PEROXIDE DEPENDENT EFFECTS IN HUMAN AIRWAY EPITHELIAL CELLS EXPOSED TO OXIDANT AIR POLLUTANTS

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ABSTRACT

Phillip A. Wages: Peroxide dependent effects in human airway epithelial cells exposed to oxidant air pollutants
(Under the direction of James M. Samet)

Air pollution is a global public health problem. The induction of oxidative stress, or the cellular response to avoid cytotoxicity due to an increase in reactive oxygen species or decrease in antioxidants, is frequently cited as a mechanism of toxicity for air pollutants. In addition to the role of oxidative stress in disease, there is growing evidence that oxidative processes including the generation of reactive oxygen species is essential for normal cellular function. This dissertation provides evidence that the reactive oxygen species, hydrogen peroxide, is a key mediator in air pollutant-induced adverse cellular responses. First, it is demonstrated that the divalent metal, zinc, induces hydrogen peroxide-dependent adaptive gene expression in human airway epithelial cells. This builds upon previous work to establish that zinc-induced pro-oxidant effects and electrophilic activity are both critical in its mechanism of toxicity. We next show that the organic component, 1,2-naphthoquinone, increases protein sulfenylation of regulatory proteins via hydrogen peroxide. This is the first report that protein sulfenylation is effected by an environmentally relevant exposure, establishing a potential new mechanism of toxicity as well as a new biomarker for future studies. Finally, two approaches to utilize readouts of oxidative stress in a translational manner are discussed. Specifically, the biological basis of a genetic risk factor of a susceptible population to air pollution is explored using a primary human airway epithelial cell culture. We report that there is an intimate relationship between hydrogen peroxide and

glutathione in the air pollutant-induced outcomes, and that the genetic risk factor, GSTM1-null, enhances the effect of 1,2-NQ to induce the novel readout protein sulfenylation. Furthermore, we were able to use a live cell imaging analysis of oxidative stress to rank the toxicity of fibers of importance to respiratory toxicity and show that asbestos fibers obtained from the Libby Montana Superfund Site have similar toxicity to that of crocidolite asbestos fibers. Together the data suggests a vital and important role of hydrogen peroxide in air pollutant-induced adverse responses and provides the basis to use redox-based readouts as biomarkers to improve public health.

To all the mentors that have helped me along my path.

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LIST OF ABBREVIATIONS

1,2-NQ	1,2-naphthoquinone
$^1O_2$	Singlet oxygen
2-AAPA	2-acetylamino-3-[4-2-acetylamino-2-carboxyethylsufanylthio-carbonylamino phenylthiocarbamoylsufanyl] propionic acid
4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxyguanosine
A-2	Aldrithiol-2
ARE	Antioxidant response element
BEBM	Bronchial epithelial basal media
BEGM	Bronchial epithelial growth media
Biotin azide	PEG4 carboxamide-6-azidohexanyl biotin
BSO	Buthionine sulfoximine
cpYFP	Circularly permutated YFP
C_P	Peroxidatic Cysteine
C_R	Resolving Cysteine
Cu_2SO_4	Copper sulfate
CuAAC	Copper-catalyzed alkynyl-azo cycloaddition
d_6-dimedone	Deuterated-dimedone
DTT	Dithiothreitol
EGFR	epidermal growth factor receptor
E_{GSH}	Glutathione redox potential
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GFP	Green fluorescent protein
Gpx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HAEC	Human airway epithelial cells
$HO^{\cdot}$	hydroxyl radicals
HO-1	Heme oxygenase-1
I-dimedone	Iodo-dimedone
KBM	Keratinocyte basal media
KEAP1	Kelch-like ECH-associated protein 1
KGM	Keratinocyte growth media
LDH	Lactate dehydrogenase
NAC	N-acetylcysteine
N-HRP	NeutrAvidin-HRP
$NO^{\cdot}$	Nitric oxide
Nox	NADPH oxidase
Nrf2	NF-E2-related factor 2
$O_2^{\cdot -}$	Superoxide
$ONOO^-$	Peroxynitrite

PBS	Phosphate buffered saline
PDI	Protein disulfide isomerases
PM	Particulate matter
Prx	Peroxiredoxin
PTP	Protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase 1b
PTM	Posttranslational Modification
PYRI	Pyrithione (2-mercaptopyridine N-oxide sodium salt)
Redox	Reduction-oxidation
RNS	Reactive nitrogen species
roGFP	Redox sensitive green fluorescent protein
ROS	Reactive oxygen species
rxYFP	Redox sensitive yellow fluorescent protein
SA	Sodium ascorbate
SOD	Superoxide dismutase
TBTA	*Tert*-butyl 2,2,2-trichloroacetimidate
Trx	Thioredoxin
YFP	Yellow fluorescent protein
Zn^{2+}	Zinc sulfate

CHAPTER 1: Introduction

1.1: Air Pollution

Outdoor air pollution is a global public health problem, responsible for approximately 3.3 million premature deaths per year (1). Air pollution originates from both anthropogenic sources including the burning of carbon-based fuels as well as natural sources including volcanic eruptions. The negative health impact of air pollution on public health was first noted during the industrial revolution. By the mid-1900's cities including London, UK (2) and Donora, Pa, USA (3) were impacted by an increase in human morbidity and mortality that was closely linked to ambient air pollution levels. Despite continuing improvements over the past decades to improve air quality (4) there is still evidence to suggest air pollution contributes to the adverse health outcomes in humans throughout the world (5).

1.1.1: Global Public Health Burden

The initial target of air pollution is the respiratory system. Even though there is epidemiological evidence for air pollution-induced acute respiratory disease, the mechanistic data to reinforce such findings is lacking (6). There is little argument that the nares and lung are the initial targets of air pollution exposure, but there is sufficient epidemiological evidence that suggests air pollution is linked to the prevalence of other adverse health outcomes including cancer (7), diabetes (8), and cardiovascular disease (9). In addition, there is strong experimental evidence in animal models (10,11) and controlled human exposure (12,13) and panel studies (14) that air pollution leads to negative systemic effects, especially with regards to cardiovascular

disease. These findings correlate well with mechanistic data afforded by *in vitro* studies that suggest a potential link between air pollution exposure and adverse health outcomes (15-17). The involvement of air pollution in many adverse health outcomes including both acute and chronic human disease supports the need to understand the fundamental basis of toxicity associated with air pollution. Of particular concern are those individuals living in developing countries as they are at higher risk to air pollution, due to fewer regulatory and engineering safeguards (18).

1.1.1.1: Susceptible Populations

In addition to the impact of air pollution on the general population, there are specific subpopulations that are susceptible to the negative health effects associated with exposure to air pollution. Individuals with preexisting disorders such as asthma (19) and cardiovascular disease (20) are at particular risk to air pollution. In fact, it has been documented that high air pollution days correlate with increases in hospital visits (21-23). Persons with existing ailments are not the only populations negatively affected by air pollution, as certain genetic polymorphisms can also lend to susceptibility (24). One such polymorphism leads to perturbed translation of the glutathione S-transferase mu 1 (GSTM1) gene. The prevalence of the GSTM1-sufficient allele is actually less prevalent than the GSTM1-null allele, with approximately 40% of the global population being GSTM1-null (25). Epidemiologically, GSTM1-null individuals are particularly susceptible to the acute effects of inhaled pollutants (26-28). Although developing countries have a significant health burden associated with air pollution derived from many different combustion sources including those linked with industrial processes, developed countries still observe a significant amount of mortality and morbidity linked to pollutants in the air (5). A major source

of current air pollution are mobile sources, which is prevalent in developed countries and tends to be localized to large metropolitan areas.

1.1.2: Traffic Related Air Pollutants

Broadly speaking air pollution is a complex mixture of both gaseous and particulate components, of which the U.S. EPA monitors and regulates six. Although five are well defined (ozone, nitric oxides, sulfur oxides, carbon monoxide and lead) the sixth regulated air pollutant is particulate matter (PM), which can be defined by size or composition. Currently PM is monitored by size with regulations set at coarse (> 10 μm) and fine (10 uM-2.5 μm). However, PM is characteristically different with regards to composition geographically and based on source (29,30). Air pollution of large metropolitan areas, as mentioned previously, is largely influenced by the surrounding traffic (31).

Air pollution originating from traffic primarily fall under two categories: the combustion of fuels (gasoline and diesel) and the wear and tear of surfaces (tires, brake-pads, and the road itself). Traffic-related pollution leads to an increase in both primary and secondary air pollutants. Primary pollutants are those that are immediately produced, while secondary pollutants are generated through the interaction of primary pollutants with environmental factors including sunlight, as in case of ozone. The incomplete combustion of fuels is a major source of primary pollutants including PM, as it provides the carbon core necessary for many particulates to form. Metals, both essential and heavy, are distributed throughout the air by the wear and tear of solid components. These metals as well as other organic materials can either anneal to particulates or can originate from the particulates eroded from the solid components of vehicular parts. Although air pollution, and even traffic-related air pollution, is a combination of many different

components there are individual components that are of particular interest in regards to human health.

1.1.2.1: Zinc

Zinc is a ubiquitous metallic component of air pollution primarily derived from the wear and tear of galvanized vehicular parts as well as tires (32), where tires are approximately 0.4-4.3% zinc by weight (33). Epidemiological and observational studies have implicated zinc as the causative metallic pollutant for the adverse health effects attributed to air pollution (34-37). Furthermore, zinc-induced health effects can be modeled by the occupational health disorder known as "metal fume fever." Individuals that work with metal, such as welders, experience a self-limiting flu-like state when they are exposed to zinc particulates in the air (38). This demonstrates the capacity for inhaled zinc to cause a systemic inflammatory response in humans. It should be noted that zinc is an essential nutrient and when ingested is utilized physiologically, even at high chronic exposure (39,40). However, the inhalation of zinc and zinc particulate-laden particulates represents a route of exposure that causes an adverse toxicological response (41).

At the cellular level, zinc can induce an adverse inflammatory response. The cellular and molecular mechanisms of zinc-induced inflammatory responses have been described (42). First, zinc is known to be thiol reactive allowing it to interact with important cysteine residues on proteins to alter the protein's function (43). One such example is zinc's interaction with the catalytic cysteine of protein tyrosine phosphatases (PTP) to inhibit their ability to dephosphorylate proteins (44-46). This can lead to an increase in the activation of signaling as kinases can freely activate pathways through phosphorylation without the balance of PTPs to inhibit or slow the activation of the kinases.

The second mechanism through which zinc elicits an inflammatory response is by elevating intracellular reactive oxygen species (ROS). Unlike most transition metals, zinc cannot redox cycle under physiological conditions to generate ROS, but it can interfere with mitochondrial respiration which leads to an accumulation of ROS (41,47). These ROS can then lead to the induction of adaptive and signaling responses (46,48). Even though both of these mechanisms could occur simultaneously within cells exposed to zinc, the relative contribution of the initiating effects of zinc-induced adverse responses is unknown.

1.1.2.2: 1,2-Naphthoquinone

1,2-naphthoquinone (1,2-NQ) is an organic component of air pollution that people are exposed to through direct and indirect sources. 1,2-NQ is primarily generated through the incomplete combustion of diesel exhaust (49), but its presence in the air can also be linked to industrial operations and second hand smoke (50). Additionally, naphthalene, the most prevalent organic chemical in the air, can be metabolized to 1,2-NQ through three secondary enzymes (51,52). Although the toxicity of 1,2-NQ has not been well described in humans, it is considered to have similar toxicity as 1,4-naphthoquinone, which has been demonstrated to be pro-inflammatory and described as a potential carcinogen when inhaled (53).

Whereas the pathophysiological effects of 1,2-NQ are not as well characterized as other organic chemicals and quinones, the effects of 1,2-NQ at the cellular and molecular level have been studied. Similar to most quinones, 1,2-NQ has two distinct mechanisms of toxicity: direct electrophilic attack by Michael addition (54,55) and generation of ROS through redox cycling (56,57). 1,2-NQ contains an electrophilic carbon that can attack nucleophilic sites on biological macromolecules including amino acids such as cysteine, histidine, and lysine (53). Additionally,

1,2-NQ redox cycles through the semiquinone and hydroquinone state (Figure 1) to provide electrons to generate ROS which could cause cellular stress (58). Of particular interest, with regards to 1,2-NQ's ability to redox cycle is that when adducted to proteins it can continue to redox cycle to generate ROS as harmful by-products, which other quinones including 1,4-naphthoquinone cannot (59). This would suggest that 1,2-NQ has the unique capability of being redox active even when adducted to proteins.

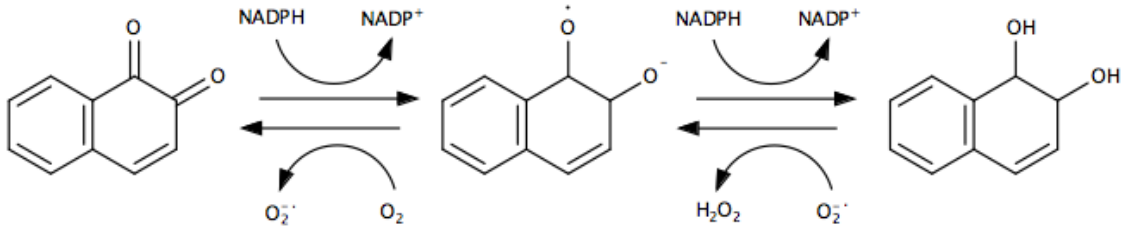

Figure 1: Quinone Redox Cycling Reaction. *1,2-naphthoquinone (left) is converted to the semiquinone (center) and hydroquinone (right) by electron transfer from the action of flavor-containing enzymes. The chemical can push electrons to oxygen and superoxide to generate reactive oxygen species, where the reaction between the semiquinone and oxygen is more favored over the hydroquinone and superoxide reaction. The hydroquinone is able to interact with oxygen to generate superoxide; however, this is even less chemically favored than the interaction between the hydroquinone and superoxide.*

Mechanistically, 1,2-NQ can induce a variety of signaling pathways within the cell either through its electrophilic or pro-oxidant activity, leading to unwanted biological outcomes. Regarding physiological outcomes, exposure to 1,2-NQ has been observed to induce a pro-inflammatory response in mice (60). Additionally, 1,2-NQ can lead to the inhibition of important cellular processes including dephosphorylation of kinases by targeting phosphatases, which result in tracheal contraction (61) and suppression of vasorelaxation in blood vessels (62). However, like zinc, due to the multiple mechanisms of toxicity of 1,2-NQ, the relative contribution of 1,2-NQ's electrophilic and pro-oxidant toxicity is unknown.

1.2: Redox Biology

Similar to how physiological processes at the tissue level can be explained by cellular adaptations and activities, many cellular functions can be explained through the biochemical transfer of electrons. This transfer of electrons is described through the tuning of reduction-oxidation (redox) state of biological macromolecules (nucleic acid, lipid, protein). As in most cases with regards to balance in biology this is a homeostatic process, wherein the cell actively avoids a loss of viability due to an excess of either oxidized or reduced molecules within the cell (63). Homeostasis is maintained through both constitutive factors as well as adaptive processes. Constitutive factors include the microenvironment of the macromolecule such as sterics and subcellular characteristics including pH, which directly influences the protonation state of atoms in molecules. For example the subcellular pH of mitochondria matrix is markedly more alkaline (pH ~8) than the cytosol (pH~7.2), which allows for a location specific function of a protein or molecule (64). Additionally, the cell can utilize adaptive processes such as the induction of the antioxidant peptide glutathione in response to an elevation of ROS, where the ROS are largely responsible for the dynamic nature of redox-dependent processes.

1.2.1: Reactive Oxygen Species

Reactive oxygen species (ROS) have a high capacity to oxidize other molecules (65-67). Reactive nitrogen species (RNS) are distinguished from ROS by the content of a nitrogen group. However, for this document RNS will be referred to as ROS, wherein ROS will define any small chemical that is an oxidant. Within this work the ROS are separated by their function in the cells as some are promiscuously active and able to react readily with any available site such as hydroxyl radicals ($HO^{\cdot}$), superoxide ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) (68,69). These promiscuous

species are relatively less functional in redox-dependent processes that are dynamic or regulatory; however, other ROS such as nitric oxide ($NO^{\cdot}$) and hydrogen peroxide (H_2O_2) have chemical characteristics that make them more capable to serve as effectors of cellular processes (70). These relatively less reactive species can also serve as second messengers. For instance $NO^{\cdot}$ is a gaseous second messenger that is notably important in vasodilation (71). Despite this physiological role, if the levels of these ROS exceed the homeostatic capacity of the cell they will exert deleterious effects that may lead to cytotoxicity.

1.2.1.1: H_2O_2

H_2O_2 is arguably the most important ROS in regards to redox-dependent signaling. H_2O_2 is "intentionally" produced through the enzymes NADPH oxidase (Nox) and superoxide dismutase (SOD) (70,72). The Nox enzymes facilitate the transfer of electrons from intracellular NADPH or FAD to the extracellular compartment by reducing molecular oxygen to H_2O_2 (73). The H_2O_2 generated from this reaction has been linked to many cellular processes, and mutations within the Nox enzymes that perturb the generation of H_2O_2 is linked to pathophysiology (74). Intracellular H_2O_2 also originates from the mitochondria through the metabolism of $O_2^{\cdot-}$ generated from complex 1 and 3 of mitochondrial respiration (75). The mitochondria maintain a high basal level of the antioxidant protein SOD, which rapidly and efficiently converts $O_2^{\cdot-}$ to H_2O_2 (76,77). Under some circumstances, the levels of H_2O_2 exceed the peroxidatic activity available in the mitochondria, and are able to diffuse to the surrounding cytosol and subcellular compartments. As H_2O_2 is generated in multiple sites in the cell, there are localized elevations of H_2O_2 that has both essential and potentially harmful effects (78).

