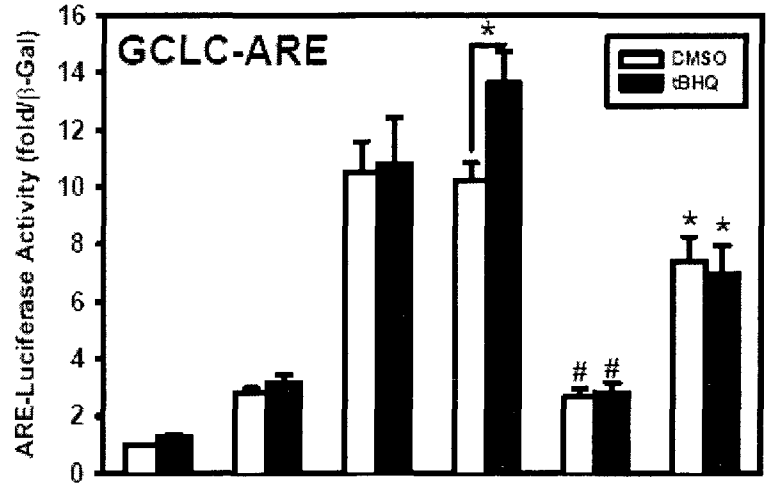
overriding the Nrf2/ARE reporter activity, with no additive effect of the Nrf2 on the reporter activity of both forms of Nrf1 observed (Figure 5.2B). These results are consistent with the idea that p65Nrf1 (Wang *et al.*, 2007) and p120Nrf1 are efficient inhibitors of the Nrf2-ARE driven gene expression and are in good agreement with the stimulatory effect of Nrf1 removal on *GCLC* and *GCLM* expression (Figure 5.1).

5.4 The DNA-binding Activities of Nrf1 and Nrf2 Diminish with Age

Having established the prevailing effect of Nrf1 on the Nrf2/ARE-mediated gene expression in the cultured cell lines, we then delved into examination of the Nrf1 and Nrf2 function in mouse models. The protein binding to the labeled ARE from *GCLM* was accessed using lung nuclear extracts of the young (6 month) and old (18 month) animals, subjected to the ambient air particles for 10 weeks, at 15 hours per week. As expected from the previous work in aged rats (Suh *et al.*, 2004), aged animal lung nuclear protein pools contained considerably lower ARE-binding activity (Figure 5.3). Importantly, the treatment with particles reversed the loss of the DNA-binding to AREs in aged animals. The use of antibodies raised against Nrf1 and Nrf2 factors suggested that, not only Nrf2, but also Nrf1 contributed to the ARE binding observed in the nuclear extracts.

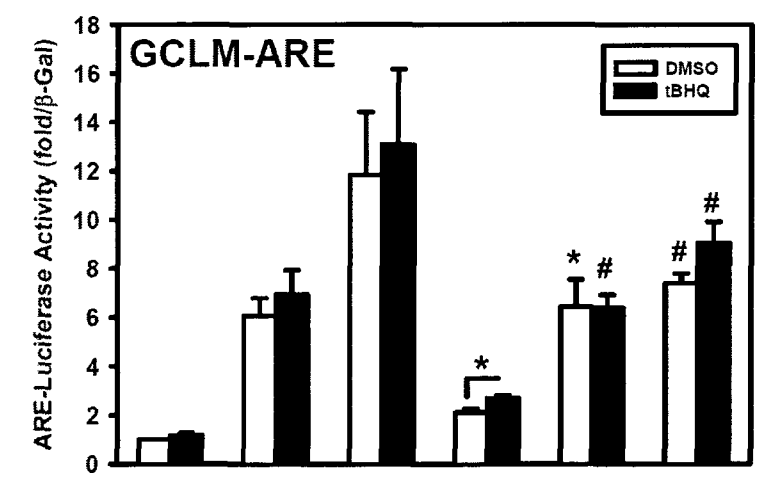
Figure 5.2. The Full-length (p120Nrf1) and 65-kDa (p65Nrf1) Forms of Nrf1 Suppress the Nrf2-mediated Transcription of ARE-controlled Gene Expression. HEK293 cells were transfected with the indicated amounts of the empty vector (pCR3.1), p65Nrf1, p120Nrf1 and Nrf2 along with the luciferase reporter vector with the ARE from GCLC (**A**) or GCLM (**B**). A β-galactosidase plasmid was used as the transfection efficiency control and the luciferase signal was normalized to β-galactosidase activity. Shown are means ± S.E.M. of at least three independent experiments ($n \geq 3$). Asterisks (*) and the number signs (#) indicate statistically significant differences from controls at the $p < 0.05$ and $p < 0.001$ levels, respectively.

A



pCR3.1	1.0 μg			1.0 μg		
p65Nrf1		1.0 μg			1.0 μg	
p120Nrf1			1.0 μg			1.0 μg
Nrf2				1.5 μg	1.5 μg	1.5 μg

B



pCR3.1	1.0 μg			1.0 μg		
p65Nrf1		1.0 μg			1.0 μg	
p120Nrf1			1.0 μg			1.0 μg
Nrf2				2.0 μg	2.0 μg	2.0 μg

5.5 Nrf1 and Nrf2 Protein Levels Change in the Opposing Fashion with Age

The EMSA results, pointing to the contribution of both Nrf1 and Nrf2 factors to ARE DNA-binding, could be explained by either lowered DNA binding affinity of the factors or their diminished nuclear presence. To investigate the latter possibility, immunoblotting was performed (Figure 5.4). Two different antibodies were used to detect Nrf1 and both showed qualitatively similar results. Thus, an increased expression of the band, migrating with an apparent molecular weight of 65 kDa, with a similar mobility to that of an exogenously-expressed Nrf1-FLAG positive control, was notable in aged and particle-treated animals (Figure 5.4A, top panel). A similar trend was seen for the long form of Nrf1, migrating with an approximate molecular weight of 95 kDa (Figure 5.4A, middle panel), and for the Nrf1 band detectable with the C-terminal antibody and migrating as a 55-kDa polypeptide (Figure 5.4A, bottom panel). On the other hand, the nuclear levels of Nrf2 were decreased in aged mice, but were increased by the particulate exposure in both young and aged animals, with the effect being more pronounced in the 6-month mice (Figure 5.4C and D). These results indicate that: i) aging regulates the Nrf1 and Nrf2 levels in the opposite direction, reminiscent of the Yin-Yang type of pattern; ii) the administration of exhaust particulate matter to mice invokes increased expression of both Nrf1 and Nrf2 and iii) that the stimulatory effects of particulate exposure on the Nrf1 and Nrf2 protein expression is more pronounced in the young animals.

5.6 The Inducibility of the Nrf1/2-ARE-controlled Genes and the Cellular Antioxidant Defence Capacity is Impaired with Age

Finally, the expression of an ARE-target gene *NQO1*, as well as the cellular capacity of mouse lung to mount an efficient antioxidant defence upon the exposure to the airborne particulate matter, was examined using real-time qPCR and oxygen radical absorbance capacity (ORAC) assay. The mRNA expression of *NQO1*, an established subject of the Nrf1/2-ARE regulation (Dinkova-Kostova and Talalay, 2010), was markedly increased by particulate treatment of the young, but not old, mice (Figure 5.5A). A similar trend was noticeable with the ORAC assay, showing that only young animals could increase their total antioxidant defence (oxygen radical scavenging) capacity when challenged with airborne particles (Figure 5.5B). These results are indicative of impaired ability of the Nrf2-ARE pathway to elicit an appropriate response to the pro-oxidant stimulus.

5.7 The Proposed Model for the Effect of Aging and Particulate Exposure on the Nrf1 and Nrf2 Function in Mice

The current study demonstrates, for the first time, that both full-length and short form of Nrf1 have an inhibitory effect on the ARE-driven gene expression, mediated by Nrf2, as evidenced by the experiments with Nrf1 silencing and Nrf1/Nrf2 overexpression in cultured human cells. Thus, the removal of *Nrf1* by RNA interference increased the gene expression of *GCLC* and *GCLM*, in accord with the previous report from the Yamamoto lab (Ohtsuji *et al.*, 2008), examining hepatocyte-specific *Nrf1* knockout mice model. In that study, *Nrf1* deficiency increased the expression of multiple ARE/Nrf2-controlled genes, including *GCLC*, *NQO1* and *HO-1*. More importantly, this stimulatory effect of

the *Nrf1* disruption was completely abolished in the livers of the *Nrf1:Nrf2* double knockout mice (Ohtsuji *et al.*, 2008), suggesting that Nrf1 may act as an inhibitor of the basal Nrf2-driven gene expression, in good agreement with our data. Similarly, the co-transfection of the HEK293 cells with p65Nrf1 or p120Nrf1 together with Nrf2 completely abrogated the Nrf2-driven *GCLC*-ARE-luciferase reporter activity and fully overrode both basal and inducible *GCLM*-ARE-driven luciferase expression, confirming our hypothesis that Nrf1 has an inhibitory effect on the Nrf2 transactivation activity. Mechanistically, the dominance of Nrf1 over Nrf2 may be attributed to the differences in their transactivation domains, such that only one and not the other may recruit all the necessary components of the basal transcription machinery to transcribe a given gene. This, apparently, is the case for the metallothionein 1 (*MT1*), whose expression is controlled exclusively by Nrf1 and not Nrf2, despite the fact that both factors bind the *MT1*-ARE with equal binding affinity (Ohstuji *et al.*, 2008). Another possibility is that the inhibitory effect of Nrf1, revealed by the *Nrf1* silencing and overexpression is due to the short form of Nrf1, p65Nrf1, which lacks the AD1 transactivation domain and may act as a repressor of Nrf2 by competing with Nrf2 for DNA-binding to the ARE (Wang *et al.*, 2007). The existence of such a negative control by p65Nrf1 is hypothesized to be in place to avoid the unrestrained expression of ARE target genes, which can be carcinogenic (Wang *et al.*, 2007). Our luciferase assay results with p65Nrf1 confirmed the data from the Chan lab (Wang *et al.*, 2007); in addition, we have shown that not only p65Nrf1, but also p120Nrf1, is capable of achieving marked reduction of both Nrf2 basal and tBHQ-inducible transactivation activity.

