ABSTRACT

RAY, PAUL DELANE. The Role of Histone H3 Phosphorylation in the Transcriptional Activation of Antioxidant Response Element (ARE)-Regulated Genes. (Under the direction of Dr. Yoshiaki Tsuji.)

Oxidative stress is implicated in the pathogenesis and progression of several disease states. In order to prevent oxidative stress related toxicity, the cell mounts an effective antioxidant defense response. This response consists of enzymes that reduce levels of reactive oxygen species (ROS). Therefore, in order to mount an effective antioxidant defense, transcriptional regulation of antioxidant genes must be tightly regulated. Elucidating the molecular pathways of antioxidant gene transcription will aid in designing therapeutic strategies for oxidative stress related diseases. Promoters of antioxidant genes such as heme oxygenase-1 (HO-1) feature an upstream enhancer element that regulates transcriptional activation. This antioxidant response element (ARE) is activated in response to oxidative stress, and while activation of the ARE by the transcription factor NF-E2-related factor 2 (Nrf2) has been studied, the role of chromatin remodeling in activation of the ARE has been relatively unexplored. Post-translational modification of histones plays a key role in chromatin-mediated regulation of gene transcription. However, the role of histone phosphorylation in gene transcription has not been fully elucidated.

We sought to determine the role of histone phosphorylation in ARE activation and in the transcriptional regulation of ARE-containing genes by employing the environmental contaminant arsenite, a known inducer of oxidative stress, which potently stimulates histone H3 serine 10 phosphorylation (H3S10P) and HO-1 expression. We report that arsenite induces H3S10P enrichment of the HO-1 AREs and HO-1 expression in an oxidative stress-dependent manner. We also observed that the c-Jun N-terminal kinase (JNK) contributes to arsenite-mediated induction of H3S10P and HO-1 expression. Furthermore, we demonstrate that Nrf2 regulates H3S10P enrichment at the HO-1 AREs. We also found that arsenite induces histone H3 threonine 11 phosphorylation (H3T11P), both at the global level and at the HO-1 AREs. Additionally, we demonstrate that the DNA damage-responsive kinase Chk1 mediates H3T11P in response to arsenite, as well as regulating arsenite induction of
HO-1. Collectively, these results suggest that histone H3 S10 and T11 phosphorylation may play a role in HO-1 transcription in response to arsenite.
The Role of Histone H3 Phosphorylation in the Transcriptional Activation of Antioxidant Response Element (ARE)-Regulated Genes

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Toxicology

Raleigh, North Carolina

2012

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DEDICATION

This one is for my parents.
Paul D. Ray was born in West Monroe, LA on November 29, 1972. He graduated from Farmerville High School in 1990, and in 1995 he enlisted in the United States Army and attended Basic Training and Advance Individual Training for Military Police at Ft. McClellan, AL. He was subsequently stationed in the Republic of Korea at Camp Humphries until 1996 when he was transferred to Ft. Polk, LA and in 1999 earned the rank of Sergeant. Receiving an honorable discharge in 2000, he returned to Monroe, LA, and at the University of Louisiana at Monroe, entered into the undergraduate Toxicology program. Upon graduation in 2004, he accepted a position as Toxicologist at Industrial Hygiene and Safety Technology in Dallas TX, screening compounds for Texas Instruments. He also worked as an industrial hygienist for the company before returning to ULM to enter the PhD program in Toxicology. In 2006 he was accepted into the PhD program in Toxicology at North Carolina State University and joined the lab of Dr. Yoshiaki Tsuji.
ACKNOWLEDGMENTS

I would like to thank my mentor and supervisor, Dr. Yoshiaki Tsuji. Dr. Tsuji has, from the very beginning of my graduate school experience, personally instructed me in almost every aspect of the process, from learning those first tentative experimental procedures to advice and guidance for my future career. He was always available at all hours of the day, no matter how busy he was, to individually instruct and assist me in whatever I brought to him. Many people go into the process of training and developing a scientist, but Dr. Tsuji I can say was the central figure in this process. I found great value in his seemingly unlimited patience, not only in matters of science, but also in dealing with whatever personal issues that beset me. I am indebted to Dr. Tsuji most of all for teaching me how to reason and think like a scientist, which greatly changed me for the better in all aspects of my life.

I would also like to thank my wife Audra, without whose support I could not have achieved my goals. I would like to thank those with whom I worked and associated with in the department during the last few years. Specifically, Bo-Wen Huang and Dr. John House. Bo-Wen has been a fellow soldier in the trenches; he has been my constant friend, and I have drawn on his scientific knowledge and optimistic personality to aid me and buoy me up during difficult times. Dr. John House was also a source of support and a friend who I relied upon greatly. I thank both of them and feel that the whole experience would have been much more difficult and dismal- certainly devoid of laughter- without them. I would also like to thank my former and present laboratory members for their assistance and guidance; Dr. Kenta Iwasaki, Dr. Kensuke Sakamoto, Alexander Bogdan, Dr. Masaki Miyazawa, and Dr. Kazunori Hashimoto. I would also like to thank my committee members Dr. Jun Ninomiya-Tsuji, Dr. James Bonner, and Dr. Christine McGahan. A special thanks to Dr. Robert Smart, who I found to be a valuable counselor.

Lastly, I would like to thank the American taxpayer, who funded this work.
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GENERAL INTRODUCTION

1. Reactive Oxygen Species (ROS) and Oxidative Stress

Reactive Oxygen Species (ROS) are oxygen radicals formed by the incomplete reduction of oxygen, such as super oxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH•). In the cellular environment, ROS may be produced endogenously or arise from exogenous sources. For example, superoxide anion is generated as a by-product of mitochondrial oxidative phosphorylation (1), and membrane bound NADPH oxidase complexes in phagocytic cells produce ROS as a response to bacterial invasion (2). Examples of exogenous sources include heavy metals such as chromium (3) and pesticides such as paraquat (4). The reactivity of ROS with proteins, lipids, and nucleic acids varies between species, with superoxide anion being less reactive compared to the highly reactive hydroxyl radical (5). At low levels, ROS play a role in cellular homeostasis. Oxidative regulation of redox sensitive cysteine groups by ROS, for example, result in structural and/or functional changes in proteins (6). In this manner ROS serve as signaling messengers, affecting cell proliferation and differentiation (7). However, ROS in excess amounts are cytotoxic, reacting with and damaging cellular components. The presence of endogenous ROS and contact with exogenous sources of ROS necessitates a system by which cells may regulate ROS levels, thus fine tuning the more beneficial cellular homeostatic role of ROS, and mitigating the cytotoxic effects. Collectively, the various processes by which cells maintain redox homeostasis are known as an antioxidant response, or antioxidant defense system (6). When there exists an imbalance between ROS levels and the cellular antioxidant capability, oxidative damage occurs and this is known as oxidative stress.

Oxidative stress has been implicated in the pathogenesis and/or progression of various diseases, including neurodegeneration (8), cancer (9), diabetes and artherosclerosis (10), and aging (11), as a result of ROS-induced macromolecular damage or aberrant cellular signaling. Circumventing the detrimental effects of excess ROS is therefore important in the prevention and/or treatment of oxidative stress related diseases. Thus, a clear understanding
of the regulation of the cellular antioxidant defense system is paramount. The antioxidant defense system can be viewed as a group of diverse mechanisms by which ROS levels are controlled through the detoxification, or reduction, of ROS. Detoxification can be viewed simplistically as enzymatic versus non-enzymatic antioxidants. Non-enzymatic antioxidants are compounds such as glutathione and ascorbic acid (12), which react with and quench ROS. Enzymatic antioxidants are those enzymes that catalyze the reduction of ROS directly, or indirectly through secondary effects. For example, superoxide dismutase (SOD) directly converts superoxide anion to hydrogen peroxide; the enzyme catalase then converts hydrogen peroxide to water (12). Another direct antioxidant enzyme is NAD(P)H Quinone Oxidoreductase 1 (NQO1), which mediates the two electron reduction of quinones into hydroquinones, preventing the single electron reduction of quinones that results in the production of ROS (13). Indirect removal of ROS consists enzymes such as heme oxygenase-1 (HO-1), which catalyzes the breakdown of heme into biliverdin, iron, and CO₂. The antioxidant capabilities of HO-1 stem from the by-products of heme catabolism, rather than the direct enzymatic activity of HO-1 on ROS (14). Ferritin H (FH), an iron binding protein, prevents the production of the toxic hydroxyl radical not through direct enzymatic activity on ROS, but by encapsulating excess iron, preventing iron from reacting with hydrogen peroxide to form hydroxyl radicals (15). Since these antioxidant enzymes play such major roles in redox homeostasis it is no surprise that many of them are highly inducible in response to oxidative stress.

2. The Antioxidant Response Element

The necessity for a timely and capable antioxidant defense requires detailed control over the levels of antioxidant enzymes. While some are regulated at the translational level, such as the iron binding protein ferritin through the iron regulatory protein-iron responsive element (IRP-IRE) system (15), most are highly inducible at the transcriptional level in response to oxidative stress. Transcriptional activation of genes is a multistep process, of which the recruitment and activation of RNA Polymerase II (RNAPII) is central. Briefly, general transcription factors (GTF) bind to a core promoter sequence located at the
transcription start site (TSS), forming a pre-initiation complex (PIC). This results in the recruitment and binding of RNAPII at the TSS (16). RNAPII is then phosphorylated at serine 5 (RNAPII-S5) in the C-terminal domain (CTD), initiating RNA synthesis. After this first step of recruitment and initiation, RNAPII undergoes phosphorylation at serine 2 in the CTD (RNAPII-S2), which results in productive transcriptional elongation along the coding region, ultimately ending in termination (17).

In conjunction with the core promoter function, enhancer elements, specific DNA sequences that serve as binding sites for a variety of transcription factors, play a primary role in transcription. RNAPII recruitment, activation, and subsequent activity are highly regulated by enhancer elements, which can be located anywhere on the gene and at any distance from the TSS (18). Specific transcription factors bind to each enhancer element and modulate gene expression. Transcription factors bound to enhancer elements recruit co-activators resulting in the formation of a large multi-subunit complex known as Mediator (19), which bridges the enhancer element and the RNAPII complex through DNA looping.

Enhancer activity is regulated by a wide variety of stimuli. Antioxidant genes are no exception; many are transcriptionally regulated by the activity of an oxidative stress–responsive enhancer element, appropriately termed the antioxidant response element (ARE) (20). The ARE is a cis-acting element with a core AP-1-like consensus sequence of TGA(C/T)nnnGCA (21,22) and is located in the upstream promoter regions of many antioxidant genes, including ferritin (23), NQO1(24), glutathione S-transferase (GST) (22), and HO-1 (25). Location of the ARE varies; the NQO1 promoter contains an ARE at -520bp (26) while both ferritin (23) and HO-1 feature AREs located -4kb to -5kb upstream of the TSS, with the HO-1 promoter containing a second functional ARE at -10kb (25). AREs are readily activated by a variety of reactive or electrophilic stimuli, including ROS (23), ROS-generating compounds such as rotenone (27) and heavy metals such as arsenic (28), lipid aldehydes (29), the antioxidant phenolic compounds resveratrol (30) and tert-butylhydroquinone (31), and hemin (32). In addition, the ARE is also responsible for the basal expression of several antioxidant genes (23). Thus, the ARE is poised to act as a
transcriptional trigger of antioxidant genes in response to alterations of the cellular antioxidant status. Elucidating the mechanism by which the ARE is activated, and its role in transcriptional activation of antioxidant genes, is vital in understanding how the cell mounts an effective antioxidant response. Regulation of the ARE by transcription factors and the resulting effect on gene expression has been an active area of research. ARE activation by transcription factors has been the topic of comparatively intensive study. The primary transcription factor involved in ARE activation is NF-E2-related factor 2 (Nrf2).

3. Nrf2

Nrf2 is a potent activator of the ARE in response to oxidative stress and as such is the central key in the antioxidant response. Nrf2 is a member of the Cap’n’collar (Cnc) family of transcription factors who share among them a conserved basic leucine zipper (bZip) region (33,34). Nrf2 consists of six Nrf2-ECH homology domains (Neh); Neh1 contains the bZip region, necessary for DNA binding and dimerization, Neh2 is a negative regulatory domain which binds Kelch-like Erythroid-derived CNC homology (ECH)-associated protein 1 (Keap 1) which will be discussed later, Neh3 is a C-terminal domain involved in transactivation as are the N-terminal Neh4 and 5 domains, while Neh6 functions as a degron that directs initiation of protein degradation (35,36). Neh5 was shown to be the primary transactivation region of Nrf2, binding the transcriptional co-activator CREB (c-AMP-response-element-binding protein) binding protein (CBP) and the chromatin remodeler Brahma-related gene 1 (BRG1) in the transcriptional induction of the HO-1 gene; loss of this region resulted in decreased antioxidant gene expression (36). Nrf2 also binds several other co-activators through the Neh1 domain, but first a discussion on the regulation and activation of Nrf2 is necessary.

Under non-stressed conditions, Nrf2 resides in the cytoplasm (though this prevailing view has been challenged (37)) with a rapid turnover rate (t1/2 ~15 min) due to ubiquitin dependent proteolytic degradation, the result of Nrf2 association with Keap1. Keap1 binds to Nrf2 by interaction with the Nrf2 Neh2 domain (38). Keap1 also binds the cullin-3 E-3 ubiquitin
ligase (Cul3) (39); therefore, Keap1 acts as a binding scaffold for both Nrf2 and Cul3, promoting association between Nrf2 and Cul3, thereby resulting in the proteosomal degradation of Nrf2 through ubiquitination (40). However, the role of Keap1 in Nrf2 regulation is two-fold; besides mediating the constitutive degradation of Nrf2, Keap1 controls Nrf2 localization in response to oxidative stress and so itself is a sensor of oxidative stress and a modulator of the antioxidant response. When Nrf2 is released from the Keap1/Cul3 complex in response to oxidative stress, it translocates into the nucleus and binds the ARE, activating transcription of numerous antioxidant genes. The release of Nrf2 from Keap1 is mediated by the oxidation of Keap1 redox-sensitive cysteine residues (Cys-151, 273, and 288) by ROS (41,42), which is thought to result in structural changes in Keap1, thereby decreasing the affinity between Nrf2 and Keap1 (43). Nrf2 nuclear localization is directly regulated by ROS at this stage through a redox-sensitive cysteine (Cys-183) located in a NES domain.

Nrf2 binding to the ARE has been amply demonstrated in several ARE-regulated genes, including ferritin, NQO1, HO-1, GST, and glutamate cysteine ligase (GCL) (23,36). Nuclear accumulated Nrf2 does not bind to the ARE singly, or as a homodimer; instead, it forms heterodimers with small Maf protein family members (Maf-F, Maf-G, and Maf-K), which are bZip transcription factors (44-46). Nrf2 has also been shown to form heterodimers with Jun (c-Jun, Jun-D, and Jun-B) proteins in ARE activation (47). While it is predominantly thought that Nrf2-Maf heterodimers activate the ARE, certain Maf family homodimers repress the ARE (48). The bzip transcriptional repressor BTB and Cnc homolog 1 (Bach1) constitutively binds and represses the ARE as well (49). Bach1 must dissociate from the ARE before the Nrf2/Maf heterodimer can bind and activate transcription. Bach1 release is also regulated by oxidative stress. Bach1 contains a redox sensitive Cys-574 that upon oxidation causes Bach1 to dissociate from the ARE and undergo cytoplasmic translocation (50). Thus, all stages of ARE activation are directly regulated by oxidative stress.
In addition to ROS, Nrf2 activity is also subject to post-translational modifications, including phosphorylation by mitogen activated protein kinase (MAPK) members c-jun N-terminal kinase (JNK) and p38 kinase (51), as well as protein kinase C (PKC) (43). However, the role of Nrf2 phosphorylation in transcriptional regulation was recently challenged (51). Nrf2 is also acetylated by the co-activator CBP, increasing Nrf2-ARE interaction (52). Nrf2 activates the ARE in response to a variety of pro-oxidant or antioxidant stimuli, such as ROS, xenobiotics, heavy metals, and UV (48), but also regulates the ARE-mediated basal expression of antioxidant genes (43); this challenges the view that Nrf2 is primarily a cytosolic protein under non-stressed conditions. The essential role of Nrf2 as an ARE activator in the antioxidant response is demonstrated by the striking decrease in ARE-gene expression in nrf2 -/- mice (44,53) and cells transfected with Nrf2 siRNA (54), and also the occurrence of oxidative stress-related disease states nrf2 -/- mice display (43).

While Nrf2 activation of the ARE and resultant gene expression has been extensively studied, little is known of the mechanisms by which activated ARE induces transcription of genes. One possible area of research that may begin to answer this question is the role of chromatin remodeling in transcription, as it was demonstrated that chromatin remodeling factors, recruited to the HO-1 ARE in a Nrf2 dependent manner, resulted in the recruitment of RNAPII (25) to the HO-1 core promoter.

4. Chromatin Remodeling and Histone Modification

In order to accommodate the entirety of genomic DNA into the nucleus, DNA is packaged into chromatin. The central protein component of chromatin is the nucleosome, 147bp of DNA wrapped ~1.7 superhelical turns around an octamer of the core histone proteins H2A, H2B, H3, and H4 (55). This unit undergoes compaction with adjacent nucleosomes and higher order folding to ultimately form chromosomes (56). The histones share a highly conserved histone fold domain and the presence of an N-terminal tail of variable length that extends outward from the core of the nucleosome and can be post-translationally modified (55), as will be discussed later. While chromatin in dividing cells is highly compacted into discrete chromosomal structures, non-dividing cells contain chromatin
that is partitioned into condensed regions known as heterochromatin and more “relaxed,” accessible regions called euchromatin (57). The accessibility of DNA within the overall chromatin structure is vital when one realizes that chromatin is not simply a packaging mechanism; any cellular function involving DNA is inherently affected by the chromatin structure. For example, the DNA repair process is regulated by chromatin structure in which repair enzymes need access to damaged sites; alternatively, in gene regulation large transcriptional complexes or transcription factors must have access to the transcription start site, as well as distal enhancers. If DNA lesions, or gene promoter enhancer elements, are tightly bound within compacted chromatin and inaccessible, repair or transcription cannot take place. The modulation of chromatin structure in cellular homeostasis is known as chromatin remodeling. Gene regulation, DNA replication, repair, condensation, and segregation are all highly regulated by chromatin remodeling. The remainder of this discussion will focus on the role of chromatin remodeling in gene regulation.

Chromatin remodeling is primarily mediated by 1) ATP-dependent chromatin remodeling enzymes and 2) the post-translational modification of N-terminal histone tails. ATP-dependent chromatin remodeling enzymes remove, or shift, nucleosomes so that specific regions of DNA are exposed and become accessible to transcriptional regulatory factors (58). Switching defective/sucrose non-fermenting (SWI/SNF), imitation SWI (ISWI), nucleosome remodeling and deacetylation (NuRD), and inositol requiring 80 (INO80) families are the most well studied ATPase chromatin remodeling families (58) and have been demonstrated to regulate both transcriptional activation and repression. Usually consisting of an large, multi-subunit complex with ATPase activity, ATP-dependent chromatin remodelers utilize ATP-hydrolysis to exchange, evict, or shuffle nucleosomes from or along DNA, possibly by disrupting the contact between DNA and histones (59). The initiation of transcription begins with the formation of the pre-initiation complex (PIC) and recruitment of RNAPII. While some constitutively expressed genes feature nucleosome free regions at the TSS, the presence of nucleosomes at the TSS of inducible genes present a barrier to transcription and so it is necessary for ATP dependent chromatin remodelers to displace the
nucleosomes (60). ATPase-dependent nucleosome displacement also extends to enhancer elements (18) in order to facilitate transcription factor binding.

The second mechanism of chromatin remodeling is the reversible post-translational covalent modification of N-terminal histone tails. There are several distinct classes of histone modification: lysine acetylation, methylation, ubiquitylation, and sumoylation; arginine methylation and deamination; proline isomerization and glutamate poly-ADP ribosylation; and lastly, serine and threonine phosphorylation (61). The most well characterized modifications are acetylation, methylation, and phosphorylation. Histone modifications serve in varied capacities, from directly disrupting DNA-histone/nucleosome contacts, to recruiting and binding non-histone proteins such as ATP-dependent chromatin remodeling complexes and histone modifiers. In the latter instance, histone modifications act as binding platforms. There exists cross-talk among histone modifications, known as the “histone code.” Histone modifications induce, by recruitment of chromatin modifiers, additional histone modifications on the same histone tail, or a neighboring histone or nucleosome (62). Histone modifications are mediated by histone acetyltransferases (HAT) and deacetylases (HDAC), serine and threonine kinases and phosphatases, and histone methyltransferases and demethylases (63). In addition to the complexity afforded by the addition and removal of multiple histone modifications, singular modifications can offer even more intricacy by the mono, di, or even tri-methylation of a single lysine residue or the symmetrical or asymmetrical di-methylation of arginines (63). This complexity, while daunting, is beneficial in that histone modification-mediated processes can be fine tuned, whether we are speaking of the DNA damage response or gene regulation.

As mentioned, histone modifications may disrupt contact between DNA and histones/nucleosomes, which is necessary for exposing desired regions of DNA, or for shuffling and/or evicting nucleosomes. How do histone modifications carry out this task? The most simplistic explanation is that of the role of histone lysine acetylation and methylation. The acetylation of N-terminal histone lysines neutralizes the basic charge of the
lysine residue, disrupting attraction with the negatively charged phosphate backbone of DNA, resulting in the “relaxation” of chromatin (64,65). This is historically correlated with activation of gene transcription, though this is an extremely simplistic view and subject to several exceptions. In opposition, methylation of histone lysine residues (preceded by lysine deacetylation via HDACs) maintain a positive charge, thus maintaining or inducing DNA-histone contact, resulting in the “tightening” or compaction of chromatin, though it may be argued that the recruitment of heterochromatin-forming proteins to methylated lysines contribute to this effect (66). Again, though histone lysine methylation is generally correlated with transcriptional repression, it is subject to several exceptions, most notably the location of the methylated lysine; for instance, histone H3 lysine 9 tri-methylation (H3K9Me3) is correlated with heterochromatin formation and transcriptional repression while histone H3 lysine 4 methylation (H3K4Me) is correlated with transcriptional activation (67).

In most cases, however, the disruption of DNA-histone contact is not mediated solely by the direct effect of a single (or multiple) histone modification, but in conjunction with the recruitment of non-histone proteins to the modified histone residue. These non-histone proteins may be histone modifiers, such as HATs or protein kinases which result in additional histone modifications, or ATP-dependent chromatin remodeling complexes which facilitate chromatin restructuring, or other chromatin binding proteins of variable function.

Single histone modifications, or multiple modifications (an example of the combinatorial effect of histone modifications, or the “histone code”) recruit non-histone proteins by binding to specific domains located in the non-histone protein. For example, acetylated lysines are bound by bromodomains; the acetyllysine residue inserts into the bromodomain pocket, or cleft, and is held in place by hydrogen bonding interactions with pocket residues. Neighboring histone modifications may enhance or hinder this bromodomain pocket binding (68). HATs contain bromodomains, and by binding to acetylated histones, in turn acetylate neighboring histones, spreading histone acetylation marks. Through binding of acetylated lysine residues, the PIC member TFIID is necessary for the recruitment of RNAPII. TFIID contains several bromodomains that bind acetylated histone H4 lysine residues (69).
Methylated histone lysine residues are bound by two main classes of domains, the plant homeo domain (PHD) family and the Royal superfamily (70). All methyllysine binding domains form a “cage” of aromatic residues around the methylammonium group of the methylated lysine. The Royal family includes chromodomains, double chromodomains, tudor and tandem tudor domains, and malignant brain tumor (MBT) domains (70). For example, heterochromatin protein 1 (HP-1) binds to H3K9Me3 via a chromodomain and facilitates heterochromatin formation (71). Phosphorylated histone serine and threonine residues are bound by WD40 repeats and 14-3-3 modules. Binding is stabilized by a series of hydrogen bonds between the N-terminal histone tail and the binding pocket side chains, with the charge of the phosphate group neutralized (70). Of note is the effect of histone phosphorylation on the binding aptitude of neighboring residues, an example of the “histone code.” Histone H3 serine 10 phosphorylation (H3S10P) decreases the affinity of the HP-1 chromodomain for H3K9Me3 during mitosis, causing the ejection of HP-1 from histone H3 (71,72).

Through these binding domains, non-histone effectors bind histone modifications, resulting in further chromatin remodeling and mediation of several cellular processes, including gene regulation. Transcriptional activity is governed by histone modifications, but the number of histone modifications and recruited effectors is vast and the multitude of transcriptional processes affected by modified histones is complex and beyond the scope of this discussion. There are several excellent reviews articles (18,63,73-77) compiling the research of the past few decades with the regard to the role of histone acetylation and methylation in gene regulation, but for the purposes of this discussion, and the scope of this dissertation, only the role of histone phosphorylation in gene regulation will be expounded upon.