Due to its relative stability and reactivity compared to other ROS, H_2O_2 is considered a better candidate to act as a second messenger than most other ROS. For these reasons H_2O_2, evolutionarily, has likely become a tightly regulated ROS within the cell (79,80). H_2O_2, as mentioned above, is generated through the function of the family of Nox enzymes as well as a byproduct of mitochondrial respiration (78). In addition to non-specific antioxidant molecules such as glutathione that serve as sinks for oxidant species, the cell expresses enzymes that specifically target H_2O_2. These enzymes are more efficient mechanism to lower H_2O_2 levels within the cell. Peroxiredoxins (Prx) have a high reactivity with H_2O_2; glutathione peroxidases (Gpx) reduce H_2O_2 through the interaction of glutathione; and catalase catalyzes the decomposition of H_2O_2 to molecular oxygen and water (81,82). The combination of these three enzyme families (Prx, Gpx, and catalase) serve as the first line defense to lower H_2O_2 levels as they are more energetically and kinetically efficient to metabolize H_2O_2 than the direct interaction of H_2O_2 with antioxidant small molecules such as glutathione. Accordingly, levels of these major enzymes, which are differentially expressed at the tissue and subcellular level, largely dictate the intracellular levels of H_2O_2. Furthermore, the induction of these proteins or inhibition of their function can dramatically impact redox-dependent signaling, since it would ultimately affect H_2O_2's second messenger functions.

H_2O_2 is electrophilic and as such acts on nucleophilic sites on biological molecules including the thiol (SH) of glutathione (83) and nucleophilic amino acids including methionine and cysteine (84). The interaction between H_2O_2 and cysteine is a highly studied topic in many research groups (85). When the thiol (SH) group of the cysteine is deprotonated, usually as a result of pH of the subcellular compartmental or the microenvironment of the molecule, it forms the more reactive thiolate (S^-) group. This thiolate on the cysteine can be oxidized directly by

H_2O_2 leading to the formation of a sulfenic acid (SOH) (86). The oxidation of cysteine thiols to the sulfenic acid of glutathione or proteins is an important regulator of redox-dependent processes (87).

1.2.2: Protein and Peptide Thiols

The sulfenylation of intracellular thiols by H_2O_2 is vital and necessary for physiology (87). Even though the elevation of H_2O_2 is the event leading to these redox-dependent processes, it is also important to discuss the importance of the target: cysteine thiols. The availability of cysteine is negligible within the cell and consequently is the limiting substrate to synthesize glutathione (88). Cysteine is also one of the rarest, and most conserved amino acids in biological systems (84,85). Despite the nucleophilic capability of the thiol portion of the cysteine to serve in oxidant reactions in the cell, it is restricted by availability. Thus, it is imperative to understand that protein sulfenylation, while an essential physiological process, is rare because of the availability of cysteine thiol targets as well is being tightly regulated through the generation and decomposition of H_2O_2.

1.2.2.1: Glutathione

Glutathione is the most prevalent non-protein thiol that serves as an antioxidant in mammalian cells (89). Frequently glutathione is present in concentrations in the millimolar range (90). Structurally, glutathione is a three amino acid peptide consisting of cysteine, glutamate, and glycine. It is synthesized in two steps, the first links cysteine to L-glutamate by the enzyme glutamate cysteine ligase to form gamma-glutamylcysteine. The second step adds glycine to gamma-glutamylcysteine via the enzyme glutathione synthetase. Although glutathione exists as

an important cellular antioxidant, by itself it has a weak interaction with H_2O_2. Even though, thermodynamically H_2O_2 should be able to oxidize the thiol of glutathione, the pKa of the thiol makes the reaction chemically unfavorable and slow (91).

Despite glutathione's slow reaction with H_2O_2 by itself, through the enzymatic interaction of Gpx, glutathione serves as an important physiological antioxidant target for H_2O_2 (92). Gpx enzymatically reacts with two molecules of reduced glutathione (GSH) with one molecule of H_2O_2 to form glutathione disulfide (GSSG) and water. GSSG is then reduced to glutathione by glutathione reductase (GR) at the cost of energy, specifically NADPH. In fact the cell expends a significant amount of energy to maintain glutathione in its reduced form (90). A quantitative measurement of GSH/GSSG is the glutathione redox E_{GSH} (mV), as defined by the Nernst Equation:

$$E_{GSH} = E^{\circ\prime}_{GSH} - \frac{RT}{2F}\ln\left(\frac{[GSH]^2}{GSSG}\right)$$

The E_{GSH} can be utilized as an important marker of redox metabolism that has been linked to a many cellular states including differentiation and apoptosis; however, it should be noted that E_{GSH} in itself is a poor marker of redox-dependent signaling (91).

1.2.2.2: Oxidative Posttranslational Modifications

As opposed to the interaction between H_2O_2 and glutathione, it is currently thought that the interaction between protein thiols and H_2O_2 is a spontaneous, non-enzymatic reaction (93). However, the number of cysteines in the proteome is finite and this number is further restricted as many of those cysteines are sterically unavailable for oxidation (85). In addition to the minimal number of protein thiols available for H_2O_2 to form sulfenic acids, there is an additional hierarchical level of sulfenylation of thiols based on their respective microenvironments (94).

The thiol that becomes sulfenylated is referred to as the peroxidatic residue, and its oxidation is dictated by mutliple factors including its pKa. The relative reactivity of the peroxidatic thiol is a major factor dictating the ranking of which proteins are targeted by H_2O_2 (94).

The spontaneous oxidation of thiols to the sulfenic acid is also characteristically short-lived. However, sulfenic acids serve as a precursor for the formation of other post-translational modifications (PTM) (Figure 2). One of the most important PTMs is the disulfide bond between two cysteines. The oxidation of the peroxidatic cysteine (C_P) interacts with another cysteine, the resolving cysteine (C_R), to form a relatively more stable disulfide bond. This disulfide bond can then be reduced back to the thiol through enzymes including thioredoxin (Trx) or glutaredoxin (Grx). Sulfenic acids can also interact with other resolving residues, such as protein amines, to form a sulfenamide residue (Figure 2). The final oxidative PTM to discuss is S-glutathionylation leading to the formation of a mixed disulfide bond between glutathione and the cysteine thiol. All of these oxidative PTMs either stabilize the effect of the oxidation of the thiol, which results in a longer lasting effect on the protein, or can result in a completely different protein conformation leading to a different function.

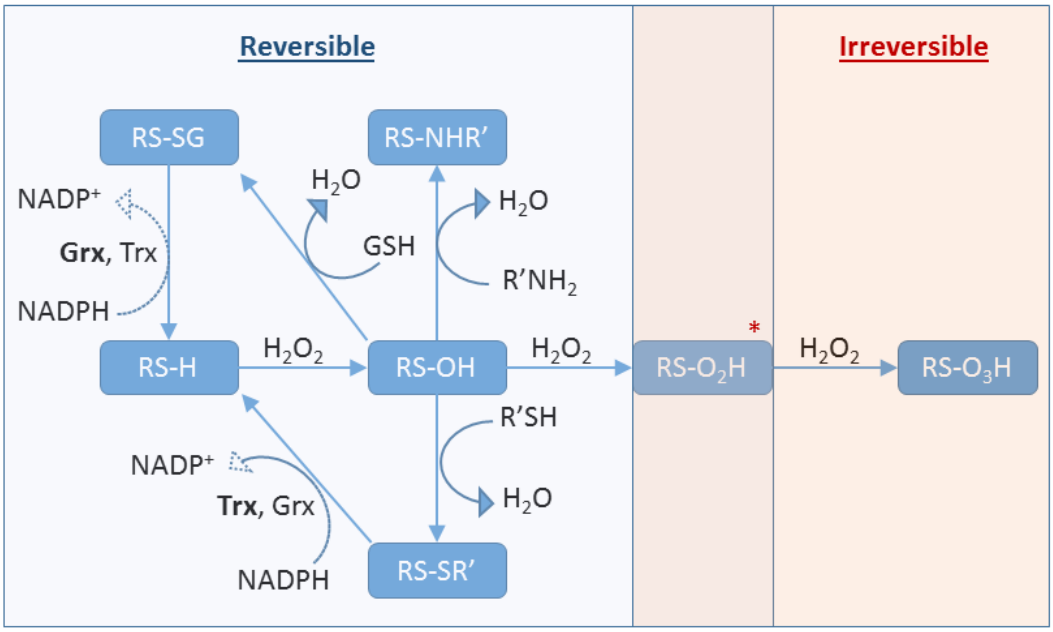

Figure 2: Cysteine Posttranslational Modifications*. Protein thiols (RS-H) can undergo oxidation to the sulfenic acid (RS-OH), which then can react with intracellular glutathione to form a mixed disulfide (RS-SG) or with another protein moiety such as another thiol to form a disulfide bond (RS-SR') or an amine to form a sulfenamide (RS-NHR'). These modifications are reversible and can be reduced back to the thiol, enzymatically, at the expense of NADPH. The sulfenic acid can be further oxidized to the irreversible modifications sulfinic acid (RS-O_2H) and sulfonic acid (RS-O_3H). *It has been demonstrated that the hyperoxidized sulfinic acid in the peroxiredoxin can be enzymatically reduced to the sulfenic acid at the expense of ATP, and as such it is possible that other unidentified proteins may utilize a reversible sulfinic acid in their function.(95)*

Proteins known to be sulfenylated are frequently referred to as "redox switches" (96). This term, "redox switches," is used to describe these proteins because their activity is regulated by their redox state. In other words, the protein's constitutive function is completely reversed once oxidized (97,98). The other important factor of "redox switches" is their reversibility, so the protein's function can be turned "on" or "off" without permanently effecting the protein. However, this dynamic nature can be influenced by subsequent oxidation by ROS, including H_2O_2, to the sulfinic acid and sulfonic acid (Figure 2). The hyperoxidation of proteins is irreversible and is frequently linked to pathology and cytotoxicity.

1.3: Redox Toxicology

Using ROS as the regulators of redox biology comes at a risk as they also have the potential to be harmful. Under physiological conditions the cell is able to replete ROS levels through multiple cellular antioxidant defense mechanisms. However, when ROS levels are elevated beyond the antioxidant capacity of the cell, the resulting outcome can be pathologic or toxicological in nature (67). Virtually all diseases (cancer, cardiovascular, neuro-degeneration, metabolic syndrome, etc.) (99-101) as well as most environmental (102,103) and pharmacological (104) exposures have been reported to have an associated oxidative stress component. For instance, even a slight change in the redox status(es) of the cell can lead to perturbed cellular function that if unchecked leads to cellular senescence by irreversibly damaging the cellular machinery (105). In other words there appears to be two distinct thresholds when one discusses redox toxicology, the first inappropriate activation of physiological cellular processes, while the second is overt cytotoxicity, which results in irreversible damage (106,107).

1.3.1: Oxidative Stress

Of the two thresholds of redox toxicology, the irreversible damage has been studied more extensively. This form of redox toxicology is often referred to as oxidative stress. The term oxidative stress as a descriptor has been used nebulously referring to both electrophilic and oxidative species and lacking no definitive meaning to characterize the effect of the stress. The vagueness of the term oxidative stress is partially due to the methodological approaches that were available to characterize and define it (108). However, for the purpose of this dissertation, oxidative stress will be defined as the cellular response to avoid cytotoxicity due to an increase in ROS or oxidation of cellular macromolecules. Arguably the most heavily studied cellular

response to oxidative stress is the induction of the antioxidant response element (ARE) by NF-E2-related factor 2 (Nrf2) (Figure 3).

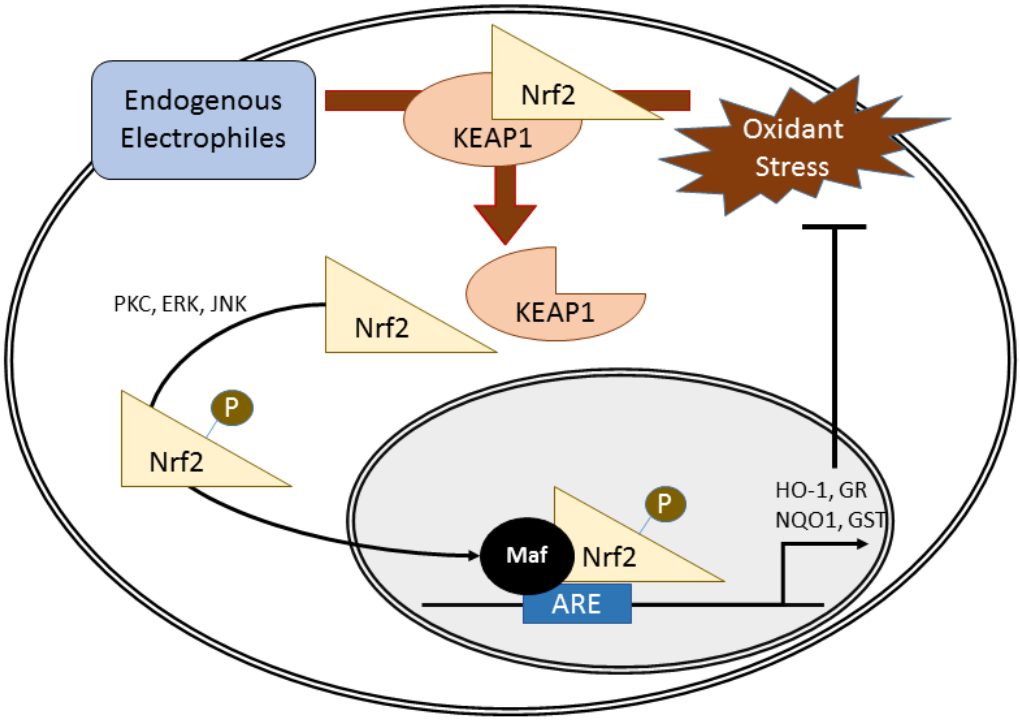

Figure 3: Nrf2-Depdenent Activation of the Antioxidant Response Element*. Endogenous electrophilic interaction or exogenous oxidant stress leads to KEAP1 release of Nrf2. Nrf2 is then phosphorylated via PKC, ERK, or JNK to facilitate translocation to the nucleus where interaction with a Maf protein allows for binding to the antioxidant response element (ARE) to induce gene expression of proteins critical in the antioxidant response.*

1.3.1.1: Antioxidant Response Element

The antioxidant response element or ARE is an important transcription factor binding element that exists in the promoters of many cytoprotective genes including heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1, GR, GSTs, Trx, metallothioneins, and sulfotransferases (109). All of these genes when translated have active roles in reducing proteins and peptides or participate in the detoxification of reactive xenobiotics. Of particular importance in xenobiotic-induced oxidative injury is HO-1. The inducible protein HO-1 is involved in the catalysis of heme to carbon monoxide, iron, and bilirubin, all three of which have “antioxidant”

capabilities by either acting as a non-specific target for ROS or inhibitors of cellular oxidative processes (110). Increased HO-1 expression in the respiratory system as a result of exposure to PM leads to improved lung function by improving mucociliary function and inhibiting neutrophil infiltration that could lead to fibrotic lesions (111). The universal role of HO-1 in responding to oxidative injury has made it a valuable marker of oxidative stress.

Of the transcription factors that bind to the ARE, the most notable is Nrf2, which activates ARE and BACH1, a repressor of ARE activity (112). At rest, BACH1 associates with the ARE and must be displaced in order for ARE activation by Nrf2 (112). Nrf2 is normally bound to Kelch-like ECH-associated protein 1 (KEAP1) in the cytosol. KEAP1 serves as an adaptor protein for the cullin 3/ring box 1-E3 ubiquitin ligase complex to target Nrf2 for degradation by ubiquitination (113). However, during oxidative stress KEAP1 releases Nrf2 which allows the translocation of Nrf2 to the nucleus to induce the antioxidant response (114). Once Nrf2 reaches the nucleus it forms a heterodimer with a transcription factor of the musculoaponeurotic fibrosarcoma (Maf) family to bind to the ARE (112).

Cellular oxidant stress leads to a separation of KEAP1 and Nrf2, allowing for Nrf2 activation of ARE-related genes. There are two distinct mechanisms through which this can occur. The first is through an increase in electrophilic species in the cell including divalent metals, $NO^{\cdot}$, and aldehydes which can all target specific cysteine residues on KEAP1, which compromises its ability to form disulfides with Nrf2 (115). The second mechanism is phosphorylation of Nrf2 by extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinase (JNK) (Xu 2006) or protein kinase C (PKC) (116). Phosphorylation of Nrf2 leads to its release from KEAP1 and is also important in its subsequent targeting to the nucleus (113). It is

argued by some researchers that Nrf2-induced ARE gene expression requires both electrophilic attack of KEAP1 and phosphorylation of Nrf2 (117).