These *in vitro* results provided an invaluable starting point in the attempt to study the existence of potential Nrf1/Nrf2 interaction in mouse models of pro-oxidant exposure. Previously, it was reported that airborne particles from the vehicular traffic contained stable, carbon-centered radicals and the particulate treatment evoked pronounced inflammatory responses in mice and glutamatergic neurotoxicity in rat brain slices and cultured primary neurons (Morgan *et al.*, 2011). Taking these findings into account, it was expected that mouse lung is a good *in vivo* model of the Nrf2-ARE pathway responsiveness to the airborne traffic particles, according to the hierarchical oxidative stress model (Li *et al.*, 2010). According to this model, explaining the pro-oxidant effects of diesel exhaust particles, the Nrf2-ARE pathway constitutes the first tier of cellular defense and it is activated at the stress levels below those needed to activate proinflammatory responses, the second cellular defense against exhaust particles (Li *et al.*, 2010). Therefore, given the report (Morgan *et al.*, 2011) of certain inflammatory changes as a result of the particle treatment of mice in the brain, an organ which is not expected to be in such a direct contact with the particles as the lung is, it was reasonable to expect that that the treatment would affect the Nrf2-ARE pathway, allowing us to dissect potential contribution of Nrf1 and Nrf2 to the aging and the cellular antioxidant defense against the particles.

The notable effects of the particle treatments compared to the controls (air) included: i) restoration of the age-impaired Nrf1- and Nrf2-ARE binding, ii) increased protein expression of Nrf2, more pronounced in the young animals, iii) increased protein expression of Nrf1, iv) increased *NQO1* mRNA content in young mice and v) increased

oxygen radical scavenging capacity in the samples from young, but not old animals. All these are indicative of the induction of the Nrf1/2-ARE pathway to counter the deleterious effects of the airborne particles, albeit such an effect was more pronounced in younger mice. It can be envisioned that, due to the accumulation of the oxidative damage over time (Sohal an Weindruch, 1996), the Nrf1/2-ARE pathway in aged animals can already be in its induced state, as suggested by the fact that "basal" *NQO1* (Figure 5.5A) and *HO-1* (data not shown) mRNA levels and antioxidant capacity measured by the ORAC assay in aged animals were already higher compared to the young subjects even without particulate matter treatment. The prominent effects of aging included: i) the decrease in the Nrf1/2-ARE binding, ii) decreased protein expression of Nrf2, iii) increased *NQO1* mRNA expression, and iv) increased oxygen radical scavenging capacity. Effects i) and ii) are indicative of the impairment of the Nrf2-ARE pathway, such that diminished ARE binding could be related to the lowered Nrf2 content in agreement with previous study in aged rats (Suh *et al.*, 2004). However, it is noteworthy that the Nrf1 and Nrf2 protein expression changed with age in the opposing fashion, such that the Nrf2/Nrf1 ratio decreases with age. It is currently unclear what regulatory event(s) is(are) responsible for the paradoxical reduction of the Nrf2 protein content in aged mice despite the increased oxidative environment and thus, greater demand for the enhancement of the cellular antioxidant gene expression. One possibility is that the redox-sensing capacity of the INrf2 (also known as Keap1), an E3 ubiquitin ligase adaptor, facilitating the ubiquitination of Nrf2 and its rapid proteasomal degradation, is impaired with age (Suh *et al.*, 2004). However, studies in the cultured mouse fibroblasts showed that subjecting cells to more oxidizing extracellular redox potential (-46 and 0

mV), to approximate the age-dependent redox changes, still induced Nrf2 translocation from the cytoplasm to the nucleus (Imhoff and Nansen, 2009), suggesting that alteration of the cellular redox status alone fails to explain the decline of Nrf2 expression with age. Alternatively, an INrf2-independent mechanism of Nrf2 control is known, involving the phosphorylation of some Ser residues of the Neh6 domain of Nrf2 by glycogen synthase kinase-3β (GSK-3β), stimulating the Nrf2 exclusion from the nucleus (Salazar *et al.*, 2006). The GSK-3β activity is, itself, inhibited by the Akt-mediated phosphorylation at the Ser9 residue and Akt inhibition stimulates nuclear translocation of Nrf2 (van Weeren *et al.*, 1998; Rojo *et al.*, 2008). A more recent work reported that the levels of the phosphorylated GSK-3β and phosphorylated (inactive) Akt decreased with age in the liver of mice models of accelerated aging (Tomobe *et al.*, 2011), suggesting that the GSK-3β activation could be responsible for the reduced nuclear Nrf2 content.

Unlike its well-studied Nrf2 CNC-bZIP family member, the mechanisms of Nrf1 control remain largely enigmatic. The full-length Nrf1 shows a double band on the Western blot, corresponding to 120- and 95-kDa proteins (p120Nrf1 and p95Nrf1, respectively). The p95Nrf1 represents the non- or unglycosylated Nrf1, while the p120Nrf1 is the glycosylated form (Zhang *et al.*, 2007). The factor is localized primarily to the ER and nuclear envelope (Zhang *et al.*, 2009). In addition, the ER redox status and Nrf1 glycosylation status are hypothesized to cause Nrf1 relocation to the nucleus from the ER (Zhang *et al.*, 2009). Nrf1 may also be activated by the proteasomal cleavage of its N-terminus to remove the inhibitory NTD, producing smaller, more active forms of Nrf1 (Schultz *et al.*, 2010; Chepelev *et al.*, unpublished). The anchoring of Nrf1 to the

ER by the NTD domain is thought to be weakened by the stimulation of Nrf1 Tyr phosphorylation by pro-oxidants, stimulating the nuclear import of the otherwise ER-bound Nrf1 (Zhang *et al.*, 2009). In addition, there is a short form of Nrf1, migrating as a 65-kDa protein (p65Nrf1) on the SDS-PAGE, presumably arising from the *Nrf1* translation from an internal start codon (Chan *et al.*, 1993) The p65Nrf1 acts as a dominant negative inhibitor of Nrf2-mediated, ARE-driven luciferase reporter activity, competing with Nrf2 for the DNA binding (Wang *et al.*, 2007). The upstream signalling factors, regulating Nrf1, have not been elucidated in great detail. The regulation of Nrf1 by the proteasome has recently been described (Steffen *et al.*, 2010; Radhakrishnan *et al.*, 2010). Thus, the ER-bound Nrf1 appears to be controlled by the ER-associated protein degradation (ERAD) system, involving ubiquitin-dependent proteasomal degradation of its protein substrates (Steffen *et al.*, 2010). To our best knowledge, there has not been a single report of the induction of the Nrf1 protein expression by treatments other than protesomal inhibition (Zhang *et al.*, 2009; Steffen *et al.*, 2010; Radhakrishnan *et al.*, 2010), except for one study, revealing arsenic-induced stabilization of the full-length Nrf1 (Zhao *et al.*, 2011). It is known that aging is associated with the proteasomal dysfunction, partly due to the accumulation of oxidized proteins and protein aggregates that could overload the proteasome-dependent functions (Grimm *et al.*, 2011). Therefore, an attractive hypothesis is that the age-dependent Nrf1 stabilization observed here could be due to impaired Nrf1 degradation by the proteasome. As Nrf2 is also a subject of the proteasomal degradation, albeit by a different proteasomal-degradation pathway than Nrf1, the differences in Nrf1 and Nrf2 age-dependent stability can reflect differential changes in sensitivity of the two proteasomal clearance pathways during aging.

Considering the inhibitory effect of Nrf1 on the Nrf2 transactivation function (Figures 5.1 and 5.2), it seems reasonable to hypothesize that the age-associated decline in the homeostatic [Nrf2]/[Nrf1] ratio may be the underlying cause for the decreased responsiveness of the Nrf2-ARE pathway to the stimulation with the airborne particles. Our working model for the combined effect of the particulate exposure and aging on the ARE pathway is summarized in Figure 5.6. In conclusion, this is the first study, describing the Yin-Yang pattern of the Nrf1/Nrf2 protein expression associated with aging. Future studies will reveal the exact mechanisms responsible for Nrf1 age- and airborne exhaust particle-dependent stabilization with the impairment of the ERAD pathway being one possible hypothesis. More importantly, the analysis of Nrf1 and Nrf2-bound co-activators or co-repressors by such techniques as immunoprecipitation and/or mass spectrometry is expected to yield invaluable clues to understand the inhibitory effect of Nrf1 on the Nrf2/ARE-driven gene expression.

Figure 5.3 Aging is Accompanied by Decreased Protein Binding to the ARE. Mouse lung nuclear protein extract (7.5 μg per lane) was incubated with the biotinylated *GCLM* ARE probe and the protein binding to the ARE was examined using horseradish peroxidase-conjugated streptavidin and chemiluminescence. The specificity of the doublet band, representing specific ARE-protein complexes, was deduced using free probe only and by the addition of an excess of unlabelled (cold) probe to the reactions. The involvement of Nrf1 and Nrf2 in the ARE-protein complex formation was revealed using normal mouse pre-immune serum (IgG, negative control) or Nrf1- or Nrf2-specific antibodies. P.M. and N.E. designate the samples from particulate matter-treated animals, and non-exposed controls, respectively. For the antibody-supershift experiments, 20 μg of protein extract from 6-month, air-treated animals per lane was used. The representative results of three independent experiments are shown.