5. Histone Phosphorylation

As with other histone modifications, histone phosphorylation is involved in several cellular processes, such as DNA damage repair, the condensation of DNA during apoptosis and mitosis, and gene regulation (78). The key players in histone phosphorylation are protein kinases. In response to stimuli, signaling pathways activate kinases that phosphorylate serine,
threonine, and tyrosine residues on the N-terminal histone tail. The interaction between kinases and histones in many cases is the result of kinase co-localization with transcription factors and co-activators recruited to promoter elements (79). This affords targeted histone phosphorylation at specific regions of the DNA (80,81), mediating the recruitment of additional histone modifiers and chromatin remodelers that are necessary for the activation of core promoter or distal enhancer elements (82). So far, only phosphorylation of histones H2B, H3, and the histone variant H2AX have been extensively studied. H2AX is phosphorylated at serine 139, and this is a crucial event in the DNA damage response pathway (83). H2B has two major phosphorylation sites, serine 14 and 36, which are phosphorylated by the mammalian sterile twenty kinase (MST1) and AMP-activated protein kinase (AMPK), respectively. Phosphorylation of serine 14 is correlated with apoptosis (84) while serine 36 has been associated with transcriptional activation (85).

Histone H3 has several phosphorylation sites currently identified; serines 10 and 28, and threonines 3, 6, 11, and 45 (86). There are multiple kinases involved in histone H3 phosphorylation, including the H3S10 kinases proto-oncogene serine/threonine-protein kinase (PIM1) (80), IkB kinase (IKK) (86), p90 ribosomal S6 protein kinase (RSK) (86), protein kinase B (Akt) (86), mitogen and stress activated protein kinase (MSK1) (86), the MAPKs JNK2/3 (81) and p38 (87), and the mitotic Aurora B kinase (86). H3S28 kinases include Aurora B, MSK1, JNK1, and mixed lineage kinase-like mitogen activated protein triple kinase (MLTK) (86). Only three kinases have been identified for H3T11; protein kinase C-related kinase (PRK1), checkpoint kinase 1 (CHK1), and the mitotic death associated protein kinase homolog kinase (Dlk/ZIP) (86).

Many histone kinases are activated by stimuli-initiated signaling pathways and so a diverse range of stimuli have been found to induce histone phosphorylation. For example, H3S10 is phosphorylated in response to heavy metals such as arsenite (88) and nickel (89), cytokines (90), growth factors (91,92), UV (93,94), phorbol ester tumor promoters (95), and ethanol (96). Of interest is stress-induced histone phosphorylation, suggesting a role in cellular stress response. This of course begs the question of whether histone phosphorylation is involved in
the antioxidant response by regulating ARE-gene expression. As with histone lysine acetylation and methylation, histone phosphorylation is a reversible event. Histone phospho-marks are removed by protein phosphatases, and it appears that the type 1 and 2 protein phosphatase (PP1/2) families regulate H3S10P (86,97).

While histone phosphorylation in transcription has been an area of intense research in the past decade, early interest in histone H3 phosphorylation was due to its role in mitosis. After S phase DNA replication, chromatin undergoes condensation during the G2 phase, which is essential for the formation and later segregation of chromosomes. Histone H3S10/28 phosphorylation first appears during late G2 phase in pericentromeric regions and then as mitosis progresses, spreads along the entire chromosome. Each phospho-histone mark appears to have varying functions in mitosis, though overlap exists. H3S10 and 28 seem to primarily regulate chromatin condensation; beginning in late G2 phase, H3S10/28P peaks during metaphase and ends in late anaphase. Histone H3T3 and T11 have slightly different roles, being enriched in centromeric regions during metaphase (78,98-100). Mitotic H3S10 and H3S28 phosphorylation is mediated by the Aurora B kinase while Dlk/ZIP mediates phosphorylation of H3T11; histone H3T3 is phosphorylated by the Haspin kinase (86).

In regards to transcriptional regulation, H3S10P was first characterized to be associated with immediate early gene expression (IEG). The “nucleosomal response,” as it was called, referred to the rapid induction of immediate early genes such as c-fos and c-jun that occurred in conjunction with phosphorylation of H3S10 in response to growth factors (91,99). The induction of only a subset of genes suggested that H3S10 phosphorylation was not an overall general transcriptional mechanism, but gene and possibly promoter site-specific. Mahadevan and colleagues first demonstrated the extreme rapidity of histone phosphorylation in response to growth factors and phorbol esters even in the presence of transcriptional inhibitors, suggesting that histone phosphorylation was not a secondary result of transcriptional activation (91). H3S10P was correlated with rapid induction of c-fos and c-jun in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) and anisomycin, regulated by the extracellular
signal regulated kinase (ERK) and p38, respectively (101). In HepG2 cells, TPA treatment had the interesting effect of decreasing global H3S10P while increasing H3S10P levels at the low density lipoprotein (LDL) promoter; the use of inhibitors and in vitro kinase assays demonstrated that protein kinase C (PKC) phosphorylated H3S10P, and PKC chemical inhibitor decreased both LDL promoter H3S10P enrichment and LDL mRNA expression, suggesting that H3S10P plays a role in TPA induction of LDL (95). Induction of mitogen-activated protein kinase phosphatase 1 (MKP-1) gene expression in response to arsenite was correlated with an increase in global and promoter enriched H3S10P mediated by the p38 and MSK1 signaling pathway (93). Yamamoto and colleagues demonstrated that IKK-α was a H3S10 kinase, and suggested that H3S10P is involved in tumor necrosis factor α (TNFα) mediated activation of the IκBα gene in mouse embryonic fibroblasts (MEF) (90). MSK1 was demonstrated to induce c-fos expression through H3S10P by employing msk −/− MEF cells (102). Enhancer specific phosphorylation of H3S10 was shown to be mediated by recruitment of MSK1 to enhancer elements on the jun, cox-2, and fosI1 promoters (103), while MSK1 phosphorylation of H3S10P at the c-fos promoter depended upon the presence of CREB, suggesting the MSK1 is recruited to CRE enhancer sites by CREB (104).

While the dual roles of histone H3T3, S10, and T11 phosphorylation seem diametrically opposed between the condensed chromatin of early mitosis versus the more relaxed, open chromatin state of transcription, one explanation may be that mitotic histone phosphorylation is indiscriminate and broad, while histone phosphorylation in transcription is gene and promoter specific (80). H3S10P may regulate transcription if it occurs at very specific sites, such as enhancer elements. This idea may have merit given the instances of histone kinases targeting specific regions of the promoter by being recruited to the chromatin via enhancer binding transcription factors (80,81,103). Of interest is the research conducted into the role of H3S10P in tumorigenesis, which should be of no surprise considering that H3S10P regulates the expression of proto-oncogenes such as c-fos, c-myc, and c-jun (91,92,99). Ras and c-myc transformed cells evidenced higher levels of H3S10P (92). Ablation of H3S10P through chemical inhibition, kinase knockdown, or the use of a transfected histone H3S10A mutant,
results in decreased cell transformation in several models (80,105-107). Therefore, the study of histone H3 phosphorylation in transcription is important, not only to increase our basic knowledge of the mechanisms of transcription, but in developing therapeutics to prevent or treat diseases that originate or progress through aberrant gene expression. For example, discovering that histone phosphorylation regulates ARE-driven antioxidant gene expression may offer new therapeutic targets in oxidative stress-related diseases. Before exploring the possibility of histone phosphorylation in ARE-regulated genes, our current knowledge of the molecular mechanisms of histone phosphorylation in regulating gene transcription must be understood.

The last two decades have been replete with studies correlating H3S10P with gene activation, however it wasn’t until 2009 that Zippo and colleagues demonstrated the mechanisms by which H3S10P induces gene activation. A previous study by the same group two years earlier showed that H3S10P regulated the c-Myc target genes fosL1 in response to serum and vascular endothelial growth factor (VEGF) treatment; ablation of H3S10P also had the effect of inhibiting c-Myc induced cell transformation, confirming the role of H3S10P induction of immediate early genes (IEG) in tumorigenesis (80). This group established that PIM1 is a H3S10 kinase in vitro and in vivo. PIM1 was shown to form a complex with c-Myc and colocalize with c-Myc to actively transcribing regions. The recruitment of PIM1 by c-Myc to a downstream enhancer element on the fosL1 promoter resulted in H3S10P of the enhancer (80). PIM1 silencing with the use of shRNA resulted in a striking decrease in downstream enhancer H3S10P and FosL1 expression, corroborated with a decrease in the presence of promoter bound RNAPII and RNAPII phosphorylated at serine 2, strongly suggesting that H3S10P may regulate the not only the recruitment of RNAPII, but the elongation phase of transcription. This group then elucidated the mechanisms by which H3S10P regulates transcriptional elongation: Serum induces enhancer-enriched H3S10 phosphorylation by the action of the PIM1 kinase; 14-3-3 is recruited and binds to enhancer-enriched H3S10P, in turn recruiting the males absent on the first (MOF) acetyltransferase which acetylates H4K16 (82). This phospho-acetylation platform then
recruits the positive elongation complex bromodomain containing protein-4 (BRD4)/positive elongation factor b (pTEFb), which through enhancer looping, comes into contact with RNAPII and through phosphorylation of the CTD serine 2, releases RNAPII into productive elongation, resulting in expression of fosL1 (82). These two reports provide the most detailed explanation of how H3S10P regulates IEG induction. However, whether H3S10P regulates non-IEG transcription is unknown.

While H3S10P has been demonstrated to regulate induction of IEG through the release of RNAPII into the elongation phase, histone H3T11P phosphorylation has been correlated with transcriptional initiation. Phosphorylation of H3T11 by t PRK1 was demonstrated to regulate androgen receptor (AR)-mediated transcription of kallikrein 2 (KLK2) and prostate specific antigen (PSA) genes (108). Of interest was the observation that PRK1 knockdown resulted in decreased RNAPS5, the initiated form of RNAPII. This suggests that H3T11P regulates transcriptional initiation (108). Another report demonstrated that the DNA damage responsive-Chk1 kinase constitutively interacted with the promoters of the cell cycle genes cyclin B1 and cdk1 (109), and enriched these regions with H3T11P. H3T11P recruited the phospho-histone binding HAT GCN5, resulting in transcriptional activation. After DNA damage however, Chk1 dissociated from the gene promoters, resulting in decreased promoter enrichment of H3T11 and transcriptional repression (109).

These reports of the last decade begin to answer the questions that were raised when histone H3 phosphorylation first garnered attention as possibly mediating transcriptional regulation. However, the transcriptional role of H3S10P, and especially H3T11P, has not been fully revealed. For while it appears that H3S10P regulates the elongation phase of transcription by recruitment of pTEF-b, and this clearly explains why H3S10P induces IEG expression, there still are many questions. One being, is elongation the only transcriptional stage regulated by H3S10P? For while it was demonstrated that H3S10P ablation by PIM1 silencing reduced coding region bound RNAPII-S2, the recruitment of unphosphorylated RNAPII to the TSS was reduced by three fold (80), suggesting that H3S10P may aid in the
recruitment of RNAPII and so regulate transcriptional initiation in addition to elongation. Indeed, it was also shown that H3S10P regulated the recruitment of TATA-binding protein (TBP) in yeast, raising the question of whether H3S10P is involved in PIC formation (110). This would explain the H3S10P-mediated recruitment of RNAPII, and provide two points at which H3S10P could regulate transcription. A second question, much more basic, is whether H3S10P regulates expression of non-IEGs. In response to epidermal growth factor (EGF), H3S10 phosphorylation is induced within five minutes (91), and expression of c-fos mRNA is detectable at ten to twenty minutes (111). The ARE-regulated antioxidant gene HO-1 is highly inducible and expression is relatively rapid when compared to other ARE genes such as FH; HO-1 mRNA expression in response to arsenite is detectable at two hours, while significant FH mRNA expression is detectable only after twelve hours (data not shown). Also, given that stressor stimuli and stress responsive signaling pathways induce H3S10P, it raises the question as to whether H3S10P regulates many of the stress response genes that are targeted by these stress responsive pathways, such as HO-1. As will be discussed presently, HO-1 is an important enzyme in cellular homeostasis and the antioxidant response system and is noteworthy due to being highly induced in response to stimuli that also potently stimulate H3S10 phosphorylation.

6. Heme Oxygenase-1

Heme oxygenase-1 is a 32 kD protein with the primary enzymatic function of heme catabolism, the byproducts of which are biliverdin, iron, and CO (112). Continual turnover of red blood cells raises the intracellular levels of free heme, which generates highly toxic hydroxyl radicals through the Fenton reaction (113). Therefore, HO-1 is essential in cellular homeostasis. As discussed earlier, it is through the by-products of heme catabolism that HO-1 exerts antioxidant effects; HO-1 has no discernable direct antioxidant enzymatic function though one could argue that removal of heme is in effect an enzymatic antioxidant function. Biliverdin is rapidly converted to bilirubin, which is a potent antioxidant (114). While excess iron is very toxic because it reacts with H2O2 in the Fenton reaction to produce the highly reactive hydroxyl radical, heme catabolism by HO-1 usually produces a concomitant
upregulation of the iron binding ferritin protein (115,116). Therefore, the removal of free heme, the production of bilirubin, and increased expression of ferritin contribute to the antioxidant capability of the cell as a result of the actions of HO-1.

Many signal transduction pathways and transcription factors that are regulated by oxidative stress induce HO-1 expression (14). For example, Nrf2 regulates HO-1 expression through two AREs (E2, -10kb and E1, -4kb upstream of the HO-1 TSS) that are constitutively bound by the ROS-regulated transcriptional repressor BACH1 (28,117-120).

In addition to canonical ARE transcription factors, chromatin remodeling has also been demonstrated to play a role in HO-1 expression. Brahma-related gene 1 (BRG1) is an ATPase of the SWI2/SNF2 family that is recruited to enhancer elements by interacting with transcription factors such as c-Myc (121). It was demonstrated that in response to oxidative stress, BRG1 co-localizes with Nrf2 to the HO-1 E2 and E1 AREs and facilitates the recruitment of RNAPII to the HO-1 TSS via formation of a left-handed Z-DNA structure in the area of the HO-1 promoter, strongly suggesting that chromatin remodeling of the HO-1 promoter is essential for formation of the RNAPII transcriptional complex (25). This report also demonstrates a role for Nrf2 in the recruitment of chromatin remodelers; this suggests a possible mechanism by which the ARE activates gene transcription. Brg1 formation of Z-DNA to facilitate transcription by RNAPII recruitment was not evidenced in other ARE genes such as NQO1 (25), suggesting that chromatin remodeling plays a much more substantial role in HO-1 transcription than other ARE genes. A previous report demonstrated that the E1, E2, and TSS regions of the HO-1 promoter evidence hypereacetylated histones H3 and H4, which increases after heme treatment (122). Given the dynamic crosstalk between acetylated and phosphorylated histone residues it may be possible that the HO-1 promoter is enriched with phosphorylated histones. The induction of HO-1 by stimuli that also induces histone phosphorylation (123,124), the regulation of HO-1 induction by kinases known to phosphorylate histones (125,126), and the involvement of chromatin remodeling in HO-1 gene expression all raise the question as to whether histone phosphorylation plays a
major role in HO-1 transcriptional activation. The most potent inducer of both H3S10P and HO-1 expression is the environmental contaminant sodium arsenite.

7. Arsenite

Sodium arsenite is the trivalent, form of the heavy metal arsenic, a naturally occurring element. Arsenite is an Highly toxic environmental contaminant found in drinking water, soil, and food. Arsenite exposure, in addition to acute cellular toxicity, has been implicated in several pathological disease states, such as disorders of the cardiovascular system, diabetes, neuropathy, and cancer (127). While it has not been conclusively determined whether arsenite is a tumor initiator (perhaps unlikely, given that arsenite is not mutagenic), or is involved the promotion and progression stages of tumors, arsenite exposure has been irrevocably linked to cancers of the liver, bladder, kidney, and most notably, skin (128). Arsenite exposure induces hyperkeratotic lesions, and these lesions are highly implicated as skin cancer precursors (129). Arsenite toxicity is thought to be primarily the result of high reactivity towards protein sulfhydryl groups, the main target of ROS. Indeed, it has been hypothesized that arsenite mediates carcinogenesis, in part, through the generation of ROS (128,130). Arsenite is known to stimulate superoxide anion and H$_2$O$_2$ production in a variety of cell lines including immortalized human keratinocytes (HaCaT) (126), though the mechanisms of arsenite-induced ROS is unknown. There is evidence suggesting that arsenite generates ROS through mitochondrial dysfunction, activation of NADH oxidase, and even the direct oxidation of arsenite to arsenate (130).

Arsenite activates multiple signaling pathways, including oxidative stress-responsive pathways. Arsenite activates the MAPKs JNK, p38, and Erk, thus influencing activator protein-1 (AP-1) and nuclear factor kappa B (NFκB) activity (128). Several ARE-regulated genes are transcriptionally activated in response to arsenite, including GCL (131), HO-1 (28), NQO1 (132), and FH (data not shown), through arsenite activation of the Nrf2-ARE pathway (133). In addition to activating MAPK pathways, arsenite also dramatically upregulates c-fos and c-jun IEG expression (134). H3S10P regulates IEG expression, so it is no surprise that
arsenite is also a potent inducer of histone H3S10 phosphorylation. He and colleagues demonstrated that arsenite induced H3S10P in mouse epidermal JB6 cells. Through the use of *in vitro* kinase assays, dominant negative kinases, and *RSK*-/− MEF cells, this group showed that H3S10 is phosphorylated by the Akt1, Erk, and RSK kinases in response to arsenite, while dominant negative MSK1 had no effect (88). However, MSK1 phosphorylates H3S10P in response to arsenic trioxide (135). H3S10P was induced by arsenite in mouse C310T1/2 cells in a p38-dependent manner, but not Erk (96). while H3S10P was induced by arsenite in an Erk-dependent manner (136) in human diploid fibroblasts. As a whole, these reports definitively demonstrate that arsenite induces H3S10P in multiple models, through the activation of a diverse set of stress responsive kinases.

Several reports, such as the potent induction of both HO-1 and H3S10P in response to arsenite, the role of chromatin remodeling in HO-1 induction, HO-1 transcriptional regulation by pathways and kinases involved in H3S10P, and the timely expression pattern of HO-1, suggests that HO-1 may be regulated by H3S10P; therefore, as will be presented in the next section, we formulated the general hypothesis that H3S10P is involved in arsenite mediated HO-1 induction through the ARE.
HYPOTHESIS

Chapter 1: The Role of Histone H3 Serine 10 Phosphorylation in Arsenite-Mediated Heme Oxygenase-1 Transcription

A vigorous antioxidant response is essential in countering oxidative stress and preventing oxidative stress-related disorders. Antioxidant enzymes are the foundation of the antioxidant response, and so transcriptional activation of antioxidant genes is a highly regulated process. Alterations of the chromatin environment are key determinants in all stages of transcription (75). H3S10P is required for the elongation phase of immediate early gene transcription (82) and is phosphorylated by multiple kinases in response to diverse stimuli (88,89,93,94,137).

Several oxidative stress-responsive MAPKs, in addition to regulating ARE-gene expression, phosphorylate H3S10P (81,86,87,101). This led us to pose the question of whether H3S10P regulates transcription of antioxidant ARE-genes. The metalloid arsenite is a potent inducer of H3S10P (88) as well as generating oxidative stress (138), activating MAPK signaling (139,140), and strongly inducing HO-1 expression (126). Therefore, we hypothesized that H3S10P regulates arsenite-mediated HO-1 transcriptional activation. The association between arsenite exposure, hyperkeratosis, and skin cancer (129), plus reports demonstrating that arsenite induces ROS (126) in immortalized human keratinocytes (HaCaT) led us to choose this cell line as a relevant model system. To test this hypothesis, we designed experiments to address the following main questions:

1. Does arsenite induce H3S10P concomitantly with HO-1 expression HaCaT cells?
2. Does arsenite enrich HO-1 AREs with H3S10P?
3. Does oxidative stress regulate H3S10P?
4. Are oxidative stress-responsive kinases responsible for arsenite-induced H3S10P?
5. Does ablation of H3S10P result in decreased HO-1 expression?
6. Does arsenite-mediated H3S10P regulate Nrf2-ARE binding?
Chapter 2: Arsenite-Induced Histone H3 Threonine 11 Phosphorylation in Heme Oxygenase-1 Expression

In response to DNA damage, cells undergo cell cycle arrest and the DNA damage repair pathway is initiated. During this time cells are vulnerable to stress. Given that cells are constantly exposed to endogenous and exogenous sources of ROS, cells undergoing DNA damage and repair may be sensitive to oxidative stress. There exists the possibility that in response to DNA damage, cells mount an antioxidant response through transcriptional activation of ARE-genes. Arsenite induces oxidative DNA damage (130,141), though it is unknown whether the expression of ARE-genes such as HO-1 is the result of arsenite-generated oxidative stress, or oxidative DNA damage and the resultant activation of the DNA damage response. The DNA damage responsive kinase Chk1 is one of three known H3T11 kinases, and H3T11P activated cell cycle genes in a Chk1 dependent manner (108). It is unknown, however, whether H3T11P is involved in transcriptional regulation of ARE-genes. We hypothesized that H3T11P regulates arsenite-mediated HO-1 gene activation through activation of the Chk1 kinase in HaCaT cells. We designed experiments to answer the following questions:

1. Does arsenite induce H3T11P?
2. Does arsenite induce H3T11P concomitantly with HO-1 expression?
3. Does arsenite enrich the HO-1 AREs with H3T11P?
4. Is the Chk1 kinase phosphorylated in response to arsenite?
5. Is Chk1 the responsible kinase for arsenite-mediated induction of H3T11P?
6. Does Chk1 regulate HO-1 expression in response to arsenite?
Chapter 1:

The Role of Histone H3 Serine 10 Phosphorylation in Arsenite-Mediated Heme Oxygenase-1 Transcription

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ABSTRACT

Heme Oxygenase-1 (HO-1) is an antioxidant gene expressed in response to oxidative stress that significantly contributes to the cellular antioxidant response. The environmental contaminant sodium arsenite transcriptionally upregulates HO-1 expression by NF-E2-related factor 2 (Nrf2)-mediated activation of the antioxidant response element (ARE). Arsenite is a potent inducer of histone H3 phosphorylation. Gene transcription is regulated coordinately by a transcription factor and chromatin environment proximal to the cis-acting element; however, the role of histone modifications in the regulation of Nrf2 and ARE enhancer activity remains largely uncharacterized. We hypothesized that arsenite activates HO-1 transcription through phosphorylation of histone H3 serine 10 (H3S10P). In response to arsenite, global and HO-1 promoter enriched H3S10P levels were increased concomitantly with HO-1 expression in immortalized human keratinocytes (HaCaT). N-acetylcysteine (NAC) and the c-jun N-terminal kinase (JNK) inhibitor SP600125 reduced not only global and promoter levels of H3S10P, but HO-1 expression as well. Furthermore, Nrf2 binding to the ARE preceded H3S10P enrichment at the ARE, yet ablation of H3S10P and HO-1 mRNA by NAC or SP600125 had no effect on Nrf2 nuclear translocation or binding to the ARE. Conversely, we observed decreased H3S10P enrichment at the ARE in Nrf2 -/- mouse embryonic fibroblasts as well as in Nrf2 knockdown HaCaT cells treated with arsenite. Given the effect of SP600125 on H3S10P, we knocked down JNK expression with siJNK and observed decreased global levels of H3S10P and HO-1 expression, as well as a decrease in HO-1 promoter-enriched H3S10P, suggesting that JNK contributes to H3S10P on the HO-1 promoter. Collectively, our results suggest that arsenite induces H3S10P through an oxidative stress-JNK dependent pathway that is involved in HO-1 expression. We also provide evidence suggesting that Nrf2 regulates H3S10P at the HO-1 promoter, a novel role for Nrf2 in transcriptional activation of ARE-genes.
INTRODUCTION

In response to adverse conditions and deleterious stimuli, cellular survival and homeostasis depends upon an effective stress response. Oxidative stress is a continual threat to cells due to the endogenous generation of reactive oxygen species (ROS), and environmental stressors such as heavy metals and xenobiotics that alter the redox status of the cell (1). Combating oxidative stress depends upon the antioxidant defense capability of the cell. Antioxidant enzymes contribute to the antioxidant defense system by removal of ROS or the prevention of further ROS generation. Fine tuned expression of antioxidant genes is therefore essential in reducing oxidative stress, which has been implicated in several disease states including neurodegeneration (2) and cancer (3). While some antioxidant genes may be regulated at the translational level, the majority are regulated by transcriptional activation. Enhancer elements play a major role in gene transcription, regulating both basal and inducible expression of genes (4). The antioxidant response element (ARE) is a cis-acting upstream enhancer element with the conserved sequence of TGA(C/T)nnnGCA (5,6) that regulates the expression of multiple antioxidant genes in response to oxidative stress, including ferritin (7), NAD(P)H quinone oxidoreductase-1 (NQO1) (8), and heme oxygenase-1 (HO-1) (9). The activation of the ARE is primarily mediated by the transcription factor NF-E2-related factor 2 (Nrf2) (10), an oxidative stress-sensitive basic leucine zipper (bZip). In response to oxidative stress, cytosolic Nrf2 is released from the Kelch-like ECH-associated protein 1 (Keap1)/cullin-3 E-3 ubiquitin ligase (Cul3) inhibitory complex and translocates into the nucleus where it forms a heterodimer with small Maf proteins and binds to the ARE (11).