1.3.2: Perturbation of Redox-Dependent Processes

Oxidant-induced signaling events such as the activation of the KEAP1/Nrf2 pathway are likely in response to overt oxidative stress. As discussed previously, there are many other redox-dependent processes within cellular physiology that are largely controlled by "redox switch" proteins that can be targeted by H_2O_2 (118). Many exposures are known to elevate intracellular ROS including H_2O_2. Thus it is possible that these exposures could affect these redox-dependent processes without leading to overt cytotoxicity. Among these processes include activation of signaling pathways, regulation of bioenergetics, cytoskeletal maintenance, and the regulation of the cell cycle (119). The focus of this dissertation will be restricted to the effect of toxicological exposures on signaling and bioenergetics via redox-dependent mechanisms.

1.3.2.1: Activation of Signaling Pathways

One of the first recognized redox-dependent mechanisms in physiology was the inhibition of PTPs (120). PTPs contain a catalytic cysteine that can be sulfenylated by H_2O_2, which inhibits the protein's ability to dephosphorylate other proteins (121,122). This has major implications for the activation of signaling pathways as PTPs and other phosphatases are more active than their kinase counter-parts (123). This provides an intracellular imbalance of proteins that are not phosphorylated, so when H_2O_2 inhibits PTPs it allows for the stabilization of signaling activation (124). It should also be noted that when the catalytic cysteine of PTPs, such

as that in protein tyrosine phosphatase 1B (PTP1B), is sulfenylated it rapidly interacts with a nearby amine forming a stable sulfenamide and abrogates PTP activity (121).

It has been shown through previous work that PTPs can be inhibited by targeting the catalytic cysteine (125). The thiolate can be directly attacked by electrophiles, which compromises the protein's ability to dephosphorylate targets (45). More recently, work in our lab has demonstrated that reducing intracellular H_2O_2 levels specifically modulated zinc- and 1,2-NQ-induced gene expression (56). It is likely that xenobiotic-induced H_2O_2 enhances signaling through the sulfenylation or possibly hyperoxidaiton of PTPs.

The most prominent PTPs in physiology as well as pathology is PTP1B. PTP1B is expressed in may tissues and is known to interact with many important targets including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor, c-Src, and Janus kinase 2 (126). Because of the widespread physiological role of PTP1B, it is a prominent candidate for toxicological targeting of redox-dependent processes. It is known that ROS and electrophilic compounds, including zinc (46) and 1,2-NQ (54), target PTP1B's catalytic cysteine. However, it is not known whether ROS contribute to zinc and 1,2-NQ's ability to target PTP1B, since it is also known that zinc and 1,2-NQ can also elevate H_2O_2 levels.

1.3.2.2: Bioenergetics

Another major cellular process that is regulated by redox-dependent proteins is bioenergetics. Bioenergetics is the ability of cells to actively provide, maintain, and utilize energy. This process is arguably the most responsive cellular process, reacting within seconds or minutes to environmental factors such as pO_2 and pH (127). David Wilson illustrates this best with the analogy that the DNA and epigenetic factors serve as the blueprint for the cell, while the

bioenergetic processes are the programs of the cell (128). Bioenergetics is functionally localized to the mitochondria through oxidative phosphorylation (129). Although oxidative phosphorylation can be considered the central processing unit that regulates all the "programs of the cell" (128), oxidative phosphorylation is regulated by the citric acid cycle to produce the needed energy to drive ATP synthase. Although citric acid cycle funnels energy to maintain oxidative phosphorylation, the citric acid cycle is fine-tuned and regulated by glycolysis, which ultimately supplies the cell with energy by converting glucose to pyruvate.

Although there is a variety of proteins involved in bioenergetics, one of the most important is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH functions as a critical step in glycolysis to utilize glucose for energy, and it is essential for cellular viability (130). Furthermore, GAPDH is expressed constitutively at high levels in the cytosol. Due to the role of GAPDH in the cells it is frequently referred to as a housekeeping protein and in many studies is utilized as an experimental control. Although GAPDH has an essential role in glycolysis, in recently published work GAPDH has been demonstrated to have other "moonlighting" functions such as the regulation of nuclear proteins, stabilization of mRNA, and even as a receptor (130).

A regulating factor of GAPDH activity is the reversible oxidation of a cysteine in one of its glyceraldehyde 3-phosphate binding domains. Sulfenylation of GAPDH leads to its inability to maintain its glycolytic function (131). It is hypothesized that this confers improved antioxidant potential to the cell as this ultimately leads to an increase in NADPH generation, which is essential for the glutathione/Grx and Trx systems to provide antioxidant protection (132). Additionally, the cysteine that regulates GAPDH activity has a substantially higher reactivity than other protein thiols such as the catalytic cysteine found in PTP1B (94). These two factors

together suggest that GAPDH could also serve an antioxidant function as well. However, the important consideration in regards to toxicological exposure is that elevation of ROS perturbs basic glycolytic function by inhibiting GAPDH through sulfenylation of its active site cysteine, even if it is at the cost of reducing elevated ROS by providing energy through the pentose phosphate pathway.

1.4: Approaches to Study Redox Toxicology

Despite the need to better understand redox biology and the interface of redox biology and environmental health, there have been few techniques available to reliably obtain information. There are two distinct issues at hand that complicate the ability to study redox toxicology. The first is that the initiating events for redox-dependent processes are rapid and frequently localized, while the spatiotemporal resolution of most techniques is inadequate to provide insightful data (133,134). In the case of techniques that are able to detect a changes in the redox status of cells, such as the case in electron spin resonance, the resources are technically and cost prohibitive for most experiments, especially once applied to toxicological experiments (135). The other disadvantage of traditional techniques in redox biology is that interventions utilized to decipher the effect of ROS use many "antioxidants" that provide unspecific targeting of redox species and processes, as is the case with N-acetylcysteine (NAC) which not only up regulates the synthesis of glutathione, but it itself can react with free radicals and ROS, thus providing a weak causal link in the physiology (136). Another issue with developing such causal arguments is that that the antioxidant used could become an oxidant itself, further complicating the explanation of a redox-dependent process.

Although the last few decades have provided considerable technical advancements in the field of redox biology, which will be discussed in this section, there are still challenges in implementing these techniques in toxicology (137,138). The primary concern is that most of these cutting-edge technologies are founded on the homeostatic redox environment (133,139). For redox biology this characteristic is advantageous; however, toxicological exposures have the potential to dramatically alter physiological processes that may compromise the functionality of these tools. Thus it is essential to implement controls that not only test the experimental conditions but also technical conditions to ensure the exposure does not impact the readout of the technique (140).

1.4.1: Small Molecule Redox Sensors

Small molecule redox sensors are chemicals, sometimes in the form of dyes, which change characteristic properties upon interaction with ROS or antioxidants. The first generation of these sensors lacked appropriate specificity for rigorous testing. For instance, 2′,7′-di-chlorofluorescein (DCF) is a small chemical molecule that was reported to increase fluorescence upon interaction with H_2O_2, yet after increased scrutiny it was shown to react with multiple ROS including $HO^{\cdot}$, $O_2^{\cdot-}$, and $NO^{\cdot}$ (141). Additionally, DCF has been demonstrated to induce oxidative stress itself by redox cycling, which makes it a poor sensor to understand the actual role of redox-dependent processes (142). More specific, selective sensors have been developed including Peroxy green-1 (PG-1), Peroxy orange-1 (PO-1), Peroxy yellow-1 (PY-1), and Peroxyfluor-6-acetoxymethyl ester (PF6-AM). These sensors use a boronate ion in their structure that interacts with H_2O_2, specifically, and have not been demonstrated to effect redox-dependent processes directly (143). Although these sensors, especially the newer generation sensors, have

their utility, they also have their weaknesses (142). Amongst these weaknesses is that they are unable to reversibly interact with their target ROS, and as such cannot capture the dynamic nature of redox-dependent cellular processes.

1.4.2: Genetically Encoded Redox Sensors

The discovery of green fluorescent protein (GFP) opened many avenues for biological and biomedical research (144), and further expanded with modifications to GFP changing its fluorescent color, including yellow fluorescent protein (YFP) (145). One of the most important characteristics of GFP-based molecules is that they can be expressed in subcellular regions in specific cell types (146). Furthermore, GFP expression can be conditionally controlled, which can be a valuable characteristic in certain experiments. These reasons led to the development of redox-based sensors based on GFP and its variants.

1.4.2.1: HyPer

Through the insertion of circularly permutated YFP (cpYFP) into the H_2O_2 sensing protein OxyR1A, the Belousov group developed a fluorogenic genetically encoded sensor, named HyPer, that responds to intracellular H_2O_2 levels (147). HyPer can be excited at dual wavelengths (404 and 488 nm) and emits in the green at ~520 nm. The excitation maximum is different for each wavelength based on the redox status of the sensor. Specifically, the 404 nm excitation reaches maximum excitation when reduced, and upon oxidation loses fluorescence. The opposite holds true for HyPer's 488 nm excitation as it reaches maximum excitation in the presence of high levels of intracellular H_2O_2, and loses fluorescence under reducing conditions. This characteristic of dual excitability is highly advantageous as the emissions from both

excitations can be used to calculate a ratio, which controls for many common issues with fluorescence microscopy including variable sensor distribution in the cell and photobleaching.

This sensor's experimental value was quickly ascertained by targeting it to a variety of subcellular organelles including the mitochondria (148), and successful expression in the widely used small-vertebrate model, zebrafish, revealed the role of H_2O_2 in development and wound healing (149,150). HyPer has also been chimerically linked to the H_2O_2 producing protein EGFR allowing for the measurement of micro-domain increases in H_2O_2 (151). Importantly, experimental results using HyPer have demonstrated that xenobiotic induction of H_2O_2 rarely is globally elevated and in most cases leads to an increase in specific subcellular compartments (56,148)

HyPer is sensitive to pH at a physiologically relevant range (147). Thus it is imperative to monitor HyPer concurrently using pH sensors such as pHred (152). One can also use side-by-side experiments such as pHluorin or pHluorin2 (153). However, the ideal pH control to utilize in HyPer experiments is SypHer, which is structurally a point mutation of HyPer that makes the sensor insensitive to H_2O_2 while retaining sensitivity to changes in pH (154). A newer version of SypHer, SypHer2 has been developed with improved experimental responsiveness (155).

1.4.2.2: rxYFP, Oba-Q Proteins and roGFP

YFP was made redox sensitive through two point mutations leading to the development of rxYFP (156). rxYFP monitors the E_{GSH} metabolically through the interaction of glutaredoxin-1 (Grx1). Grx1 reduces oxidized proteins through transfer of electrons between glutathione. This attribute was utilized to construct a chimeric protein linking rxYFP to Grx1 that resulted in a sensor that rapidly equilibrated with the E_{GSH} as it no longer was limited by endogenous levels

of Grx1 (138). Expression of rxYFP could be targeted to a variety of subcellular compartments; however, both the parent sensor and its chimeric form are highly sensitive to pH changes (157,158). This sensitivity to pH is further complicated by its characteristic excitation at only one wavelength, making it a technically difficult sensor to use in live cell imaging experiments because of the extensive need of controls. Despite this disadvantage rxYFP has been successfully used in redox-based immunoblots (159). A group of proteins similar to rxYFP have recently been developed known as oxidation balance sensed quenching (Oba-Q) proteins (160). Although these sensors are only excitable at one wavelength, they are all based on variant chromophores of GFP including Sirius (Oba-Qs), CFP' (Oba-Qc), and BFP (Oba-Qb) allowing for expression of multiple sensors in the same cell without overlap of fluorescent emission spectra.

The redox sensitive version of GFP (roGFP) was developed using two point mutations (161). The family of roGFPs have been developed each with its distinct midpoint potential and dynamic range (133) that are functional in a variety of subcellular compartments (Figure 4). Of the available family members, roGFP1 and roGFP2 are currently the most used. roGFP1 is not sensitive to pH while roGFP2 is, but has a significantly larger dynamic range (161). Both roGFP1 and roGFP2 have dual excitation wavelengths (404 and 488 nm) and emit in the green, affording the benefit of ratiometric analysis. However, unlike HyPer the roGFP sensors maximum fluorescence emission from 404 and 488 nm are reversed, where 488 nm reaches maximum fluorescence under reducing conditions and the 404 nm wavelength reaches strongest fluorescence under oxidizing conditions. Additionally, one group has utilized the same point mutations as the super-folder GFP to roGFP producing roTurbo (162).

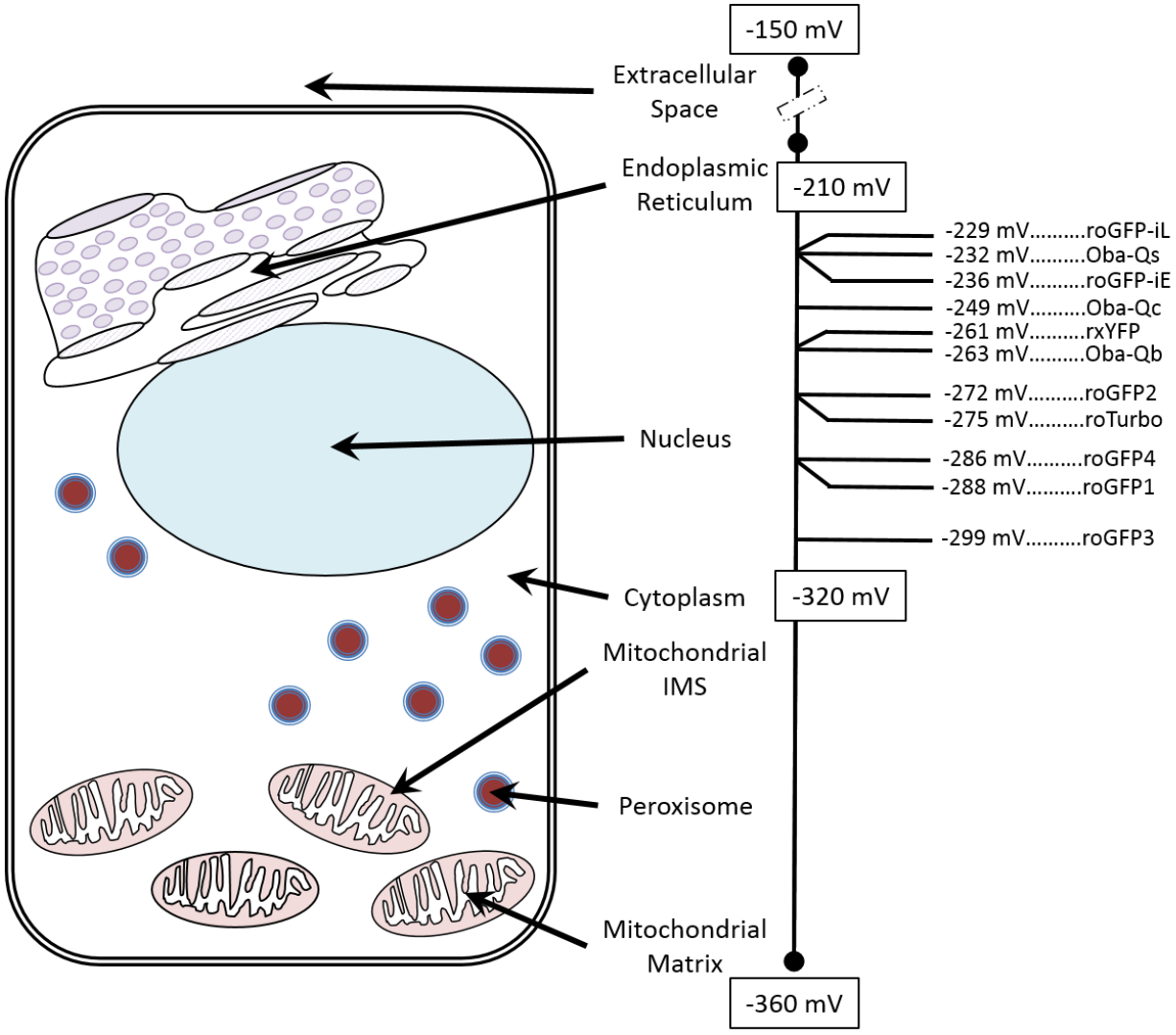

Figure 4: Midpoint Potentials for E_{GSH} Sensors. *The average redox potentials for subcellular compartment are shown with the mitochondrial matrix as the most reducing compartment (-360 mV) and the extracellular space the most oxidizing compartment (-150 mV). Of the available EGSH sensors only a few have a high enough midpoint potential to be functional in the endoplasmic reticulum (roGFP-iL, roGFP-iE (163), Oba-Qs (160)). There are many sensors that can be effectively targeted to the nucleus, cytoplasm, mitochondrial intermembrane space (IMS), peroxisome, or mitochondrial matrix (Oba-Qc, Oba-Qb (160), rxYFP (156), roGFP1-4 (161), roTurbo (162)). The roGFP3 (161) will have improved accuracy in highly reducing compartments such as the mitochondrial matrix.*

Although roGFP is a highly efficient sensor, it is limited by its slow equilibration with the cellular environment as it is like rxYFP and the Oba-Q proteins in that it metabolically monitors E_{GSH} through the interaction of Grx1. To improve the utility of roGFP, Dick's group developed a

chimeric protein linking roGFP2 to Grx1 (roGFP2-Grx1), which dramatically improved sensor kinetics (164). The same research group further improved the subcellular localization of the sensor as well as responsiveness to changes in E_{GSH} by switching the order of the chimeric protein (Grx1-roGFP2) (165,166). It is likely that Grx1-roGFP2 had improved sensor characteristics compared to roGFP2-grx1 due to the elimination of steric hindrances improving the kinetic reaction between roGFP2 and Grx1. Another key study is the development of the chimeric protein that linked roGFP2 to mrx1, the analog of Grx1 in mycobacterium. This chimeric protein (roGFP2-mrx1) was able to successfully monitor the E_{MSH} of mycobacterium as a readout for an effect on the host as well as assess the efficacy of antibiotics (167). This development established the potential to use roGFP as a way to monitor the redox metabolism of prokaryotic models as well.