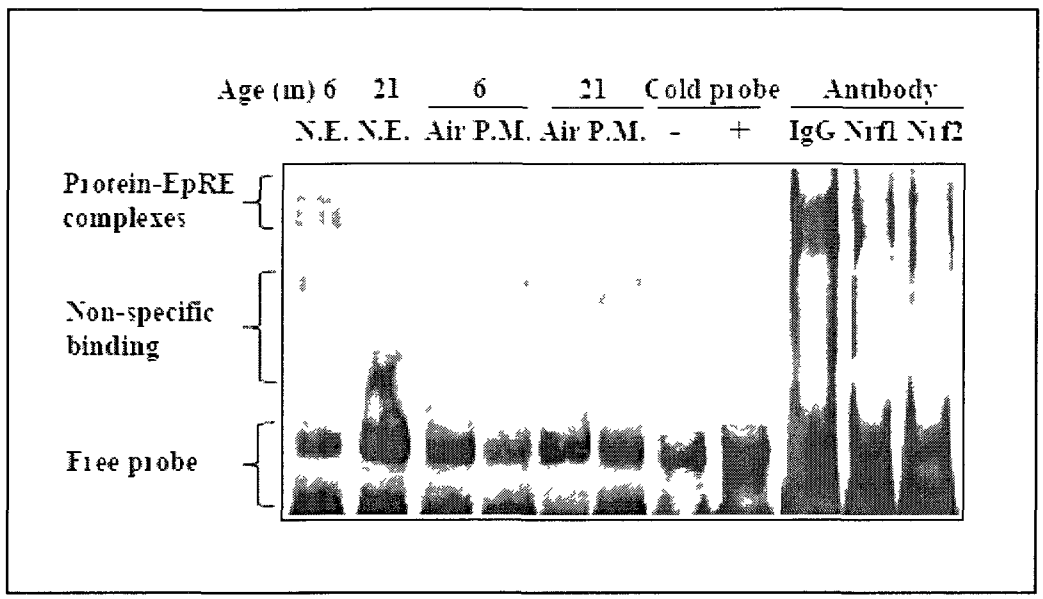

Age (m) 6 21 6 21 Cold probe Antibody
N.E. N.E. Air P.M. Air P.M. - + IgG Nrf1 Nrf2
Protein-EpRE complexes
Non-specific binding
Free probe

Figure 5.4. Nrf1 and Nrf2 Protein Levels Change in the Opposing Fashion with Age. The protein expression of Nrf1 (**A**) and Nrf2 (**C**) in the lung of young and old mice, treated with airborne particles, was studied with Western blotting. The lysates of the HEK293 cells, transfected with an empty vector (pCR3.1) or Nrf1-FLAG were used as a positive control for Nrf1 expression (**A**). Graphical determination of Nrf1 (**B**) and Nrf2 (**D**) densitometry with respect to loading controls is presented. Data are presented as means ± S.E.M. and results shown are representative of at least three ($n \geq 3$) independent experiments.

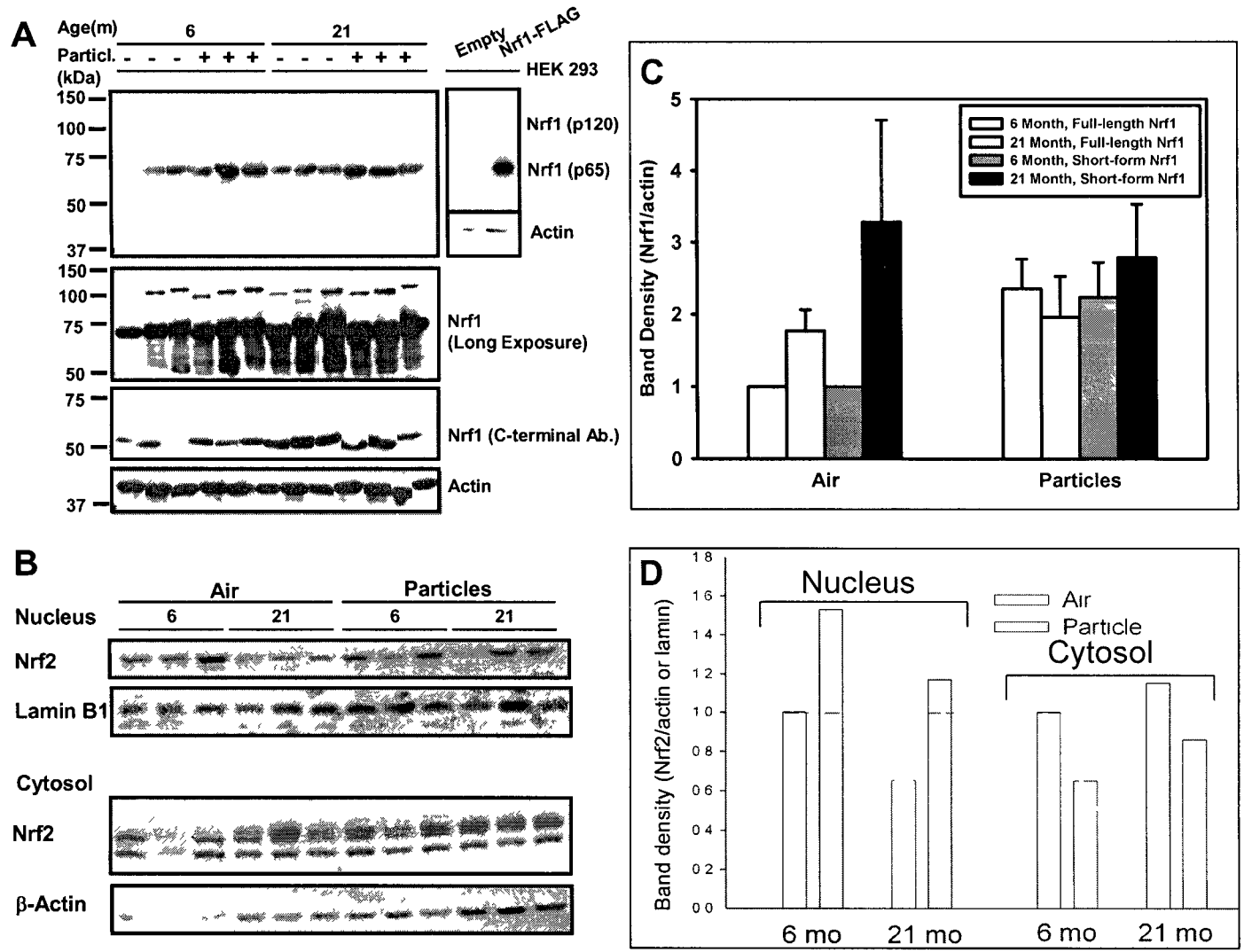
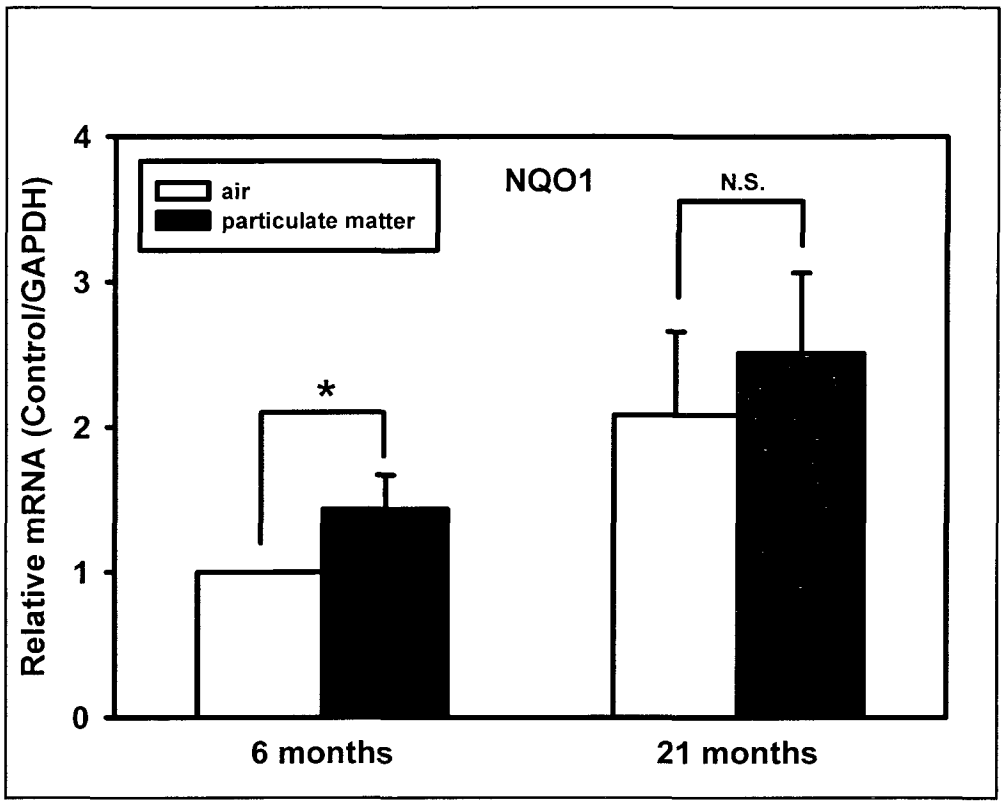
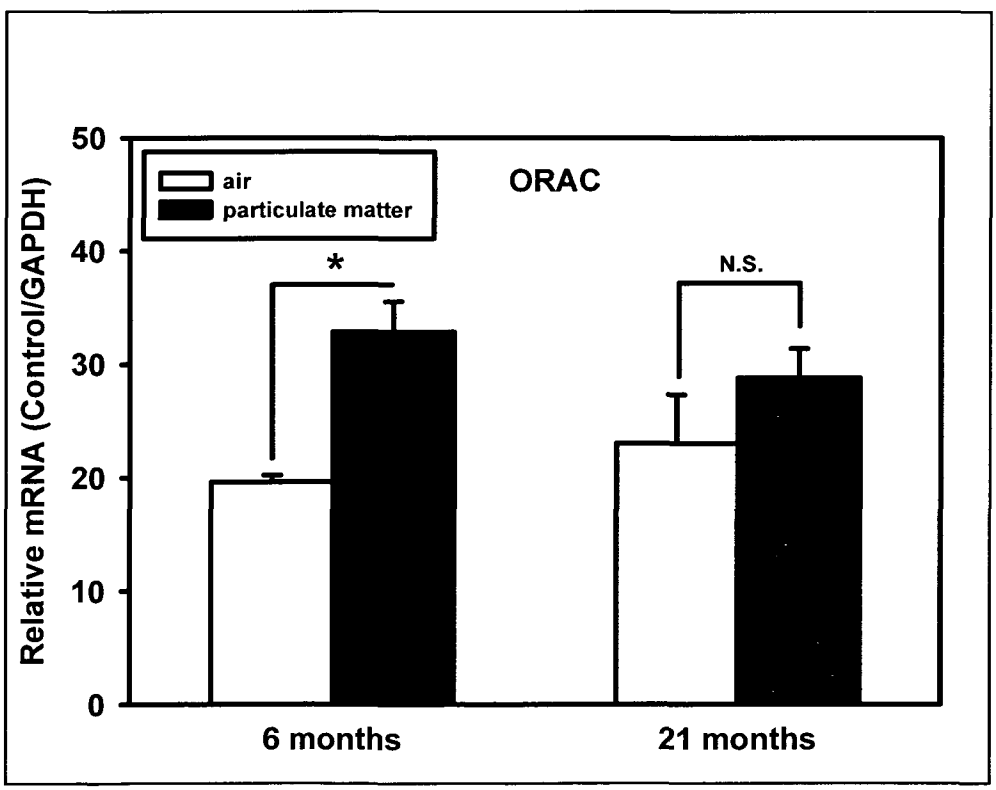