Chromatin is the nucleic acid-protein complex by which the genome is structured and packaged. The primary unit of chromatin is the nucleosome, which consists of DNA coiled around a histone octamer composed of the core histones H2A, H2B, H3, and H4 (12). The N-terminal tails of histones extend outward from the nucleosome and can be post-translationally modified. Histone modifications include lysine acetylation, arginine and lysine methylation,
and serine and threonine phosphorylation (13). Histone modifications have been demonstrated to regulate all stages of gene transcription, depending upon the specific residue and the location of the modified histones within the gene-chromatin structure. For example, proximal promoter regions enriched with histone H3 tri-methylated at lysine 4 (H3K4Me3) are associated with transcriptional activation (14), whereas tri-methylation of histone H3 lysine 27 (H3K27Me3) surrounding the transcription start site (TSS) are involved in repression (15). Phosphorylation of histone H3 at serine 10 (H3S10P) has been linked with the rapid induction of immediate early genes (IEG) such as c-myc (16), c-fos, and c-jun (17,18). H3S10P enrichment of FosL1 specific enhancer elements by the proto-oncogene serine/threonine-protein kinase (PIM1) controlled transcriptional elongation of the FosL1 gene in response to growth factors (19). Furthermore, enhancer-specific H3S10P recruited a phospho-binding protein/histone acetyltransferase complex which resulted in phosphorylation of RNA polymerase II (RNAPII) by elongation factors (20).

In addition to PIM1, H3S10 is phosphorylated by the p38(21) and c-Jun N-terminal kinases (JNK) (22), members of the oxidative stress-responsive mitogen activated protein kinase (MAPK) signaling pathways. Given the role of p38 and JNK pathways in ARE-gene expression (23,24), and the involvement of chromatin remodeling in ARE-gene regulation (9,25,26), it raises the question as to whether H3S10P is involved in the transcriptional regulation of ARE-genes such as HO-1. Highly inducible, HO-1 catalyzes the breakdown of heme into Fe^{2+}, carbon monoxide (CO), and biliverdin (27). The byproducts of heme catabolism are thought to be responsible for the antioxidant effect of HO-1 (28). The upstream promoter region of the HO-1 gene contains two AREs, -10kb (E2), and -4kb (E1), which are activated by Nrf2 in response to oxidative stress (29-31). The metalloid arsenite is an environmental contaminant, and exposure has been linked with human skin cancer as well as other disorders (32). Arsenite has been demonstrated to induce oxidative stress (33,34), and as such, dramatically upregulates HO-1 expression through the Nrf2-ARE pathway (35). Arsenite also stimulates the phosphorylation of H3S10 through the MAPKs extracellular signal regulated kinase (ERK) (36) and p38 (37). We therefore hypothesized that
transcriptional activation of the HO-1 gene in response to arsenite is regulated by phosphorylation of H3S10.

Employing immortalized human keratinocytes (HaCaT) as a biologically relevant model, we observed that arsenite induces H3S10P at the HO-1 promoter concomitantly with HO-1 expression. We also demonstrate that arsenite induces H3S10P through an oxidative stress-JNK dependent pathway. Furthermore, our results suggest that Nrf2 may mediate H3S10P-enrichment of the HO-1 ARE, proposing a novel role for Nrf2 in chromatin remodeling. Collectively, our results imply that H3S10P plays a role in ARE-mediated HO-1 transcription.
MATERIALS AND METHODS

Cell Culture and chemical reagents- Immortalized human keratinocyte HaCaT cells (38) were cultured in Dulbecco’s Modified Eagle’s Medium (Mediatech, VA) supplemented with 10% fetal bovine serum (Mediatech, VA) and 1% penicillin-streptomycin solution (Mediatech, VA). Mouse embryonic fibroblasts cultured from wild type and Nrf2-deficient mouse lines (39), a gracious gift from Dr. Jefferson Chan (University of California, Irvine CA), were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 0.05uM 2-mercaptoethanol (EMD), 1mM non-essential amino acids (Mediatech, VA), and 15% fetal bovine serum (Mediatech, VA). Both cell types were incubated at 37°C in a humidified 5% carbon dioxide atmosphere. Sodium arsenite (NaAsO₂) was purchased from Thermo Fisher Scientific and dissolved in water. SP600125, SB203580, and U0126 were purchased from Calbiochem (LaJolla, CA) and dissolved in DMSO. N-acetyl L-cysteine (NAC) was purchased from Sigma and dissolved in 1M Tris (pH 7.6). Menadione was purchased from Sigma and dissolved in ethanol.

Antibodies- Antibodies utilized in Western blotting and/or chromatin immunoprecipitation assay (ChIP) were purchased from the following companies: anti-heme oxygenase-1 (sc-7695), anti-Nrf2 (sc-13032X), anti-JNK (sc-571), normal rabbit IgG (sc-2027), normal mouse IgG (sc-2025), anti-RNAPII (sc-899X), Santa Cruz Biotechnology; anti-laminB1 (Ab-1), Oncogene; anti-lactate dehydrogenase (AB1222), Chemicon; anti-H3S10P (3377S), Cell Signaling Technology; anti-H3S10P (ChIP) (ab14955), anti-RNAPSer2 (ab5095), anti-RNAPSer5 (ab5131), Abcam; anti-β-actin (A5441), Sigma.

Whole cell extracts, nuclear and cytoplasmic fractionation, and Western blotting- Whole cell extracts (WCE) were prepared by washing cells with 1X ice cold PBS and lysed with cell lysis Buffer A (150mM NaCl, 10mM Na₂HPO₄, 1% Triton-X, 0.5% Deoxycholic Acid, 0.1% SDS, 0.2% Sodium Azide; pH 7.4). Nuclear and cytoplasmic extracts were prepared using a nuclear extraction kit (Active Motif) and following the manufacturers direction. Cell lysates
were electrophoretically separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels then transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific) and probed with primary antibodies. After overnight incubation, membranes were washed and incubated at room temperature with horseradish peroxidase conjugated secondary antibody (CalBiochem) and subsequently incubated in chemiluminescent HyGlo reagent (Denville Scientific) and visualized by exposure to x-ray film (Denville Scientific).

Northern blotting- Total RNA was isolated with TRI Reagent RT (Molecular Research Center) according to manufacturer’s protocol. 5-10µg of total RNA was separated on 1.1% agarose gel with 5% formaldehyde in 3-(N-morpholino)-propanesulfonic (MOPS) acid buffer, followed by overnight capillary transfer to an Protran® BA85 nitrocellulose transfer membrane (Whatman). 32p-labeled human Heme Oxygenase-1 cDNA probe prepared with MegaPrime DNA Labeling Kit (GE Healthcare) was hybridized with membrane at 42°C overnight and washed with buffer (0.1% SDS in 0.5X SSC buffer) at 52°C. The dry membranes were subjected to autoradiography and visualized on x-ray film (Denville Scientific). Staining RNA with ethidium bromide was used for equal RNA loading.

Small interfering RNA (SiRNA) transfection- 100 pmol of siNrf2 (J-003755-11, sense 5’-UGGAGUAAGUCGAGAAGUAU-3’, anti-sense 5’-UACUUCUCGACUACUCAAU-3’; Dharmacon) or siJNK, (Hs_MAPK8_13, sense 5’-CCAGUAAUAUAGUAGUAAATT-3’, anti-sense 5’-UUUACUACUAAUACUGGGC-3’; Qiagen), or non-targeting siControl (D-001210-01, sense 5’-UAGCGACUAAACACAUAUU-3’, anti-sense 5’-UUGAUGUGUUAGUCGCUAUU-3’; Dharmacon) was mixed with 10µl Lipofectamine RNAiMAX (Invitrogen) in 800µl FBS/antibiotic-free OptiMEM (Invitrogen) containing sodium bicarbonate. The resulting RNA-lipid complexes were incubated at room temperature for 20 min. The growth media of either 4 x 10⁶ HaCaT cells in 100-mm dishes, or 2 x 10⁶ HaCaT cells in 60-mm dishes was removed and replaced with OptiMEM. 400 µl of Lipofectamine/siRNA mix was added to each 100-mm dish (50 pmol), or 200µl was added to each 60-mm dish (25 pmol) and incubated for 6 hours, after which time the
OptiMEM/siRNA mixture was removed and replaced with serum-free DMEM. 48 hours later cells were treated with sodium arsenite and harvested for Western and Northern blotting, and/or ChIP assays.

**Chromatin Immunoprecipitation Assay and Quantitative Real Time Polymerase Chain Reaction/Semiquantitative PCR and Autoradiography**- Chromatin immunoprecipitation (ChIP) assay was carried out according to the “Fast ChIP” protocol (7). HaCaT cells transfected with siRNA or arsenite treatment were subjected to chromatin cross-linking with 1.42% formaldehyde which was subsequently quenched with 125mM glycine, and lysed according to protocol. Cell lysates were sonicated 12 times (12 seconds on; 20 seconds rest) to shear chromatin DNA. Sonicated lysates were subjected to chromatin immunoprecipitation by incubating with control IgG or applicable target antibodies at 4°C in a chilled sonication bath (Branson 2510, 40mHz) for 15 min and then incubated with protein A agarose/SSDNA bead slurry (Millipore 16-157) for 45 minutes. After washing and decrosslinking, the isolated genomic DNA was subjected to SYBR Green qPCR with iQ™ SYBR® Green Supermix (Bio-rad) by using the primer pairs flanking the HO-1 E2, E1, TSS, Exon 3 (9), and AP-1 regions (AP-1 F’-TCCAGGAAAGATCAACCACTG; R’-AGGTAAATCTGTCTGGGTCTGGT). The relative efficiency of each primer set was determined using input genomic DNA. Immunoprecipitated genomic DNA in each sample was normalized to input (ΔCt), then normalized to control samples and presented as fold enrichment. Alternatively, the isolated genomic DNA was subjected to semiquantitative PCR in the presence of 0.1µCi of [α-32P] d CTP and electrophoretically separated on a polyacrylamide gel and subjected to autoradiography.
RESULTS

Enrichment of histone H3S10P and Nrf2 binding to the ARE in the HO-1 gene following sodium arsenite treatment

To address the question whether histone H3S10 phosphorylation is involved in the ARE enhancer activity of the human HO-1 gene, we first treated HaCaT cells with 0-50 uM sodium arsenite for 8 hr (Fig. 1A), or 10 uM sodium arsenite for 0-24 hrs (Fig. 1B) and examined HO-1 expression as well as phosphorylation of H3S10 by Western and Northern blots. 5-50 uM sodium arsenite for 8hr induced expression of HO-1 mRNA and protein in a dose dependent manner (Fig. 1A, top). This profile of induction was similarly observed in H3S10 phosphorylation (Fig. 1A, bottom). The induction of both HO-1 and H3S10 phosphorylation was detected by 4 hr at 10 uM sodium arsenite in HaCaT cells and further increased until 12-24 hrs (Fig. 1B).

Then, to test whether H3S10P is enriched at the HO-1 ARE region, HaCaT cells treated with 10 uM sodium arsenite for 4 hr were analyzed by ChIP assay using anti-H3S10P, anti-Nrf2, and anti-RNA pol II antibodies. We employed primer sets covering the ARE E2 (-10kb ARE), ARE E1 (-4kb ARE), transcription start site (TSS), and the exon 3 regions (5kb downstream from TSS) of the human HO-1 gene (Fig. 2A, top). As expected, Nrf2 enrichment after sodium arsenite treatment was specific to the E2 and E1 ARE enhancers with marginal change of interaction between Nrf2 and TSS or the exon 3 region (Fig. 2A). Consistently, arsenite significantly induced the recruitment of RNA pol II to the TSS and the downstream exon 3, in which both the initiated RNA pol II (Ser5-phosphorylated: RNAPIIS5P) and elongating pol II (Ser2-phosphorylated: RNAPIIS2P) were increased, suggesting that HO-1 transcription was activated at 4 hr with sodium arsenite. In contrast, phosphorylation of H3S10 on the HO-1 promoter was promoter wide. The E1 ARE, however, demonstrated the highest increase of H3S10P enrichment, with a ~3-fold increase as compared to the ~ 2-fold increase at the E2 ARE, TSS, and exon 3 regions.
Our results of increased H3S10P at the E1 and E2 AREs of the HO-1 gene together with previous reports of protein kinases being recruited by transcription factors to enhancer elements (19,40) led us to elucidate whether Nrf2 binding to the E1 and E2 AREs precedes the enrichment of H3S10P around the AREs. A time course ChIP analysis up to 4hr of sodium arsenite treatment showed that Nrf2 recruitment to the ARE was induced by 15 min of arsenite treatment, whereas H3S10P induction detectable starting at 1hr (Fig. 2B). These results indicate that increased H3S10P on the HO-1 AREs is an event preceded by Nrf2 binding to the AREs.

*Induction of H3S10P by arsenite in an oxidative stress-dependent manner*

Given the fact that oxidative stress activates various signaling pathways (41) and that arsenite stimulates oxidative stress (42), we asked whether arsenite-mediated oxidative stress induced H3S10P. To this end, we employed the widely used antioxidant N-acetyl cysteine (NAC) in HaCaT cells to test the effect of altering cellular redox status on arsenite-induced phosphorylation of histone H3S10. Pretreatment of HaCaT cells with NAC resulted in the significant decrease of 10 uM or 30 uM arsenite-induced H3S10P levels (Fig. 3A). Furthermore, NAC pretreatment blocked HO-1 protein and mRNA expression (Fig. 3B), suggesting the involvement of oxidative stress in arsenite-induced H3S10P levels and HO-1 expression.

It was previously demonstrated that arsenite induces superoxide anion in HaCaT cells (34). Our results showing potential involvement of ROS in histone H3S10 phosphorylation (Fig. 3A) persuaded us to test the effect of other ROS generators on H3S10 phosphorylation. We treated HaCaT cells with menadione, a superoxide generator (43), or H2O2, and carried out Western blotting to measure H3S10 phosphorylation. As shown in Fig. 3C, menadione induced histone H3 S10 phosphorylation and this induction was blocked by pretreatment with NAC, while H2O2 had no induction of H3S10 phosphorylation. These results suggest
that arsenite-induced histone H3S10 phosphorylation is dependent on oxidative stress and may utilize a similar mechanism to menadione through the involvement of superoxide anion.

These results also suggest that the H3S10 kinases regulated by arsenite may be oxidative stress-activated Ser/Thr protein kinases. Some of the MAPK family including JNK and p38 were demonstrated to be activated by oxidative stress as well as to induce histone H3S10 phosphorylation (21,22). To characterize the protein kinase responsible for arsenite-induced histone H3S10 phosphorylation, HaCaT cells were pretreated with pharmacological inhibitors of either p38 (SB203580), JNK (SP600125), or ERK (U-0126) 1 hr prior to arsenite treatment, followed by Western blotting to measure H3S10 phosphorylation. As shown in Fig. 4, SP600125, but not SB203580 or U0126, blocked arsenite-induced histone H3S10 phosphorylation, suggesting the primary involvement of the JNK pathway. In fact, SP600125 also blocked arsenite-induced HO-1 mRNA and protein expression (Fig. 5A). Next we explored the possibility that the JNK inhibitor may block the nuclear accumulation of Nrf2 after arsenite treatment through which HO-1 expression was inhibited. To address this question, HaCaT cells were treated with 10 uM SP600125 for 1 hr, then treated with 10 uM sodium arsenite for 4 hr, and cytoplasmic and nuclear fractions were analyzed by Western blot with anti-Nrf2 antibody. Interestingly, even though the JNK inhibitor SP600125 blocked HO-1 expression (Fig. 5A), it did not affect Nrf2 nuclear accumulation (Fig. 5B). To further characterize the status of HO-1 AREs with regard to Nrf2 binding and enrichment of phosphorylated histone H3S10, ChIP assays for the E1 and E2 AREs and the TSS region of the HO-1 gene were conducted under the same pretreated conditions prior to arsenite challenge for 4 hr. We observed that induction of Nrf2 binding to the E1 and E2 AREs was unaffected by SP600125, while the enrichment of phosphorylated histone H3S10 on the E1 and E2 AREs was significantly decreased by SP600125 (Fig. 5C). These results suggest that ablation of histone H3S10 phosphorylation did not affect Nrf2 binding to the HO-1 AREs, and that SP600125 blocked the transcriptional mechanism of HO-1 that occurred after Nrf2 recruitment to the AREs. In addition, NAC mimicked SP600125 (Fig.
supported by our results in Fig. 3 that NAC blocked arsenite-induced histone H3S10 phosphorylation and HO-1 expression.

Relationship between histone H3S10 phosphorylation and Nrf2 on the HO-1 AREs

Our initial hypothesis was that histone H3S10 phosphorylation regulates Nrf2 binding to the ARE. However, our observation that arsenite-induced Nrf2 recruitment to the HO-1 ARE precedes the enrichment of phosphorylated histone H3S10 (Fig. 2B) and that inhibition of histone H3 phosphorylation blocked HO-1 expression without affecting Nrf2 recruitment to the ARE (Fig. 5) prompted us to explore the possibility of phosphorylation of histone H3S10 being regulated by Nrf2. To this end, we employed Nrf2 knockout mouse embryonic fibroblasts (MEF) and tested whether Nrf2 deficiency affects histone H3S10 phosphorylation following arsenite treatment. As expected, HO-1 induction by arsenite was significantly decreased in Nrf2-/− MEF cells (Fig. 6A). Utilizing ChIP assay with anti-Nrf2 and anti-phospho-histone H3S10 antibodies (H3S10P), we observed a loss of not only Nrf2 binding to the ARE but also enrichment of phosphorylated histone H3S10 in Nrf2-/− MEF compared to wild type Nrf2+/+ MEF cells (Fig. 6B). These results suggest that Nrf2 regulates histone H3S10 phosphorylation on the mouse HO-1 ARE in response to arsenite.

This observation may be the result of an adaptive response of the Nrf2 knockout MEF cells. To exclude this possibility, and to reproduce this observation in our human keratinocyte model, we performed knockdown of Nrf2 in HaCaT cells with siNrf2RNA. As shown in Fig. 7A, Nrf2 knockdown blocked arsenite-induced HO-1 expression but total histone H3S10 phosphorylation was unaffected. Under this condition, ChIP assays demonstrated that Nrf2 knockdown decreased phosphorylated histone H3S10 enrichment along with decreased Nrf2 binding to the HO-1 E2 ARE (Fig. 7B), supporting our initial observation in Nrf2 knockout MEF cells. Taken together, these results suggest that the enrichment of the ARE with phosphorylated histone H3S10 is dependent upon Nrf2 recruitment to the ARE by arsenite.
**JNK and histone H3S10 phosphorylation on the HO-1 gene**

The JNK inhibitor SP600125 inhibited arsenite-induced HO-1 expression and phosphorylation of histone H3S10 on the AREs (Fig. 5). JNK was activated by arsenite in HaCaT cells (44) and very recently JNK has been identified as a direct histone H3S10 kinase in the transcriptional regulation of genes associated with cell differentiation (22). We therefore asked whether JNK is the kinase responsible for the arsenite-induced HO-1 expression and phosphorylation of histone H3. To address this question, we attempted to knockdown JNK in HaCaT cells and performed protein and ChIP analysis after treatment with arsenite. When JNK protein expression was knocked down in JNK siRNA-transfected HaCaT cells, HO-1 induction by arsenite was decreased without affecting increased Nrf2 protein levels (Fig. 8A). ChIP assays in Fig. 8B demonstrated that JNK knockdown partially decreased phosphorylated histone H3S10 present on the E1 ARE (33% decrease) but marginal to the E2 ARE (8% decrease), TSS (15%), and exon 3 (13%). JNK knockdown also decreased (28%) H3S10 phosphorylation associated with a functional AP-1 site located 0.9kb upstream from TSS in the HO-1 gene that was shown to mediate HO-1 transcriptional activation through c-jun and JNK (45). These results suggest that JNK is, at least in part, involved in HO-1 expression following arsenite exposure through enrichment of histone H3S10 phosphorylation on the E1 ARE and AP-1 sites. In consistent with the effect of SP600125 on Nrf2 (Fig. 5C), arsenite-induced Nrf2 recruitment to the HO-1 AREs was marginally affected by JNK knockdown in HaCaT cells (Fig. 8C). Collectively, these results suggest that JNK is involved in HO-1 expression through arsenite-mediated enrichment of histone H3S10 phosphorylation on the HO-1 E1 ARE together with the AP1 site.
Cells are in continual battle against oxygen radicals. ROS, while at low levels beneficial signaling molecules (41), in excess engage in constant action against cellular constituents. Cellular antioxidant response relies heavily upon antioxidant proteins, and timely upregulation of these proteins necessitates a high level of control over transcriptional activation. One of the main “control switches” that regulates antioxidant gene expression is the oxidative stress-responsive enhancer element ARE. While activation of the ARE through the transcription factor Nrf2 has been extensively studied (46), little is known about the contribution of chromatin remodeling in ARE-mediated gene activation. Histone modifications play a major role in transcription, but reports detailing histone modifications in ARE-dependent gene regulation are limited (26). Understanding the way in which histone modifications regulate AREs may lead to an attractive approach in oxidative stress-related disease therapeutics, in which the enzymes that posttranslationally modify histones, such as kinases and phosphatases that regulate histone H3S10, can be targeted. Thus, revealing the role of histone modifications in regulation of ARE-antioxidant genes, and their direct effectors, provide concrete targets for the purpose of modulating cellular antioxidant status.

In this study we set out to investigate whether histone H3S10 phosphorylation regulates the human HO-1 gene through the AREs. We employed the oxidative stress-inducing metalloid arsenite in immortalized human keratinocytes as a biologically relevant system, given that arsenite induces hyperkeratotic lesions (47) that are implicated as skin cancer precursors, a disease state to which arsenite-induced oxidative stress is thought to contribute. We demonstrate for the first time that the HO-1 ARE regions along with proximal AP-1 site are enriched with phosphorylated histone H3S10 when transcription of the HO-1 gene was activated in response to arsenite.
Our results in this study demonstrated that activation of the Nrf2-ARE pathway by arsenite is independent of histone H3S10 phosphorylation; namely, blocking H3S10 phosphorylation by SP600125 or NAC, failed to inhibit Nrf2 nuclear accumulation or Nrf2 binding to the HO-1 AREs (Fig. 5A & C). In contrast, histone H3S10 phosphorylation on the ARE was dependent on the presence of Nrf2; namely Nrf2 knockout MEF cells or Nrf2 knockdown HaCaT cells showed significantly decreased phosphorylated histone H3S10 associated with the HO-1 ARE (Fig. 6 & 7). One possible explanation for these observations is that Nrf2 is recruiting a H3S10 kinase to the AREs, where upon the kinase, associated with chromatin, phosphorylates H3S10. This is not without precedence; it has been demonstrated that transcription factors such as c-myc (19), CREB (48), and Elk1 (40) recruit H3S10 kinases to enhancer regions. This would suggest a novel role for Nrf2 in chromatin remodeling, as well as directly correlate ARE with histone phosphorylation.

It should be noted that the core promoter TSS region upon which the transcription apparatus is formed as well as the exon 3 coding region also displayed increased phosphorylated histone H3S10 following arsenite treatment (Fig. 2). This may be consistent with the reports demonstrating that H3S10 phosphorylation regulates transcriptional elongation (ref 23, 24). It is unknown, however, whether increased H3S10 phosphorylation is necessary for efficient elongation in the coding region. Our observation of broader distribution of histone H3S10 phosphorylation appears to be consistent with the previous reports showing the gene-wide global distribution of specific histone modifications (49).

Our results suggested that oxidative stress induced by arsenite, in particular, menadione-type of generation of superoxide anion is responsible for the induction of histone H3S10 phosphorylation (Fig. 3C) that is supported by the previous report demonstrating the production of superoxide anion by arsenite in HaCaT cells (34,42). Employing superoxide scavengers would strengthen the evidence that points to a role of superoxide anion in the induction of H3S10 phosphorylation in response to arsenite; however, our preliminary experiments using TEMPOL and MnTMPyP in HaCaT cells were not reproducible enough to
conclude the role of superoxide anion. However, given that both compounds are superoxide dismutase (SOD) mimetics, they may actually contribute to oxidative stress by increasing intracellular H$_2$O$_2$.

The apparent role of oxidative stress in the induction of histone H3S10 phosphorylation led us then to investigate the role of JNK. The JNK inhibitor SP600125, as well as JNK knockdown blocked arsenite-induced HO-1 expression along with enrichment of histone H3S10 phosphorylation associated with the ARE and AP-1 sites of the HO-1 gene (Fig. 5 & 8). Our results with siJNK suggest that JNK is a responsible kinase for HO-1 expression and histone H3S10 phosphorylation following arsenite treatment in HaCaT cells (Fig. 8). During the preparation of this manuscript, it was reported that JNK is a direct histone H3S10 kinase that is involved in cellular differentiation target gene transcription (22), suggesting that JNK directly phosphorylates histone H3S10 in our context.