1.4.3: Dimedone-Based Chemical Probes

Detecting ROS and antioxidants directly is a powerful method to interrogate the role of redox toxicology in adverse health outcomes; however, elucidating the identity of those targets as well as how those outcomes are propagated can be just as important. Altered protein sulfenylation is a good candidate to characterize the effect of oxidant events, especially if intracellular H_2O_2 levels are affected. However, as mentioned previously, sulfenylation is a rapid, short-lived modification and due to the scarcity of cysteines available to be sulfenylated, it is physiologically rare (168). To detect sulfenylation one must have a highly specific and sensitive method of detection (169). Many of these experiments utilize dimedone to detect sulfenic acids (170). Dimedone is a small cell permeable molecule that is tolerated by cells that specifically and irreversibly labels sulfenic acids. Importantly, dimedone is unreactive towards

all other cysteine-based PTMs (137). This allows for the detection of sulfenic acids by mass spectrometric or immunoblot methods targeted to identify the dimedone-thioether bond on the protein.

1.4.3.1: DYn-2 and DAz-2

Dimedone-based approaches to detect sulfenic acids have beneficially impacted the field of redox biology, yet many of these approaches have a low signal-to-noise ratio. In response, Kate Carrol's group developed DYn-2 and DAz-2, which have chemical structures that have the dimedone warhead with either an alkynyl or azide tail, respectively (171). These probes are slightly more reactive than dimedone, but their real utility is that they can be coupled by copper-catalyzed alkynyl-azo cycloaddition (CuAAC) to attach biotin to the DYn-2 or DAz-2 labeled sulfenic acids (172-174). This allows one to take advantage of the biotin-avidin binding to dramatically improve sensitivity. This approach, rather than directly linking dimedone to biotin, is preferred, since biotin cannot cross the cell membrane (175). However, a drawback to using CuAAC in biological samples is that it can damage the proteins that are to be detected (176,177).

1.4.4: *In Vitro* Models

Although many of these techniques can be implemented *in vivo*, *in vitro* cell culture models currently provide the highest spatial and temporal resolution to mechanistically link oxidant events to a specific adverse outcome. When researching the effects of air pollution on cell toxicity, it is vital to utilize the most appropriate cell type. It has already been discussed that air pollution has the capacity to affect multiple organs and tissue types, but the initial target of air pollution is the nasal and bronchial epithelium of the respiratory system. The most representative

model for such studies are primary human airway epithelial cultures grown at an air-liquid interface (178). However, primary cell cultures are notoriously difficult to use in mechanistic studies as they are resistant to gene induction techniques and due to varying genotypes lead to low signal-to-noise results.

Even though immortalized cell lines exist, many researchers are hesitant to utilize them in toxicological studies due to concerns of relevance to clinical scenarios. These concerns are certainly warranted, especially since established cell lines have neoplastic characteristics and may be considered to have progressed towards transformation away from normal, healthy cells. Relevant to redox biology, transformed cells have markedly different oxidant processes as compared to their respective background, normal cells (179). Thus, it is important to validate whether an immortalized cell line used in redox studies have a similar oxidant capacity as their respective tissue found in intact, normal individuals. For instance the established human bronchial epithelial cell line, BEAS-2B (180,181) has been demonstrated to maintain a similar oxidative and antioxidative capacity as primary human bronchial epithelial cells (182). With these considerations in mind, the BEAS-2B cell line are a powerful model to elucidate the effect of air pollution on the human bronchial epithelia.

1.5: Summary

Air pollution is recognized as an increasing global public health problem. A mechanistic contributor to air pollutant-induced adverse human health outcomes is oxidative stress. Oxidative stress can be defined as a pathological increase in ROS, such as H_2O_2, that leads to impaired cellular functions. Of particular interest is H_2O_2-induced sulfenylation of the regulatory proteins PTP1B and GAPDH, which are involved in cellular signaling and bioenergetics, respectively. It

is known that many air pollutants can lead to an increase in H_2O_2, but the role of air pollutant-induced H_2O_2 has yet to be thoroughly studied. Thus, a hypothesis was formulated that exposure to air pollution alters cellular redox homeostasis through the elevation of H_2O_2 resulting in oxidant-dependent activation of adaptive and signaling pathways. To test this hypothesis three goals were undertaken: 1) determine the role of pollutant-induced H_2O_2 in inflammatory and adaptive gene expression, 2) examine the role of protein sulfenylation in air pollutant-induced signaling, and 3) explore the application of H_2O_2-induced oxidant events as translational biomarkers in air pollution exposures.

CHAPTER 2: Role of H_2O_2 in the Oxidative Effects of Zinc Exposure in Human Airway Epithelial Cells[1]

2.1: Introduction

Human exposure to ambient particulate matter (PM) is a public health concern of global proportions. Observational studies demonstrate an association between exposure to PM and elevated rates of cardiovascular morbidity and mortality (183-187). Despite the association between these adverse health effects and ambient PM levels, the constituents in PM responsible for its toxicity and the underlying mechanisms remain largely unknown. Epidemiological (36) and toxicological (35,188) studies have specifically implicated the particle-associated transition metal zinc (Zn^{2+}) as a contributor to PM health effects. Although zinc is an essential nutrient and vital to many physiological processes, inhalational exposure to zinc is associated with a number of adverse health outcomes (41).

The health effects of zinc inhalation are modeled by metal fume fever, an occupational disease characterized by a self-limited febrile flu-like condition with airway inflammation resulting from inhalation of ZnO particles generated during welding (38). The mechanisms responsible for the pathophysiological effects of Zn^{2+} inhalation have been investigated in cultured human airway epithelial cells (HAEC) by our laboratory (48,189,190) and by other groups utilizing diverse *in vitro* models (191-194). Observations from these studies show that

[1] This chapter previously appeared as an article in the Journal of Redox Biology. The original citation is as follows: Wages, P., Silbajoris, R., Speen, A., Brighton, L., Henriquez, A., Tong, H., Bromberg, P., Simmons, S., & Samet, J. "Role of H_2O_2 in the oxidative effects of zinc exposure in human airway epithelial cells." *Redox Biology* 2014; 3:47-55.

Zn^{2+} induces inflammatory and adaptive gene expression through processes that involve the deregulation of signaling cascades. Specifically, Zn^{2+} is thought to perturb multiple signaling pathways by direct interaction with thiol groups on key regulatory proteins, including protein tyrosine phosphatases (PTP) (44-46). Zn^{2+} is a known mediator in signaling pathways, including the Keap1/Nrf2/ARE pathway (115,195).

Unlike other transition metals associated with PM (e.g., Fe, Ni, Cu, V), Zn^{2+} lacks two adjacent valence states and, therefore, does not support single electron transfers to produce reactive oxygen species (ROS), meaning that ROS generated during Zn^{2+} exposure are derived from cellular metabolism. Zn^{2+} interferes with mitochondrial respiration at multiple points (41) and consistent with this, we recently reported that exposure of HAEC to Zn^{2+} results in increased intracellular generation of H_2O_2 of mitochondrial origin (196). Physiologically, H_2O_2 serves as a second messenger that plays pivotal roles in the reversible inactivation of regulatory proteins, most notably PTP (78,81,82,197). Thus, there is evidence that toxicological Zn^{2+} exposure can induce gene expression through signaling mechanisms by direct interaction as well as through the generation of H_2O_2.

In order to determine the dependence of Zn^{2+}-induced responses on H_2O_2, the present study expanded our previous live-cell imaging approach to monitor oxidative changes in the cytosol and mitochondria of HAEC exposed to Zn^{2+} (56,140). We utilized cytosolic overexpression or ectopic mitochondrial expression of the H_2O_2 scavenging enzyme catalase in BEAS-2B cells bearing the genetically-encoded fluorogenic ratiometric sensors HyPer or roGFP2, which report on H_2O_2 and the glutathione redox potential (E_{GSH}), respectively (133,147,198). In this study we examined the link between oxidative events associated with Zn^{2+} exposure and signaling events, using the level of HO-1 gene expression as a downstream readout

of the adaptive response to oxidant xenobiotic exposure (111,199,200). This study reveals that exposure of HAEC to Zn^{2+} leads to multiple oxidative effects that are exerted through H_2O_2-dependent and independent mechanisms.

2.2: Materials and Methods

2.2.1: Reagents

Tissue culture media and supplements were purchased from Lonza (Walkersville, MD, USA). Phenol red-free keratinocyte basal media (KBM) with or without glucose was acquired from Cell Applications, Inc (San Diego, CA, USA). X-tremeGENE 9 DNA Transfection Reagent was obtained from Roche Applied Science (Indianapolis, IN, USA). Adenoviral vectors were obtained from the Gene Therapy Center Virus Vector Core Facility (University of North Carolina at Chapel Hill, USA). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): Hydrogen Peroxide (H_2O_2), Aldrithiol-2 (A-2), Dithiothreitol (DTT), 2-mercaptopyridine N-oxide sodium salt (Pyrithione, PYRI), Zinc Sulfate (Zn^{2+}), 2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsufanylthio-carbonylamino) phenylthiocarbamoylsufanyl] propionic acid (2-AAPA), buthionine sulfoximine (BSO). Basic laboratory supplies were obtained from Fisher Scientific (Raleigh, NC, USA).

2.2.2: Cell culture

SV40 large T antigen-transformed HAEC (BEAS-2B, subclone S6 (181)) were cultured as previously described (140,201).

2.2.3: Viral transduction

Plasmids for the genetically encoded redox sensors roGFP2 and HyPer were the generous gift of S.J. Remington (University of Oregon, Eugene, OR, USA) and purchased from Evrogen (Axxora, Farmingdale, NY, USA), respectively. Cytosolic (Cyto) and mitochondrial (Mito) targeted versions of both plasmids were introduced into lentiviral vectors as described previously (56). Human catalase targeted to the mitochondrial inter-membrane space was introduced into a lentiviral vector. Stable expression of Mito-Catalase, Cyto-roGFP2, Mito-roGFP2, Cyto-HyPer, and Mito-HyPer was achieved using lentiviral transduction as previously described (140). Mitochondrial localization of Mito-HyPer was verified by co-localizing its fluorescence with that of MitoTracker ® Red CMXRos (Invitrogen, Grand Island, New York, USA) (Supplemental Figure 1). The subcellular localization of Mito-roGFP2 has been validated previously (196). Lentiviral mitochondrial fluorogenic sensor transduction efficiency was approximately 70%, while that of the cytosolic forms of the sensors was approximately 40% efficient, requiring enrichment by cell sorting, conducted by the UNC Flow Cytometry Core Facility utilizing an iCyt Reflection maintained under sterile conditions. Mitochondrial localization of catalase was confirmed immunocytochemically using an anti-catalase antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and by immunoblot analysis with anti-GAPDH (Santa Cruz Biotechnology) and anti-Cytochrome C (Cell Signaling, Beverly, MA) antibodies as cytosolic- and mitochondrial-specific controls. Briefly, for immunoblot analysis mitochondria were isolated in a sucrose solution (250 mM sucrose, 15 mM NaCl) followed by centrifugations at 500 x *g* for 5 min and 20,000 x *g* for 10 min; the pellet containing the mitochondrial fraction was resuspended in RIPA (1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 150 mM Tris-HCl, pH 8.0) for immunoblotting. For cytosolic catalase overexpression, cells were transduced with an adenoviral

vector encoding human catalase driven by a CMV promoter at a MOI of 500 for 4 hrs, followed by a 2-day incubation in KGM (202). An empty vector driven by CMV was used as an experimental control. The pH-specific fluorogenic sensor pHred, created by the laboratory of G. Yellen (152), was obtained as a construct through Addgene (Cambridge, MA, USA) for expression into BEAS-2B cells via transient transfection of 1-2 µg plasmid DNA using the suggested X-tremeGENE 9 protocol.

2.2.4: Live Cell Imaging

Immediately before exposure, roGFP2 or HyPer expressing cells, were placed in KBM without phenol red and analyzed using a Nikon Eclipse C1si spectral confocal imaging system and 404 nm, 488 nm, and 561 nm primary laser lines (Nikon Instruments Corporation, Melville, NY, USA; (140)). Sequential scans of each laser line were performed at a frequency of 60 secs with at least 10 cells expressing the biosensor in the field of view, with results calculated as a ratio of the respective 525/30 nm emission for the 404 nm and 488 nm excitation of each sensor. Baseline data points were collected 10 min prior to the addition of 50 µM Zn^{2+} and 1 µM PYRI. To normalize for variability in the dynamic range of the sensors expressed in individual cells, HyPer expressing cells were treated with 50 µM H_2O_2 for 5 min followed by treatment with 1 mM H_2O_2 for 3 min and finally with DTT for 3 min, while roGFP2 expressing cells were treated with 1 mM H_2O_2 for 5 min followed by addition of 100 µM A-2 for 3 min and finally with DTT for 3 min to quantify the span of sensor responsiveness. Data were expressed normalized to the maximum sensor response (i.e., the maximum response elicited by H_2O_2) recorded during the experiment set as 100%, with the average starting baseline response set as 0%. Cell viability was determined using retention of intracellular Calcein-AM (Molecular Probes, Eugene, OR, USA).

2.2.5: Gene Expression Analysis

BEAS-2B cells with or without cytosolic catalase overexpression or ectopic expression of mitochondrial catalase were exposed to 0-50 μM Zn^{2+}/1 μM PYRI for 5 min in KBM, washed and incubated at 37 ºC, 5% CO_2, 100% humidity for 2 hrs in KBM. Relative gene expression was quantified using the real-time PCR, ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described (56). GAPDH mRNA was used for normalization. Oligonucleotide primer pairs and fluorescent probes for HO-1 were as follows: (5'CAGCAACAAAGTGCAAGATTCTG3', 3'ACTGTAAGGACCCATCGGAGAAG5'); and for GAPDH: (5'GAAGGTGAAGGTCGGAGTC3', 3'GAAGATGGTGATGGGATTTC5'). Oligonucleotides were designed using a primer design program (Primer Express, Applied Biosystems) and obtained from Integrated DNA Technologies (Coralville, IA). The catalase primer/probe set was a TaqMan Gene Expression Assay (Hs00156308_m1) obtained from Applied Biosystems.

2.2.6: Statistical Analysis

All imaging data were quantified using NIS-Elements AR software (Nikon). Data are expressed as the mean ± SEM of at least three separate experiments. Determination of statistical significance ($p<0.05$) of all imaging data was made using linear regression, comparing the slopes of linear portion of the time-course plots. Comparison of gene expression were conducted using Student's t-test with Bonferroni correction for multiple group comparisons. ANCOVA analysis was utilized when appropriate to test for multiple interactions, with $p<0.05$ considered

statistically significant. PRISM (GraphPad Software, La Jolla, CA, USA) and R (R Core Team, Vienna, Austria) software packages were used for statistical tests.

2.3: Results

2.3.1: Catalase Expression Ablates Zn^{2+}-Induced H_2O_2 Generation

The Zn^{2+}-specific ionophore PYRI used at 1 µM, was found to reduce intercellular variability in the response of BEAS-2B cells to Zn^{2+} exposure (196) and was, therefore, included in all zinc exposure experiments in this study. Exposure to 1-100 µM Zn^{2+} , 1 µM PYRI (ZnP) did not result in overt cytotoxicity in BEAS-2B cells over the time periods used in this study, as assessed by the cellular release of calcein-AM (Figure 6).

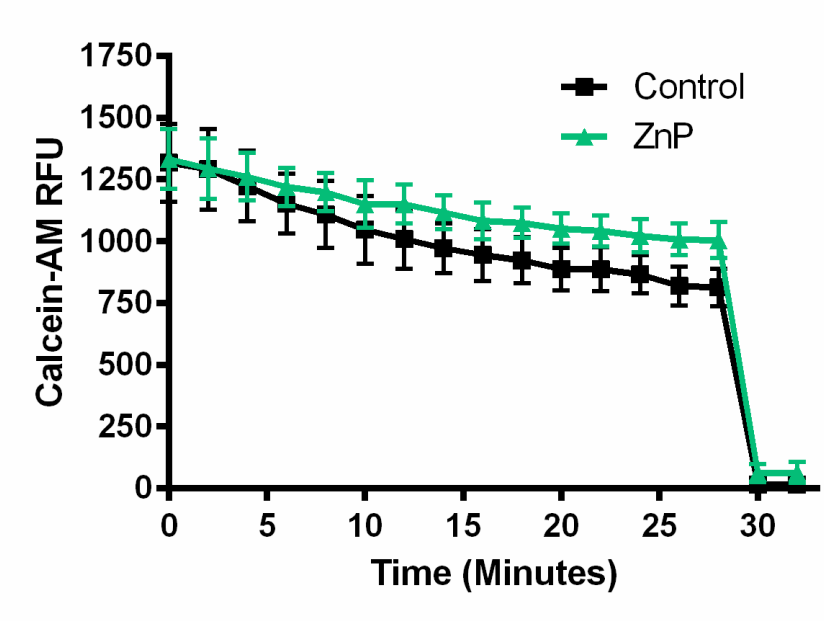

Figure 5: Zinc Pyrithione Does Not Induce Cytotoxicity*. Cells loaded with the fluorescent compound calcein-AM were analyzed by confocal live cell imaging. Either 50 µM Zn^{2+}/1 µM PYRI (ZnP) or water (control) was added to cells at minute 5. Cell membrane integrity was maintained throughout the exposure as calcein-AM remained in the cell. As a positive control to disrupt membrane integrity, 0.1% tritonX was added at minute 30. Values are presented as mean ± SE (*n =3*)*

Exposure to 50 µM ZnP rapidly increased intracellular H_2O_2 levels in both the cytosol and the mitochondria, as reported by HyPer fluorescence in each of these cellular compartments (Figures 6, 7). In contrast PYRI alone did not induce a change in H_2O_2 (Figure 8). The fluorescence emission intensity of the HyPer fluorophore is known to be affected by pH of its surroundings. Therefore, we monitored pH changes induced in cells exposed to ZnP using pHred (152). As shown in Figure 9, pH remained unchanged over the 10 min post ZnP exposure period during which the HyPer signals were monitored.