A
Age(m)
6
21
Particl. - - - + + + - - - + + +
(kDa)
Empty
Nrf1-FLAG
HEK 293
150
100
75
50
37
Nrf1 (p120)
Nrf1 (p65)
Actin
Nrf1
(Long Exposure)
Nrf1 (C-terminal Ab.)
Actin
B
Air
Particles
Nucleus
6
21
6
21
Nrf2
Lamin B1
Cytosol
Nrf2
β-Actin
C
Band Density (Nrf1/actin)
6 Month, Full-length Nrf1
21 Month, Full-length Nrf1
6 Month, Short-form Nrf1
21 Month, Short-form Nrf1
Air
Particles
D
Band density (Nrf2/actin or lamin)
Nucleus
Air
Particle
Cytosol
6 mo
21 mo
6 mo
21 mo

Figure 5.5. Age-dependent Decline in the Inducibility of the Nrf1/2-controlled Gene Expression and Oxygen Radical Scavenging Capacity. The lung samples of young and old mice, subjected to the ambient air or particulate exposure, were analyzed for the expression of the ARE-regulated enzyme *NQO1* by the real-time qPCR (**A**) and for their ability to scavenge the peroxyl and alkoxyl radicals generated by AAPH in the ORAC assay format (**B**). Data is presented as means ± S.E.M. of the relative mRNA expression, normalized to *GAPDH*, and to the controls (**A**) or as μmoles of Trolox equivalents per μg of cytoplasmic protein (**B**). Asterisks (*) indicate significant difference from the corresponding controls ($p < 0.05$, $n \geq 3$).

A


NQO1
air
particulate matter
N.S.
*
Relative mRNA (Control/GAPDH)
0
1
2
3
4
6 months
21 months

B


ORAC
air
particulate matter
*
N.S.
Relative mRNA (Control/GAPDH)
0
10
20
30
40
50
6 months
21 months

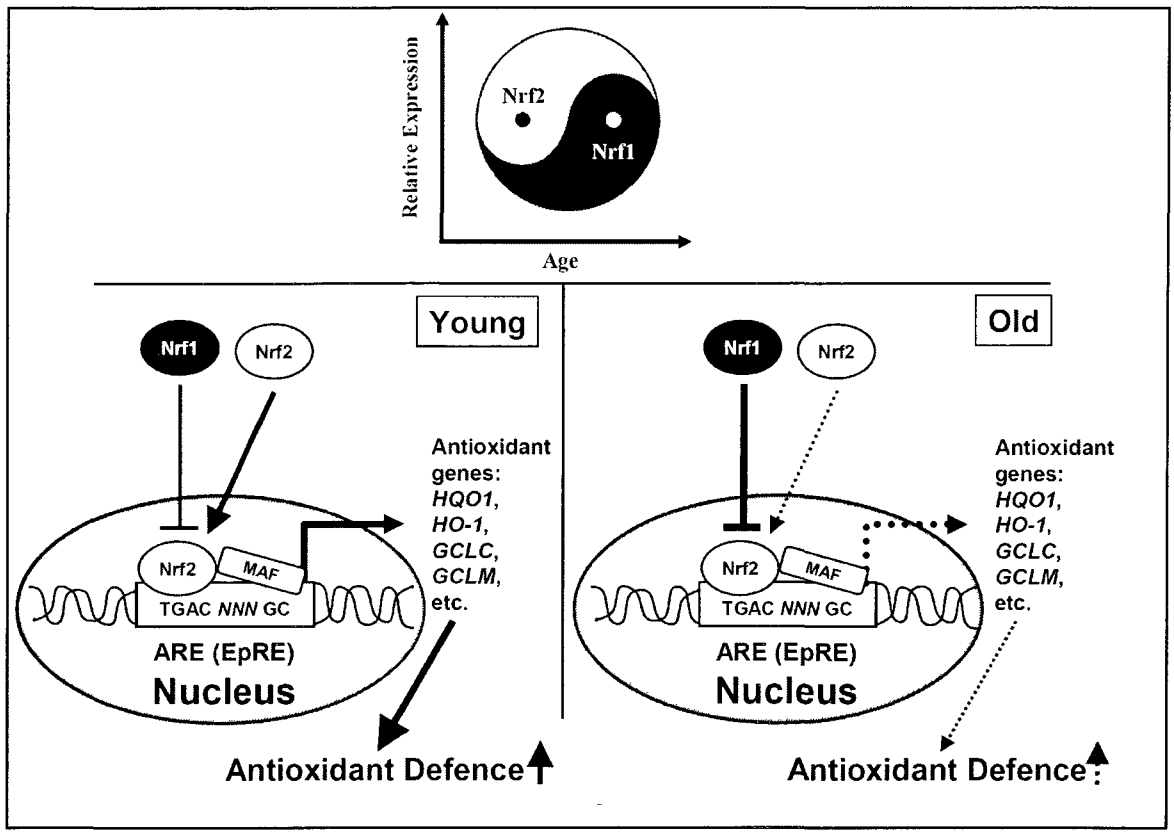


Figure 5.6. The Proposed Model for the Combined Effect of the Particulate Exposure and Aging on the Nrf1/2-ARE Pathway. The ratio of Nrf1 (thought to act as an Nrf2/ARE inhibitor) and Nrf2 (the activator of the ARE-driven gene expression) is proposed to be the underlying cause for the reduced responsiveness of the ARE pathway to airborne particle-induced oxidative stress. In young animals, the inhibitory effect of Nrf1 on the ARE-driven gene expression, observable with *Nrf1* silencing (Figure 5.1A) is not seen clearly due to relatively high Nrf2 content. This situation changes dramatically in old animals, where the Nrf1/Nrf2 ratio is increased as a result of lower Nrf2 and higher Nrf1 protein expression. As a result, the inhibitory potential of Nrf1 on both basal and oxidative stress- (created by the exposure to the airborne particles) inducible expression of the ARE target genes is more pronounced. See the text for further details.

6 Chapter: General Discussion

6.1 Towards Understanding the Physiological Functions of Nrf1

Since the beginning of this project in 2006, a significant breakthrough in understanding the mechanisms involved in the control of Nrf1 has been achieved. Nearly 50% of the articles on Nrf1 (17 publications) have been published since 2006, out of 36 publications in total since the initial discovery of Nrf1 in 1993 by the Jefferson Chan group (Chan *et al.*, 1993). Compared to Nrf1, the number of publications on Nrf2 seems to be growing exponentially, totaling nearly 4,000 since Nrf2 discovery in 1994 (Figure 6.1). One can argue that such a discrepancy stems from the fact that Nrf2 global knockout mice are viable, while Nrf1-deficient mice die during embryonic development (Chan *et al.*, 1998).