We chose the ARE-regulated HO-1 gene as a model to study H3S10P in transcription not simply because the HO-1 stimulator arsenite induced H3S10P as well, but due to the temporal expression pattern of HO-1 mRNA induction. While HO-1 is by no means an immediate early gene, when compared to other ARE-regulated genes however, HO-1 expression is relatively rapid. We have demonstrated (data not shown) that HO-1 mRNA expression is induced as early as 2 hours in response to arsenite, while ferritin was only substantially induced 10 hours later. This of course raises the question whether HO-1 is regulated by paused RNAPII, which allows for extremely rapid induction. H3S10P is known to induce the release of pause RNAPII into the elongation phase by recruiting positive elongation factor (pTEFb) (20) and so HO-1, out of several ARE genes, seemed the most likely target in studying the role of H3S10P in transcription.

In summary, we provide insight into the role of histone H3S10 phosphorylation in transcriptional regulation of the ARE-regulated HO-1 gene. Arsenite induced H3S10 phosphorylation in an oxidative stress- and JNK-dependent manner that contributed to HO-1
gene transcription. Furthermore, we provide evidence for the role of Nrf2 in histone H3S10 phosphorylation on the ARE, suggesting a novel role for Nrf2 in the activation of ARE.
ACKNOWLEDGMENTS

We thank Dr. Jefferson Chan at the University of California, Irvine CA for his kind gift of Nrf2-/− MEF cell line. This work was supported by National Institutes of Health Training Grant ES007046, and National Institutes of Health Grants RO1-GM088392, and RO1-GM095550 (to Y.T.).
REFERENCES


FIGURE LEGENDS

FIGURE 1. Sodium arsenite induces both H3S10 phosphorylation and HO-1 expression in a dose and time dependent manner.

A, HaCaT cells were treated with 1, 5, 10, 25, or 50µM arsenite for 8 h, or alternatively treated with 10µM arsenite for 4, 8, 12, or 24 h and harvested as either whole cell lysate or total RNA. Whole cell lysates were subjected to Western blot analysis with anti-HO-1 specific antibodies; β-Actin blots are shown as loading controls. 6 µg of total RNA was subjected to Northern blot analysis employing a 32P-labeled HO-1 cDNA probe; ethidium bromide staining of total RNA is shown to verify equal loading and positions of 18 and 28S ribosomal RNA are indicated. B, HaCaT cells were treated with arsenite as described above. Whole cell lysates were harvested and subjected to Western blot analysis with anti-H3S10P specific antibodies; histone H3 blots are shown as loading controls.

FIGURE 2. Induction of H3S10 phosphorylation at the HO-1 promoter.

A) HaCaT cells were treated with 10µM arsenite for 4 h, then harvested for chromatin immunoprecipitation (ChIP) and incubated with rabbit IgG, anti-H3S10P, anti-Nrf2, anti-RNAPII, anti-RNAPIIS2, and anti-RNAPIIS5 antibodies as described in Materials and Methods. Isolated genomic DNA was subjected to quantitative RT-PCR using primer pairs for the HO-1 E2, E1, TSS, and Exon 3 regions. Samples were normalized to input, then normalized to control samples and presented as fold enrichment. The average of two independent experiments is shown. B) HaCaT cells were treated with 10µM arsenite for 15 min, 30 min, 1 h, and 4 h then harvested for ChIP as described above using rabbit IgG, anti-H3S10P, and anti-Nrf2 antibodies. Isolated genomic DNA was subjected to quantitative RT-PCR using primer pairs for the HO-E1 region. Samples were normalized to input, then normalized to control samples and presented as fold enrichment. A representative of two independent experiments is shown.
FIGURE 3. **ROS regulates H3S10P in response to arsenite.**

A) HaCaT cells were pretreated with 10mM NAC for 1 h, then treated with 10 or 30µM arsenite for 8 h and harvested as whole cell lysates. Whole cell lysates were subjected to Western blot analysis with anti-H3S10P specific antibody; histone H3 blot is shown as loading control.  B) HaCaT cells were pretreated with 10mM NAC for 1 h, then treated with 10 or 30µM arsenite for 8 h and harvested as whole cell lysates or total RNA. Whole cell lysates were subjected to Western blot analysis with anti-HO-1 specific antibody. 5 µg of total RNA was subjected to Northern blot analysis employing a 32P-labeled HO-1 cDNA probe; ethidium bromide staining of total RNA is shown to verify equal loading and positions of 18 and 28S ribosomal RNA are indicated.  C) HaCaT cells were pretreated with 10mM NAC for 1 h, then treated with 25µM arsenite, 100µM H2O2, or 30µM menadione for 8 h and harvested as whole cell lysates. Whole cell lysates were subjected to Western blot analysis with anti-H3S10P specific antibody; histone H3 blot is shown as loading control.

FIGURE 4. **MAPK regulate H3S10P in response to arsenite.**

A) HaCaT cells were pretreated with 10µM SB203580, U0126, or SP600125 for 1 h, then treated with 10µM arsenite for 8 h and harvested as whole cell lysates. Whole cell lysates were subjected to Western blot analysis with anti-H3S10P specific antibody; histone H3 blot is shown as loading control.

FIGURE 5. **H3S10P induction in response to arsenite is inhibited by SP600125.**

A) HaCaT cells were pretreated with 10µM SP600125 for 1 h, then treated with 10µM arsenite for 8 h and harvested as whole cell lysates or total RNA. Whole cell lysates were subjected to Western blot analysis with anti-HO-1 specific antibodies; Lactate Dehydrogenase (LDH) blots are shown as loading controls. 7 µg of total RNA was subjected to Northern blot analysis employing a 32P-labeled HO-1 cDNA probe; ethidium bromide staining of total RNA is shown to verify equal loading and positions of 18 and 28S ribosomal RNA are indicated.  B) HaCaT cells were pretreated with 10µM SP600125 for 1 h, then treated with 10µM arsenite for 4 h and subjected to nuclear fractionation (Active Motif).
Nuclear and cytoplasmic lysates were subjected to Western blot analysis with anti-Nrf2 specific antibodies; Lamin B blot is shown as loading control. C) HaCaT cells were treated with 10µM arsenite for 4 h, then harvested for chromatin immunoprecipitation (ChIP) and incubated with rabbit IgG, anti-H3S10P, and anti-Nrf2 antibodies as described in Materials and Methods. Isolated genomic DNA was subjected to semiquantitative PCR using primer pairs for the HO-1 E2, E1, and TSS regions in the presence of 0.1μCi of [α-32P] d CTP and electrophoretically separated on a polyacrylamide gel and subjected to autoradiography. A representative of three independent experiments is shown.

FIGURE 6. Nrf2 regulates H3S10P induction at the mouse HO-1 ARE.
A) Nrf2 +/+ and Nrf2 -/- MEF cells were treated with 10µM arsenite for 8 h and harvested as whole cell lysates. Whole cell lysates were subjected to Western blot analysis with anti-HO-1 specific antibodies; β-Actin blot is shown as loading control. B) Nrf2 +/+ and Nrf2 -/- MEF cells were treated with 10µM arsenite for 4 h, then harvested for chromatin immunoprecipitation (ChIP) and incubated with rabbit IgG, anti-H3S10P, and anti-Nrf2 antibodies as described in Materials and Methods. Isolated genomic DNA was subjected to quantitative RT-PCR using primer pairs for the mouse HO-1 E2 region. Samples were normalized to input and presented as percent input. The average of three independent experiments is shown with standard error.

FIGURE 7. Nrf2 regulates H3S10P induction at the human HO-1 ARE.
A) HaCaT cells were transiently transfected with Nrf2 specific siRNA as described in Materials and Methods. After 48 h, cells were treated with 10µM arsenite for 6 h and subjected to nuclear fractionation. Nuclear and cytoplasmic lysates were subjected to Western blot analysis with anti-HO-1, anti-Nrf2, and anti-H3S10P specific antibodies; Lamin B and LDH blots are shown as loading controls. B) HaCaT cells were transiently transfected with Nrf2 specific siRNA as described in Materials and Methods. After 48 h, cells were treated with 10µM arsenite for 4 h, then harvested for chromatin immunoprecipitation (ChIP) and incubated with rabbit IgG, anti-H3S10P, and anti-Nrf2 antibodies as described in
Materials and Methods. Isolated genomic DNA was subjected to semiquantitative PCR using primer pairs for the HO-1 E2 region in the presence of 0.1µCi of $\alpha^{-32}$P dCTP and electrophoretically separated on a polyacrylamide gel and subjected to autoradiography. A representative of two independent experiments is shown.

FIGURE 8. **JNK regulates H3S10P induction on the human HO-1 promoter.**
A) HaCaT cells were transiently transfected with JNK specific siRNA as described in Materials and Methods. After 48 h, cells were treated with 10µM arsenite for 8 h and subjected to nuclear fractionation. Nuclear and cytoplasmic lysates were subjected to Western blot analysis with anti-HO-1, anti-Nrf2, anti-JNK, and anti-H3S10P specific antibodies; Lamin B and LDH blots are shown as loading controls. B,C) HaCaT cells were transiently transfected with JNK specific siRNA as described in Materials and Methods. After 48 h, cells were treated with 10µM arsenite for 4 h, then harvested for chromatin immunoprecipitation (ChIP) and incubated with rabbit IgG, anti-H3S10P (B) or anti-Nrf2 (C) antibodies as described in Materials and Methods. Isolated genomic DNA was subjected to quantitative RT-PCR using primer pairs for the HO-1 E2, E1, AP-1, TSS, and Exon 3 regions. Samples were normalized to input, then normalized to control samples and presented as fold enrichment. An average of two independent experiments is shown.
Fig. 1 Arsenite induces H3S10P and HO-1 expression in a time and dose dependent manner.
Fig. 2A Arsenite induces H3S10P on the HO-1 promoter concomitantly with ARE activation.
Fig. 2B Nrf2 localization precedes H3S10P enrichment at the HO-1 ARE.
Fig. 3A & B  ROS regulate H3S10P and HO-1 expression in response to arsenite.
Fig. 3C  Superoxide anion generator induces H3S10P.

Fig. 4  MAPK pathways regulate H3S10P in response to arsenite.
Figure 5. JNK signaling pathway regulates arsenite mediated H3S10P and HO-1 expression.
**Figure 6.** Nrf2 regulates H3S10P enrichment of the mouse HO-1 ARE in response to arsenite.
Figure 7. Nrf2 regulates arsenite mediated H3S10P on the human HO-1 ARE.
Figure 8A & B  JNK regulates arsenite mediated H3S10P and HO-1 expression.
Figure 8C. JNK regulates Nrf2 recruitment to the HO-1 ARE.
Chapter 2

Arsenite-Induced Histone H3 Threonine 11 Phosphorylation in Heme Oxygenase-1 Expression

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INTRODUCTION

While histone H3 serine 10 (H3S10) has been extensively studied in regards to transcriptional regulation, very little research has been conducted to explore the role of histone H3 threonine 11 (H3T11). There are only three known kinases proven to phosphorylate H3T11; protein kinase C-related kinase (PRK1), checkpoint kinase 1 (CHK1), and the mitotic death associated protein kinase homolog kinase (Dlk/ZIP) (1). While Dlk/Zip phosphorylates H3T11 during mitosis, Chk1 and PRK1 have been demonstrated to phosphorylate H3T11 in the context of transcriptional regulation. Chk1 is phosphorylated in response to DNA damage and regulates checkpoint cell cycle arrest (2), and was demonstrated to phosphorylate H3T11 (H3T11P) (3). Chk1 was shown to constitutively interact with the promoters of the cell cycle genes cyclin B1 and cdk1 (3). In the absence of DNA damage Chk1 phosphorylated H3T11, resulting in recruitment of the histone acetyltransferase GCN5, maintaining transcriptional activation; DNA damage resulted in the phosphorylation of Chk1 and its dissociation from the gene promoters. This event caused a decrease in H3T11P, decreased recruitment of GCN5, and ultimately transcriptional repression (3). H3T11 phosphorylation by PRK1 is also involved in androgen receptor (AR)-mediated transcription of prostate specific antigen (PSA) and kallikrein 2 (KLK2) genes (4). PRK1 co-localized with the AR to androgen response elements (AR-E) on the PSA and KLK2 promoters and phosphorylated H3T11; this novel role of PRK1 as a H3T11 kinase was confirmed in vitro, and through the use of PRK1 siRNA (4). H3T11P accelerated demethylation of H3K9 by the Jumonji C domain containing protein (JMJD2C), though PRK1 silencing did not disrupt JMJD2C localization to AR-Es. PRK1 silencing resulted in decreased RNAPolIIIS5, the initiated form of RNAPolII, but not the unphosphorylated form of RNAPolII, suggesting that unlike H3S10P, phosphorylation of H3T11 regulates the transcriptional initiation stage (4).
While these two reports greatly increase our knowledge of the role of H3T11P in transcription, the exact mechanisms of activation have yet to be uncovered. Unlike H3S10P, which is primarily associated with specific immediate early gene (IEG) induction (5,6), the two reported instances of H3T11P in gene regulation suggest that H3T11P may regulate diverse genes. This raises the question of whether H3T11P is a general transcriptional regulatory mechanism. While DNA damage resulted in the dissociation of Chk1 from chromatin and the loss of H3T11P, resulting in transcriptional repression of cell cycle genes, it is unknown whether Chk1 mediates H3T11P in the transcriptional regulation of other DNA damage and stress response genes. The environmental contaminant arsenite induces DNA damage (7,8) as well as potently stimulating H3S10P (9-11); this raises question of whether arsenite induces H3T11P as well, through activation of Chk1.

The heme oxygenase-1 (HO-1) is a highly inducible gene in response to oxidative stress (12,13) and heavy metals such as arsenite (14). In regards to the cellular stress response, HO-1 is primarily gene associated with antioxidant activity through the by-products of HO-1-mediated heme catabolism (15). A few reports indicate that HO-1 induction is correlated with oxidative stress-induced DNA damage (16-18), suggesting the possibility that HO-1 may be induced as part of the DNA damage response.

We asked whether arsenite induces H3T11P through Chk1, and whether H3T11P regulates arsenite-mediated HO-1 induction. We report that in response to arsenite, H3T11P was induced in immortalized human keratinocytes. Furthermore, we observed that arsenite treatment resulted in phosphorylation of Chk1, and Chk1 ablation resulted in a concomitant decrease in H3T11P and HO-1 expression, suggesting that H3T11P is involved in HO-1 gene expression.
MATERIALS AND METHODS

Cell Culture and chemical reagents- Immortalized human keratinocyte HaCaT cells (19) were cultured in Dulbecco’s Modified Eagle’s Medium (Mediatech, VA) supplemented with 10% fetal bovine serum (Mediatech, VA) and 1% penicillin-streptomycin solution (Mediatech, VA) and incubated at 37°C in a humidified 5% carbon dioxide atmosphere. Sodium arsenite (NaAsO₂) was purchased from Thermal Fisher Scientific and dissolved in water.

Antibodies- Antibodies utilized in Western blotting and/or chromatin immunoprecipitation assay (ChIP) were purchased from the following companies: anti-heme oxygenase-1 (sc-7695), normal rabbit IgG (sc-2027), Santa Cruz Biotechnology; anti-H3S10P (3377S), anti-H3T11P (9764), anti-Chk1 (2360), anti-phospho-Chk1 (S345) (2341), Cell Signaling Technology; anti-H3T11P (ChIP) (ab5168), anti-phospho-RNAPII (S5) (ab5131), Abcam; anti- β -actin (A5441, Sigma).

Whole cell extracts and Western blotting- Whole cell extracts (WCE) were prepared by washing cells with 1X ice cold PBS and lysed with cell lysis Buffer A (150mM NaCl, 10mM Na₂HPO₄, 1% Triton-X, 0.5% Deoxycholic Acid, 0.1% SDS, 0.2% Sodium Azide; pH 7.4). Cellular lysates were electrophoretically separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels then transferred to polyvinylidene difluoride (PVDF) membranes (Thermal Fisher Scientific) and probed with primary antibodies. After overnight incubation, membranes were washed and incubated at room temperature with secondary horseradish peroxidase conjugated antibodies (CalBiochem) and subsequently incubated in chemiluminescent HyGlo reagent (Denville Scientific) and visualized by exposure to x-ray film (Denville Scientific).
Small interfering RNA (SiRNA) transfection- 100 pmol of siChk1 (target sequence: AAGAAAGAGATCTGTATCAAT; Qiagen) or non-targeting siControl (D-001210-01, sense 5’-UAGCGACUAAACACAUAUU-3’, anti-sense 5’-UUGAUGUGUUAGUCGUAAUU-3’; Dharmacon) was transfected into 1 x 10^7 HaCaT cells using Gene Pulser X-Cell (Bio-Rad pre-optimized setting for CHO cells) in siRNA transfection medium (sc-36868, Santa Cruz Biotechnology) without serum or antibiotics. After incubation of cells in the cuvette for 10 min at room temperature, the cells were suspended in 10 ml of FBS/antibiotic-free DMEM and incubated in a 100-mm dish for 48 h, after which time cells were treated with sodium arsenite and harvested for Western blotting.

Chromatin Immunoprecipitation Assay and Semiquantitative PCR and Autoradiography-
Chromatin immunoprecipitation (ChIP) assay was carried out according to the “Fast ChIP” protocol (7). HaCaT cells transfected with siRNA or arsenite treatment were subjected to chromatin cross-linking with 1.42% formaldehyde which was subsequently quenched with 125mM glycine, and lysed according to protocol. Cell lysates were sonicated 12 times (12 seconds on; 20 seconds rest) to shear chromatin DNA. Sonicated lysates were subjected to chromatin immunoprecipitation by incubating with control IgG or applicable target antibodies at 4°C in a chilled sonication bath (Branson 2510, 40mHz) for 15 min and then incubated with protein A agarose/SSDNA bead slurry (Millipore 16-157) for 45 minutes. After washing and decrosslinking, the isolated genomic DNA was subjected to semiquantitative PCR in the presence of 0.1µCi of [α-32P] d CTP by using the primer pairs flanking the HO-1 E2 ARE, E1 ARE, and transcription start site (12) and electrophoretically separated on a polyacrylamide gel and subjected to autoradiography.
RESULTS

Arsenite induces H3T11P enrichment of the HO-1 promoter-
To investigate the possible role of Chk1 phosphorylation of H3T11 in arsenite-mediated HO-1 transcription, we first treated HaCaT cells with arsenite for 1-24 h and measured H3T11P induction by Western blotting. As shown in Fig. 1, H3T11P was significantly induced by arsenite beginning at 8 h, and after 24 h was undetectable. This result indicates that arsenite induces H3T11P. We next asked whether arsenite induces H3T11P at the HO-1 ARE regions during transcription. To address this question we treated HaCaT cells with arsenite for 1-8 h and then performed ChIP assay for the presence of H3T11P and phospho-RNAPII (S5), a marker of initiated RNAPII, at the distal HO-1 E2 (-10kb) and E1 (-4kb) AREs as well as the TSS. ChIP assay revealed that arsenite enriched the HO-1 E1 and E2 ARE with H3T11P in a time dependent manner, and to a lesser degree the TSS (Fig. 2). Additionally, arsenite induces the phosphorylation of RNAPII (S5) on the HO-1 promoter in a time dependent manner (Fig. 2). Collectively, these results demonstrate that arsenite induces H3T11P enrichment of the HO-1 promoter during transcriptional activation.

Chk1 regulates arsenite-mediated H3T11P and HO-1 expression-
We next asked whether arsenite induces H3T11P through the H3T11 kinase Chk1. To answer this question, we first determined whether arsenite induces phosphorylation of Chk1 at serine 345, a marker of Chk1 activation (2). HaCaT cells were treated with 10, 30, and 500 µM arsenite for 4 and 8 h, and phospho-Chk1 (S345) levels were measured by Western blotting. As shown in Fig. 3, 30 and 500µM arsenite induced phosphorylation of Chk1 at 4 h in a dose dependent manner without significantly effecting non-phosphorylated Chk1 levels. This result indicates that Chk1 is phosphorylated in response to arsenite. Next, to address whether Chk1 is the responsible kinase for arsenite-induced H3T11P, we performed knockdown of Chk1 by transfection of siChk1 in HaCaT cells and examined H3T11P levels by Western blotting after exposing transfected cells with arsenite. As shown in Fig. 4, Chk1 knockdown decreased levels of H3T11P, while not reducing levels of H3S10P. This result indicates that
Chk1 is the responsible kinase in arsenite-mediated H3T11P. We next posed the question of whether Chk1-mediated H3T11P regulates arsenite-stimulated HO-1 expression. As shown in Fig. 4, HO-1 inducible protein expression in decreased after Chk1 knockdown. This result indicates that Chk1 regulates arsenite-mediated HO-1 protein induction. Collectively, these results suggest that in response to arsenite, Chk1 specifically phosphorylates H3T11 as well as regulating the induction of HO-1.
DISCUSSION

We asked the question as to whether H3T11P plays a role in the transcriptional regulation of the HO-1 gene in response to arsenite, a potent inducer of both HO-1 expression H3S10P. We hypothesized that arsenite, an inducer of oxidative DNA damage, would stimulate H3T11P through the DNA damage-responsive kinase Chk1. In this study, we demonstrate that arsenite induces both global H3T11P as well as HO-1 promoter enriched H3T11P (Fig. 1 and 2). H3T11P was increased at the HO-1 E1 and E2 AREs (Fig. 2). It was reported that H3T11P regulated gene activation by inducing the phosphorylation of RNAPolII at serine 5, which is the activated form of RNAPolII. We demonstrate that while H3T11P enrichment of the HO-1 promoter occurs during transcriptional activation, as evidenced by the increase of RNAPolII (S5) on the HO-1 TSS (Fig. 2), arsenite induction of RNAPolII (S5) precedes H3T11P, suggesting that H3T11P does not regulate the transcriptional initiation phase in HO-1 gene activation.

We also demonstrate that the Chk1 kinase is phosphorylated at serine 345 in response to arsenite treatment (Fig. 3). This is puzzling; given that it was reported that phosphorylation of Chk1 at serine 345 resulted in the release of Chk1 from chromatin, resulting in decreased H3T11P at the promoters of genes involved in the DNA damage response (3). This issue needs to further addressed by ChIP assay to determine whether arsenite induces the association of Chk1 with the HO-1 promoter. To determine whether Chk1 phosphorylates H3T11 in response to arsenite, we performed knockdown of Chk1 employing Chk1 siRNA. We demonstrate that inducible H3T11P levels decrease after Chk1 knockdown, indicating that Chk1 phosphorylates H3T11 in response to arsenite. Interestingly, Chk1 knockdown had no effect on H3S10P induction by arsenite, suggesting that Chk1 is an H3T11-specific histone kinase. Lastly, we show that HO-1 induction by arsenite is impaired by Chk1 knockdown, suggesting that Chk1 is involved in HO-1 expression in response to arsenite. This result also raises the possibility that H3T11P is involved in expression of the HO-1 gene in response to arsenite.
In summary, we demonstrate for the first time that arsenite, in addition to inducing H3S10P, stimulates H3T11P in a Chk1 dependent manner and that Chk1 regulates expression the antioxidant gene HO-1.
ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Training Grant ES007046, and National Institutes of Health Grants RO1-GM088392, and RO1-GM095550 (to Y.T.).
REFERENCES


FIGURE LEGENDS

FIGURE 1. Arsenite induces H3T11 phosphorylation in a time dependent manner. HaCaT cells were treated with 10µM arsenite for 1, 2, 4, 8, 12, and 24 h, and harvested as whole cell lysate. Whole cell lysates were subjected to Western blot analysis with an anti-H3T11P specific antibody.

FIGURE 2. The HO-1 promoter is enriched with H3T11P in response to arsenite. HaCaT cells were treated with 10µM arsenite for 1, 2, 4, 6, and 8 h, then harvested for chromatin immunoprecipitation (ChIP) and incubated with rabbit IgG, anti-H3T11P, and anti-RNAPIIIS5 antibodies as described in Materials and Methods. Isolated genomic DNA was subjected to semiquantitative PCR using primer pairs for the HO-1 E2, E1, and TSS regions in the presence of 0.1µCi of [α-32P] d CTP and electrophoretically separated on a polyacrylamide gel and subjected to autoradiography. A representative of three independent experiments is shown.

FIGURE 3. Chk1 is phosphorylated in response to arsenite in a dose dependent manner. HaCaT cells were treated with 10, 30, or 500µM arsenite for 4 and 8 h, and harvested as whole cell lysate. Whole cell lysates were subjected to Western blot analysis with anti-Chk1 and anti-Chk1 (S345) specific antibodies.

FIGURE 4. In response to arsenite, Chk1 regulates both H3T11P and HO-1 expression. HaCaT cells were transiently transfected with Chk1 specific siRNA as described in Materials and Methods. After 48 h, cells were treated with 10µM arsenite for 8 h and harvested as whole cell lysate. Whole cell lysates were subjected to Western blot analysis with anti-Chk1 anti-H3T11P, anti-H3S10P, and anti-HO-1 specific antibodies. β-Actin blot is shown as loading control.
Fig. 1 Arsenite induces H3T11P in a time dependent manner.