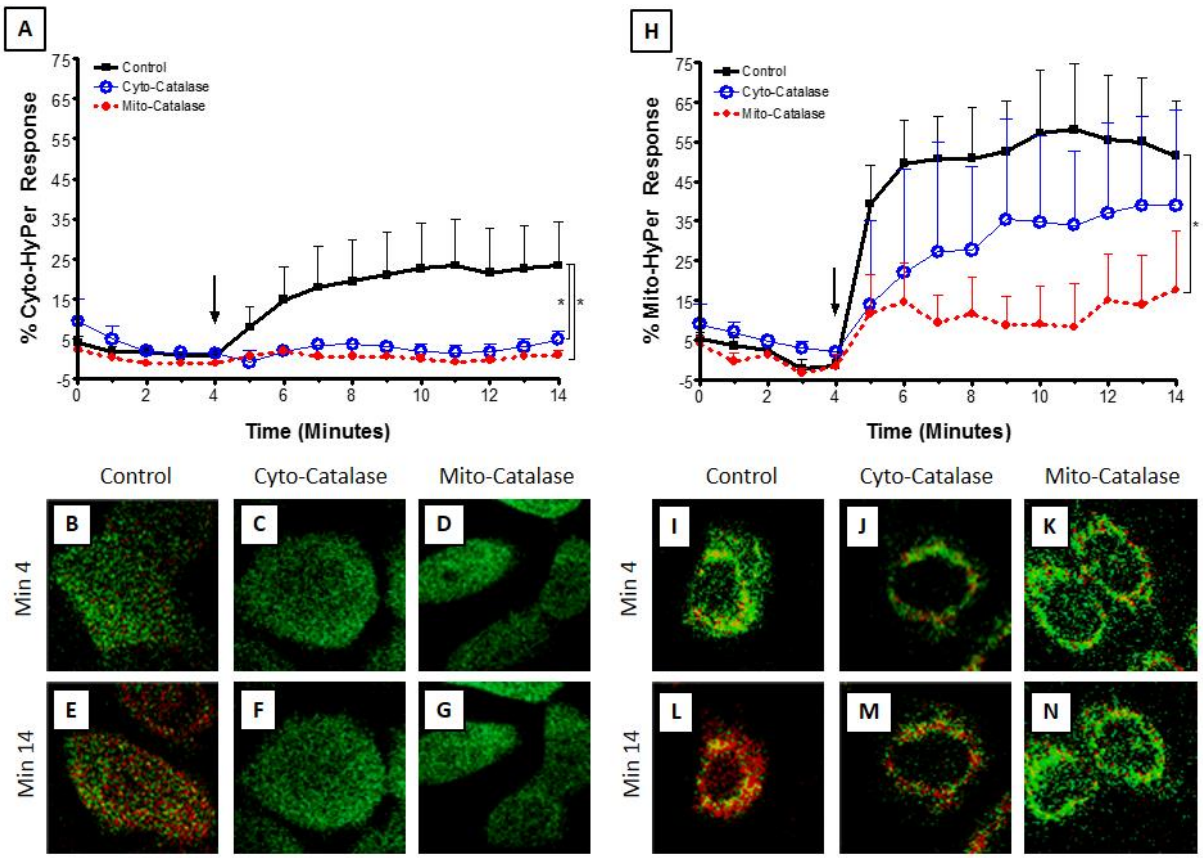

Figure 6: Targeted catalase expression abrogates Zn^{2+}-induced cytosolic and mitochondrial increases in H_2O_2 levels in BEAS cells. *BEAS-2B cells expressing HyPer in the cytosol (A-G) or mitochondria (H-N), and either over expressing catalase (cyto-catalse) or targeted-ectopic catalase expression in the mitochondria (mito-catalase) were exposed to 50 µM Zn^{2+}/1 µM PYRI at the indicated time (arrow). Ratiometric fluorescence values normalized for sensor response utilizing the baseline and maximal response H_2O_2 as the minimum and maximum responses, respectively, in either the cytosol (A) or mitochondria (H) are shown. Values are presented as mean ± SE (*n ≥ 3, where n *consists of an average of 10 distinct cells' responses), * indicates statistical significant change compared to control by linear regression. Representative cells expressing basal catalase levels (B, E, I, L), cyto-catalase (C, F, J, M), or mito-catalase (D, G, K, N) shown at baseline (min 4; B-D, I-K) and following Zn^{2+} stimulation (min 14; E-G, L-N). Fluorescence intensity color is shown as a function of increasing H_2O_2 levels:* ***low*** *H_2O_2*  ***high*** *H_2O_2.*

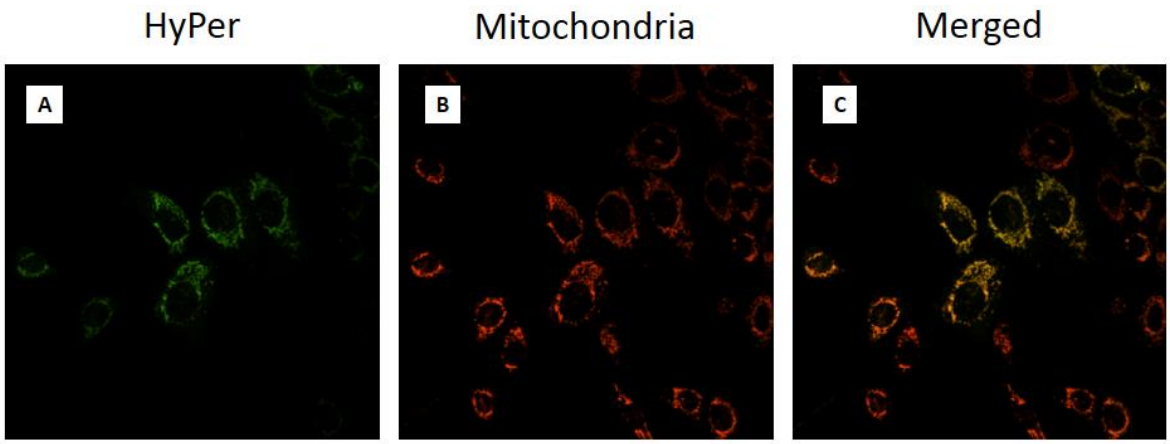

Figure 7: Mito-HyPer is specifically localized in the mitochondria of BEAS cells*. BEAS-2B cells transduced by lentivirus with mitochondrially-targeted HyPer (A) were colocalized with the mitochondria marker, MitoTracker Red (B). The two channels were merged, where the presence of yellow indicates colocalization of the HyPer construct and the mitochondria (C).*

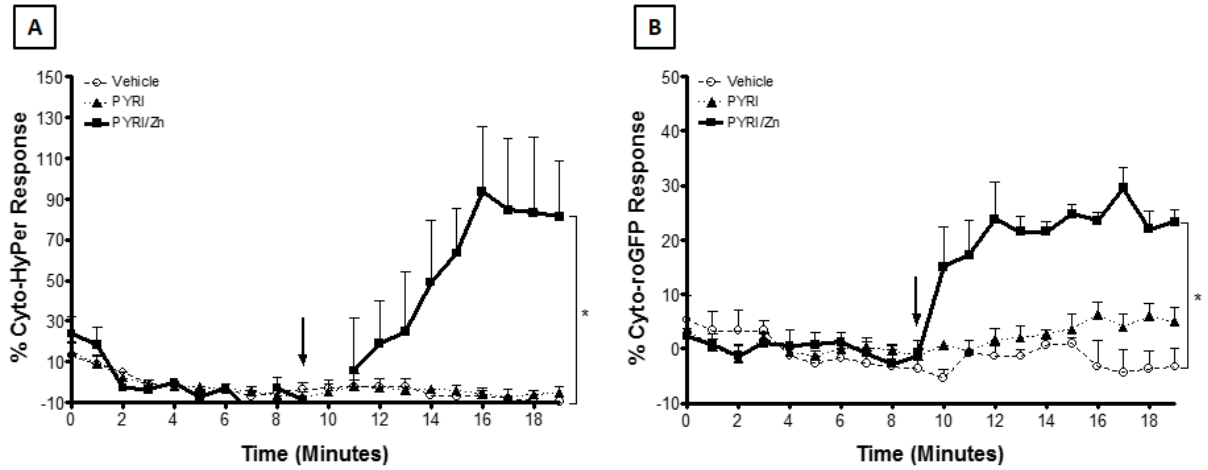

Figure 8: Exposure to pyrithione alone does not induce intracellular H_2O_2 or E_{GSH} responses*. BEAS-2B cells stably expressing cytosolic (A) or roGFP2 (B) were exposed to vehicle (open circle), 1 µM PYRI (PYRI, closed triangle), or 50 µM Zn^{2+}/1 µM PYRI (PYRI/Zn, closed square) at the indicated time (arrow). Shown are ratiometric values calculated from the fluorescent 525/30 emission of the 404 nm and 488 nm laser excitation normalized as a percent of the maximum sensor response. Values are presented as mean ± SE (*n = 3, *where* n *consists of an average of 10 distinct cells' responses), * indicates statistical significant change compared to control by linear regression.*

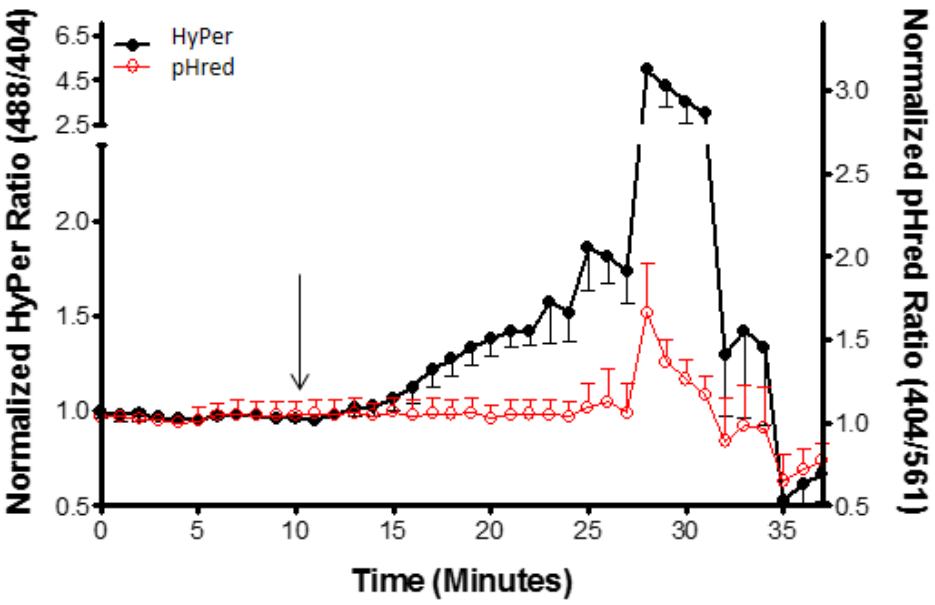

Figure 9: Zn^{2+}-induced elevation in H_2O_2 reported by HyPer are not mediated by changes in pH. *BEAS-2B cells co-expressing HyPer and the pH sensor pHred in the cytosol were exposed to 50 µM Zn^{2+}/1 µM PYRI at the indicated time (arrow). Shown are ratiometric values calculated from the fluorescent 525/30 emission obtained through excitation of the 404 nm, 488 nm, and 561 nm laser excitation normalized to the baseline. HyPer sensor responsiveness was assessed using 50 µM H_2O_2 (min 25) and 5 mM DTT (min 35). pHred sensor response was verified by addition of 10 mM acetic acid (min 28) followed by addition of 10 mM ammonium chloride (min 33). Values are presented as mean ± SE (*n *= 3, where each* n *consists of an average of 10 distinct cells' responses).*

We next tested the effectiveness of catalase expression as an interventional approach to suppress Zn^{2+}-induced increases in H_2O_2 in a cell compartment-specific manner. Expression of adenoviral mediated expression of Cyto-catalase and lentiviral encoded expression of Mito-catalase resulted in similar increase levels of catalase mRNA compared to controls (Figure 10A-B). Interestingly, expression of Mito-catalase was potentiated by exposure to 10-50 µM ZnP (Figure 10B), possibly reflecting Zn^{2+}-induced activation of the Sp1 transcription factor as reported previously in retinal pigment epithelial cells (203). CMV-driven overexpression of catalase in the cytosol effectively prevented the elevation in cytosolic H_2O_2 concentrations induced by exposure to ZnP (Figure 6A). Immunocytochemical analyses showed that the expression of catalase targeted to the mitochondria intermembrane space successfully

immunoreactive catalase to the mitochondria (Figure 10D-K). Immunoblotting of cytosolic and mitochondrial fractions confirmed the presence of catalase in the mitochondria of Mito-Catalase expressing BEAS cells but not in the mitochondria of control cells (Figure 10C). The slight elevation in catalase found in the cytosol of cells transduced with Mito-Catalase lentivirus presumably represents nascent catalase that has yet to reach the mitochondrial intermembrane space. Similar to the effect of overexpressing cytosolic catalase, cells bearing ectopic mitochondrial expression of catalase showed a marked reduction in Zn^{2+}-induced increases in cytosolic H_2O_2 (Figure 6A).

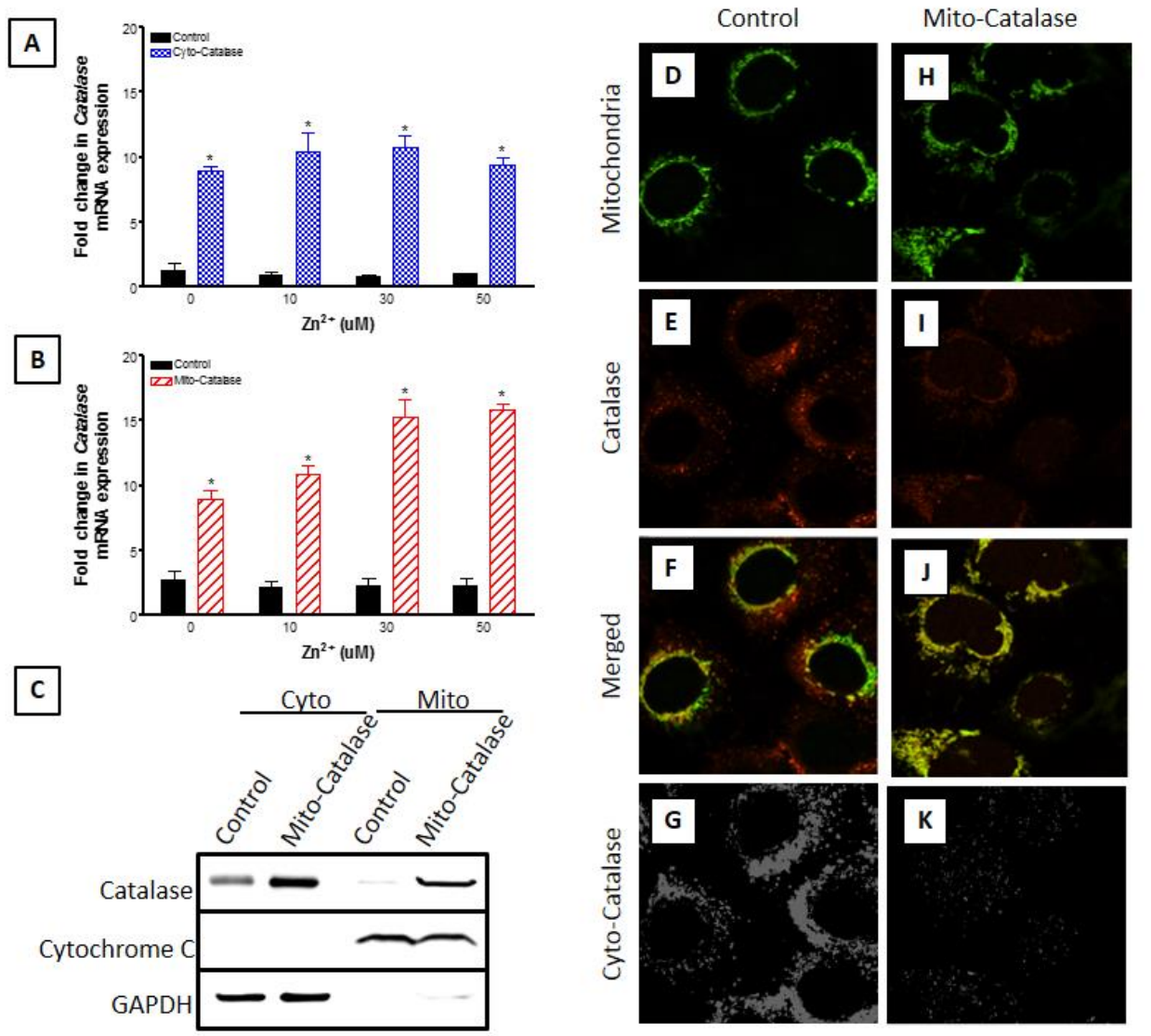

Figure 10: Ectopic expression of catalase in the mitochondria of BEAS-2B cells*. BEAS overexpressing catalase (cyto-catalase, A) or expressing ectopic mitochondrial catalase (mito-catalase, B) were exposed to the indicated zinc concentrations and 1 µM PYRI for 5 mins, washed, and then incubated for two hrs in fresh media before mRNA extraction. mRNA levels measured using TaqMan-based RT-PCR were normalized to levels of GAPDH mRNA and expressed as fold increases over basal mRNA expression in cells not exposed to media control. Values are presented as mean ± SE (*n = 3), * indicates $p < 0.05$ as *determined by Student's t-test compared to control. Mito-catalase expressing BEAS-2B cells were fractionated between cytosol (cyto) and mitochondria (mito) and immunoblotted against catalase, cytochrome C—a mitochondrial specific protein, and GAPDH—a cytosol specific protein (C). BEAS-2B cells expressing mitochondrial roGFP2 (D-K) with expression of the human catalase gene targeted to the mitochondria by a CMV promoter via lentiviral transduction (H-K) were fixed and immunostained using anti-catalase antibodies. Cells were stained with catalase primary antibody (E, I) and merged with the mitochondrial marker for colocalization analysis (F, J). For presence of cytosolic catalase localization analysis (G, K), the fluorescent intensity values across the images were converted to a binary value followed by subtraction of the mitochondria channel from the catalase channel leaving only catalase signal outside the mitochondria. Results shown are representative of three separate experiments.*