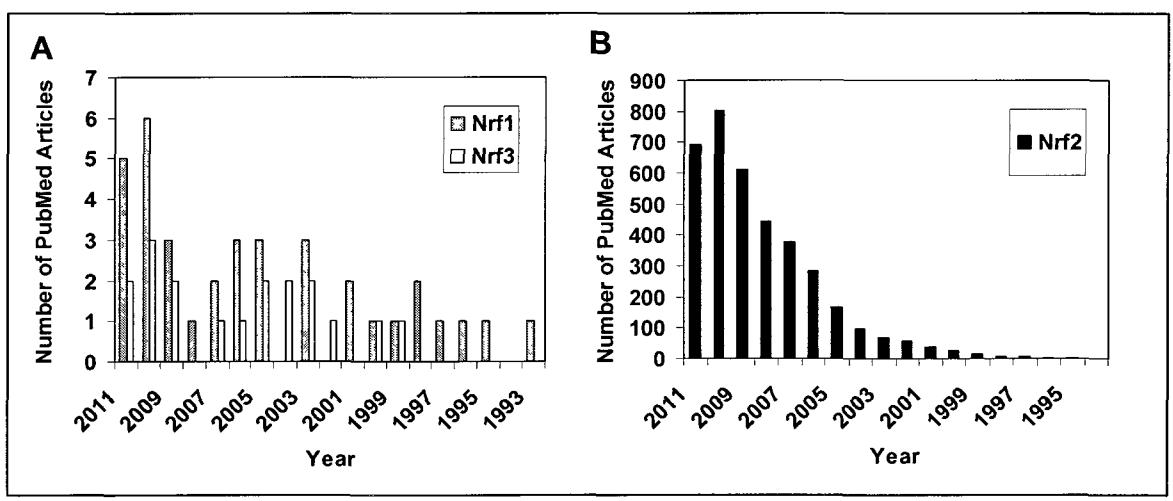


Figure 6.1. The Progress of Our Understanding of the Nrf Factors as Revealed by the Number of PubMed Publications. Review publications were not counted.

However, this view can be challenged by the rate of progress achieved with understanding the regulation and function of a closely-related CNC-bZIP family member, Nrf3. The Nrf3 factor has received even less attention than Nrf1 since its discovery in 1999 and has even been called the "Cinderella" of the CNC-bZIP family (Chevillard and Blank, 2011). Unlike Nrf1, the Nrf3 knock-out mice are viable and show no obvious phenotypic abnormalities (Derjuga *et al.*, 2004). The function of Nrf3 is also more apparent upon certain stimuli such as chemical-induced carcinogenesis (Chevillard *et al.*, 2011). One common feature of Nrf1 and Nrf3 is that they are both glycosylated and are localized to the ER through their N-terminal domain (Zhang, Kobayashi *et al.*, 2009; Zhang *et al.*, 2009). This might suggest that the physiological functions of these two factors are related to the maintenance of the ER homeostasis against the ER stress as was proposed by Zhang and co-workers (2009) and Zhang and Hayes (2010). While both Nrf1 and Nrf3 have been reported to act as activators of antioxidant gene expression (Zhang *et al.*, 2009; Chénais *et al.*, 2005), their negative control of the ARE-driven gene expression by Nrf3 (Sankaranarayanan and Jaiswal, 2004) and Nrf1 (Wang *et al.*, 2007) has also been reported previously and in this study (Chapter 5). On the other hand, Nrf2 is a well-known master regulator of ARE-inducible gene expression (Pi *et al.*, 2010). Therefore, Nrf1 and Nrf3 may simply add another level of complexity to the Nrf1/2/3-ARE pathway regulation and this complexity makes it difficult to investigate the effects of the individual factors alone. One interesting hypothesis regarding the inhibition of Nrf2 function by Nrf1 is that Nrf1 function might be necessary to turn off the unrestrained antioxidant gene expression, which would otherwise be pro-carcinogenic (Wang *et al.*, 2007). Regardless of the precise reasons for the poor characterization of

Nrf1 and Nrf3 factors, the disproportionately high focus of the scientific community on Nrf2 seems puzzling and unjustified as both Nrf1 and Nrf3 may play as an important role in human health and disease as Nrf2 does (Sykiotis and Bohmann, 2010).

Recent studies on the regulation of the gene expression of subunits of the proteasome in human Ea.hy926 cells (Steffen *et al.*, 2010) and mouse embryonic fibroblasts (Radhakrishnan *et al.*, 2010) suggested a role of Nrf1 in the maintenance of proteasomal-mediated protein degradation. Nrf1 brain-specific knockout in mice led to proteasomal impairment, further confirming the importance of Nrf1 as a translational regulator of proteasomal gene expression (Lee *et al.*, 2011). In addition, the involvement of Nrf1 in ER membrane-dependent biological processes has been proposed by Zhang and Hayes (2010). According to this proposal, the amphipathic regions of Nrf1, once integrated in the ER membrane as α-helices, can upset the organization of membrane lipids by restraining the diffusion of neighboring lipids (Zhang and Hayes, 2010). Such a hypothesis has been proposed due to the fact that liver-specific knockouts of *Nrf1* in mice resulted in the development of non-alcoholic hepatitis, presumably emanating from the increased overexpression of the cytochrome P450 4A enzymes which catalyze the oxidation of fatty acids, as well as diminished ARE-driven gene expression (Xu *et al.*, 2005). Similarly, hepatocytes isolated from the *Nrf1*-/- animals contained higher number of lipid vesicles and exhibited greater proliferation of smooth ER (Ohtsuji *et al.*, 2008; Xu *et al.*, 2005), which may be yet another indication of the role of Nrf1 in the regulation of the ER homeostasis. Other than regulating the antioxidant gene expression, Nrf1 also plays a role in: i) inflammation, where Nrf1 inhibits the expression of the inducible nitric

oxide synthase (iNOS) (Berg *et al.*, 2007); ii) bone development, activating the expression of osterix gene (Xing *et al.*, 2007); iii) ondontoblast differentiation, through the regulation of dentin sialophosphoprotein gene expression (Narayanan *et al.*, 2004); and iv) the survival of neurons upon acute brain injury (reviewed in Biswas and Chan, 2010). Taken together, these findings reveal that Nrf1 is involved in multiple physiological processes from development to antioxidant response and inflammation, representing exciting subjects for future investigation.

6.2 Assessment of the Current Models to Study Nrf1 Function

Unlike the functions of other proteins, such as enzyme-catalyzed reactions where product formation or substrate disappearance can often be easily detected and quantified, studying the function of transcription factors is far more complicated. First, there is no inherent measurable product or property that can be detected in association with a transcription factor's DNA-binding. This limits the *in vitro* studies of the properties of transcription factors to artificial systems, for example, the one in which a DNA-binding region of a factor is placed upstream of some easily measurable reporter gene, such as luciferase. Such an approach has been used in this project for the study of Nrf1 and Nrf2 functions. However, it suffers from the fact that, in mammalian cells, the transcription of a specific gene can be affected by DNA regions located hundreds or even thousands of base pairs away from the transcription initiation site. As a result, cloning only a short segment of DNA, representing a DNA-binding region of a given factor, does not take into account

the influence of other regulatory DNA regions. In addition, overexpressing an activator protein in a luciferase type of format can actually decrease rather than increase the reporter activity due to the alteration in the subcellular organization of this factor or corrupting the transcription machinery by exceeding its capacity (Xang *et al.*, 2003). Despite that, such an approach can be an invaluable starting point for future studies in more advanced systems.

To allow for more quantifiable and specific measurement of the DNA-binding function of a given factor, some commercially-available kits exist, such as TransAM systems from Active Motif (http://www.activemotif.com/catalog/180). These rely on the use of specific antibodies, recognizing a DNA-binding protein, bound to a microplate well, coated with a certain DNA segment to which the transcription factor is known to bind. While definitely being more specific than luciferase reporter assays, which can capture the contribution of other transcription factors, this approach, once again, ignores the existence of other regulatory DNA regions outside of the specific sequence recognized and bound by the transcription factor. Another, more practical disadvantage of this technique is its high cost (approximately $700 US for a set of 96 reactions) relative to other assays.

In addition, there are electrophoretic mobility shift assays (EMSAs) that allow one to measure the binding of nuclear proteins to a labelled DNA probe. Nuclear extract, isolated from cells, is incubated with the DNA probe, run on a nondenaturing gel and analyzed by autoradiography (an old and hazardous approach) or blotted over to a

membrane and detected by chemiluminescence (preferred by younger generation of scientists). The weakness of this *in vitro* technique is that, like luciferase reporter assays, only a short DNA segment is examined, the specificity of the binding must be verified by the addition of specific antibodies, expected to produce a supershift, retarding the DNA-protein complex mobility on a gel. This might be quite a challenge to achieve and often requires special grade of antibodies.

Another, more direct approach is to study the expression of a gene, known to be controlled by a specific transcription factor, with real-time quantitative PCR. A problem with that approach is that a given gene is usually controlled by a battery of transcription factors. An example of this is NQO1, which can be controlled by both Nrf1 and Nrf2, in addition to some other, unknown factors. This problem can be solved by silencing the gene expression of a transcription factor by RNAi, as was performed in Chapter 5, to study the function of Nrf1. Alternatively, a gene, coding for a transcription factor of interest, can be studied by either global or organ-specific knockouts in live animals. Naturally, these animal studies are given more credit and are viewed as the gold standard in transcription factor studies. However, even this approach suffers from some ambiguity, associated with the fact that the effect of removing the factor of interest on the expression of a given gene may be indirect and result as a consequence of some perturbations in the normal cellular homeostasis. For example, Ohtsuji and co-workers (2008) postulated that the upregulation of Nrf2-controlled genes in the livers of *Nrf1* hepatocyte-specific knockout mice can be due to the fact that the removal of Nrf1 may create oxidative stress or other stresses, such that Nrf2 becomes hyperactive to counter such a stress. Figure 6.2

illustrates how the relative confidence level of transcription factor studies depends upon the experimental design.