Fig. 2 Arsenite induces H3T11P enrichment of the HO-1 promoter.
Fig. 3 Arsenite induces Chk1 phosphorylation.

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Fig. 4 In response to arsenite, Chk1 induces H3T11P concomitantly with HO-1 expression.

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GENERAL DISCUSSION

The Role of Histone H3 Serine 10 Phosphorylation In Arsenite-Mediated Heme Oxygenase-1 Transcription (Chapter 1)

In this study, we investigated the role of histone H3 serine 10 phosphorylation (H3S10P) in transcriptional regulation of the HO-1 gene. While H3S10P has been demonstrated to regulate the transcription of immediate early genes (IEG), little research has been conducted to conclusively demonstrate that H3S10P is involved in the transcriptional activation of non-immediate early genes, such as antioxidant genes up-regulated in response to oxidative stressors. As these genes are activated by the antioxidant response element (ARE), and previous reports demonstrated that H3S10P of promoters is largely confined to specific enhancer elements, we explored the possibility of H3S10P enrichment in activation of the ARE. Based on the previous reports demonstrating that arsenite potently induces both H3S10P and HO-1 expression, we chose arsenite as a tool to enable us to study the possible role of H3S10P in HO-1 transcription. As a biologically relevant cellular model, we employed immortalized human keratinocytes (HaCaT), since it has been demonstrated that human skin cancer is correlated with arsenite exposure.

H3S10P Enrichment of the HO-1 Gene During Transcription

We first demonstrated that arsenite induces global H3S10P in HaCaT cells in a dose and time dependent manner. Examining the expression of HO-1 protein and mRNA in response to arsenite allowed us to compare the temporal patterns of H3S10P and HO-1 expression. H3S10P and HO-1 mRNA induction shared a similar expression pattern, both detectable at 4 h, thus excluding the possibility that H3S10P induction occurred after HO-1 expression (Fig. 1). However, global increases of phosphorylated histones are not necessarily correlated with phosphorylated histones at discrete gene promoters. Therefore, we examined the induction of H3S10P on the HO-1 gene. Arsenite induced gene-wide H3S10P, with induction at the distal AREs E2 (-10kb) and E1 (-4kb), as well as at the transcription start site (TSS) and even in the distal coding region of exon 3 (Fig. 2A). This promoter wide
enrichment of H3S10P did not, however, exclude the possibility that H3S10P might regulate the AREs, for it has been demonstrated that H3S10P may be enriched at different coding regions, but only enrichment of a particular enhancer significantly affects transcription. For example, Zippo et al demonstrated that on the fosL1 gene, two enhancer regions were enriched with H3S10P after treatment with VEGF, but at differing time points; the downstream enhancer element was enriched with H3S10P at 60 minutes post treatment while the upstream enhancer was enriched with H3S10P within 15 minutes. This was explained by demonstrating that the rapid H3S10P at the upstream enhancer was mediated by the stress response kinase MSK1, while the downstream enhancer was targeted by PIM1 (80); only the PIM1-regulated enhancer significantly affected transcription of the fosL1 gene. 

In addition, we observed that arsenite-induced H3S10P enrichment of the HO-1 gene occurs during ARE-activation and active transcription (Fig. 2A). Nrf2 was recruited to the E2 and E1 AREs, suggesting ARE activation. The presence of RNAPII at the TSS, as well as the initiated (RNAPII(S5)) and elongating (RNAPII(S2)) forms of RNAPII at the TSS and coding region in response to arsenite indicates a fully active transcriptional state. Thus, arsenite induces H3S10P enrichment of the HO-1 gene during transcription. In regards to our question of whether H3S10P might regulate the HO-1 ARE, we examined the recruitment of Nrf2 to the HO-1 ARE after arsenite treatment. We observed that Nrf2 binds the E1 ARE at 15 minutes, while H3S10P enrichment occurs 1 h after arsenite treatment (Fig. 2B), thus excluding the possibility that H3S10P may activate the ARE by facilitating recruitment of Nrf2.

**ROS and H3S10P**

Several H3S10 kinases are responsive to oxidative stress (142); arsenite generates reactive oxygen species, and so this raised the possibility that arsenite induces H3S10P in an oxidative stress-dependent manner. We observed that the antioxidant NAC blocked arsenite-mediated induction of global H3S10P levels, as well HO-1 expression (Fig. 3A and B), suggesting the involvement of ROS in arsenite-mediated induction of global H3S10P and HO-1 upregulation. We also observed that NAC reduced promoter-enriched H3S10P as well
(Fig. 5C). Arsenite induces superoxide anion in HaCaT cells (126). Therefore, to determine whether superoxide anion regulates H3S10P, we treated HaCaT cells with menadione, which generates superoxide anion (143); we also tested whether other ROS species may induce H3S10P by treating HaCaT cells with H$_2$O$_2$. While H$_2$O$_2$ had no effect, menadione robustly increased global H310P levels, and this increase was blocked by NAC (Fig. 3C). This suggests that arsenite induces H3S10P through generation of superoxide anion. However, further studies need to be conducted to confirm this observation, through detection of ROS and superoxide scavengers. Collectively, these results demonstrate a role for ROS in the arsenite-mediated induction of H3S10P.

**JNK Signaling Pathway and H3S10P**

Some MAPK family members are responsive to oxidative stress, as well as phosphorylating H3S10 (81,86,87,101). Therefore, we employed chemical inhibitors of MAPKs as a screening tool to identify the signaling pathway and/or specific kinase of arsenite-ROS-mediated H3S10P. We observed that while the Erk inhibitor U0126 had no effect on H3S10P, and the p38 inhibitor SB203580 demonstrated only a slight inhibitory effect, the JNK inhibitor SP600125 abolished global H3S10P levels in response to arsenite, reducing H3S10P to basal level (Fig. 4). SP600125 also decreased HO-1 protein and mRNA expression in response to arsenite (Fig. 5A). The observation that both NAC and SP600125 inhibit both H3S10P and HO-1 mRNA expression suggests that H3S10P may be involved in HO-1 transcription. The inhibitory effect of SP600125 on HO-1 expression may be due a block of Nrf2 nuclear translocation; however, SP600125 did not affect Nrf2 nuclear translocation (Fig. 5B). While Nrf2 translocates into the nucleus even after SP600125 pretreatment, there is the likelihood that this compound may prevent the recruitment of Nrf2 to the ARE. We observed, though, that SP600125 does not inhibit arsenite-mediated Nrf2 recruitment to the E2 and E1 AREs, suggesting that SP600125 targets transcriptional mechanisms that occur after Nrf2 recruitment to the ARE. This observation was also made with NAC pretreatment (Fig. 5C).
Similarly to NAC, SP600125 inhibited arsenite-mediated induction of H3S10P at the HO-1 AREs as well (Fig. 5C). These results suggest that H3S10P, as well as HO-1 expression, are regulated by a ROS-JNK dependent pathway. This provides a new role for ROS in regulating the chromatin environment of gene promoters.

**Nrf2 Regulation of H3S10P**

In the process of identifying the signaling pathway and kinase of H3S10P in response to arsenite, we made an interesting observation that while NAC and SP600125 inhibited H3S10P and HO-1 expression, they had no effect on Nrf2-ARE binding (Fig. 5C), which is the primary mechanism of ARE activation. This observation, coupled with the result demonstrating Nrf2-ARE binding precedes H3S10P enrichment (Fig. 2B), raises the possibility that Nrf2 regulates phosphorylation of H3S10P at the ARE. This is not without precedence, given the reports showing the recruitment of H3S10 kinases to enhancer regions via interaction with enhancer-specific transcription factors (80). This observation was unexpected, but compelling. To further explore the possibility of Nrf2 regulation of ARE-H3S10P enrichment, we employed Nrf2-/- MEF cells to examine the effect of Nrf2 ablation on arsenite induction of H3S10P. We observed the loss of Nrf2 recruitment to the ARE in Nrf2 -/- cells (Fig. 6B left panel), as well as decreased HO-1 induction in response to arsenite (Fig. 6A). Surprisingly, the fold induction of H3S10P at the mouse HO-1 ARE in response to arsenite was entirely abolished in Nrf2 -/- cells (Fig. 6B right panel). These results suggested that Nrf2 regulates H3S10P enrichment of the mouse HO-1 promoter, though whether this regulatory event is specific to the ARE is not clear and needs to be addressed by use of primers specific for mouse HO-1 non-ARE promoter regions.

We realized that this result might have been simply an adaptive response of the knock-out mouse line. We therefore performed knockdown of Nrf2 in HaCaT cells by employing Nrf2 siRNA. Nrf2 knockdown decreased HO-1 expression while unaffected global H3S10P levels (Fig. 7A). However, H3S10P at the human HO-1 E2 ARE was decreased after Nrf2 knockdown (Fig. 7B), confirming our initial observation in Nrf2-/- MEFs and excluding the possibility of an adaptational response. While these results require further inquiry as to the
regional specificity of the Nrf2-H3S10P relationship, collectively they suggest that HO-1 promoter-associated H3S10 is phosphorylated by a kinase and/or signaling pathway that is directly or indirectly regulated by Nrf2. Furthermore, taking into account these results, the timely promoter recruitment of Nrf2 versus H3S10P enrichment (Fig. 2B), and the observation that NAC and SP600125 decrease promoter-associated H3S10P while unaffected Nrf2 recruitment (Fig. 5C), raises the possibility that Nrf2 recruitment to the ARE may be necessary for Nrf2-regulated H3S10P of the HO-1 promoter. These results suggest a novel role for Nrf2 in recruiting histone kinases to the ARE, and altering the chromatin environment of enhancers.

**JNK Kinase in HO-1 Transcriptional Regulation**

The JNK inhibitor SP600125 inhibited arsenite induction of global and promoter-associated H3S10P concomitant with a decrease in HO-1 expression. JNK is activated in response to oxidative stress and arsenite, and has been shown to play a role in HO-1 expression, and has recently been identified as a H3S10 kinase in the transcriptional regulation of genes associated with cell differentiation (81). We therefore asked whether JNK is the kinase responsible for arsenite-mediated induction of H3S10P and HO-1 expression. We employed JNK specific siRNA in HaCaT cells, and we observed that JNK knockdown decreased both global H3S10P in addition to HO-1 protein expression (Fig. 8A).

In addressing the role of JNK in promoter enrichment of H3S10P, we also asked whether JNK may specifically target H3S10 at discrete promoter regions, and designed primers corresponding to an AP-1 (-0.9kb) site on the HO-1 promoter that was shown mediate HO-1 transcriptional activity through c-jun, which is activated by JNK (144). We demonstrated a ~33% decrease in fold enrichment of H3S10P at the HO-1 E1 ARE after JNK knockdown; however, loss of JNK expression resulted in relatively minor decreases in fold enrichment of H3S10P at the E2 ARE (~8%), TSS (~15%), and Exon 3 (~13%) regions (Fig. 8B). In addition, the HO-1 AP1 site demonstrated enrichment of H3S10P, though induction was comparable to the E2 and TSS regions; however, JNK knockdown resulted in a ~28% decrease of fold enrichment of H3S10P at the AP-1 site (Fig. 8B). Therefore, this result
indicates that JNK contributes to H3S10P enrichment of the HO-1 E1 ARE and AP-1 sites. Arsenite-induced Nrf2 recruitment to the AREs was marginally decreased after JNK knockdown; the E2 ARE recruitment of Nrf2 was decreased ~28%, while at the E1 ARE Nrf2 recruitment was decreased only ~16% (Fig. 8C), suggesting that JNK may regulate Nrf2 recruitment to the ARE in response to arsenite, given that nuclear accumulation of Nrf2 was not decreased after JNK knockdown (Fig. 8A).

To summarize, in Chapter 1, we shed light onto the role of histone H3 phosphorylation in transcriptional regulation of the HO-1 gene. Arsenite mediates H3S10P in a ROS-JNK dependent manner and may possibly contribute to HO-1 expression. Most interesting was our findings that suggests that the ARE transcription factor Nrf2 regulates promoter-enriched H3S10P, suggesting a novel role for Nrf2 in chromatin remodeling.

**Arsenite-Induced Histone H3 Threonine 11 Phosphorylation in Heme Oxygenase-1 Expression (Chapter 2)**

(Some paragraphs were adapted from Chapter 2 and modified)

We demonstrated in Chapter 1 that in response to arsenite, H3S10P was associated with the HO-1 promoter regions and was induced through an ROS-JNK dependent pathway. While H3S10P was originally correlated with IEG gene induction, our results suggest that H3S10P regulation of transcription may be more widespread. Histone H3 threonine 11 phosphorylation (H3T11P), unlike H3S10P, is not identified with transcription of only a specific subset of genes and has been demonstrated to mediate induction of androgen receptor dependent genes (108) as well as genes involved in the cell cycle (109). H3T11 is phosphorylated by the DNA damage response kinase Chk1; given that arsenite induces oxidative DNA damage, we posed the question of whether H3T11P plays a role in the transcriptional regulation of the HO-1 gene in response to arsenite.
Arsenite Induces H3T11P Enrichment of the HO-1 Promoter During Transcriptional Activation.

We demonstrate that arsenite induces both global and promoter enriched H3T11P (Fig. 1 and 2). H3T11P was dramatically increased at the HO-1 E1 ARE (Fig. 2), though the HO-1 TSS and distal E2 ARE were both enriched with H3T11P to a lesser degree. H3T11 was shown to activate transcription by inducing the phosphorylation of RNAPII at serine 5, which is the activated form of RNAPII that mediates the transcriptional initiation phase. We demonstrate that while H3T11P enrichment of the HO-1 promoter occurs during transcriptional activation, as evidenced by the increase of RNAPII(S5) on the HO-1 TSS (Fig. 2), arsenite induction of RNAPII(S5) precedes H3T11P, suggesting that H3T11P does not regulate the transcriptional initiation phase in HO-1 gene activation. This raises the question as whether H3T11P may be involved the elongation phase of transcription in a similar manner as H3S10P.

Chk1 Mediates H3T11P and HO-1 Expression in Response to Arsenite

We also demonstrate that Chk1, in response to arsenite, is phosphorylated at serine 345 (Fig. 3). It was however reported that phosphorylation of Chk1 at serine 345 resulted in the release of Chk1 from chromatin, resulting in decreased H3T11P at the promoters of genes involved in the DNA damage response (109). If phosphorylated Chk1 can no longer associate with gene promoter regions, then it seems unlikely that phosphorylated Chk1 can associate with the HO-1 promoter to induce HO-1 ARE H3T11P. However, this observation may be promoter and sequence specific. Employing ChIP assays to determine whether arsenite induces the association of Chk1 with the HO-1 promoter will provide a starting point to address this issue. We performed knockdown of Chk1 employing Chk1 siRNA to determine whether Chk1 induces H3T11P. We demonstrate that inducible H3T11P levels decrease after Chk1 knockdown, indicating that Chk1 phosphorylates H3T11 in response to arsenite. Interestingly, Chk1 knockdown had no effect on H3S10P induction by arsenite, suggesting that Chk1 is a H3T11-specific histone kinase. This raises the question of whether H3T11P and H3S10P both regulate HO-1 expression in response to arsenite. After arsenite
treatment, the HO-1 promoter is enriched with both H3T11P and H3S10P. It could very well be that H3T11, while having no effect on RNAPII phosphorylation or the recruitment of RNAPII, may aid H3S10P in regulating transcriptional elongation by virtue of their close proximity, for the elongation factor recruitment by H3S10 relies on the formation of a nucleosomal binding platform whose core is the interaction between H3S10P and the 14-3-3 protein (82). This raises the question of whether 14-3-3 can bind to H3T11P, augmenting H3S10P association with 14-3-3. We also observed that HO-1 upregulation in response to arsenite is decreased by Chk1 knockdown, suggesting that Chk1 is involved in HO-1 expression in response to arsenite, and providing preliminary evidence, though indirect, of a possible role for H3T11P in HO-1 expression in order that further experiments be conducted.

In summary, our findings indicate that arsenite mediates the Chk1-dependent phosphorylation of H3T11P in HO-1 gene activation.

**The Biological Relevance of Histone Phosphorylation Research**

The cell is in a continual battle against oxygen radicals. ROS, while at low levels beneficial signaling molecules, in excess engage in constant action against cellular constituents. Proteins, nucleic acids, and lipids are all targets of ROS reactivity, and the cell must persistently present an effective antioxidant defense against ROS to prevent macromolecular damage. For if ROS levels exceed the antioxidant defense system, widespread cellular damage occurs, and this state of redox imbalance is known as oxidative stress (6). The pathogenesis and/or progression of several disease states results from oxidative stress. Neurodegeneration, cardiovascular disorders, cancer, and even aging have been correlated with oxidative stress (145). Therefore, the prevention of oxidative stress through curtailing de novo ROS production, lowering existing ROS levels, and preventing the further generation of ROS is paramount in providing effective therapeutics for oxidative stress-related diseases. These strategies are based upon boosting the efficiency and strength of the antioxidant response. The antioxidant response relies heavily upon antioxidant enzymes, and timely upregulation of these enzymes necessitates a high level of control over
transcriptional activation. One of the main “control switches” that regulates antioxidant gene expression is the oxidative stress-responsive enhancer element ARE. The ARE is found in several antioxidant genes, regulating both basal and inducible expression of these essential genes. Therefore, unveiling the molecular mechanisms by which the ARE is activated and how it activates gene transcription, is essential in providing new molecular therapeutic targets.

While activation of the ARE through the transcription factor Nrf2 has been extensively studied, little is known about the contribution of chromatin remodeling in ARE-mediated gene activation. Histone modifications play a major role in transcription, but reports detailing histone modification in ARE-dependent gene regulation are limited. Studying the way in which histone modifications regulate not only ARE activation, but the contribution of chromatin remodeling in activation of the transcriptional apparatus by the distal ARE itself, is an attractive approach in oxidative stress-related disease therapeutics. The enzymes that post translationally modify histones, such as kinases that phosphorylate H3S10 and H3T11, can be targeted. Thus, revealing the role of histone modifications in regulation of ARE-antioxidant genes, and their direct effectors, provide concrete targets in which to therapeutically alter the redox status of the cell.

We are hopeful that our research into histone H3 phosphorylation will contribute to the current understanding of the transcriptional regulation of antioxidant genes by providing new therapeutic targets. Our studies implicate H3S10 and H3T11 in HO-1 gene activation and as such, provide two new targets that may be chemically regulated in order to manipulate HO-1 expression in organisms. Additionally, we demonstrate that Nrf2, the primary transcription factor responsible for the activation of the ARE and timely, robust induction of essential antioxidant genes, may regulate H3S10P, providing another key control point in manipulating HO-1 expression.
GENERAL REFERENCES


APPENDIX A

Reactive Oxygen Species (ROS) Homeostasis and Redox Regulation in Cellular Signaling

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Published in Cellular Signalling (2012) May, 24(5) 981-990

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ABSTRACT

Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion. Oxidative stress refers to the imbalance due to excess ROS or oxidants over the capability of the cell to mount an effective antioxidant response. Oxidative stress results in macromolecular damage and is implicated in various disease states such as atherosclerosis, diabetes, cancer, neurodegeneration, and aging. Paradoxically, accumulating evidence indicates that ROS also serve as critical signaling molecules in cell proliferation and survival. While there is a large body of research demonstrating the general effect of oxidative stress on signaling pathways, less is known about the initial and direct regulation of signaling molecules by ROS, or what we term the “oxidative interface.” Cellular ROS sensing and metabolism are tightly regulated by a variety of proteins involved in the redox (reduction/oxidation) mechanism. This review focuses on the molecular mechanisms through which ROS directly interact with critical signaling molecules to initiate signaling in a broad variety of cellular processes, such as proliferation and survival (MAP kinases, PI3 kinase, PTEN, and protein tyrosine phosphatase), ROS homeostasis and antioxidant gene regulation (thioredoxin, peroxiredoxin, Ref-1, Nrf-2), mitochondrial oxidative stress, apoptosis, and aging (p66Shc), iron homeostasis through iron-sulfur cluster proteins (IRE-IRP), and ATM-regulated DNA damage response.

Key words:
reactive oxygen species, antioxidant response element, Shc, Nrf2, ATM, iron
1. Introduction

Reactive oxygen species (ROS), such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (HO•), consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. Cellular ROS are generated endogenously as in the process of mitochondrial oxidative phosphorylation, or they may arise from interactions with exogenous sources such as xenobiotic compounds. When ROS overwhelm the cellular antioxidant defense system, whether through an increase in ROS levels or a decrease in the cellular antioxidant capacity, oxidative stress occurs. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids, and has been implicated in carcinogenesis [1], neurodegeneration [2, 3], atherosclerosis, diabetes [4], and aging [5]. However, ROS involvement in the pathogenesis of disease states is not confined to macromolecular damage. There is increasing evidence that ROS signaling contributes to disease. For example, ROS have been shown to promote tumor metastasis through gene activation [6]. While there exists ample evidence demonstrating the role of ROS in regulating cellular signaling pathways, the question that is raised is exactly how do ROS initiate cellular signaling? The “oxidative interface” is that boundary between ROS and the signaling molecules they activate; that is, the figurative region that describes how ROS directly activate oxidative stress-responsive pathways. This review seeks to explore the oxidative interface between ROS and a functionally broad selection of cellular signaling pathways regulating a variety of cellular processes (Fig. 1).
In order to understand ROS regulation of signaling pathways, the mechanism of how ROS alters protein function should be briefly addressed. The oxidative interface consists mainly of the redox regulation of redox-reactive cysteine (Cys) residues on proteins by ROS. Oxidation of these residues form reactive sulfenic acid (-SOH) that can form disulfide bonds with nearby cysteines (-S-S-) or undergo further oxidation to sulfinic (-SO₂H) or sulfonic (-SO₃H) acid; if nearby nitrogen is available sulfenic acid may also form a sulfenamide. These oxidative modifications result in changes in structure and/or function of the protein. With the exception of sulfonic acid, and to a lesser degree sulfinic acid, these redox modifications are reversible by reducing systems such as thioredoxin and peroxiredoxin [7] which is necessary given that these modifications function in redox sensing and signaling. For a more detailed overview of redox chemistry refer to Winterbourn [8] and Janssen-Heininger [9].

2. Regulation of MAPK Signaling Pathways by ROS

The mitogen-activated protein kinase (MAPK) cascades consist of four major MAPKs; the extracellular signal-related kinases (Erk1/2), the c-Jun N-terminal kinases (JNK), the p38 kinase (p38), and the big MAP kinase 1 (BMK1/Erk5). These kinases are evolutionally conserved in eukaryotes and play pivotal roles in cellular responses to a wide variety of signals elicited by growth factors, hormones, and cytokines, in addition to genotoxic and oxidative stressors. The function and regulation of the MAPK cascades have been comprehensively covered [10-13]; therefore, this review article focuses
solely on those protein kinases and phosphatases in the MAPK cascades that are
directly regulated by ROS. MAPK pathways are composed of a three-rung kinase tier;
MAPK kinase kinases (MAPKKK) phosphorylate and activate MAPK kinases (MAPKK),
which in turn phosphorylate and activate MAPKs. Among the members of the MAPK
cascades, apoptosis signal-regulated kinase 1 (ASK1) is an upstream MAPKKK that
regulates the JNK and p38 MAPK pathways leading to apoptosis through
phosphorylation of MKK4, MKK3, and MKK6 MAPKKs [14]. ASK1 is activated under
various stress conditions including oxidative stress [15]. ASK1 is homo-oligomerized
by both C- and N-terminal coiled-coil domain interaction and activation occurs through
phosphorylation of a conserved threonine (Human: Thr-838, Mouse: Thr-845) residue
in the activation loop of the human ASK1 kinase domain (Fig. 2A). There are many
ASK1-associated proteins so far identified, among which the redox protein thioredoxin
was shown to constitutively interact with ASK1 and directly inhibit its kinase
activity [16]. Only the reduced form of thioredoxin interacts with ASK1; by blocking N-
terminal, but not basal C-terminal interaction, thioredoxin inhibits complete ASK1
oligomerization and subsequent activation (Fig. 2A) [16, 17]. ASK1 is activated when
oxidants or ROS oxidize two cysteine residues in the redox center of thioredoxin,
inducing formation of an intramolecular disulfide bond between Cys-32 and Cys-35 that
results in the dissociation of thioredoxin from ASK1, allowing for the subsequent N-
terminal homophilic interaction and complete oligomerization of ASK1 (Fig. 2A), which
is enhanced by the binding of tumor necrosis factor-α receptor associated factors
The ASK1 oligomer subsequently undergoes autophosphorylation of a conserved threonine residue (Human: Thr-838, Mouse: Thr-845) located in the activation loop of ASK1 (Fig. 2A) [19], which is inactivated by protein phosphatase 5 (PP5) [20]. In addition to homo-oligomerization of ASK1, ASK1 hetero-oligomerizes with ASK2, another ASK family serine/threonine MAPKKK (Fig. 2B). ASK2 binds to the C-terminal domain of ASK1, and this interaction stabilizes ASK2, resulting in autophosphorylation of ASK2 at the conserved threonine (Human: Thr-806, Mouse: Thr-807) in the activation loop. ASK1 is then phosphorylated at Thr-838 by ASK2, resulting in activation of the hetero-oligomer (Fig 2B) [21]. ASK1-deficient mouse embryonic fibroblasts were shown to be less susceptible to TNF- or H₂O₂-induced cytotoxicity along with decreased JNK and p38 MAPK activation, suggesting that ASK1 plays a pivotal role in promoting cell death under oxidative stress [15]; however, ROS activated ASK1 mediates p38 signaling leading to non-apoptotic outcomes also, such as differentiation [22] and immune signaling [23], thus reinforcing the role of ROS signaling in cellular homeostasis.