2.3.2: H_2O_2 Contributes to Zn^{2+}-Induced Oxidant Stress

Next, we examined changes in E_{GSH} as an objective measure of the oxidant stress presented to the cell by exposure to ZnP. Following the same approach used to monitor H_2O_2 with HyPer, cytosolic- and mitochondria-targeted versions of the E_{GSH}-sensing fluorophore roGFP2 were stably expressed in BEAS-2B cells. Exposure to 50 μM ZnP induced a rapid rise in the cytosolic $E_{GSH,}$ which could be reduced substantially through overexpression of catalase in either the cytosol or the mitochondria (Figure 11A). However, neither cytosolic nor mitochondrial catalase intervention affected the Zn^{2+}-induced E_{GSH} increase in the mitochondria (Figure 11H). We also examined the possibility that roGFP2 fluorescence changes might arise from a direct interaction of Zn^{2+} with the sensor itself by using 2-AAPA to inhibit glutaredoxin, the enzyme through which roGFP2 senses E_{GSH} changes (140). Pretreatment of roGFP2-expressing BEAS-2B with 2-AAPA ablated Zn^{2+}-induced increases in E_{GSH} (Figure 12) establishing that Zn^{2+}-induced changes are not the result of a direct interaction of Zn^{2+} with roGFP2, since any Zn^{2+}-direct effect on roGFP2 would not require glutaredoxin.

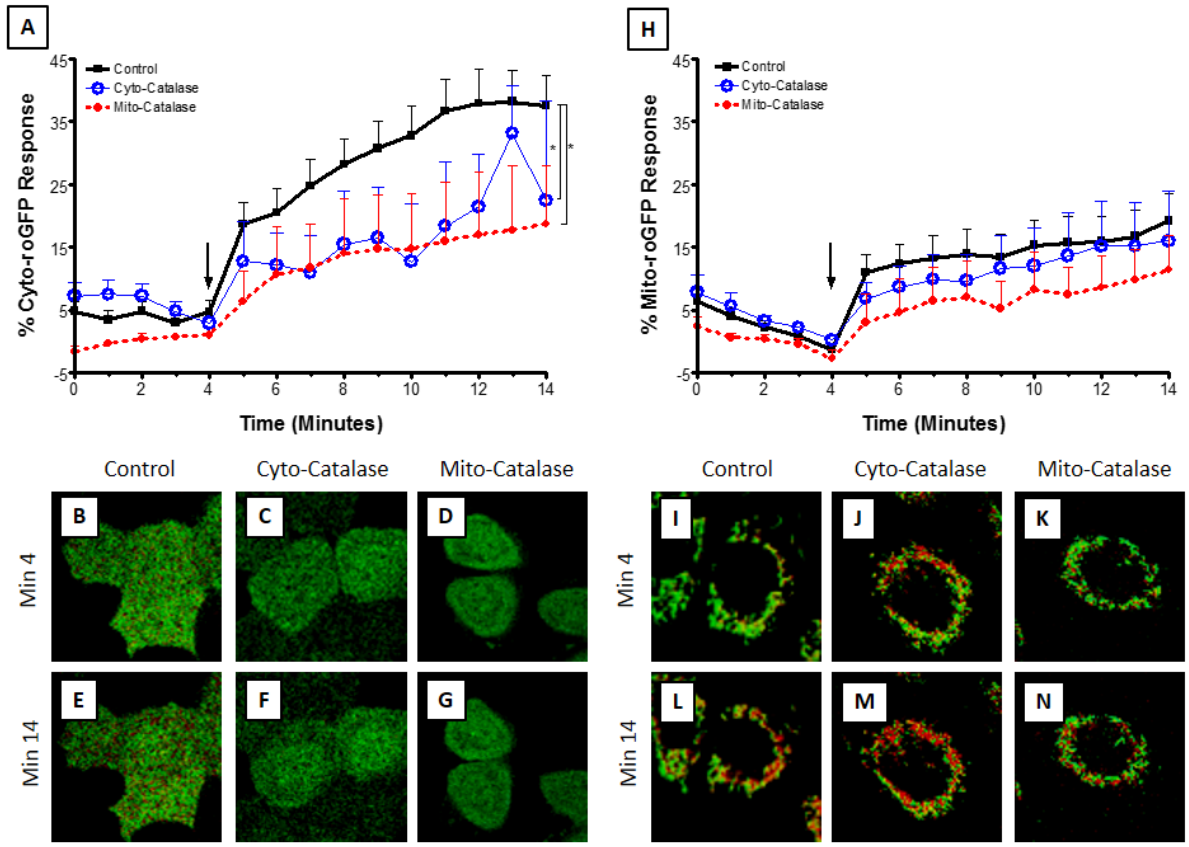

Figure 11: H_2O_2 mediates Zn^{2+}-induced elevation in cytosolic but not mitochondrial E_{GSH}. *BEAS-2B cells expressing roGFP2 in the cytosol (A-G) or mitochondria (H-N), and either over expressing catalase (cyto-catalse) or targeted-ectopic catalase expression in the mitochondria (mito-catalase) were exposed to 50 µM Zn^{2+}/1 µM PYRI at the indicated time (arrow). Ratiometric fluorescence values normalized as for sensor response utilizing the baseline and stimulus to 1 mM H_2O_2 as the minimum and maximum responses, respectively, in either the cytosol (A) or mitochondria (H) are shown. Values are presented as mean ± SE (*n ≥ 3, *where* n *consists of an average of 10 distinct cells' responses), * indicates statistical significant change compared to control by linear regression. Representative cells expressing basal catalase levels (B, E, I, L), cyto-catalase (C, F, J, M), or mito-catalase (D, G, K, N) shown at baseline (min 4; B-D, I-K) and following Zn^{2+} stimulation (min 14; E-G, L-N). Fluorescence intensity color is shown as a function of increasing E_{GSH}, also interpreted as an increase in GSSG to GSH:* ***low*** E_{GSH} ***high*** E_{GSH}.

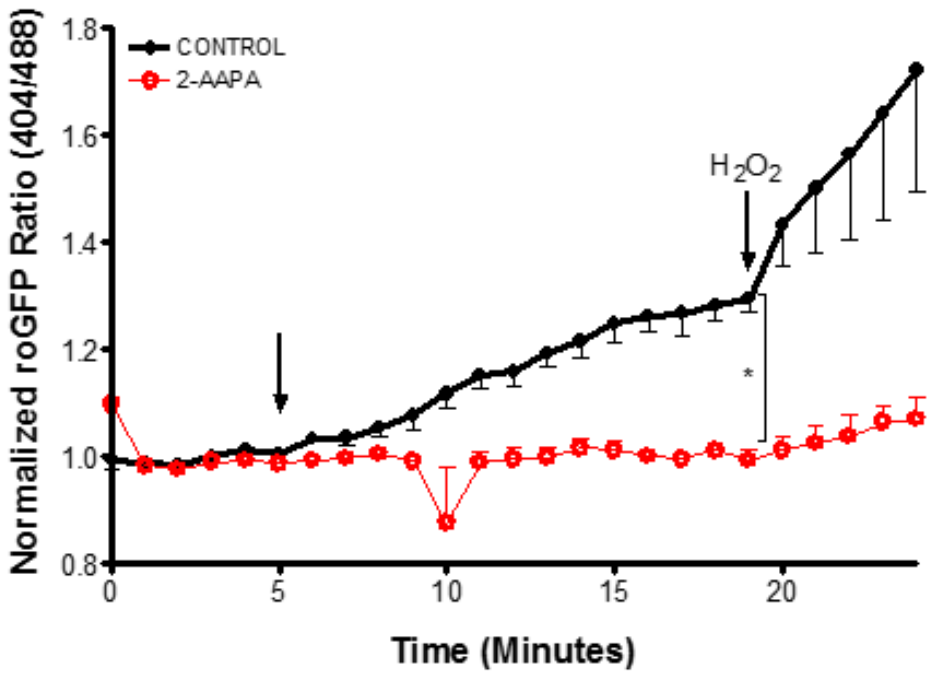

Figure 12: Zn^{2+} -induced changes in E_{GSH} are properly reported by roGFP2*. BEAS-2B cells stably expressing cyto-roGFP2 were incubated with either 100 µM 2-AAPA, a glutaredoxin inhibitor, or DMSO as a vehicle control (CONTROL) for 2 hrs. Cells were exposed to 50 µM Zn^{2+}/1 µM PYRI after five min of baseline measurements. Shown are ratiometric values (404/488) calculated from the fluorescent 525/30 emission of the 404 nm and 488 nm laser excitation normalized to baseline. To validate sensor response, cells were exposed to 1 mM H_2O_2 (min 20). Values are presented as mean ± SE (*n = 4, *where* n *consists of an average of 10 distinct cells' responses), * indicates statistical significant change compared to control by linear regression.*

2.3.3: E_{GSH} Changes Do Not Mediate HO-1 Gene Expression

In order to link the Zn^{2+}-induced oxidative stress observed in the live cell imaging experiments to a downstream cellular response, we measured the level of expression of the HO-1 gene in BEAS-2B cells exposed to ZnP. In order to avoid potential cytotoxicity associated with exposure to Zn^{2+} for the prolonged periods required to observe changes in mRNA levels, BEAS-2B cells were exposed to ZnP for 5 min, a time point that coincides with the plateau of the observed Zn^{2+}-induced H_2O_2 and E_{GSH} increases (Figures 6 and 11). This short exposure to ZnP was followed by washing and a 2 hr incubation in culture medium without added Zn^{2+} or PYRI.

As seen in Figure 13 (black bars), this "pulse" exposure to 0-10 µM ZnP resulted in concentration-dependent increases in HO-1 gene expression that were up to 45-fold over control levels exposed to 1 µM PYRI alone.

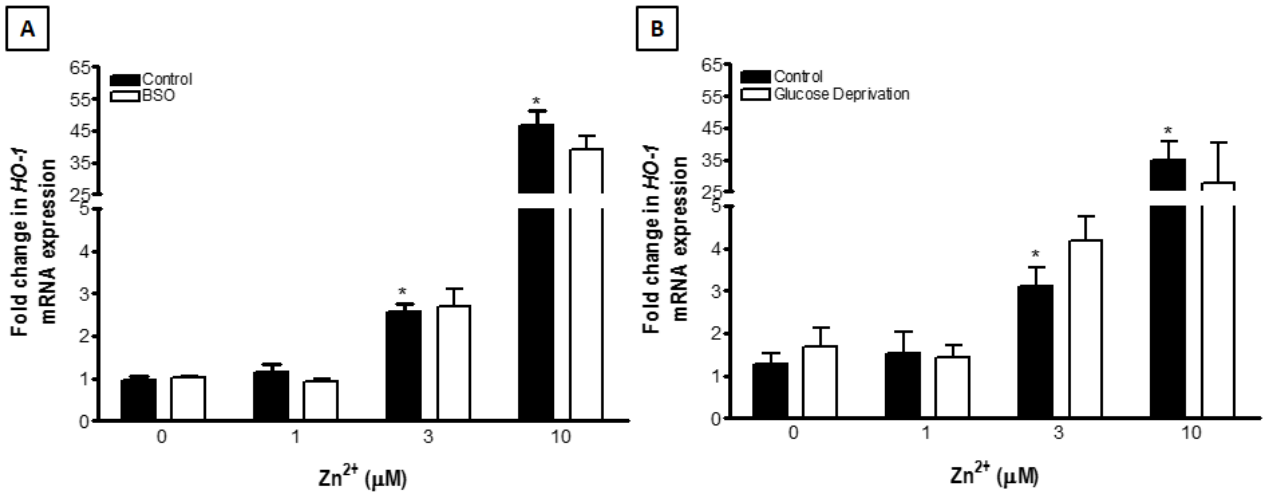

Figure 13: Depletion of glutathione levels does not alter Zn^{2+}-induced HO-1 mRNA levels*. BEAS-2B cells were incubated with 500 µM BSO for 24 hrs (A) or glucose deprived for 2 hrs (B) before exposure to the indicated Zn^{2+} concentrations and 1 µM PYRI for 5 min followed by 2 hrs in media alone. HO-1 mRNA levels were normalized to levels of GAPDH mRNA and expressed as fold increases over basal mRNA expression in control cells. Values are presented as mean ± SE (*n = 3), * indicates $p < 0.05$ as determined by ANCOVA compared to 0 µM Zn^{2+} exposure.*

To examine the role of E_{GSH} in mediating Zn^{2+} induced HO-1 expression, we next sought to sensitize the HAEC to ZnP exposure by decreasing the total glutathione content in the cells with the gamma-glutamyl synthetase inhibitor BSO. Treatment of BEAS-2B cells with 500 µM BSO alone for a 24-hr period reduced the total glutathione pool by 80% and concomitantly increased GSSG by 40% (data not shown). Treatment of BEAS-2B cells with BSO alone produced only a small 1.61±0.06-fold increase in baseline HO-1 gene expression (Figure 14), and had no effect on the subsequent HO-1 response to ZnP exposure ($p=0.07$, $F=3.6$, Figure 13A).

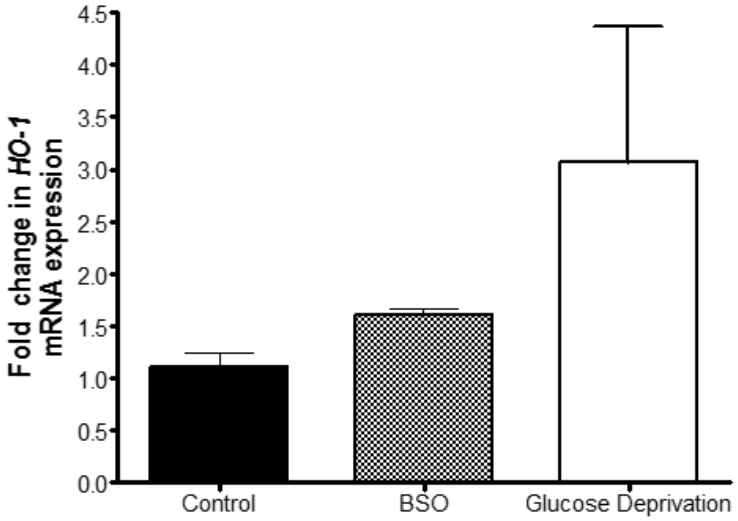

Figure 14: Depletion of glutathione levels does not lead to HO-1 mRNA expression in BEAS cells*. BEAS-2B cells were incubated with 500 µM BSO for 24 hrs or starved of glucose for 2 hrs before, washed, and then incubated for two hrs in fresh media before mRNA extraction. BSO only imposed a 1.61±0.06 (SEM) fold increase over untreated cells. Glucose deprivation induced a 3.08±1.29 (SEM) fold increase in HO-1 gene expression. mRNA levels measured using TaqMan-based RT-PCR were normalized to levels of GAPDH mRNA and expressed as fold increases over basal mRNA expression*

Since impairing glutathione synthesis was relatively ineffective at inducing HO-1, we next determined the effect of decreasing intracellular stores of NADPH through glucose deprivation alone and followed by ZnP -exposure on HO-1 expression. A 2 hr incubation in glucose-free media did not alter the total glutathione pool or change the GSH to GSSG ratio (data not shown) and induced a modest 3.08±1.29 fold increase in HO-1 expression (Figure 14), but failed to alter HO-1 expression induced by Zn^{2+} ($p=0.24$, $F=1.5$, Figures 13B).