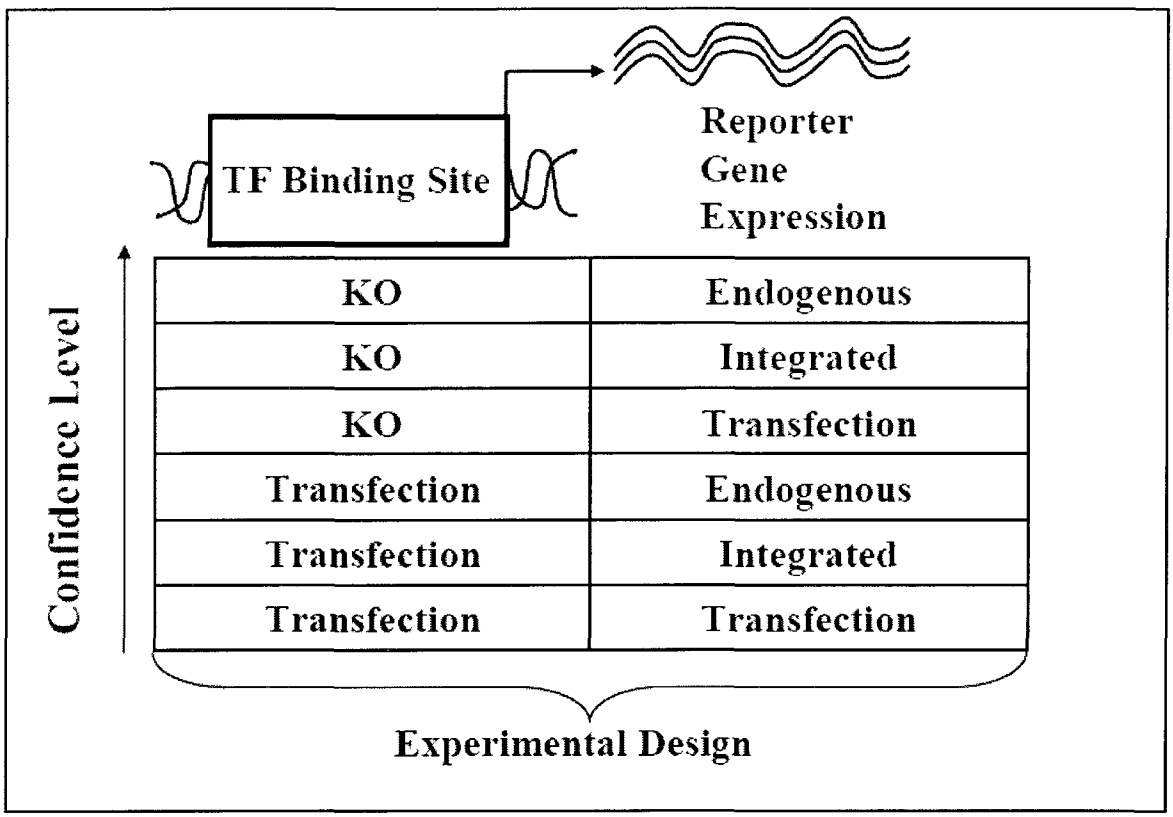


Figure 6.2. The Experimental Design Determines the Confidence in the Results of Transcription Factor Studies. The confidence level of a transcription factor study depends upon the nature of the transcription factor binding site and the type of reporter gene expression. KO refers to knockout studies. The highest confidence is obtained by detecting endogenous gene expression in knockout models. Modified from Xang *et al.* (2003).

6.3 The Potential of Manipulating Nrf1 Activity for Clinical Purposes

Given the cytoprotective nature of its target genes, the Nrf1/2-ARE pathway is thought by many researchers to be amenable to manipulations aimed at the betterment of the human health. First of all, enhanced detoxification of xenobiotics and potential carcinogens by certain compounds (later known as the Nrf2/ARE inducers) has been described as a viable strategy for chemoprevention using rodent carcinogenesis models (Slocum and Kensler, 2011). To demonstrate that such an enhanced detoxification is afforded by the Nrf2-ARE pathway, *Nrf2* knockout mice are used in conjunction with carcinogenesis or tumorigenesis models and the efficacy of the inducer is compared in wildtype and knockout animals. For instance, utilizing this technique, it was shown that oltripaz prevents benzo[a]pyrene-induced gastric tumor formation in wildtype, but not in *Nrf2-/-* animals (Ramos-Gomez *et al.*, 2003). Benzo[a]pyrene contains five benzene rings and readily forms DNA adducts. Nrf2 is thought to activate phase 2 detoxification enzymes, including epoxide hydrolase and NQO1, which, upon the epoxidation reaction, catalyzed by cytochrome p450 enzymes (phase 1 detoxification enzymes), aid in further metabolism and removal of the benzo[a]pyrene metabolites (Slocum and Kensler, 2011).

However, as noted previously, the unrestrained activation of Nrf2 can have a "dark side", namely, pro-carcinogenic effects. This is why Nrf1 control could be useful in making sure the Nrf2/ARE pathway activation only occurs when needed and not constitutively. These dark side effects of Nrf2 have been noticed in several cancer types, where the mutations in *Nrf2* or *Keap1* prevent the interactions between the two proteins

and lead to constitutive Nrf2-ARE activation (reviewed in Slocum and Kensler, 2011). The pro-carcinogenic effect of Nrf2 overactivation can be due to a) increased capacity of cellular defence enzymes and b) the enhanced ability of malignant cells to detoxify chemotherapeutic drugs by the action of phase 2 detoxification enzymes. Therefore, these undesirable effects of Nrf2 activation must be carefully considered. Even though the Nrf1 niche has been left essentially unoccupied by scientists and clinical researchers, examining virtually any understudied aspect of Nrf1 is expected to generate a great deal of attention. One very interesting question that could be considered is whether one can activate Nrf1 in the instances of cancers with increased overexpression of Nrf2. Can such interference counteract the pro-carcinogenic effects of excessive Nrf2 activity? This would require the design of a Nrf1 inducer and a good starting point would be to consider inducing Nrf1 by disrupting its association with the ER.

Despite some potentially undesirable effects of the increased Nrf2 nuclear presence, there is some suggestion that reversing or preventing the age-associated decline of Nrf2 function can be beneficial. For example, Noyan-Ashraf and co-workers (2008) reported that adding a known phase 2 detoxification enzyme inducer, 2(3)-tert-butyl-4-hydroxyanisole (tBHA) into the diet of mice reduced oxidative stress and age-associated weight gain as well as improved the locomotor function. This might suggest that adding certain Nrf2 inducers to our diets can help us achieve healthier aging. Such a view becomes quite popular even among the general public. However, more studies carefully examining the dark side of Nrf2 activation are required. Apparently, a good balance

between too much and too little Nrf2 has to be found in any attempt to use Nrf2 inducers to human health benefits.

While the benefits of the long-term activation of Nrf2 are currently understudied and the risks associated with the overexpression of Nrf2 have not been fully assessed, the activation of Nrf2 over a short period of time is expected to hold much promise in the treatment of acute diseases such as brain injury, acute lung injury, cerebral ischemia and intracerebral hemorrhage. This is evidenced using rodent models, where the benefits of Nrf2 activation, administered even after the onset of the tissue damage, were seen (reviewed by Sykiotis and Bohnman, 2010). Given the plethora of the clinical conditions where the activation of Nrf2 can be useful and given the inhibitory effect of Nrf1 on the Nrf2/ARE-controlled basal and inducible gene expression, a valid question would be whether Nrf1 inactivation can be of any benefit as well? Again, the attempts at keeping Nrf1 inert in the ER-bound, glycosylation state could provide a good starting point for these types of studies.

6.4 Conclusions and Future Directions

The results showed, for the first time, that Nrf1 is regulated by the proteasome through its continuous, ubiquitin-dependent degradation. This finding, originally reported by the author in 2009 (Chepelev and Willmore, 2009) was later confirmed by Radhakrishnan and others (2010). According to what is now known regarding the proteasomal regulation

of Nrf1, the repression of its continuous degradation by proteasomal inhibition allows Nrf1 to accumulate and activate gene expression of several proteasome genes, regulated through the ARE, including *PSMA7* and *PSMC1* (Radhakrishnan *et al.*, 2010). This project has also revealed the possibility that Nrf1 is protealytically cleaved by the proteasome at its N-terminus to produce a fragment, migrating as a 23-kDa polypeptide. This conclusion was based on the following: i) the N-terminal location of the Nrf1 region, recognized by the antibody employed; ii) the fact that p23 signal was diminished following proteasomal inhibition in both COS7 and WFF2002 cells (Figure 3.5), with concomitant increase in the p120 Nrf1 iii) the fact that the overexpression of the Nrf1, FLAG-tagged at its N-terminus, could not be seen following FLAG immunoblotting (data not shown), unlike the C-terminally FLAG-tagged Nrf1 (Figure 3.7D); iv) the observation that the p23 is observed using two different antibodies, Nrf1 Antibodies H-4 and H-285 (Santa Cruz). Conceptually, this hypothesis makes sense given a similar regulation of NF-*k*B by the proteasome, in which the inactive p105 is cleaved to the DNA-binding, p50 form of NF-κB by the 26S proteasome (Magnani *et al.*, 2001; Tanaka *et al.*, 2000). However, more experiments are required to establish that p23 is indeed a part of Nrf1. These additional experiments could include the use of alternative antibodies, mass spectrometry as well as (or in combination with) epitope tagging of Nrf1 at different N-terminal sites to establish the site of fragmentation and to reveal the identity of the p23 fragment. This same list of additional experiments can be extended to reveal the identity of the putative p250 form of Nrf1, which was seen to be modulated by oxidative stressors and hypoxia. Given that Nrf1 is involved in the ARE regulation, it would seem logical to expect some changes at the protein level of Nrf1 in response to oxidative stressors, such

as AAPH or tBHQ. The fact that p250 disappeared in response to pro-oxidants may suggest that the inhibitory effect of Nrf1 on Nrf2 (as discussed extensively in Chapter 5 of this thesis) is somehow repressed in the cell by its removal, such that the Nrf2-mediated transcription of the ARE target genes could be activated in response to a stressor; however, more experiments are required, primarily aimed at the identification of p250, to make any further conclusions. The fact that p250 is resistant to reducing and denaturing SDS-PAGE conditions suggests that covalent linkage to some other cellular macromolecule may be involved.