Evidence suggests that cGMP-dependent protein kinase (PKG) regulates MAPK activation [24, 25]. It was demonstrated that PKG1α is a redox sensor activated by ROS; oxidation of Cys-42 by H₂O₂ resulted in the formation of an active PKG1α homodimer through intermolecular disulfide bond formation [26], though it is unknown whether ROS-activated PKG1α regulates MAPK pathways. Similarly, protein kinase A (PKA) was
shown to be activated by ROS through formation of intramolecular disulfide bonds [27], and protein kinase C (PKC) activity is also regulated through redox mechanisms [28]; both kinases have been implicated in MAPK signaling.

MAPK pathways are also activated by the direct inhibition of MAPK phosphatases by ROS. ROS produced by NADPH oxidases or in mitochondria have been shown to inhibit JNK-inactivating phosphatases [29] through reversible oxidation of a catalytic-site cysteine to sulenic acid, thus sustaining JNK activation. Inhibition of phosphatases by ROS have also been shown to regulate p38 signaling [30, 31], and it was recently demonstrated that ROS generated by commensal bacteria inactivated dual-specific phosphatase 3 (DUSP3) by oxidation of Cys-124, resulting in ERK activation [32]. DUSP is a protein tyrosine phosphatase (PTP); inactivation of classical PTPs such as protein tyrosine phosphatase 1B (PTP1B) or SH2-domain containing PTP (SHP2) by ROS in a similar cysteine redox mechanism (I/V-H-C-X-X-G-X-X-R/S/T) [33-35] ultimately potentiates MAPK and growth factor signaling pathways initiated from RTK, cytokines, and stressors [33, 35-41].

Oxidation of the catalytic site cysteine in PTPs to sulenic acid (-SOH) is reversible, as are disulfide bonds and sulfenamides, but further oxidation to the generally irreversible sulfinic acid (-SO₂H) or sulfonic acid (-SO₃H) can also occur (Fig. 3). Thioredoxin or glutathione appears to be involved in reducing sulenic acid residues and reversing the oxidative inactivation of PTPs [34]. The classical PTP family includes the ligand binding transmembrane receptor-like PTPs (RPTPs) such as RPTPA. Ligand binding to RPTP induces RPTP dimerization, which results in the catalytic-inactive conformation of RPTP
ROS have been shown to inhibit human RPTPα tyrosine phosphatase activity through preferential oxidation of Cys-723 in the second catalytic domain of RPTPα rather than oxidation of Cys-433 in the first catalytic domain [43], leading to the formation of intermolecular Cys-Cys disulfide bonds as well as a reversible cyclic sulfenamide [44] and subsequent stabilization of the inactive RPTPα dimer [45]. Growth factor signaling events are frequently associated with production of ROS that are known to be important signaling molecules [46]. Oxidation and inhibition of PTPs by ROS appears to be one of the molecular mechanisms through which growth factor-induced ROS production is essential for transducing and sustaining growth factor signals.

3. Regulation of PI3K Signaling Pathway by ROS

Another signaling pathway that plays a key role in cell proliferation and survival in response to growth factor, hormone, and cytokine stimulation is the phosphoinositide 3-kinase (PI3K) pathway. The PI3K, consisting of the p110 catalytic subunit and the p85 regulatory subunit, is tightly coupled with RTKs activated by various growth factors such as EGF (Epidermal Growth Factor), PDGF (Platelet-derived Growth Factor), NGF (Nerve Growth Factor), insulin, and VEGF (Vascular Endothelial Growth Factor). PI3K is recruited to activated tyrosine-phosphorylated RTK dimers through a SH2 domain in the PI3K p85 regulatory subunit. PI3K catalyzes the synthesis of the second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3) from phosphatidylinositol 4,5 bisphosphate (PIP2), wherein the membrane bound PIP3 serves as a signaling molecule to recruit proteins containing the pleckstrin homology
(PH) domain. These PH domain proteins, such as the phosphoinositide-dependent protein kinase (PDK) and protein kinase B (AKT) serine/threonine kinases, are thus activated and mediate further downstream signaling events [47]. The synthesis of PIP3 is negatively regulated primarily by the phosphatase and tensin homology (PTEN) phosphatase, which dephosphorylates PIP3 back to PIP2 [48]. Through PTEN, the PI3K pathway is subject to reversible redox regulation by ROS generated by growth factor stimulation. H2O2 was shown to oxidize and inactivate human PTEN through disulfide bond formation between the catalytic domain Cys-124 and Cys-71 residues [49, 50]. It was also demonstrated that endogenously generated ROS following treatment with peptide growth factors such as insulin, EGF, or PDGF causes oxidation of PTEN leading to the activation of the PI3K pathway [51]. PTEN oxidation is reversed by peroxiredoxin II, a member of cytoplasmic peroxiredoxin isoforms that eliminates H2O2 generated in response to growth factors [49]. Thus the PI3K pathway is regulated by ROS in a similar manner as the MAPK pathways; at the oxidative interface, protein phosphatases are directly oxidized by ROS resulting in sustained activation of the signaling pathways.

It is noteworthy that various oxidants and ROS-producing chemicals activate transcription of a battery of antioxidant genes through a PI3K-NFE2-like 2 (Nrf2)-antioxidant response element (ARE) mechanism (Section 4), where PTEN knockdown enhances transcription of ARE-regulated antioxidant genes [52]; however, it is not known whether these oxidants induce PTEN oxidation and inhibition of phosphatase activity leading to gene activation.
4. Nrf2 and Ref1-Mediated Redox Cellular Signaling

In order to prevent oxidative stress, the cell must respond to ROS by mounting an antioxidant defense system. Antioxidant enzymes play a major role in reducing ROS levels; therefore, redox regulation of transcription factors is significant in determining gene expression profile and cellular response to oxidative stress. Redox factor-1 (Ref-1) (Fig. 4A), identified as a 37-kDa protein that facilitates Fos-Jun DNA binding activity [53], was shown to be identical to an apurinic/apyrimidinic (AP)–endonuclease named APE (AP endonuclease) [54] or HAP1 (human AP endonuclease 1) [55]. Thus Ref-1 is a multifunctional protein that not only regulates transcription factor activity, but also mediates base excision repair. The transcriptional regulatory function of Ref-1 is mediated through its redox activity on several transcription factors such as activator protein 1 (AP-1), p53, nuclear factor kappa B (NFkB), and hypoxia inducible factor 1 (HIF-1α) [56]. The N-terminus region of Ref-1 is responsible for redox activity while the AP-endonuclease activity domain is located at the C-terminal region (Fig. 4A)[57]. Cys-65 of Ref-1 appears to be a major redox active site (along with Cys-93) that is required for the reduction and increased DNA binding of targeted transcription factors [58]. Ref-1 activated the AP-1 transcription factor, Fos-Jun, through redox regulation of cysteine residues in the Fos-Jun DNA binding domains [53, 59, 60]. As shown in Fig. 4B, this cysteine is highly conserved in various human b-zip transcription factors, and except for CAATT enhancer binding protein (C/EBP) transcription factors, all may be regulated in a redox dependent manner by Ref-1, resulting in increased DNA binding and transcriptional activation of target genes. Indeed, it was demonstrated that this conserved cysteine in Nrf2 and cAMP response element binding (CREB) protein is
subject to redox regulation. Site mutagenesis of Cys-506 interfered with mouse Nrf2-antioxidant response element (ARE) binding [61], in contrast to mutation of Cys-300/310 of CREB which increased DNA binding activity [61, 62], demonstrating the importance of these redox regulated cysteine residues in transcriptional activity. Furthermore, Ref-1 was shown to be involved in the transcriptional activation of Nrf2-target genes under oxidative stress [63]. Oxidation and inactivation of b-zip transcription factors is not due to formation of intra-or intermolecular disulfide bonds, but probably the result of reversible oxidation of cysteine to sulfenic (-SOH) or sulfinic (-SO$_2$H) acids [59], though the possibility of an intermolecular disulfide bond between Cys-300 and Cys-310 in CREB cannot be entirely discounted [62]. It is generally accepted that reduction of Ref-1-targeted transcription factors results in oxidation of Ref-1 at Cys-65, possibly along with Cys-93 (Fig. 4A), although it has not been completely determined whether this oxidation event results in formation of an intramolecular disulfide bond or conversion to sulfinic or sulfinic acid [64].

The reduction of Ref-1 appears to be regulated by thioredoxin (Fig. 4A). In response to phorbol myristate acetate or ionizing radiation, thioredoxin was shown to translocate into the nucleus and interact with Ref-1, resulting in the activation of AP-1 transcriptional activity under reducing conditions [65, 66]. The interaction of thioredoxin with Ref-1 and the subsequent activation of Ref-1 target proteins appear to be regulated by the redox active Cys-32 and Cys-35 residues of thioredoxin [65-67] which are responsible for its reducing activity [68] (Fig. 4A). Ref-1 is localized in the nucleus as well as in the cytoplasm, depending on the cell type and physiological conditions. Cytoplasmic Ref-1 translocates to the nucleus under oxidative stress conditions such as H$_2$O$_2$ [69] and hypochlorous acid (HOCl) [70]. It was
demonstrated that nuclear importins interact with the N-terminal 20 amino acid region of Ref-1, mediating nuclear translocation (Fig. 4A) [71]. It remains unknown how oxidants or ROS trigger nuclear localization of Ref-1, though it has been shown that nitric oxide induces nuclear export of Ref-1 by S-nitrosation of the redox sensitive Cys-93 residue [72]; p38 may also play a role in the nuclear translocation of Ref-1 via phosphorylation of Ser-54 [73]. These reports suggest that Ref-1 nuclear translocation by oxidants or oxidative stress responsive signaling pathways may occur through modifications of regions outside the accepted nuclear localization sequence of Ref-1.

Increasing the cellular antioxidant capacity by upregulation of antioxidant detoxification genes is critical in cellular adaptation to oxidative stress and protection from oxidative damage. Ref-1 was shown to be upregulated by genotoxic agents and oxidants, such as bleomycin and H2O2, and so protected cells from DNA and oxidative damage [70]. Ref-1 upregulation seems to be a reasonable adaptive response since Ref-1 mediates both DNA repair and the redox activation of key transcription factors involved in cellular defense, such as AP-1 and NFkB. However, under oxidative stress conditions, a group of antioxidant detoxification genes such as glutathione S-transferase (GST) [74], NADPH quinone oxidoreductase-1 (NQO1) [75], heme oxygenase-1 (HO1) [76, 77], and ferritin H (FH) [78, 79] are transcriptionally activated in an AP-1/NFkB-independent manner. These antioxidant genes are regulated by a highly homologous enhancer termed the antioxidant responsive element (ARE), or electrophile response element (EpRE), located ~0.5 kb-10 Kb upstream from transcription initiation sites of these genes [74-76, 78-80]. The consensus core ARE
sequence is TGA(C/T)nnnGCA [81] and the presence of two or more copies of the ARE in close proximity to each other often serves as a bona fide ARE [80]. Various endogenous and exogenous ROS-generating and electrophilic chemicals (such as H2O2 [82], lipid aldehydes [83], arsenic [84], tert-butylhydroquinone (t-BHQ) [82], hemin [63, 82, 85], and resveratrol [86]) activate transcription of these antioxidant genes via the ARE.

The primary transcription factor involved in ARE activation under oxidative stress conditions is NFE2-like 2 (Nrf2), a cap ’n’ collar (CNC)-b-zip transcription factor (Fig. 5) [87]. Under non-stressed conditions, the majority of Nrf2 resides in the cytoplasm (though there is controversy as to the exact subcellular localization of Nrf2 [88]) and associates with a dimeric inhibitory protein, Kelch-like ECH-associated protein-1 (Keap1) [87]; Keap1 interacts with the cullin-3 E3-ubiquitin ligase (Cul3) and serves as a platform for the ubiquitination and resultant proteasomal degradation of Nrf2 (Fig. 5) [89, 90]. Reactive mouse Keap1 cysteines (Cys-151, -273, and -288) [91, 92] are redox sensors, and upon oxidation by ROS, results in the dissociation of Nrf2 from Keap1/Cul3 which allows Nrf2 stabilization and translocation into the nucleus. In the nucleus Nrf2 dimerizes with members of another b-zip family, the small Maf proteins (Maf-F, Maf-G, and Maf-K). The Nrf2-small Maf heterodimer binds the ARE enhancer and activates ARE-dependent transcription of target genes which serve as antioxidants and in processes such as electrophile detoxification, glutathione synthesis, and ROS homeostasis (Fig. 5) [80, 93]. As shown in Fig. 4B, Nrf2 contains a conserved cysteine located in the DNA binding domain (Cys-514) which is the conserved site of Ref-1-mediated redox regulation. Indeed, mutation of mouse Nrf2 at Cys-506 (equivalent to Cys-514 of the human Nrf2) to Ser-506 affected DNA binding to the ARE.
enhancer and decreased NQO1 expression [61]. It has also been demonstrated that Ref-1 nuclear localization and transcriptional activation of the ARE in the human ferritin H gene was increased following t-BHQ or hemin treatment [63], suggesting the possibility of Nrf2 redox regulation by Ref-1. Another example of possible redox regulation of Nrf2 was shown when mutation of the Cys-199 residue located in the transactivation domain of Nrf2 evidenced decreased binding to the NQO1 ARE [94].

Interestingly, the BTB and CNC homolog 1 (Bach1), a b-zip transcriptional repressor of the ARE [95], also features the Ref-1 associated conserved cysteine (Cys-574) (Fig. 4B) that is subject to redox regulation [96]. In this study, the sulfhydryl oxidizing agent diamide reversed Bach1-repressed ARE enhancer activity via Cys-574 oxidation (probably Cys-557 as well) leading to cytoplasmic translocation of Bach1 (fig. 5) [96]. Nuclear export of Bach1 during ARE-dependent transcriptional activation of the NQO1 gene after t-BHQ treatment is also facilitated through phosphorylation of the mouse Bach1 at Tyr-486 (Tyr-483 in the human Bach1) by an undetermined tyrosine kinase [97]. Collectively, these results suggest that at least two sequential redox events, 1) the oxidation or adduct formation of Keap 1 in the cytoplasm and subsequent release and nuclear translocation of Nrf2, and 2) the redox regulation of Nrf2 and Bach1 in the nucleus, appear to be critical for maximum transcriptional activation of ARE-dependent antioxidant genes via the Nrf2 signaling pathway. Thus, through upstream redox regulators such as Ref-1, transcription factor and repressor activity is modulated indirectly through ROS, while the examples of Nrf2 and Bach1 demonstrate the direct regulation of transcription factors and repressors by ROS. Both
direct and indirect control of transcriptional regulators illuminates the oxidative interface between ROS and ARE gene transcription.

6. Regulation of p66Shc, Mitochondrial Oxidative Stress, and Aging

ROS have been implicated in the process of aging. Given that the majority of endogenous ROS are generated in mitochondria [98], there has been much interest in the role that mitochondrial ROS may play in aging. Of note is the Shc adaptor protein family, encoded by the shcA locus in mammalian cells, consisting of the p66Shc, p52Shc, and p46Shc isoforms (Fig. 6) [99-101]. Expression of p66Shc and p52/p46Shc isoforms are regulated by two different promoters [102] along with alternative translation initiation or splicing [99]. All isoforms share a phosphotyrosine binding domain (PTB), and a proline-rich collagen homology domain-1 (CH1) followed by a C-terminal Src homology 2 domain (SH2) (Fig. 6). p52Shc and p66Shc also share a cytochrome c binding domain (CB), while a second CH domain (CH2) is unique to pShc66 (Fig. 6). The homozygous shcA gene knockout mice lacking shcA exons 2 and 3 are embryonically lethal by E11.5 due to congestion of blood in the heart and cardiac outflow tracts [103]. The predominant expression of Shc proteins in the developing cardiovascular system indicates the importance of Shc proteins in the development of the heart and angiogenesis [103]. Among the three Shc isoforms, p52Shc and p46Shc are adaptor proteins involved in RTK signaling pathways through recruitment of the SH2 domain to phospho-tyrosines in the cytoplasmic domain of RTKs upon growth factor stimulation.
In contrast, p66Shc was revealed to play more predominant roles in mitochondrial ROS metabolism and oxidative stress response rather than serving as an RTK adaptor protein. Pelicci and colleagues demonstrated that p66Shc-deficient mice are not only more resistant to apoptosis under oxidative stress, but also have increased life span [104]. p66Shc-deficient mouse fibroblasts also showed decreased toxicity in response to oxidative stress compared with normal p66Shc fibroblasts and in vitro results suggest that phosphorylation of p66Shc at Ser36 is critical for stress-induced apoptosis [104]. Subsequently, a fraction of p66Shc was shown to localize in mitochondria as a high molecular protein complex containing heat shock protein 70 (HSP70), and a modest increase in mitochondrial p66Shc along with its dissociation from this large protein complex were observed after UVC or H2O2 exposure [105].

p66Shc mitochondrial translocation or proapoptotic activity may be regulated by posttranslational modifications such as phosphorylation of p66Shc at Ser-36 in the CH2 domain (Fig. 6) by PKC or JNK following exposure to UV or H2O2, and/or interaction with TOM-TIM protein import complexes [105-109], although it was previously noted that p66Shc in mitochondria is not serine phosphorylated [110].

Our current understanding is that p66Shc is a proapoptotic protein involved in ROS production in mitochondria leading to mitochondrial damage and apoptosis under oxidative or genotoxic stress conditions such as H2O2 or UV exposure. What is the initial and direct impact of oxidative or genotoxic stress on p66Shc protein and how is p66Shc activated? First, p66Shc protein levels in cytoplasm as well as in mitochondria
appear to be increased under certain stress conditions [111, 112] in addition to increased serine and threonine phosphorylation of p66Shc [100, 101]. Although the molecular mechanism by which p66Shc expression is increased in response to stress signals remains largely uncharacterized, the Rac1 GTPase, which generates ROS through activation of NADPH oxidase [113], was shown to block p66Shc ubiquitination and degradation through phosphorylation of p66shc on Ser-54 and Thr-386 in a p38 dependent manner [114]. Furthermore, oxidative stress-activated PKC-β induces phosphorylation of p66Shc at Ser-36, which in turn triggers the interaction of the prolyl isomerase Pin1 with p66Shc, possibly inducing the isomerization of a p66shc phospho-Ser36-Pro37 bond, resulting in the subsequent translocation of p66Shc into mitochondria [115]. Secondly, p66Shc was shown to be pro-apoptotic in mitochondria upon redox-dependent reversible tetramerization through formation of two disulfide bonds via Cys-59 in the N-terminus CH2 domain of p66Shc (Fig. 6), leading to copper-dependent ROS generation and initiation of apoptosis [106]. The active oxidized form of p66Shc is reversibly reduced by glutathione or thioredoxin leading to inactivation [106]. The molecular mechanism through which stress-activated p66Shc induces apoptosis has not been fully elucidated; however, p66Shc was shown to serve as a redox protein that produces H₂O₂ in mitochondria through interaction and electron transfer between p66Shc and cytochrome c [110], in which mutations in the redox center of p66Shc (E132-E133 to Q132-Q133 in the CB domain, Fig. 6) impaired opening of the mitochondrial permeability transition pore and thus negating the proapoptotic
function of p66Shc [99]. It appears that p66shc induces apoptosis through generation of ROS but also may be activated by ROS. Aging is associated with a decrease in mitochondrial function such as impaired oxidative phosphorylation that results in increased generation of ROS [116]. It is interesting to speculate whether increased ROS in this context would activate p66shc to produce further ROS, resulting in apoptosis and maintaining the steady progression of aging. Regardless, p66shc remains an interesting link between ROS and aging.

7. Regulation of the IRE-IRP system and Iron Homeostasis by ROS

Iron is an essential element that plays crucial roles in cell proliferation and metabolism by serving as a functional constituent of various enzymes including ribonucleotide reductase and cytochrome P450. However, when present in excess, free iron generates ROS via the Fenton reaction [117-119], placing cells under deleterious oxidative stress. Therefore, tight regulation of iron homeostasis is not only crucial to maintain normal cellular function, but also to prevent iron-mediated oxidative stress. The expression of many genes involved in iron transport and storage are regulated by iron itself at the post-transcriptional level in which iron regulatory protein-1 and -2 (IRP1 and IRP2) interact with an iron-responsive element (IRE) in the 5’- or 3’-untranslated region (UTR) of mRNAs such as, for example, ferritin (serving as intracellular iron storage) and transferrin receptor-1 (serving as iron transport into
cells), respectively (Fig. 7). The IREs consist of a stem-loop structure composed of approximately 30 nucleotides with a 5’-CAGUG-3’ loop to which cytoplasmic IRP1 and/or IRP2 binds and determines the fate of mRNAs. In general, IRP binding to an IRE in the 5’-UTR of mRNA (e.g. ferritin) results in mRNA translational block and decreased protein expression while IRP binding to the 3’-UTR of mRNA (e.g. transferrin receptor-1) increases the stability of mRNA, increasing protein expression [117, 120]. The amino acid sequence of IRP1 is highly homologous to mitochondrial aconitase, and IRP exhibits aconitase activity when forming a 4Fe-4S iron-sulfur cluster [121, 122] and it is through this cluster that cellular iron levels control IRP1-IRE interaction. Under iron-rich conditions IRP1 retains the 4Fe-4S cluster (inactive IRP1) and therefore cannot bind to the IRE (Fig. 7). IRP2, on the other hand, is highly homologous to IRP1 but lacks an iron-sulfur cluster and has no aconitase activity. Furthermore, under iron rich conditions IRP2 is degraded via the proteasome pathway (Fig. 7). Thus, iron-rich conditions induce dissociation of IRPs from IREs, resulting in release of the translational block of ferritin mRNA and the destabilization of transferrin receptor 1 mRNA as well. As a result, iron-rich cells synthesize more ferritin for iron storage/detoxification and less transferrin receptor-1 to halt iron transport into the cells, ultimately reducing excess intracellular iron. In contrast, iron-deficient conditions facilitate the disassembly of the 4Fe-4S cluster in IRP1 while stabilizing IRP2, allowing IRP1 and IRP2 to bind IREs in 5’-UTR ferritin mRNA (translational block) and 3’-UTR transferrin receptor-1 mRNAs (mRNA stabilization). As a result, iron-deficient cells
produce less ferritin (decreasing iron storage capacity) and more transferrin receptor-1 (increasing iron transport) to maintain iron homeostasis [117, 120]. In the last decade several new iron transport and metabolism genes such as Divalent Metal Transporter 1 (DMT1) and ferroportin (Fpn) [117] were discovered. The identification of IREs present in the 5'- or 3'-UTR of Fpn and DMT1 mRNAs strengthens the view of the IRE-IRP regulatory system as the primary post-transcriptional mechanism of the majority of iron metabolism genes tightly regulated by iron (Fig. 7). In addition, other genes are regulated by the IRE-IRP system, such as NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1) [123], Alzheimer’s amyloid precursor protein (APP) [124], hydroxyacid oxidase 1 (HAO-1) [125], myotonic dystrophy kinase-related Cdc42-binding kinase alpha (MRCKα) [126], cell division cycle 14 homolog A (CDC14A) [127], delta-aminolevulinate synthase 2 (ALAS2) [128], and hypoxia inducible factor-2 alpha (HIF-2α) [129] (Fig. 7).