2.3.4: H_2O_2 Mediates Zn^{2+}-Induced Adaptive Gene Expression

The role of H_2O_2 in Zn^{2+}-induced HO-1 response was examined by utilizing the compartment-specific catalase expression strategy employed in the imaging studies. Catalase overexpression in the cytosol blunted Zn^{2+}-induced HO-1 gene expression across the range of ZnP exposures tested (10-50 µM) by approximately 50% (p=0.04, F=4.5, Figure 15A). Similarly, ectopic catalase expression in the mitochondria halved the HO-1 expression induced by the higher (30 and 50 µM) ZnP exposures (p=0.002, F=10.5, Figure 15B).

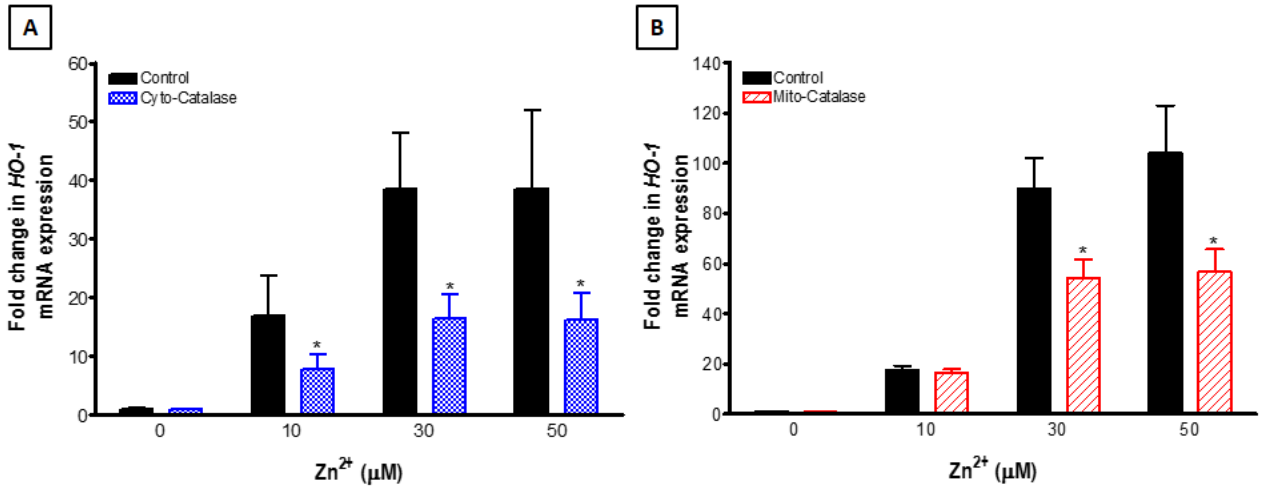

Figure 15: Targeted catalase expression blunts Zn^{2+}-induced HO-1 mRNA levels in BEAS cells. *BEAS-2B cells overexpressing catalase (A) or expressing ectopic mitochondrial catalase (B) were exposed to the indicated Zn^{2+} concentrations and 1 µM PYRI for 5 min followed by 2 hrs in media alone. HO-1 mRNA levels were normalized to levels of GAPDH mRNA and expressed as fold increases over basal mRNA expression in control cells. Values are presented as mean ± SE (n = 3), * indicates $p < 0.05$ as determined by ANCOVA compared to control.*

2.4: Discussion

The findings of this study add to previous work demonstrating the pathophysiological effects of Zn^{2+} exposure on HAEC (191,193,194,204,205). Zn^{2+} can activate signaling through direct interaction with critical cysteine residues in regulatory proteins such as Keap1 (115) or PTP (44). However, as described in our previous study (196) Zn^{2+} also acts as an oxidant

stressor by inducing mitochondrial production of H_2O_2, a ROS implicated in multiple physiological signaling processes, including the induction of Keap1 and the redox regulation of PTP (120,206,207). The present study investigated the role of H_2O_2 as a mediator of Zn^{2+}-induced oxidant responses. We show that a brief "pulse" exposure to Zn^{2+} is sufficient to commit cells to activate signaling mechanisms which lead to the induction of adaptive gene expression. Using compartment-specific expression of catalase as an interventional approach we show that Zn^{2+} acts through both H_2O_2-dependent and -independent mechanisms to induce HO-1 expression and increases in cytosolic E_{GSH}.

As recently reviewed (41), a number of studies demonstrate that Zn^{2+} exposure induces mitochondrial dysfunction through multiple mechanisms, including impairment of mitochondrial respiration through inhibition of the α-ketoglutarate dehydrogenase complex (208) and loss of mitochondrial membrane potential (209). Of specific interest is the inhibition of cytochrome c oxidase activity, which can result in blocked electron transport and production of superoxide from complexes I and III (47). In the present study, imaging-based experiments to monitor H_2O_2 levels and E_{GSH} in real time, combined with a validated strategy in the form of catalase overexpression, provide a compartment-specific perspective on the oxidative effects of Zn^{2+} exposure. These data add to the weight of evidence supporting the notion that Zn^{2+} exposure impairs mitochondrial metabolic processes leading to increased production of H_2O_2 and changes in E_{GSH} in HAEC.

Our observation that H_2O_2 scavenging with either cytosolic or mitochondrial catalase blunted Zn^{2+}-induced cytosolic E_{GSH} changes shows a H_2O_2-dependent effect of Zn^{2+} on the cytosolic glutathione pool. It is also noteworthy that Zn^{2+} is a known inhibitor of glutathione reductase (210), an effect that could potentially contribute to an accumulation of oxidized

glutathione and increase E_{GSH}. The fact that increasing the E_{GSH} with the gamma-glutamyl synthetase inhibitor BSO does not result in significant elevations in HO-1 mRNA levels argues that the peroxide-dependent effect of Zn^{2+} on E_{GSH} does not carry through to the activation of signaling pathways that regulate HO-1 expression. This interpretation is consistent with the fact that manipulation of E_{GSH} with BSO failed to induce significant HO-1 expression. In this regard, it is interesting that a 2 hr glucose deprivation, which was previously demonstrated to potentiate E_{GSH} increases induced by ozone exposure in BEAS-2B cells (140), was ineffective in promoting HO-1 expression by itself. Thus, neither BSO pretreatment nor glucose deprivation potentiated Zn^{2+}-induced HO-1 expression. Taken together, these findings argue that Zn^{2+}-induced increase in E_{GSH} is not sufficient to activate signaling leading to HO-1 expression (Figure 8). This agrees with the previous proposed view that a change in E_{GSH} should not be considered as an effector but rather as an indicator of intracellular redox metabolism (91). On the other hand, the inability of catalase expression to ablate Zn^{2+}-induced changes in E_{GSH} completely, suggests the presence of a H_2O_2-independent mechanism that may involve direct Zn^{2+} reactivity with unknown protein thiol targets.

The observation that HO-1 expression was only partially ablated by increased catalase expression in either the cytosol or mitochondria implies the presence of additional signaling mechanisms leading to Zn^{2+}-induced adaptive responses. It is well established that the Keap1/Nrf2/ARE signaling pathway regulates HO-1 expression and, therefore, it is plausible that signaling intermediates along this pathway are involved in the response to Zn^{2+} (112,114,211). One possibility is an effect on the nuclear transcription factor repressor Bach1, which is targeted for degradation in response to treatment with Zn-mesoporphyrin (212). In this regard, the notion that the combination of Zn^{2+} and PYRI used in this study might be a structural mimic of Zn-

mesoporphyrin is intriguing. However, utilizing the exposure conditions of the present study, experiments in our laboratory show ZnP does not affect Bach1 protein levels in HAEC (Speen, unpublished observations). Another possibility is presented by the presence of an active Zn^{2+}-binding or broadly "metal(loid)"-specific sensor in Keap1 (115). Preliminary studies in our laboratory using a version of Keap1 in which the amino acids that constitute the Zn^{2+} sensor have been substituted, suggest that direct Zn^{2+} binding of Keap1 is in fact a contributing mechanism leading to HO-1 expression in Zn^{2+}-exposed BEAS-2B cells (Silbajoris, unpublished). Additional studies will be needed in order to further characterize H_2O_2-independent pathways leading to adaptive gene expression in HAEC exposed to Zn^{2+}.

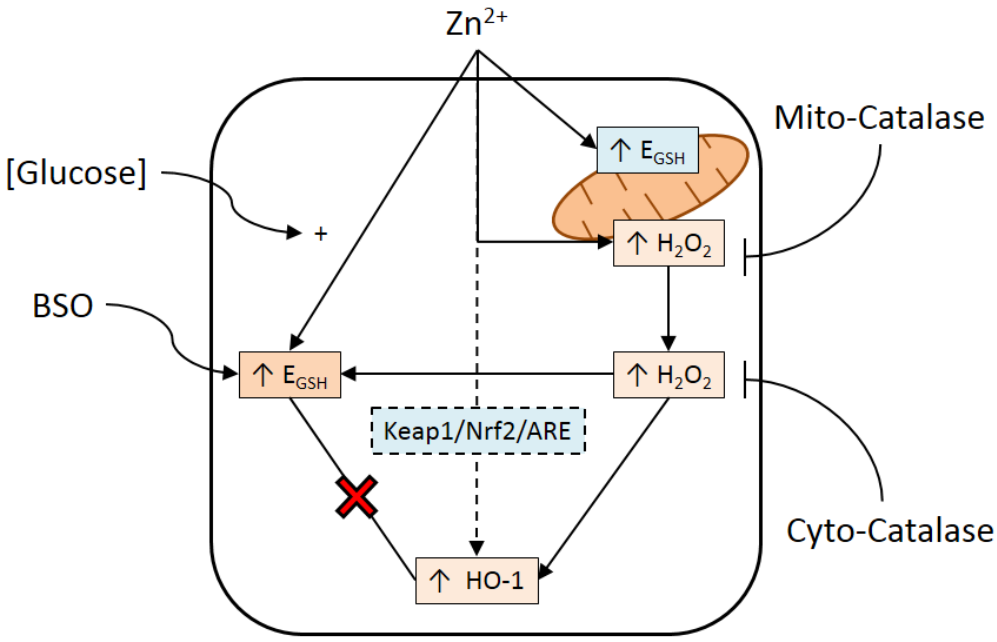

Figure 16: H_2O_2 mediates Zn^{2+}-induced increases in cytosolic E_{GSH} and HO-1 mRNA expression. *Zn^{2+} exposure induces mitochondrial H_2O_2 production and elevates E_{GSH} in both the cytosol and mitochondria. Cytosolic catalase overexpression (Cyto-Catalase) ablates H_2O_2 levels in the cytosol, and reduces cytosolic E_{GSH} and HO-1 mRNA expression. Ectopic catalase expression in mitochondria (Mito-Catalase) ablates H_2O_2 levels in the mitochondria and cytosol as well as reduces cytosolic E_{GSH} and HO-1 mRNA expression. Increased E_{GSH} does not induce HO-1 mRNA expression as determined through BSO and glucose deprivation ([Glucose]) treatments. Dashed line indicates hypothesized mechanism accounting for H_2O_2-independent Zn^{2+}-induced HO-1 mRNA expression*

2.5: Summary

The data presented in this study demonstrate that H_2O_2 is a critical mediator of the oxidative effects induced by exposure to Zn^{2+} as schematized in Figure 16, while revealing the presence of multiple contributing pathways leading to adaptive responses by HAEC. These findings are relevant to understanding the mechanistic basis and potential mitigation of the adverse health effect of Zn^{2+} inhalation.

CHAPTER 3: Protein Sulfenylation: A Novel Readout of Environmental Oxidant Stress[2]

3.1: Introduction

A number of epidemiological and toxicological studies conducted in recent years have shown an association between exposure to diesel exhaust and the incidence of cardiovascular and respiratory morbidity and mortality (19,213-217). Diesel exhaust is a complex mixture of gaseous and particulate components, including pro-oxidant organic compounds such as quinones (53). Quinones, as exemplified by 1,2-naphthoquinone (1,2-NQ), are toxic via two mechanisms of action. 1,2-NQ can covalently adduct with macromolecules through Michael addition (52,54,55). It can also undergo redox cycling reactions to form reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) that contribute to oxidative stress (56,57). Investigations to date have predominantly focused on the effect of 1,2-NQ adduction, while the biological ramifications of 1,2-NQ-induced oxidative stress have yet to be fully investigated.

Oxidative stress has been identified as a mechanistic feature of a broad range of environmental exposures (102). Evidence of environmental oxidative stress typically invovles the analysis of the oxidant-damaged biomolecules, such as the oxidation of protein thiols to the sulfonates (Figure 17) as well as the formation of ROS such as H_2O_2. However, there is a growing appreciation that intracellular H_2O_2 is a closely regulated second messenger with pivotal

[2] This chapter previously appeared as an article in the Journal of Chemical Research in Toxicology. The original citation is as follows:
Wages, P., Lavrich, K., Zhang, Z., Cheng, W., Corteselli, E., Gold, A., Bromberg, P., Simmons, S. & Samet, J. "Protein Sulfenylation: A Novel Readout of Environmental Oxidant Stress." Chemical Research in Toxicology 2015; 28(12):2411-8.

roles in cellular processes ranging from the regulation of cytoskeletal function to bioenergetics and signaling (63,84,85,96,134). Specifically, H_2O_2 has been shown to hydroxylate cysteinyl thiols to form protein sulfenic acids. Sulfenylation of cysteines is now regarded as a critical step in the formation of inter- and intramolecular disulfide bonds as well as the formation of mixed disulfides with glutathione (Figure 17). Importantly, these formed disulfides can be reduced to the basal thiol level through the activity of proteins like glutaredoxin or thioredoxin supporting the role of thiol oxidation as a hub of redox signaling (85,96). The oxidation of protein cysteinyls to sulfenates has been directly linked to the regulation of signaling and metabolic processes (93,120,171,218-221).

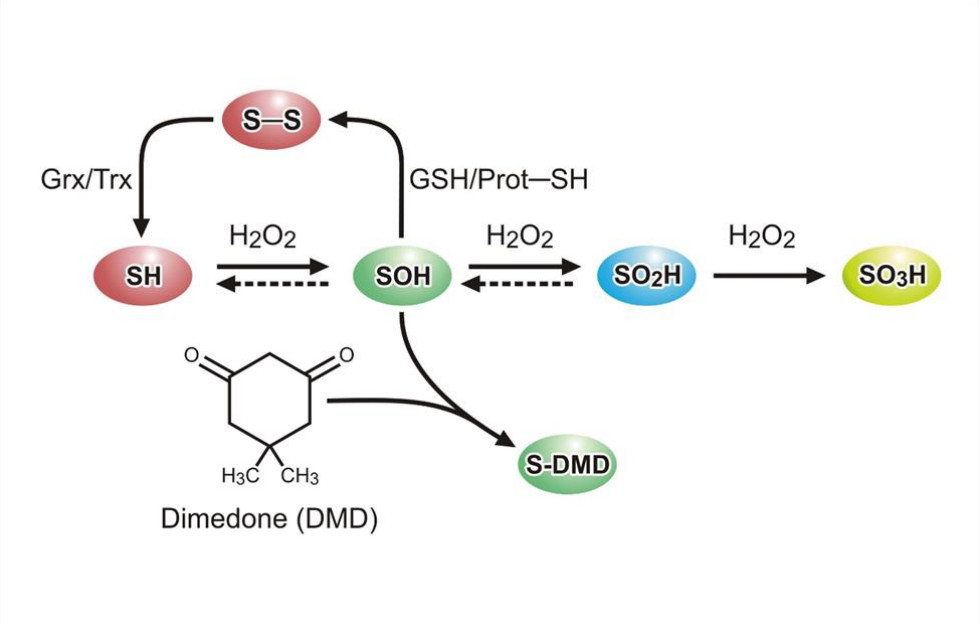

Figure 17: Schematic of cysteinyl-thiol post-translational modifications*. Dashed line indicates possible reversibility of thiol oxidation. Dimedone (shown) and dimedone-like molecules, including DAz-2 and DYn-2, specifically and irreversibly label sulfenic acids (SOH). Glutaredoxin (Grx) and thioredoxin (Trx) reduce disulfide bonds or mixed disulfide bonds with glutathione to the thiol. Continual presence of H_2O_2 hyperoxidizes the sulfenic acid to the sulfinic (SO_2H) and sulfonic (SO_3H) acids, which are largely irreversible under physiological conditions.*

The inventory of cellular proteins known to be sulfenylated, recently referred to as the "sulfenome," continues to expand with the development of new techniques and analytical

strategies (119,173,174,222). Yet, to our knowledge the effect of an environmental exposure on protein sulfenylation has not been investigated. We report here that the environmentally relevant exposure to 1,2-NQ induces sulfenylation of regulatory proteins in human airway epithelial cells (HAEC).

3.2: Materials and Methods

3.2.1: Reagents

Tissue culture media and supplements were purchased from Lonza (Walkersville, MD). Phenol red-free keratinocyte basal medium (KBM) was acquired from Cell Applications, Inc. (San Diego, CA). Adenoviral vectors were obtained from the Gene Therapy Center Virus Vector Core Facility (University of North Carolina at Chapel Hill, Chapel Hill, NC). The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): hydrogen peroxide (H_2O_2), 1,2-naphthoquinone (1,2-NQ), Me_2SO, dimedone, DTT, *tert*-butyl 2,2,2-trichloroacetimidate (TBTA), copper sulfate (Cu_2SO_4), sodium ascorbate (SA). Biotin azide (PEG4 carboxamide-6-azidohexanyl biotin) was obtained through Life Technologies (Grand Island, NY). Basic laboratory supplies were obtained from Fisher Scientific (Raleigh, NC).