In addition to proteasomal regulation, Nrf1 was found to respond positively to the stimulation of intracellular phosphorylation as judged by the luciferease reporter results (Figure 3.8A) while the repression of PKC kinases had the opposite effect. This data suggests that Nrf1 is regulated by phosphorylation, but, it says nothing about the phosphorylation status of Nrf1 itself. Indeed, some other regulatory protein target of phosphorylation may affect Nrf1 activity, such that the phosphorylation status of the cell may determine the transactivation activity of Nrf1 indirectly. Mass spectrometry analysis might be a useful tool to reveal the identity of phosphorylation sites on Nrf1 by using phosphoprotein enrichment through metal-affinity chromatography (Collins *et al.*, 2005). However, this approach might be unable to pinpoint the exact residue(s) undergoing phosphorylation especially among the 25 Ser residues located in the NST region of Nrf1 of which 14 Ser residues are located in a row (see Figure 6.3) as the phosphate group can be transferred amongst adjacent phosphorylation sites (J. Smith, personal communication). Similarly, affinity purification (performed in a manner, similar to the

MLSLKKYLTEGLLQFTILLSLIGVRVDVDTYLTSQLPPLREIILGPSSAYTQTQFHNLR
NTLDGYGIHPKSIDLDNYFTARRLLSQVRALDRFQVPTTEVNAWLVHRDPEGSVSGSQP
NSGLALESSSGLQDVTGPDNGVRESETEQGFGEDLEDLGAVAPPVSGDLTKEDIDLIDI
LWRQDIDLGAGREVFDYSHRQKEQDVEKELRDGGEQDTWAGEGAEALARNLLVDGETGE
SFPAQVPSGEDQTALSLEECLRLLEATCPFGENAEFPADISSITEAVPSESEPPALQNN
LLSPLLTGTESPFDLEQQWQDLMSIMEMQAMEVNTSASEILYSAPPGDPLSTNYSLAPN
TPINQNVSLHQASLGGCSQDFLLFSPEVESLPVASSSTLLPLAPSNSTSLNSTFGSTNL
TGLFFPPQLNGTANDTAGPELPDPLGGLLDEAMLDEISLMDLAIEEGFNPVQASQLEEE
FDSDSGLSLDSSHSPSSLSSSEGSSSSSSSSSSSSSSASSSASSSFSEEGAVGYSSDSE
TLDLEEAEGAVGYQPEYSKFCRMSYQDPAQLSCLPYLEHVGHNHTYNMAPSALDSADLP
PPSALKKGSKEKQADFLDKQM[redacted]
[redacted]RDIRRRGKNKMAAAQNCRKRKLDTILNLERDVEDLQRDKARLLREKVEFLRSLR
QMKQKVQSLYQEVFGRLRDENGRPYSPSQYALQYAGDGSVLLIPRTMADQQARRQERKP
KDRRK

Legend:
Yellow = LXXLAP Sequence where X is Any Amino Acid
Light Blue = Poly-Serine Region
Green = Leucine Zipper
Dark Grey = Basic Domain
Light Grey = Asp/Glu-rich (Acidic) Domain
Blue = CNC Domain
Red = Met321 and Met326, the Proposed Start Sites of Internal Translation of Nrf1
Underlined = the Recognition Region (aa. 191-475) of Anti-Nrf1 Antibodies H-4 and H-285 (Santa Cruz) and the proposed site for the Nrf1 proteolytic cleavage.

Figure 6.3. The Amino Acid Sequence of Nrf1 Along with Its Domains and Features.

LXXLAP. Shown are some interesting structural features of Nrf1, some of which require future experiments, including the proposed internal translation sites (Met321 and Met326) and putative oxygen-dependent degradation motif (LXXLAP) found in certain proteins such as HIF-α, ALAS2 and pyruvate kinase M2. Whether LXXLAP is involved in the O_2-dependent regulation of Nrf1 is unknown. The region, recognized by the antibody used in this project and where the proteolytic cleavage of Nrf1 is thought to occur to produce p23, a 23-kDa fragment of Nrf1, is underlined. The predicted phosphorylation sites are located primarily within the poly-Ser region.

co-immunoprecipitation shown in Figure 3.3C) could be used in conjunction with mass spectrometry to identify the sites of ubiquitin attachment on Nrf1. In the search for the sites of Nrf1 ubiquitination, characteristic shifts of predicted peptide masses of + 114.1 and/or + 383.2 atomic mass units are expected to occur at the Lys residues, where ubiquitin is attached by the ubiquitin ligases (Denis *et al.*, 2007).

The hypoxic induction of Nrf1-FLAG (Figure 3.7A) was found to correlate well with the decreased expression of the inhibitory p65 form of Nrf1 (Figure 3.7E and F). While the documented relationship allows us to hypothesize that the removal of p65 is the underlying cause of the hypoxic activation of Nrf1, more data is required to substantiate this claim. The p65 form has been proposed to arise either through proteolysis or the translation of Nrf1 (Chan *et al.*, 1993; See Figure 6.3), but the exact mechanism of p65 generation has not been elucidated yet. To test the hypothesis that hypoxic activation of Nrf1 is due to the destabilization of p65, site-directed mutagenesis to abolish the internal translation Met codons can be used and the response of wild-type and mutant Nrf1 can be compared. This will be a useful approach only if p65 is, indeed, generated by the internal translation, which should be seen by immunoblotting as the disappearance of the p65 form. If the p65 form of Nrf1 arises due to internal cleavage sites, another approach should be used to disentangle the exact mechanisms by which p65 is generated. For example, mutating different portions of Nrf1 near the putative cleavage site, in combination with immunoblotting, may reveal the regions of Nrf1, if not specific residues, where proteolytic processing takes place.

The activation of Nrf1 and Nrf2 (Figure 4.4) and the induction of the ARE-target genes *Nqo1* and *HO-1* (Table 4.1) by BPA can be also studied in greater detail to gain a better mechanistic insight into the phenomenon. The transcriptional (Table 4.1) and post-translational modifications of the factors observed (Figure 4.5) provide a wide variety of possible mechanisms that can be addressed. Very little is currently known about the transcriptional regulation of Nrf1 and Nrf2 genes, other than that, under certain circumstances, the gene expression of these two factors is regulated in the opposite fashion as in the case of whole mouse treatment with butylated hydroxytoluene, a known antioxidant and food additive (Chevillard *et al.*, 2010). Investigating the exact mechanisms by which BPA affects the Nrf1/2-ARE pathway is apparently a more ambitious task than elucidating the types of post-translational modifications of Nrf1, but a global approach using gene microarrays, aimed at identification of other genes affected by BPA, is currently being undertaken at Health Canada and may provide some meaningful insights into the mechanisms of BPA action. What is currently known, using this data, is that a number of the ARE-controlled genes are affected by high concentrations of BPA only. Other pathways affected include a) linoleic acid metabolism, b) glycine, serine and threonine metabolism, and c) cell cycle control of chromosomal replication (Chen *et al.*, in preparation). In addition, microarray analysis of gene expression in human lung fetal fibroblasts revealed that the gene expression of ubiquitin-conjugating enzyme UBE2T is downregulated almost 2-fold in response to 100 μM BPA (Chen *et al.*, in preparation). The downregulation of the ubiquitin-proteasomal pathway can stabilize both Nrf1 and Nrf2 in response to BPA and this is a very attractive

hypothesis that can be tested by examining the ubiquitination state of Nrf1/2 with the help of affinity chromatography and mass spectrometry, as suggested above.

The formation of putative HO-1 dimer is also a very intriguing result, which holds a lot of potential. Since the putative dimer appears to be stable under reducing and denaturing SDS-PAGE conditions, attempts can be made to digest it in gel with trypsin and the generated peptides can be subjected to mass spectrometry analysis. The use of alternative antibodies should be employed, as well, to confirm the identity of the dimer. In addition, the assay of HO-1 activity in which the production of biliverdin and bilirubin from heme under the action of heme oxygenases, can be applied to BPA-treated samples as described by Ryter and colleagues (1998) in order to test the functional significance of HO-1 dimerization. It is expected that dimerization would enhance the HO-1 catalytic activity as suggested by Hwang and others (2009).

Finally, the competition between Nrf1 and Nrf2 was studied. The inhibitory potential of Nrf1 on the Nrf2-driven gene expression was seen under the conditions of *Nrf1* silencing by RNAi and in the luciferase reporter format (Figures 5.1 and 5.2). To gain a better confidence in these important and somewhat counter-intuitive results (which have been supported only by two previously-published reports as discussed in Chapter 5), it is desirable that the effect of *Nrf1* silencing be confirmed with another set of RNAi oligos.

The data described in this thesis also showed that not only Nrf2, but also Nrf1 contribute to the ARE binding in young animals and that age is associated with the loss of the ARE-protein binding (Figure 5.3). Interestingly, the exposure of animals with airborne particles abolished age-dependent loss in the ARE binding, which is reminiscent of the concept that ROS are involved in the intracellular signalling pathways and the concept of mitohormesis can be invoked, according to which, the stimulation of mitochondrial ROS generation to a certain extent is believed to have pro-longevity effects (Ristow and Schmeisser, 2011). There is a large body of evidence, suggesting that the stabilization of Nrf2 has may extend the life span in organisms (reviewed by Lewis *et al.*, 2010). The stimulatory effect of particle exposure was seen as an increased expression of Nqo1 and increased oxidative radical absorbance capacity (Figure 5.5). Paradoxically, this effect of particles was less pronounced in old animals, perhaps, due to the increased stability of Nrf1 (especially its inhibitory p65 form) and the concomitant decrease in Nrf2 expression (Figure 5.4) and it has been discussed extensively throughout the thesis that p65 is known to an act as the Nrf2-ARE pathway inhibitor. Even though there is some evidence that other proteins, such as Bach1, are also stabilized in the samples of aged animals (Zhang *et al.*, in preparation), the inhibitory effect of Nrf1 might be of great interest to the scientific community given the immense attention Nrf2 has received. Nrf1 and Nrf2 appear to have a similar binding affinity to the ARE (Ohtsuji *et al.*, 2008) and the mechanisms by which Nrf1 can act as an inhibitor are likely to be due to the differences in the transactivation domains of these two factors (Ohtsuji *et al.*, 2008). Once again, Nrf1/2-other protein interactions can be studied by mass spectrometry, upon

affinity purification, to reveal the binding partners, of Nrfs which are expected by be chiefly transcriptional co-activators and co-repressors.