The fact that IRP1 contains a 4Fe-4S cluster implies that it may be subject to redox regulation. Indeed, H$_2$O$_2$ was shown to convert, or destabilize, the 4Fe-4S cluster of IRP1 (inactive) to a 3Fe-4S cluster (active) (Fig. 7) through loss of a single iron [122], and ferritin protein expression was transiently downregulated after H$_2$O$_2$ exposure [78, 130] through increased IRP1 binding to the IRE, though followed by upregulation of ferritin by transcriptional activation of the ferritin gene via the ARE [78]. The question that arises is whether increased IRP1-IRE binding is the direct effect of H$_2$O$_2$ on the 4Fe-
4S cluster. When IRP1 was directly incubated with H₂O₂ in vitro, there was no increase in IRP1 binding to IRE [122, 130], suggesting that destabilization of the 4Fe-4S cluster is not sufficient for IRP1 binding to IREs in response to H₂O₂, implying that H₂O₂ activates an alternate signaling pathway leading to additional posttranslational modifications of IRP1 for increased IRE binding. Nitric oxide (NO) was also found to increase IRP1 binding to the IRE through destabilization of the 4Fe-4S cluster [131, 132] (Fig. 7). The redox-regulated PKC was shown to phosphorylate IRP1 at Ser-138 [133], and it was later demonstrated that phosphorylation of Ser-138 results in destabilization of the 4Fe-4S cluster and increases IRP1 binding to IRE [134]. Unlike IRP1, IRP2 does not contain a Fe-S cluster and its binding to IREs is primarily decreased in iron-rich cells through iron-dependent proteasomal degradation mediated by F-box/LRR repeat protein 5 (FBXL5) [135] (Fig. 7), resulting in downregulation of iron transporting proteins (destabilization of such mRNAs as Tfr1 and DMT1) and upregulation of iron storage and export proteins (release of translational block of such mRNAs as ferritin and ferroportin) [136]. It has recently been demonstrated that IRP2 is subject to redox regulation, in which oxidative stress caused by glucose deprivation in HEK293 cells induced oxidation of Cys-512 and Cys-516 in IRP2 that in turn decreased IRP2 binding to IREs [137], and decreased IRE binding ability of IRP2 was correlated with decreased transferrin receptor-1 expression that may allow cells to limit iron transport and hindering subsequent iron-mediated ROS production. However, in contrast, a recent report demonstrated that ROS increased IRP2-IRE binding [138] in addition to
protecting IRP2 from iron-mediated degradation, an effect similar to that shown under hypoxic conditions [139] (Fig. 7).

Taken together, the IRE-IRP regulatory system is not only regulated by cellular iron status but also regulated by ROS, in which cells elicit a defense mechanism against iron toxicity and iron-catalyzed oxidative stress.

8. ROS and DNA-Damage Response

Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia and Rad3-related (ATR) are PI3K-like serine/threonine protein kinases activated under genotoxic stress conditions and phosphorylate various proteins involved in cell proliferation, cell death and survival, and DNA repair [140, 141]. These two signaling proteins were initially thought to be activated by a particular type of DNA damage therefore serving in parallel signaling pathways; however, accumulating evidence suggests that the ATM- and ATR-pathways communicate and cooperate in response to DNA damage [141]. ATM, preferentially activated by DNA double strand breaks, has been shown to serve as a sensor of oxidative stress in which ATM-deficient cells were more susceptible to oxidative stress-inducing agents as well as DNA damaging agents [142]. However, it has recently been demonstrated that the molecular mechanisms of the activation of ATM by DNA damage and oxidative stress are different. Upon double strand DNA break induction by agents such as bleomycin, cells recruit the Mre11-Rad50-Nbs1 (MRN)
complex to damaged sites together with ATM, which in turn triggers autophosphorylation of ATM at Ser-1981 and activates ATM protein kinase activity leading to phosphorylation of downstream signaling proteins such as checkpoint kinase 2 (Chk2) at Thr-68 and p53 at Ser-15 (Fig. 8). Phosphorylation of ATM at Ser-1981 and its kinase activity are reversibly regulated by protein phosphatase 2A (PP2A)[143]. Cells exposed to H$_2$O$_2$ also feature ATM activated via Ser-1981 phosphorylation [144-146], although Guo et. al showed that H$_2$O$_2$ activates ATM in an MRN/Ser-1981 autophosphorylation-independent manner (Fig. 8) based on their results that 1) ATM was activated by H$_2$O$_2$ equivalently in both wild type and mutant Mre11 cells, and 2) H$_2$O$_2$ activated both wild type and Ser-1981 to alanine mutant purified dimeric ATM in vitro [144]. Noncovalently associated dimeric (non-active) ATM is known to be dissociated into active monomers in response to DNA damage; however, Guo et. al showed that purified ATM protein incubated with H$_2$O$_2$ in vitro migrated slower in SDS-PAGE due to formation of covalent dimers that were sensitive to reducing agents, and given the fact that N-acetyl-cysteine (NAC) blocked ATM activation induced by H$_2$O$_2$ in vitro [144], these results suggest that H$_2$O$_2$ activates ATM through formation of active ATM dimers via intermolecular disulfide bond(s). Further characterization demonstrated that Cys-2991, located near the kinase domain of human ATM, is primarily involved in the disulfide bond formation and oxidative activation of ATM (Fig. 8) [144]. It is noteworthy that a C2991A ATM mutant was fully activated by the MRN-DNA complex but not by H$_2$O$_2$ in vitro[144]. Thus H$_2$O$_2$ and possibly other ROS, elicit
ATM activation not through the DNA damage and MRN mediated pathway, but directly by ATM dimer formation via Cys-2991 oxidation and intermolecular disulfide bridge formation (Fig. 8).

The fact that ATM deficient cells accumulate ROS and are sensitive to oxidative damage [142] suggests that ATM is crucial to a cellular oxidative stress defense program. What downstream signaling events are regulated by activated ATM in response to ROS? One clue to address this question has recently been presented [145] in which cytoplasmic ATM autophosphorylated at Ser-1981 in response to oxidative stress activates a liver kinase B1 (LKB1)-AMP activated protein kinase (AMPK) cascade (Fig. 8). Autophosphorylated cytoplasmic ATM activates LKB1 via phosphorylation of Thr-366, which activates AMPK through Thr-172 phosphorylation. Activated AMPK in turn activates the tuberous sclerosis complex 2 (TSC2) tumor suppressor protein via phosphorylation at Thr-1271 and Ser-1387, leading to inhibition of mammalian target of rapamycin complex 1 (mTORC1), thereby suppressing protein synthesis and inducing autophagy under oxidative stress (Fig. 8) [145]. The activation of autophagy through this pathway may be a cellular defense mechanism in response to ROS.
9. Conclusions

The disease states in which ROS signaling and toxicity have been implicated are areas of intensive research in regards to prevention and therapy. Unveiling the molecular mechanisms of disease pathogenesis and progression is therefore essential in providing relevant targets in order to develop innovative therapeutic strategies. In this context it is worthwhile not only to investigate ROS signaling in disease, but to also to reveal how ROS instigate cellular signaling under homeostatic conditions. Having a clear understanding of how ROS directly regulate signaling pathways that are found to play a key role in the pathogenesis and progression of disease will allow us to understand how ROS may cause or contribute to disease and uncover new therapeutic targets. For example, ROS regulates proliferative and apoptotic pathways, and aberrant regulation of proliferation and apoptosis is essential in tumorigenesis; therapeutic strategies exploiting the role of ROS in those pathways are being developed [1, 147]. While therapeutic development has primarily been concerned with reducing ROS levels to prevent toxicity, as in neurodegeneration [148], atherosclerosis, and diabetes [149], it remains to be seen whether targeting the redox sensitive molecules and signaling pathways activated by ROS will produce viable therapeutics in the prevention and alleviation of ROS-mediated disease states.
Acknowledgement

This work was supported in part by NIH grant number R01 GM-088392 and R01 GM-095550 from the National Institute of General Medical Sciences to Y. Tsuji.
References


Figure Legends

Fig. 1. Cellular signaling pathways regulated by ROS
Reactive oxygen species (ROS) regulate several signaling pathways through interaction with critical signaling molecules, affecting a variety of cellular processes, such as proliferation, metabolism, differentiation, and survival (apoptosis signal-regulated kinase 1 (ASK1), PI3 kinase (PI3K), protein tyrosine phosphatase (PTP), and Src homology 2 domain-containing (Shc)); antioxidant and anti-inflammatory response (thioredoxin (TRX), redox-factor 1 (Ref-1), and NFE2-like 2 (Nrf-2)); iron homeostasis (iron regulatory protein (IRP)); and DNA damage response (ataxia-telangiectasia mutated (ATM)).

Fig. 2 Activation of ASK kinases in response to oxidative stress
A) Oxidation of thioredoxin (TRX) results in disulfide bond formation between Cys-32 and Cys-35 and subsequent dissociation from ASK1. ASK1 undergoes complete homo-oligomerization and subsequent autophosphorylation at Thr-838 located in the kinase domain. B) Hetero-oligomerization of ASK1 and ASK2 stabilizes ASK2, resulting in 1) the autophosphorylation of ASK2 at Thr-806, and 2) the subsequent phosphorylation of ASK1 at Thr-838 by ASK2.

Fig. 3. Mechanism of ROS-mediated protein tyrosine phosphatase inactivation
Tyrosine kinases, activated by growth factors, cytokines, and hormones, phosphorylate target proteins. Phosphorylation can be reversed by protein tyrosine phosphatases (PTP); ROS inactivates PTP by oxidation of catalytic cysteine residues resulting in the formation of the sulfenic acid (-SOH) intermediate that can form disulfide bonds or sulfenamide residues. Further oxidation of sulfenic acid results in formation of sulfinic (-SO2H) or sulfonic acid (-SO3H), which are relatively irreversible.

Fig. 4. Ref1-mediated redox cell signaling
A) The human Ref-1 N-terminal region consists of the redox domain (REDOX; residues 1-127) and a 20 amino acid nuclear localization sequence (NLS); the C-terminal region contains the apurinic/apyrimidinic endonuclease domain (APE; residues 162-318). Cysteine -65 and -93 are major redox active sites. Under oxidative stress, Ref-1 translocates into the nucleus by NLS-importin-dependent or -independent pathways where Ref-1 regulates the activity of b-zip transcription factors (bZIP-TF) by redox mechanisms. Oxidized Ref-1 is subject to redox regulation by nuclear translocated thioredoxin (TRX) through the TRX catalytic center (Cys-32 and -35). B) The conserved redox-active cysteine of various human b-zip transcription factors is indicated in red color and labeled with residue number. In addition to the b-zip family, other transcription factors such as p53, NFκB, and HIF-1α are also regulated by Ref-1 [56].
Fig. 5 Redox regulation of the Nrf2-ARE pathway
ROS oxidation of cysteines (Cys-151, -273 and -288) in the mouse Kelch-like ECH-associated protein-1 (Keap1) results in the release of Nrf2 from the Keap1/cullin-3 E3-ubiquitin ligase (Cul3) complex, preventing Nrf2 degradation. Nrf2 subsequently undergoes nuclear translocation. In the nucleus, a hetero-dimer of Nrf2 and small Maf members (Maf-F, Maf-G, Maf-K) binds the antioxidant-responsive element (ARE); oxidation of a b-zip transcriptional repressor of ARE, the human BTB and CNC homolog 1 (Bach1) at Cys-557 and -574 results in cytoplasmic translocation of Bach1, both leading to activation of the ARE.

Fig. 6. Isoforms of p66Shc, p52Shc and p46Shc
Schematic representation of three isoforms produced from the human ShcA gene (CH1 and 2, proline-rich collagen homology domain-1 and 2; CB, cytochrome c binding domain; PTB, phosphotyrosine binding domain; and SH2, Src homology 2 domain); numbers define the regions of different domains according to human p66Shc amino acid sequence; Met-111 and -156 of p66shc are equivalent to the first methionines of p52Shc and p42Shc. ROS-mediated phosphorylation sites are at Ser-36 and -54; Thr-386; pro-apoptotic residues are Cys-59 and Glu-132/133.

Fig. 7. Regulation of IRP-IRE interactions
Under iron rich conditions, IRP1 contains a [4Fe-4S] cluster and is unable to bind to the IRE, though loss of iron from the cluster (destabilization) under iron deficient conditions allows IRP1 to bind to the IRE. IRP2 does not contain a [4Fe-4S] cluster and is degraded by F-box/LRR-repeat protein 5 (FBXL5)-dependent ubiquitination. Iron chelators, nitric oxide (NO), hypoxia, and hydrogen peroxide (H₂O₂) increase IRP/IRE interaction. H₂O₂ destabilizes the [4Fe-4S] cluster of IRP1 and also stabilizes IRP2 protein by preventing FBXL5-dependent ubiquitination. Increasing IRP-IRE interaction in 5'UTR results in translational block of ferritin (Ft), ferroportin (Fpn), aminolevulinic acid synthase-2 (ALAS2), hypoxia inducible factor-2α (HIF-2α), amyloid precursor protein (APP), and NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1) genes, but in the 3'UTR it results in mRNA stabilization of transferrin receptor (TfR), divalent metal transporter 1 (DMT1), hydroxyacid oxidase 1 (HAO-1), myotonic dystrophy kinase-related Cdc42-binding kinase alpha (MRCKα), and CDC14 cell division cycle 14 homolog A (CDC14A).

Fig. 8. Schematic of ATM signaling upon oxidative stress and double-strand DNA breaks
Double-strand DNA breaks mediates the phospho-ATM-Chk2 pathway; however, oxidative stress elicits both the phospho-ATM-LKB1 and the ATM-homodimer pathways (Ataxia-telangiectasia mutated (ATM); liver kinase B1 (LKB1); AMP activated protein kinase (AMPK); Tuberosus sclerosis complex 2 (TSC2); Mammalian target of rapamycin complex1 (mTORC1)).
Fig. 1

FIGURE 1. Cellular signaling pathways regulated by ROS

A) **Inactive**

B) **Active**

Fig. 2

FIGURE 2 Activation of ASK kinases in response to oxidative stress
FIGURE 3. Mechanism of ROS-mediated protein tyrosine phosphatase inactivation
FIGURE 4. Ref1-mediated redox cell signaling
**FIGURE 5** Redox regulation of the Nrf2-ARE pathway

**FIGURE 6.** Isoforms of p66Shc, p52Shc and p46Shc
FIGURE 7. Regulation of IRP-IRE interactions
FIGURE 8. Schematic of ATM signaling upon oxidative stress and double-strand DNA breaks
APPENDIX B

ROLE AND REGULATION OF FERRITIN H IN ROTENONE-MEDIATED MITOCHONDRIAL OXIDATIVE STRESS

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Published in Free Radical Biology & Medicine (2008) 44(9) 1762-1771

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ACKNOWLEDGEMENTS

We would like to thank Janet Dow for expert guidance and technical support of our flow cytometry experiments. We are grateful to Drs. Frank and Suzy Torti for graciously sharing mouse ferritin H reporter plasmids with us. We are also thankful
to Dr. Emily Omori for sharing her protocol and reagents for ROS detection in live cells with us, and Dr. Sarah Ross at Caner Research UK for her invaluable suggestions regarding siRNA transfection. This work was supported in part by a National Institutes of Health Grant DK-60007 to Y. Tsuji.
ROLE AND REGULATION OF FERRITIN H IN ROTENONE-MEDIATED MITOCHONDRIAL OXIDATIVE STRESS

ABSTRACT

Tight regulation of intracellular iron levels in response to mitochondrial dysfunction is an important mechanism that prevents oxidative stress, thereby limiting cellular damage. Here, we describe a cytoprotective response involving transcriptional activation of the ferritin H gene in response to the mitochondrial complex I inhibitor and neurotoxic compound, rotenone. Rotenone exposure increased ferritin H mRNA and protein synthesis in NIH3T3 fibroblasts and SH-SY5Y neuroblastoma cells. Transient transfection of a ferritin H promoter-luciferase reporter into NIH3T3 cells showed that ferritin H was transcriptionally activated by rotenone through an antioxidant responsive element (ARE). Chromatin immunoprecipitation assays showed that rotenone treatment enhanced binding of Nrf2 and JunD transcription factors to the ARE. In addition, rotenone induced production of reactive oxygen species (ROS), and pretreatment with N-acetylcysteine abrogated ferritin H mRNA induction by rotenone, suggesting that this response is oxidative stress-mediated. Furthermore, reduced ferritin H expression by siRNA sensitized cells to rotenone-induced apoptosis with enhanced ROS production and annexin V positive cells. Taken together, these results suggest that ferritin H transcription is activated by rotenone via an oxidative stress-mediated pathway leading to ARE activation, and may be critically important to protect cells from mitochondrial dysfunction and
oxidative stress. Keywords: ferritin, rotenone, mitochondria, iron, oxidative stress
INTRODUCTION

Iron is a vital element mandatory for metabolic processes and the function of many enzymes, including cytochrome P450s and ribonucleotide reductase, however, a surplus of free iron leads to the formation of reactive oxygen species (ROS) via the Fenton reaction, which is potentially harmful to the cells [1, 2]. Iron is also involved in the catalysis of oxidation of important cellular proteins, lipids, and DNA. Thus, excess iron may lead to an increase in the oxidative stress burden of the cell in several ways [3]. Oxidative stress has been implicated in the pathogenesis of numerous conditions, including cancer, inflammation, and neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease [4-6]. Therefore, cells have intricate systems to control intracellular iron levels and to detoxify ROS.

Ferritin is a nanocage protein that functions to sequester free intracellular iron that may become toxic to cells. In vertebrates, there are two ferritin subunits, heavy and light, which coassemble in 24 subunit bundles forming a channel that encloses iron [7]. The heavy, or H, subunit is catalytically active, and induces oxidation of ferrous iron Fe(II) to ferric iron Fe(III) and aggregation of the oxidized iron inside the core, while the light (L) subunit does not have ferroxidase activity but may serve a structural function [2, 8]. In this manner, ferritin functions to protect cells against iron-mediated toxicity [9, 10]. Because of its important role, ferritin is tightly regulated at both the transcriptional and translational levels. Ferritin protein levels are controlled by a well-characterized translational repression system in response to
free iron levels in the cells. The 5’ untranslated region of ferritin message contains
an iron responsive element (IRE), to which the iron regulatory proteins, IRP1 and
IRP2, bind in low iron concentrations, thereby blocking translation when necessary
[11]. In addition to iron, ferritin levels are also altered by other stimuli. TNFα was
shown to stimulate ferritin H expression, but not ferritin L, through transcriptional
activation via an upstream promoter region containing an NFκB site [12, 13]. Our
subsequent studies revealed an antioxidant responsive element (ARE) in the far
upstream region of the promoter that is necessary for the transcriptional activation of
the ferritin gene in response to various oxidative stressors, including H2O2, tBHQ
(tert-butylhydroquinone), and hemin [14-16]. A similar ARE element was identified in
ferritin L gene [17]. The ARE is a highly conserved enhancer element in various
phase II genes involved in detoxification or with antioxidant properties, allowing for
the activation of a battery of antioxidant genes including glutathione-S-transferases,
NADH quinone oxidoreductase 1, and heme oxygenase 1 under chemical and
oxidative stress conditions [18]. The ferritin H ARE contains a bidirectional AP1 like
and AP-1/NFE2 sequence, to which basic leucine zipper (bZip) transcription factors,
including JunD and NFE2-related factor 2 (Nrf2), bind [14, 16]. This facilitates
activation of ferritin H gene transcription under conditions of oxidative stress.

Upregulation of ferritin in response to increased iron levels and oxidative stress may
be involved in preventing the cellular damage caused by the excess iron and
oxidative stress that has been observed in numerous neurodegenerative diseases,
like Parkinson’s disease. A large body of evidence indicates a correlation between increased oxidative stress and the incidence of Parkinson’s disease (PD) [4, 19]. Recent studies have shown that iron chelators can block 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP) induced dopaminergic cell death [20]. Excess free iron may be the catalyst in the production of deleterious ROS that are responsible for damage to DNA, lipids, and proteins, ultimately leading to the deletion of dopaminergic neurons [21]. Furthermore, the insults that induce neuronal degeneration are intimately connected with oxidative stress [5]. Rotenone, a mitochondrial complex I inhibitor, induces oxidative stress; however, despite its universal effect, it leads to the specific deletion of dopaminergic neurons [22], suggesting that they are highly sensitive to oxidative stress.

We hypothesized that rotenone would evoke cellular defense mechanisms through increased expression of ferritin H via an oxidative stress-pathway. In our present study, we show that ferritin H expression was increased at the transcriptional level following rotenone exposure. We further characterized that rotenone specifically activated the ferritin H ARE, and increased the binding of the oxidative stress-responsive bZip transcription factors, JunD and Nrf2, to it. Furthermore, rotenone-mediated transcriptional activation of the ferritin H gene was oxidative stress-dependent. Finally, knocking down ferritin H expression by siRNA propagated generation of ROS and sensitized cells to rotenone-mediated apoptosis, suggesting
that rotenone-induced transcriptional activation of the ferritin H gene via the ARE is cytoprotective.
MATERIALS AND METHODS

Cell culture - NIH3T3 mouse fibroblasts and SH-SY5Y human neuroblastoma cells were obtained from American Type Culture Collection. NIH3T3 cells were cultured in Dulbecco’s Modified Eagle’s Medium containing 1mM sodium pyruvate, 4mM L-glutamine, and 4.5g/L glucose with 10% bovine calf serum (Hyclone), and Penicillin/Streptomycin. SH-SY5Y cells were cultured in a 1:1 mixture of Eagle’s Minimum Essential Medium and F12 medium supplemented with non-essential amino acids and 10% fetal bovine serum (Mediatech). These cells were incubated at 37°C and 5%CO₂ in a humidified atmosphere. Rotenone and tert-butylhydroquinone (tBHQ) were purchased from Sigma and were dissolved in DMSO. H₂O₂ and N-acetylcysteine (NAC) were purchased from Calbiochem. H₂O₂ was diluted in sterile PBS, and NAC was reconstituted in medium and adjusted to pH 7.4 with NaOH.

Plasmids and DNA transfections - pGL3-0.22kb mouse ferritin H luciferase (mFH-Luc) was constructed by digesting pGL3–4.8kb mFH-Luc with Smal to remove the upstream 4.6 kb and then self-ligating the remaining vector and 0.22kb promoter sequence. ARE, and double mutant ARE-Luc plasmids were obtained by blunt end ligation of the oligonucleotides into the –0.22kb mFH-Luc plasmid following oligonucleotide purification by urea denaturing polyacrylamide gel electrophoresis, Sephadex G-25 column purification, and subsequent annealing. The sequences are: wt ARE SENSE:
5’- 
CATGACAAAGCACTTTTGAGCCCAACCCTCCAAAGGAGCAGAATGCTGAGTCACGG-3’

wt ARE ANTISENSE: 
5’-
CCGTGACTCAGCATTCTGCTCCTTTGGAGGGTTGGGTCTCCAAAAGTGCTTTGTCATG-3’
mARE SENSE: 
5’-
CAACAACAAGCACTTTTGAGCCCAACCCTCCAAAGGAGCAGAAACTGAGTCACGG-3’
mARE ANTISENSE: 
5’-
CCGTGACTCAGTGTTCTGCTCCTTTGGAGGGTTGGGTCTCCAAAAGTGCTTTGTG-3’

NIH3T3 cells were transfected with 5ug of the indicated plasmid DNA via electroporation with a BioRad GenePulser XL using NIH3T3 preset conditions, $5 \times 10^6$ cells/0.2cm cuvette. Cells were then plated into 35mm dishes (10 dishes) with $5 \times 10^5$ cells, 0.5ug DNA per dish. Following a 24 hr recovery period after electroporation, cells were treated as indicated for 24 hours, and preparation of cell lysates and luminometry were performed with luciferase assay kit (Promega).
Gel retardation and ChIP Assays -

Nuclear extract preparation, binding reactions, and electrophoretic mobility shifts were previously described [23]. Chromatin immunoprecipitation (ChIP) assays were performed according to a minor modification of Upstate Biology's ChIP assay protocol as previously described [14]. A total of $1 \times 10^6$ cells/100-mm plate were treated with 1 uM rotenone for 4 hrs, followed by formaldehyde cross-linking of chromatin and preparation of lysates. (Upstate Biology). 2 ul of the following antibodies were used for immunoprecipitation: rabbit IgG (Alpha Diagnostics), anti-Nrf2 (sc-722X), anti-JunD (sc-074X, Santa Cruz Biotechnology). Quantitative PCR was performed in the presence of 0.1uCi $\alpha^{32}$P-dCTP/reaction, while using specific primers designed to amplify a 230 bp region within the mouse ferritin H promoter that contains the ARE:

Forward:

5'GGCCCCTCTGTTCTGTACAA
\hspace{1pt}TACTAGCTC-3'

Reverse:

5'TAACCACAAAACCACAGCCCTCCAG-3'

PCR reactions were run on an 8% acrylamide gel and visualized by autoradiography.

Northern Blotting - Total RNA from NIH3T3 or SH-SY5Y cells was isolated using TRIzol (Invitrogen). 5-10 ug RNA was separated on a 1.1% agarose, formaldehyde gel, and separated RNA was transferred to a 0.45 micron nitrocellulose Protran
BA85 membrane (Whatman, Schleicher & Schuell) by capillary transfer. Northern blotting was performed using an α-\(^{32}\)P-dCTP random primer labeled 0.9 kb fragment of ferritin H human cDNA as a probe.

*Western blotting* - Total cell lysates were subjected to electrophoresis with 15% or 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), followed by wet transfer of separated proteins to an Immobilon-P membrane (Millipore). Membranes were blocked for one hour in 5% skim milk in Tris buffered saline with 0.1% Tween 20, and subsequently incubated overnight at 4°C with anti-ferritin H antibody (Abcam 16875). Recombinant human ferritin H and L were purchased from Calbiochem.