In summary, the studies described here have raised some very interesting questions regarding Nrf1 regulation and function. First, what is the identity of the p250 form of Nrf1, which responds to multiple stimuli, including hypoxia, pro-oxidants and aging? This seems to represent a common mechanism of Nrf1 responsiveness to various stressors. To solve this mystery, affinity purification followed by mass spectrometry, could be helpful in elucidating the modification(s) of Nrf1 or its protein-protein or protein-membrane interactions, responsible for its higher molecular weight. Secondly, the same combination of techniques could be instrumental in answering the following questions. As both Nrf1 and Nrf2 bind to the same ARE sequence, what is the difference between their co-activators and co-repressors that determine the transcriptional specificity of these two factors? Third, what can explain the age-related increases in Nrf1 content in the cell? Again, this might be the consequence of the diminished proteasomal performance due to the age-accentuated overload of the proteasome with oxidatively damaged proteins, such that Nrf1 becomes more stable. The increased stability of Nrf1 could activate the gene expression of proteasomal subunits and relieve the burden on the proteasome, due to the accumulation of oxidatively-damaged and misfolded proteins whose population is known to increase with age (Grim *et al.*, 2011). To answer this question, one would have to not only show that the activity of the ERAD system, responsible for Nrf1 degradation declines with age, but also to demonstrate a cause-effect relationship between the declined function of the ERAD and Nrf1 stability. This is not a

straightforward task since proteasomal inhibitors will stabilize Nrf1, as shown in this project (Chapter 3). Last, a very ambitious approach would be to design a Nrf1 activator, useful in the types of cancer with abnormally high Nrf2 activity or to design a Nrf1 inhibitor designed to activate Nrf2 in acute clinical conditions such as brain injury, acute lung injury or cerebral ischemia.

Now is the exciting time for the research on Nrf1 and, especially its interaction with Nrf2. Studies aimed at revealing the molecular details of Nrf1 regulation are slowly entering the exponential phase to catch up with the research done on Nrf2. The findings on the dominant role of Nrf1 over Nrf2, described in Chapter 5, might facilitate this trend. Future work will determine how the functions of the CNC-bZIP factors are related to each other and what their combined effects are on ARE-driven gene expression.

Gloria Patri, et Filio, et Spiritui Sancto, Amen!

Appendices

Appendix A

A.1 List of Publications

The following publications, manuscripts and conference presentations were produced during this project.

Research Papers

Chepelev, N., Bennitz, J., Wright, J., Smith, J., and Willmore, W. 2009. Oxidative inactivation of citrate synthase and protection with novel antioxidants. *J. Enzyme Inhib. Med. Chem.* 24, 1319-31.

Cao, X.-L., Corriveau, J., Popovic, S., Coughlan, M., **Chepelev, N.**, Willmore, W., Schrader, T., and Jin, X. 2010. Background bisphenol A in experimental materials and its implication to low-dose *in vitro* study. *Chemosphere.* 81, 817-20.

Chepelev, N., Bennitz, J., Huang, T., McBride, S., and Willmore, W. (2011) The NRF1 CNC-bZIP protein is regulated by the proteasome and activated by hypoxia. Submitted to PLoS ONE on July 20, 2011; submission number: PONE-S-11-17302.

Chepelev, N., Enikanolaiye, M., Chepelev, L., Chen, Q., Scoggan, K., Coughlan, M., Jin, X., and Willmore, W. (2011) Bisphenol A activates Nrf1/2-antioxidant response element pathway in HEK293A cells. Submitted to Chemical Research in Toxicology on March 31, 2011, submission number: tx-2011-00132j.

Conference Proceedings

Chepelev, N., Wright, J., and Willmore, W. 2006. Oxidative modification and inactivation of citrate synthase by peroxyl radicals and protective effects of novel antioxidants. *Free Radic. Biol. Med.* 41(S1), S133. Award-winning poster presentation at the 13th Annual Meeting of the SFRBM, Denver, CO.

Chepelev, N., and Willmore, W. 2009. Regulation of Nrf1 levels and ARE binding activity during hypoxia in COS7 cells. Abstract for award-winning oral presentation at the 6th Meeting of the Canadian Oxidative Stress Consortium, Winnipeg, MB.

Chepelev, N., and Willmore, G. 2009. The Nrf1 CNC/bZIP protein is regulated by the proteasome and activated by hypoxia. *Free Radic. Biol. Med.* 47(S1), S14-S15. Award-winning abstract for poster presentation, the 16th Annual Meeting of the SFRBM, San Francisco, CA.

Chepelev, N., Enikanolaiye, M., Chen, Q., Scoggan, K., Jin, D., and Willmore, W. 2010. Human Antioxidant Response Element-Nrf1/2 pathway-mediated defence against BPA

exposure. *Free Radic. Biol. Med.* 49(S1), S127-S128. Abstract for poster presentation at the 17th Annual Meeting of the SFRBM, Orlando, FL.

Reviews

Chepelev, N., Chepelev, L., Alamgir, M., and Golshani, A. 2008. Large-scale protein-protein interaction detection: past, present, and future. *Biotechnol. Biotechnol. Equip.* 22, 513-29.

Chepelev, N., and Willmore, G. 2011. Regulation of iron pathways in response to hypoxia. *Free Radic. Biol. Med.* 50, 645-66.

Book Chapter

Chepelev, L., **Chepelev, N.**, Shadnia, H., Willmore, G., Wright, J., and Dumontier, M. 2009. Chapter 7. Development of Small-Molecule Ligands and Inhibitors (p. 115-147). In: Protein Targeting with Small Molecules. Hyroyuki Osada (edit.). John Willey and Sons, Inc. Hoboken, NJ.

Manuscript in preparation

Chepelev, N., Zhang, H., Liu, H., Willmore, W., Morgan, T., Finch, C., Davies, K., and Forman, H. The Yin-yang of Nrf1 and Nrf2 in aging mouse lung.

News Releases

"Carleton PhD Student Invited to Work With World Experts on the Aging Process." Carleton University News Release, March 2, 2011. http://www1.carleton.ca/newsroom/category/news-releases/page/11/

"He'll try to unravel the Nrf1 riddle." Metro Ottawa, March 3, 2011. http://www.metronews.ca/ottawa/local/article/792386--he-ll-try-to-unravel-an-nrf1-riddle

"Kanata resident works with world experts on aging." Kanata Kourier, March 16, 2011. http://www.yourottawaregion.com/news/local/article/967553--kanata-resident-works-with-world-experts-on-aging

A.2 Ten Simple Rules for Surviving Graduate School

This section is written in hope that the experience gained by the author during his graduate work might be helpful for other graduate students.

1. During the first year of your studies, try to write a review article. The subject of the review should be based on or be very close to your proposal. Your supervisor is an expert in his field, but he or she often lacks time, necessary for writing. On the other hand, you have some spare time and, under the guidance of your supervisor, can easily review relevant literature and put that into a written form. You will have to do this for your proposal anyways and writing a review is a good way to put everything together. Importantly, our understanding will be assessed by peer-review process, which will give you a good writing and publishing experience.

2. Attend conferences! Early on, find out what key scientific conferences, relevant to your field of research are out there. Plan to gather enough data, make a poster and present your findings at such a conference. Besides, such conferences often have some stimulating awards, such as travel awards, to help students.
3. Read the "Ten Simple Rules" series of articles, such as "Ten Simple Rules for Graduate Students", freely available at: http://www.ploscompbiol.org/article/info:doi/10.1371/journal.pcbi.0030229
4. Try to think about where you see yourself in the future. This should help you develop the skills necessary for success.
5. When experiments do not work out well, do not give up. Try to look at your data in slightly different way. Maybe, you see something else, other than what you expected? Read the phdcomics: http://www.phdcomics.com/comics.php
6. Develop yourself as a writer and reviewer. You can help your supervisor review a manuscript, submitted for a publication and assigned to your supervisor for reviewing or critically review your supervisor's grant proposal.
7. Become a member of some graduate society/council and socialize with your peers. This helps you develop overall as a person. Try to balance your research with other activities. This will give you more energy to be a more productive researcher and being more productive will give you satisfaction and so on.
8. Time management is essential. Enjoy your life and try to spend each and every moment of it wisely! Avoid downtime. When experiment A does not work, try to work on experiment B at the same time.

9. Read a lot of articles. Importantly, if something is unclear or if you have an alternative interpretation to what is given in an article, do not hesitate to contact the corresponding author. I found these contacts stimulating and helping myself think in a slightly different manner, especially if the corresponding author is a top expert in the field.
10. Work on the weekends as often as possible. This is a very quiet and productive time. "When I was younger, I always worked in a laboratory on Saturdays. I used to call these my Saturday specials. Sometimes there was an unexpected discovery that was more important than the original objective." These are the words of Theresa Stadtman, a world-renowned biochemist.

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The fear of the LORD is the beginning of knowledge (Proverbs 1:7)