\(^{35}\)S-labeling/immunoprecipitation- Exponentially growing NIH3T3 cells were treated with either rotenone or ferric ammonium citrate for the indicated times. Following treatment, medium was removed and methionine/cysteine deficient DMEM containing 10% dialyzed bovine calf serum was added. Simultaneously, 10uCi/ml of \(^{35}\)S-methionine/cysteine (GE Healthcare) was added to each dish. Cells were incubated under normal culture conditions for 1 hr. Total cell lysates were prepared, and pre-cleared with rabbit serum (CAPEL) and protein A agarose (Calbiochem) overnight. Total incorporation of \(^{35}\)S was measured using TCA precipitation and scintillation counting. Input protein for immunoprecipitation was determined by adding equal counts (1x10^6 cpm) for each immunoprecipitation reaction. 6 ul of anti-Ferritin antibody (DAKO, A133) and 20 ul of protein A agarose were utilized for
overnight immunoprecipitation at 4°C. Finally, the resulting immunoprecipitates were subjected to SDS-PAGE as described above, the gel was dried, and exposed to film at –86°C.

**SiRNA transfection** - Ferritin H siRNA was purchased from DHARMACON, Inc, siGENOME duplex D-045965-01 mouse FTH, NM_010239.

Sense: 5'CAAGAAUGAUCCCCACUUAUU-3'

Antisense: 5'PUAAGUGGGGAUCAUUCUUGUU-3'

Briefly, 1x10^5 cells/35 mm dish were plated and cultured under normal conditions in serum-containing, antibiotic-free medium (2 ml) until cells reached approximately 80% confluency. 16 ul DharmaFECT3 (Dharmacon) in a final volume of 200 ul OPTI-MEM medium (Invitrogen) was added to 7.5 ul of 20 uM siRNA dissolved in 200 ul of OPTIMEM and the mix was incubated for 20 min. After formation of RNA complexes, the 400 ul mix was added to the dishes and cultured under normal conditions (the final concentration of siRNA was 60 nM). After 24 hrs, cells were split into one, 6-well plate for treatment. Whole cell extracts were obtained and subjected to Western blotting as described above to examine ferritin H protein expression. In other cases, cells were trypsinized and assessed for ROS production and apoptosis using flow cytometry.
Detection of ROS - NIH3T3 cells in 35 mm dishes were incubated with 5 uM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Molecular Probes) dissolved in molecular biology grade DMSO (Calbiochem) in phenol red-free DMEM containing 10% fetal bovine serum for 30 minutes. After loading, the unincorporated dye was washed out with phenol red-free complete medium, and sublethal concentrations of rotenone (12.5 uM and 25 uM) and H$_2$O$_2$ (50 uM) were added for 0.5 or 2 hrs. Following treatment, cells were trypsinized and resuspended in phenol red-free complete medium, and cell pellets were collected after centrifugation at 2000 rpm for 3 min. The resultant pellets were suspended in 500 ul phenol red-free complete medium for flow cytometry on a Becton-Dickinson FACSCalibur.

Detection of Apoptosis - Annexin V conjugated with AlexaFluor647 (Molecular Probes-Invitrogen) was utilized to detect rotenone-mediated apoptosis. Annexin V detects the presence of phosphatidylserine on the outside of apoptotic cells. NIH3T3 cells in 35 mm dishes were treated with 12.5 or 25 uM rotenone for 8h. Cells were trypsinized, and resulting cell pellets were stained according to manufacturer’s protocol, with the following modification: 2.5 ul Annexin V AlexaFluor 647 conjugate (Molecular probes) was added to the 100 ul cell suspension. AlexaFluor 647 fluorescence (indicating Annexin V positive cells) was assessed via flow cytometry on a Becton-Dickinson FACSCalibur.
RESULTS

*Rotenone induces ferritin H expression.*

To investigate the effect of the mitochondrial complex I inhibitor, rotenone, on ferritin H expression, we examined the levels of ferritin H mRNA following rotenone exposure by Northern blotting. As shown in Fig. 1a, treatment of NIH3T3 cells with increasing concentrations of rotenone (0-5 uM) for 24 hrs induced ferritin H message. The inductive effect of rotenone was less than that of tBHQ, an electrophilic compound that has been shown to induce various antioxidant and detoxification genes [18] including ferritin [15], therefore used as a positive control. Ferritin protein synthesis also showed a dose responsive increase following 24 hr treatment with 0.1, 0.5, and 1uM rotenone in NIH3T3 cells (Fig. 1b). In SH-SY5Y neuroblastoma cells, rotenone induced ferritin H mRNA and protein expression (Figs. 1c and 1d) similarly but at much lower concentrations (nanomolar) than those used in experiments with NIH3T3 cells. Micromolar ranges of rotenone were used throughout the experiments in NIH3T3 cells without severe cell damage (see Fig. 6 and the legend). Our previous studies demonstrated that H2O2 treatment transiently activated IRP within 1-2 hrs thereby inhibiting ferritin translation prior to transcriptional activation of the ferritin H gene [15]. Since inhibition of mitochondrial complex I by rotenone has a potential to induce production of ROS and H2O2 [24], we next investigated whether ferritin H protein synthesis was transiently repressed following rotenone treatment. We exposed NIH3T3 cells to 1 uM rotenone for various times over a period of 24 hrs, and subsequently subjected them to 35S-
methionine/cysteine pulse-labeling and ferritin immunoprecipitation. As shown in Fig. 1e, increased ferritin protein was observed after 1 hr of rotenone treatment, and was maintained for 24 hrs, suggesting that transient translational repression of ferritin synthesis via IRP was not involved during rotenone treatment.

**The Ferritin H gene is transcriptionally activated by rotenone through the ARE.**

Since we had observed increased levels of mRNA and protein synthesis following rotenone treatment, we were interested in whether or not ferritin H was transcriptionally activated. To test this, we transiently transfected NIH3T3 cells with a firefly luciferase reporter fused to 4.8kb or 0.22kb of the 5’ region of the mouse ferritin H promoter, and treated for 24 hrs with rotenone. Increasing concentrations of rotenone specifically activated the -4.8kb promoter in a dose-dependent manner, but had no effect on the -0.22kb promoter (Fig. 2a). This suggests that the increase in ferritin H mRNA results from transcriptional activation.

The -4.8kb region of the mouse ferritin H promoter that was activated by rotenone contains an antioxidant responsive element (ARE), located 4.1kb upstream of the transcription start site [23]. Next we asked whether or not the ARE was involved in transcriptional activation of ferritin H by rotenone. We cloned the wt-ARE or double mt-ARE, in which both the AP-1 like and AP-1/NFE2 sites contain critical mutations that abrogate transcription factor binding [15, 16], into the minimum 0.22kb promoter reporter, and employed them for transient transfection assays in NIH3T3 cells. Fig. 2b shows that insertion of the wt ARE was sufficient for promoter activation by
rotenone treatment, while introduction of mutations in the AP-1 like and AP-1/NFE2 sites abrogated rotenone mediated promoter activation. This suggests that not only is the ARE activated by rotenone treatment, but that the AP-1 binding sequences are also critical for rotenone-mediated transcriptional activation of the ferritin H gene.

**Binding of Nrf2 and JunD to the ferritin H ARE is enhanced by rotenone treatment.**

Given that the mutations of the AP-1 like and AP-1/NFE2 sites blocked transcriptional activation of ferritin H in our transient transfection assays, we investigated whether transcription factor binding to the ARE was altered following rotenone treatment. First, gel retardation assays demonstrate that total protein binding to the ARE was increased by rotenone treatment as well as tBHQ in NIH3T3 cells (Fig. 3a). To assess the role of Nrf2, a major b-zip transcription factor responsible for regulation of the ARE in various phase II genes [25], and JunD, another b-zip family member that regulates the ferritin H ARE [16], we performed ferritin H ARE ChIP assay. As shown in Fig. 3b, *in vivo* binding of Nrf2 and JunD to the ARE was increased following rotenone treatment, suggesting that they are involved in the transcriptional activation of the ferritin H gene in response to rotenone treatment.

**ROS production is involved in rotenone-mediated ferritin H induction**

Given the results of rotenone-mediated ferritin H ARE activation in this study, we then hypothesized that rotenone induces ferritin H in an oxidative stress dependent...
manner. To test this hypothesis, we assessed rotenone’s propensity to produce ROS, using the dye CM-H$_2$DCFDA, which is taken up by cells and is non-fluorescent in its acetylated, reduced form. Once localized in the cell, intracellular esterases deacetylate the dye, allowing for its oxidation by ROS. The oxidized dye exhibits a shift in its emission spectra to the fluorescein range. Treatment of NIH3T3 cells with 5 uM rotenone for 0.5 hr resulted in a significant increase in the percent fluorescent positive cells, indicating that rotenone induces production of ROS and has the potential to cause oxidative stress (Fig. 4a). To reveal the role of oxidative stress in the induction of ferritin H by rotenone, we assessed the ferritin H mRNA levels of NIH3T3 cells treated with vehicle, rotenone, or rotenone following pre-administration of N-acetylcysteine (NAC), which is known to prevent production of reactive oxygen species by raising levels of glutathione. Indeed, NAC pretreatment abrogated the increase in ferritin H mRNA by rotenone treatment, but NAC treatment alone had negligible effects on ferritin mRNA levels (Fig. 4b). Taken together, these results suggest that rotenone activates the ferritin H transcription by an oxidative stress-dependent mechanism.

*Ferritin H knockdown increases ROS production and sensitizes cells to rotenone-induced apoptosis*

Our observation that oxidative stress is required for the induction of ferritin H by rotenone, led us to assess the role of ferritin H in protecting cells from rotenone induced oxidative stress and apoptosis. To determine whether ferritin H is
cytoprotective against rotenone mediated oxidative damage, we utilized ferritin H siRNA to transiently decrease ferritin expression. Ferritin H siRNA (FH) decreased the expression of ferritin H protein in NIH3T3 cells by 50% compared to non-targeting siRNA (NT) (Fig 5a). Following either Non-targeted (NT) or FH siRNA transfection, cells were treated with sublethal concentrations of rotenone and examined for ROS production. As shown in Fig. 5b, FH siRNA NIH3T3 transfectants had increased ROS production following rotenone treatment compared to NT siRNA transfectants. We then asked if ferritin H knockdown cells are more susceptible to cytotoxicity induced by rotenone. To this end, rotenone-induced apoptosis was detected using fluorescently labeled annexin V, a protein that binds externalized phosphatidylserines. FH siRNA transfected cells had higher levels of annexin V staining, indicating that a greater percentage of the ferritin H knockdown cells were undergoing apoptosis (Fig. 6). Taken together, these results suggest that decreased ferritin H expression increased ROS production and sensitized cells to rotenone, and that a deficient ferritin H response to rotenone-induced oxidative stress caused cell death.
DISCUSSION
Maintenance of iron homeostasis is critically important in preventing oxidative cell damage via the Fenton reaction. Ferritin is a major protein involved in the regulation of intracellular free iron levels [10]. It sequesters iron within its shell, thereby blocking iron from participating in reactions that generate free radicals [1, 26]. In some instances where cellular oxidative load is increased, cells may be more susceptible to iron mediated production of ROS and damage. We, along with others, demonstrated that ferritin is upregulated in response to a battery of different oxidative stressors [14, 15, 17]. Overexpression of ferritin H in cells reduced free iron levels and increased cellular resistance to H₂O₂ toxicity [27, 28]. Deficiencies in ferritin lead to cellular profiles of oxidative stress and iron accumulation [9].

Oxidative stress and iron also have both been implicated in the pathogenesis of Parkinson’s disease. Increased iron concentrations and oxidative damage have been observed in damaged regions of the substantia nigra in both human cases of Parkinson’s disease (PD) and in animal models [29, 30]. Taken together with rotenone-induced experimental models of Parkinson's disease [22], we hypothesized that rotenone may increase levels of ferritin H. In fact, we observed that ferritin H is induced following rotenone exposure at the mRNA and protein synthesis levels in NIH3T3 fibroblasts and SH-SY5Y neuroblastoma cells (Fig. 1). Our previous studies demonstrated that H₂O₂ produces a transient activation of IRP binding to the IRE, thus conferring a temporary reduction in ferritin H synthesis [15].
Our results in this study demonstrated that ferritin H synthesis was not reduced at any time point following rotenone exposure, suggesting that IRP was not activated by rotenone. Like rotenone, tBHQ has a potential to produce reactive oxygen species [31] but did not activate IRP (unpublished data). This may potentially be due to the differences in the ROS produced by each stressor. Rotenone and tBHQ produce ROS indirectly by leakage of electrons from the electron transport chain and redox cycling, respectively [32]. H2O2 on the other hand, a ROS precursor, may directly interact with IRP, leading to its activation.

We were interested in the mechanism of ferritin increase by rotenone. Upregulation of ferritin following chronic MPTP administration in mice was demonstrated by cDNA microarray [33], but little is known about the mechanism of transcriptional activation of ferritin, or the signaling pathway responsible for the inductive effect of neurotoxicants. We showed that the ferritin H gene is transcriptionally activated by rotenone in a dose dependent manner through an ARE (Fig. 2). Furthermore, this activation was mediated through the ARE. Wild type ARE insertion alone was sufficient for rotenone-mediated activation similar to that observed by the 4.8kb promoter. In addition, mutation of the AP-1 like and AP-1/NFE2 binding sites abrogated the activation by rotenone (Fig. 2), suggesting the importance of these AP1 sites in the activation of transcription of the ferritin H gene. Indeed, enhanced total protein binding to the ARE was observed following rotenone treatment, in which increased Nrf2 and JunD binding to the ARE was detected by ChIP assay (Fig. 3).
This led us to propose that oxidative stress may be downstream of the complex I inhibition by rotenone in the cascade of events that lead to ferritin H transcriptional activation. Confirming our hypothesis, addition of the glutathione precursor, NAC, blocked rotenone-mediated ferritin H mRNA induction (Fig. 4), suggesting that oxidative stress is a necessary cue in ferritin H activation by rotenone. Several recent studies have reported that in some conditions, especially hypoxia, rotenone reduces mitochondrial ROS generation [34-36]. To date there is no clear consensus about the effect of rotenone on mitochondrial ROS generation under various conditions; however, such contradictory reports support the need for further investigation into the intricacies of mechanisms of mitochondrial ROS generation and suppression. Recently, two studies demonstrated a protective function of Nrf2 in mitochondrial dysfunction and oxidative stress caused by the complex II inhibitor, 3-nitropropionic acid [37, 38]. These findings, along with our results in this study suggest that ferritin H may be a target gene in the response to mitochondrial dysfunction and oxidative stress produced by rotenone insult.

To investigate the cytoprotective role of ferritin against rotenone toxicity, we examined the effect of ferritin H knockdown on cellular sensitivity to rotenone by siRNA transfection. Ferritin H siRNA transfection caused increased production of ROS and apoptosis in response to sublethal concentrations of rotenone treatment compared to non-targeting siRNA transfected cells (Fig. 5a). In this experiment we observed that knocking down ferritin H expression resulted in induction of a slower-
migrating ferritin in NIH3T3 cells (Fig. 5a). This may be ferritin L (mouse ferritin L protein migrates slower than ferritin H in SDS-PAGE—H and L ferritins from mouse migrate in the reverse order of that observed for human ferritin H and L [28, 39].) because a recent study reported by Cozzi et. al., showed that ferritin H knockdown increased ferritin L expression in HeLa cells [9]. In this study the increased ferritin L expression did not affect iron availability [9]. These results suggest that alterations in ferritin H expression may directly contribute to the altered iron metabolism and increased oxidative stress present during the disease progression of PD. In fact, it was reported that overexpression of ferritin H prior to MPTP exposure in a mouse model of PD conferred resistance to the neurotoxic insult [20]. This seems to be consistent with the present study showing that ferritin H knockdown resulted in an increased percentage apoptotic cells following rotenone treatment (Fig. 6).

However, it should also be noted that prolonged ferritin accumulation in the same mouse PD model has recently been shown to contribute to a progressive age-related neurodegeneration [40]. In this study the authors discussed that iron-saturated ferritin in the aged brain may release more iron during ferritin turnover, which would be neurotoxic rather than neuroprotective.

In brain tissue from individuals afflicted with Parkinson’s Disease, the Fe(II)/Fe(III) ratio is 1:2 compared to 2:1 in a normal substantia nigra pars compacta [41]. This indicates that an increased amount of Fenton reactions occur during the progression of PD. Also, regions of iron accumulation colocalize with those of neuronal death.
Furthermore, iron chelators prevent alpha-synuclein translocation and mitochondrial aggregation, two hallmark events in the pathogenesis of PD [43]. In addition to ferritin, a number of other metal-regulatory/antioxidant genes have been implicated in PD. For instance, metallothionein overexpression was shown to be cytoprotective against neurotoxic insult [44]. Like ferritin H, metallothionein also contains an ARE and is regulated by both oxidative stress and iron [45]. It seems likely that a common ARE-mediated mechanism of phase II gene activation is a critical cytoprotective response to neurotoxicants. Ferritin H, as a major iron sequestering protein, may be an important component among the battery of activated genes. Our results in this study may shed light on the potential role of ferritin regulation in the pathogenesis of PD, as well as providing information as a potential target for chemoprevention.
ACKNOWLEDGEMENTS

We would like to thank Janet Dow for expert guidance and technical support of our flow cytometry experiments. We are grateful to Drs. Frank and Suzy Torti for graciously sharing mouse ferritin H reporter plasmids with us. We are also thankful to Dr. Emily Omori for sharing her protocol and reagents for ROS detection in live cells with us, and Dr. Sarah Ross at Cancer Research UK for her invaluable suggestions regarding siRNA transfection. This work was supported in part by a National Institutes of Health Grant DK-60007 to Y. Tsuji.
FIGURE LEGENDS

Figure 1  Effect of rotenone treatment on ferritin H expression.
a) NIH3T3 cells were treated with either 0, 0.5, 1 or 5 uM rotenone or 10 uM tBHQ for 24 hrs. Total RNA was isolated and subjected to Northern blotting with a ferritin H cDNA probe. The resulting ferritin H band is indicated. To assess equal loading and integrity of total RNA, ethidium bromide staining is shown below. The positions of 18S and 28S ribosomal RNA are also indicated. A representative Northern blot result of 5 independent experiments is shown. b) NIH3T3 cells were treated with 0, 0.1, 0.5, or 1 uM rotenone or 10 uM tBHQ for 24 hrs and subjected to in vivo 1 hr pulse labeling of newly translated proteins with $^{35}$S-methionine/cysteine labeling. $1 \times 10^6$ TCA insoluble counts were subjected to immunoprecipitation with anti-ferritin antibody and subsequently separated by SDS-PAGE. A representative image from 3 independent experiments is shown.  c, d) SH-SY5Y cells were treated with 0.1-2.5 nM rotenone or 10 uM tBHQ for 24 hrs, and c) total RNA was isolated and subjected to Northern blotting with a ferritin H cDNA probe or d) preparation of whole cell lysates and Western blotting with the anti-ferritin H antibody (Abcam 16875).  e) NIH3T3 cells were incubated with 1uM Rotenone for 0, 1, 2, 8, and 24 hrs, and subsequently subjected to in vivo labeling with $^{35}$S-methionine/cysteine, immunoprecipitation with anti-ferritin antibody and SDS-PAGE. NIH3T3 cells were treated with 5ug/ml ferric ammonium citrate (FAC) as a positive control for ferritin protein induction and $^{35}$S-labeled at 0 and 24 hrs. The resulting ferritin H (FH) and ferritin L (FL) bands are indicated.
**Figure 2** Transcriptional regulation of the ferritin H gene by rotenone treatment.

a) NIH3T3 cells were transfected via electroporation with 0.5 ug each of either -0.22kbFH or -4.8kbFH firefly luciferase promoter reporter construct. After a 24 hrs recovery period, cells were treated with 0, 0.1, 0.5, or 1.0 uM rotenone, or 10 µM tBHQ and incubated for 24 hrs. Cell lysates were collected and assessed for transcriptional activation via luminometry. -4.8kbFH-Luc without rotenone treatment was set to 1 to calculate mean fold induction. Standard errors of means (S.E.M.) are shown, where n=5 independent experiments. b) NIH3T3 cells were transfected with -0.22kbFH, wt ARE insertion, or mt ARE insertion fused to a firefly luciferase reporter construct. Cells were treated for 24 hrs with either 1.0 µM rotenone or 10 µM tBHQ. Samples were normalized for recovery and transfection efficiency differences using a co-transfected internal control pRL-EF (elongation factor-renilla luciferase plasmid). The -0.22kbFH-Luc control value was set to 1 to calculate mean fold induction. S.E.M. shown, n=4 independent experiments. Asterisks denote statistical significance compared to a) 0uM rotenone, or b) control (no treatment), defined as p<0.001 by Student’s t-test.
Figure 3  Nrf2, JunD transcription factor binding to the ARE after rotenone treatment.

a) 50 ug of nuclear extracts from NIH3T3 cells treated with 1uM Rotenone or 10 uM tBHQ for 4hr, or untreated cells (control) were subjected to gel retardation assay using a probe for the AP-1/NFE2 site ferritin H ARE. Addition of 50X excess cold probe competitor to the right lane is indicated by Comp +.  b) NIH3T3 cells untreated (control) or treated with 1 uM Rotenone or 10 uM tBHQ for 4hr, were used for ferritin H ARE ChIP assay. Primers specific to a region of the mouse ferritin H promoter that contains the ARE were employed for PCR with the input DNA or the DNA obtained following immunoprecipitation with either rabbit IgG, Nrf2 specific antibody or a JunD specific antibody. The resulting 230 bp product is shown. -4.8kbFH plasmid indicates the use of the mouse 4.8kbFH plasmid DNA as template as a positive control, and no template as a negative control. Representative images are shown of 3 and 4 independent experiments for a) and b), respectively.

Figure 4  Involvement of ROS production in rotenone-mediated ferritin H mRNA induction.

a) NIH3T3 cells were preloaded with the ROS sensitive dye, CM-H2DCFDA for 0.5 hr, and then treated with 5 uM rotenone or 50 uM H2O2 or left untreated (Control) for 0.5 hr. Levels of ROS, as indicated by FITC fluorescence, were assessed via flow cytometry. A representative histogram of ROS levels showing number of counts vs. relative FITC fluorescence is shown on the top. Mean fold induction of
the number of ROS positive cells from 3 independent experiments was calculated by setting the control levels to 1. Asterisks denote statistical significance compared to control, p<0.001 by Student’s t-test. b) NIH3T3 cells were treated with 0 or 1 uM rotenone for 24 hr (indicated by – or + respectively, lower row, marked rotenone), following a 2 hr pre-treatment with 0 or 10 mM NAC (indicated by – or + respectively, upper row, marked NAC). Resulting total RNA was subjected to Northern blotting with a ferritin H cDNA probe. Ethidium bromide RNA staining is shown below for loading and RNA integrity. The positions of 18S and 28S RNA bands are indicated. A representative image of 3 independent experiments is shown.

**Figure 5 The effect of ferritin H knockdown on ROS production.**

a) NIH3T3 cells were transfected with non-targeted (NT) siRNA or ferritin H (FH) siRNA. Resulting ferritin H protein levels were assessed by Western blotting with anti-ferritin H antibody. The corresponding β-actin expression is shown as a loading control. A representative result of Western blotting is shown. Recombinant human ferritin H (rec hFH) was included as a Western blotting control. b) NT and FH siRNA transfectants were treated with sublethal concentrations of rotenone (12.5, and 25 uM) for 0.5 hr following loading for 0.5 hr with CM-H2DCFDA, and ROS levels were analyzed by flow cytometry. Representative histograms with counts (y-axis) vs. ROS FITC fluorescence (x-axis) are shown at right. The shaded area represents the histogram of NT siRNA transfectants (siNT), the thick black line is the FH siRNA transfectants (siFH). The mean fold increase in ROS positive cells between NT and
FH siRNA transfectants is summarized at left. The difference between NT and FH siRNA transfectants left untreated was set to 1.0. Standard error is shown for n=3 independent experiments. Asterisks denote statistical significance compared to control, p<0.005 by Student’s t-test.

**Figure 6 The Effect of ferritin H knockdown on rotenone-induced apoptosis.**

NIH3T3 cells were transfected with non-targeted (NT) siRNA or ferritin H (FH) siRNA as described in Fig. 5 and the section of experimental procedures. NT and FH siRNA transfectants were then treated with 0, 12.5, or 25 uM rotenone for 8 hrs, and apoptosis was quantified using flow cytometry to measure the fluorescence of AlexaFluor 647 conjugated to Annexin V. The mean fold increase in percent Annexin V positive cells was determined by setting NT and FH siRNA transfectants left untreated was set to 1.0 in each experiment. Means of Annexin staining-positive cells were 9.2% (rotenone 0), 15.0% (rotenone 12.5 uM), 16.0% (rotenone 25uM) in NT siRNA transfected cells, and 8.9% (rotenone 0), 20.4% (rotenone 12.5uM), 22.5% (rotenone 25uM) in FHsiRNA transfected cells. Standard error is shown for n=3 independent experiments.
REFERENCES


FIGURES

Figure 1. Effect of rotenone treatment on ferritin II expression
Figure 2. Transcriptional regulation of the ferritin H gene by rotenone treatment

Figure 3. Nrf2, JunD transcription factor binding to the ARE after rotenone treatment
Figure 4. Involvement of ROS production in rotenone-mediated ferritin H mRNA induction
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