3.2.2: Cell Culture

SV40 large T antigen-transformed HAEC (BEAS-2B, subclone S6, passage 54-60) were cultured as previously described (201) in keratinocyte growth medium (KGM). Cells at 80% confluence were deprived of growth factors overnight by changing cell media to KBM. Cells were exposed to KBM containing H_2O_2 or 1,2-NQ (dissolved in Me_2SO, final Me_2SO concentration <1:1000) at the indicated concentrations for ten min or as otherwise noted. Cells

were then quickly rinsed with PBS and then labeled in KBM with 5 mM dimedone or dimedone analog (DYn-2 or DAz-2) with Me_2SO at 1:200 dilution for one h.

3.2.3: Viral Transduction

Plasmid for the genetically encoded H_2O_2 sensor, HyPer, was purchased from Evrogen (Axxora, Farmingdale, NY). The genetically encoded pH sensor, SypHer, was created as described by Poburko et al.(223) through a single point mutation of HyPer at Cys199Ser. Both plasmids were introduced into lentiviral vectors as described previously (56). Stable expression of Hyper and SyPher in the cytosolic compartment of BEAS-2B cells was accomplished using lentiviral transduction. For cytosolic catalase overexpression, cells were transduced with an adenoviral vector encoding human catalase driven by a cytomegalovirus promoter at a MOI of 500 for 4 h, followed by a 1-day incubation in KGM(202). The pH-specific fluorogenic sensor pHred, created by the laboratory of Yellen (152), was obtained as a construct through Addgene (Cambridge, MA) for expression in BEAS-2B cells via transient transfection of 1-2 μg plasmid DNA using the suggested X-tremeGENE 9 protocol (Roche Applied Science, Indianapolis, IN).

3.2.4: Detection of Intracellular Sulfenylation

3.2.4.1: Staudinger Ligation

Cells labeled with DAz-2 underwent Staudinger Ligation for fluorescent detection utilizing the recommended protocol of the Sulfenylated Protein Cell-Based Detection Kit (Cayman Chemical, Ann Arbor, MI). Labeled cells were then detected by fluorescence microscopy using a Nikon Eclipse C1si with laser excitation at 488 nm and 525/30 nm emission with identical laser settings. Images were then imported to ImageJ (NIH, Bethesda, MD) for

Lookup Table editing to enhance visual determination of fluorescence by converting images to 16-bit monochromatic images, implementing Green Lookup Table edited to an interpolated 4 color table (0 Green, 150 Green, 175 Green, 255 Green) followed by a median filter of 2.0 pixel radius.

3.2.4.2: Immunoblotting

Cells labeled with dimedone were washed three times with ice-cold PBS and lysed in a mild-detergent buffer—1% NP40, 150 mM tris-HCl, pH 7.4, 150 mM NaCl, 5 mM DTT supplemented with Calbiochem protease inhibitor cocktail set III (EMD Millipore, Temecula, CA)—for 20 min and then centrifuged at 4˚C, 12000g for 10 min. Supernatant was collected and normalized for protein concentration via Bradford assay. Samples were prepared for Western Blotting with 4X Laemmli Sample Buffer and boiled for 10 min before loading into Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) alongside Precision Plus Protein Kaleidoscope Standards (Bio-Rad) and then gel electrophoresed for size separation. Gels were transferred using the Trans-Blot Turbo Transfer System onto nitrocellulose membranes (Bio-Rad). Membranes were then blocked with 5% Milk in TBST for one h at room temperature followed by incubation with primary antibody overnight at 4˚C, then secondary antibody incubation for one h at room temperature. The following antibodies were used: anti-sulfenic acid modified cysteine (2-Thiodimedone-Specific Ig) antibody (Millipore), and anti-GAPDH (6C5), anti-Catalase (A-7), goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP (all from Santa Cruz, Dallas, TX). After antibody incubation, membranes were set in Clarity Western ECL Blotting Substrate for 5 min followed by detection with LAS-3000 FujiFilm Imager.

3.2.4.3: Copper Catalyzed Azide Alkyne Cyclo-addition

Cells labeled with DYn-2, which was prepared as described by Paulsen et al(171), were washed three times with ice cold PBS, then lysed with mild-detergent buffer described in *Immunoblotting* for 20 min and centrifuged at 4˚C, 12000g for 10 min. Protein supernatant was normalized to 1.5 mg/mL and pre-cleared of endogenous biotin by agitation in a 150 µL slurry of Pierce NeutrAvidin agarose (N-agarose, Life Technologies). Cleared samples were labeled with biotin using copper catalyzed azide alkyne cyclo-addition (CuAAC) by agitation for one h in a buffer containing the following (final concentrations): biotin azide (0.2 mM), TBTA (0.1 mM), Cu_2SO_4 (1.0 mM), SA (1.0 mM). An aliquot of each sample was immunoblotted for total sulfenylation probed against Pierce High Sensitivity NeutrAvidin-HRP (N-HRP, Life Technologies). Remaining post-CuAAC sample was separated into equal aliquots for immunoprecipitation overnight at 4˚C with anti-GAPDH (6C5) (Santa Cruz) or anti-PTP1B (H-135) (Santa Cruz) followed by rotating for 2 h with Protein A-agarose. Samples were then prepared for immunoblotting and probed against N-HRP (Life Technologies), and anti-GAPDH (6C5) or anti-PTP1B (H-135) (both from Santa Cruz).

3.2.5: Detection of Recombinant Protein Sulfenylation

Deuterated-dimedone (d_6-dimedone) and iodo-dimedone (I-dimedone) were synthesized as described by Seo and Carroll (224). Recombinant GAPDH from rabbit muscle (Sigma-Aldrich) was reduced in solution of Tris-HCl (50 mM, pH 7.4) with 10 mM DTT on ice for 30 min and then purified with Micro Bio-Spin Columns Bio-Gel P-6 (Bio-Rad) before use in sulfenylation assays. 25 µM GAPDH was stimulated with a range of molar equivalents of H_2O_2 or 1,2-NQ dissolved in Tris-HCl (50 mM, pH 7.4) in the presence of 10 mM d_6-dimedone.

Samples were then reduced with DTT, isolated with P-6 column, and labeled with 20 mM I-dimedone. Samples of modified GAPDH were digested at 37 ^{o}C overnight using Trypsin Gold (Promega, Madison, WI) and *Rapi*Gest™ surfactant (Waters Corp., Milford, MA) according to manufacturer specifications. The resulting mixture was analyzed for the presence of modified peptides by injection onto an Agilent 1200 HPLC system (Milford, MA) coupled to an Agilent 6520 Accurate Mass Q-TOF mass spectrometer. Peptides were separated on a 150 mm x 2.1 mm Agilent PLRP-S 5 µm analytical column using a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 200 µL per min, and data were collected in positive mode while scanning from 100 m/z to 3200 m/z.

3.2.6: Live Cell Imaging

Immediately before exposure, HyPer or SypHer expressing cells were placed in KBM without phenol red. Fluorescence in cell cultures was imaged using a Nikon Eclipse C1si spectral confocal imaging system under illumination with 404, 488, or 561 nm primary laser lines (Nikon Instruments Corporation, Melville, NY). Sequential scans of each laser line were performed at a frequency of 60 s with 10 cells expressing the biosensor in the field of view, and results calculated as a ratio of the respective 525/30 nm emission for the 404 and 488 nm excitation of each sensor. Baseline fluorescence was established for 5 min prior to the addition of 0-10 µM 1,2-NQ. To normalize for variability in the dynamic range of the sensors expressed in individual cells, 100 µM H_2O_2 was added at min 30, 1 mM H_2O_2 was added at min 33, followed by 5 mM DTT at min 35. Data were expressed normalized to the maximum sensor response (% oxidized HyPer) or as raw ratiometric values normalized to baseline. HyPer cells expressing pHred were

analyzed similarly but with an additional excitation at 561 nm allowing for a ratiometric analysis of pH between the 404 and 561 nm excitations and 605/75 emission.

3.2.7: Statistical Analyses

All imaging data were quantified using NIS-Elements AR software (Nikon). Data are expressed as the mean ± SEM of at least three separate experiments. Statistical significance ($p>0.05$) of immunoblot results was determined through one-way ANOVA with Dunnett's post-test. PRISM (Graphpad Software, La Jolla, CA) was used for statistical analyses.

3.3: Results

3.3.1: Exposure to 1,2-Naphthoquinone Induces Protein Sulfenylation in BEAS-2B Cells

Dimedone is a cell permeable molecule that can be used to label sulfenic acids specifically and irreversibly (Figure 17). A number of dimedone analogs have been generated to meet a range of analytical goals (137). We used an azide based dimedone derivative, Daz-2, to biotinylate protein sulfenic acids using a commercially available assay that enables their detection as a fluorescent readout in fixed BEAS-2B cells exposed to 3-100 µM 1,2-NQ. As shown in Figure 18A, exposure of BEAS-2B cells to concentrations of 1,2-NQ as low as 3 µM for 10 min resulted in a marked increase in the concentration of protein sulfenylation, the magnitude of which approximated that induced by exposure to 1000 µM H_2O_2. In accord with a previous report (56), exposure of BEAS-2B cells to 0-100 µM 1,2-NQ for 10 minutes did not result in cytotoxicity.

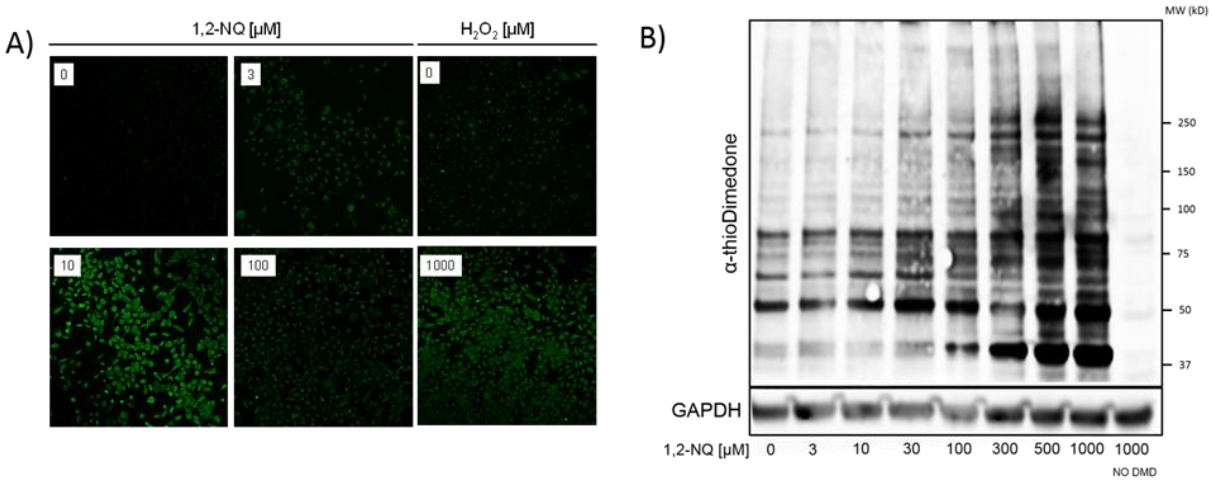

Figure 18: 1,2-naphthoquinone induces intracellular protein sulfenylation*. (A) BEAS-2B cells were exposed to H_2O_2 (0-1000 μM) or 1,2-NQ (0-100 μM) for 10 minutes, labeled with DAz-2 followed by conjugation to FITC by Staudinger Ligation. (B) BEAS-2B cells were exposed to the indicated concentration of 1,2-NQ for 10 min and then immunoblotted for sulfenylation using α-thiodimedone antibody. GAPDH was utilized as loading control.*

In order to assess the range of proteins sulfenylated in response to 1,2-NQ exposure, we next subjected protein extracts of BEAS-2B cells treated with 3-1000 μM 1,2-NQ for 10 minutes to immunoblotting using an antibody that detects the dimedone-protein thioether complex (225). The results (Figure 18B) show that 1,2-NQ induces a dose-dependent increase in protein sulfenylation of multiple proteins varying in molecular weight between 37 and 250 kD.

3.3.2: 1,2-Naphthoquinone-Induced Protein Sulfenylation is Dependent on H_2O_2

Sulfenylation of proteins can occur by the reaction of peroxynitrite, hypohalous acids, haloamines, and hydroperoxides with cysteine residues (94). We have previously reported that exposure to environmentally relevant concentrations of 1,2-NQ results in elevation of H_2O_2 levels in BEAS-2B cells (56). We therefore examined the role of H_2O_2 generation in 1,2-NQ-induced protein sulfenylation in BEAS-2B cells. In agreement with our previous studies using the H_2O_2 sensor, HyPer, 10 μM 1,2-NQ induced a rapid increase in cytosolic H_2O_2 (Figure 19).

The responsiveness of HyPer to H_2O_2 is known to be influenced by pH. We, therefore, conducted control experiments using BEAS-2B cells expressing the pH sensors SypHer or pHred, which showed that the HyPer fluorescence intensity changes observed in response to 10 µM 1,2-NQ over a 30 minute time period were not attributable to changes in cytosolic pH (Figure 19).

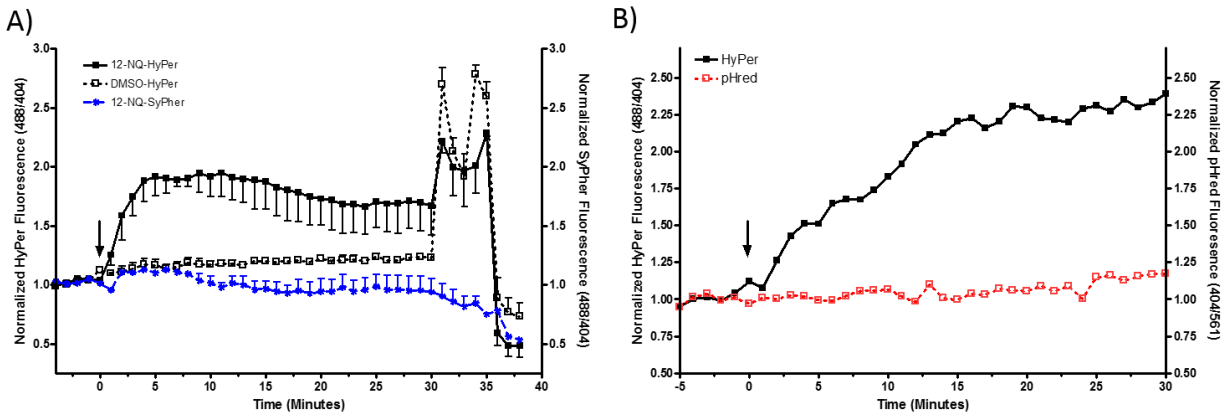

Figure 19: 1,2-naphthoquinone induces a rapid increase in cytosolic intracellular H_2O_2. *(A) 10 µM 1,2-NQ (black square) or respective vehicle control (DMSO, open square) was added at minute 0 as indicated by arrow to BEAS-2B cells expressing the H_2O_2-specific fluorogenic sensor, HyPer. 10 µM 1,2-NQ was similarly added to BEAS-2B cells expressing the pH-specific fluorogenic sensor, SyPher (blue circles). 100 µM H_2O_2 was added at minute 30, 1 mM H_2O_2 was added at minute 33, and 5 mM DTT was added at minute 35 to determine sensor dynamic range. The normalized ratio to baseline is shown obtained from an average of ten individual cells for each experiment. Error bars indicate SEM (n=3). (B) 10 µM 1,2-NQ was added at minute 0, as indicated by arrow, to BEAS-2B cells coexpressing the H_2O_2-specific fluorogenic sensor, HyPer and the pH-specific fluorogenic sensor, pHred. The normalized ratio to baseline is shown obtained from an average of five individual cells.*

We next examined the role of H_2O_2 in 1,2-NQ-induced protein sulfenylation by increasing the rate of catabolism of H_2O_2 by overexpressing catalase in BEAS-2B cells. Catalase overexpression ablated the increase in H_2O_2 concentrations induced by treatment of BEAS-2B cells with either 3 or 10 µM 1,2-NQ throughout the experiments, confirming the efficacy of catalase overexpression as a means to blunt H_2O_2 levels induced by 1,2-NQ exposure in BEAS-2B cells. Immunoblotting assays showed that catalase overexpression blunted protein sulfenylation induced by treatment of BEAS-2B cells with 3 or 10 µM 1,2-NQ (Figure 20). We

then examined the temporal relationship between 1,2-NQ-induced production of H_2O_2 measured using HyPer and protein sulfenylation levels quantified using immunoblotting. Exposure to 10 µM 1,2-NQ induced a rapid peak in protein sulfenylation that coincided with the sharp rise in cytosolic H_2O_2 levels reported by HyPer (Figure 21). Protein sulfenylation then appeared to decrease at a rate faster than that observed for H_2O_2 concentrations, although protein sulfenylation rebounded with 10 µM 1,2-NQ (but not with 3 µM) reaching its highest level at 30 min of exposure. Supporting the earlier experiments (Figure 20) BEAS-2B cells overexpressing catalase showed diminished levels of protein sulfenylation induced by exposure to 3 or 10 µM 1,2-NQ (Figure 21). These results established that 1,2-NQ induced protein sulfenylation depends on the generation of H_2O_2 in BEAS-2B cells.

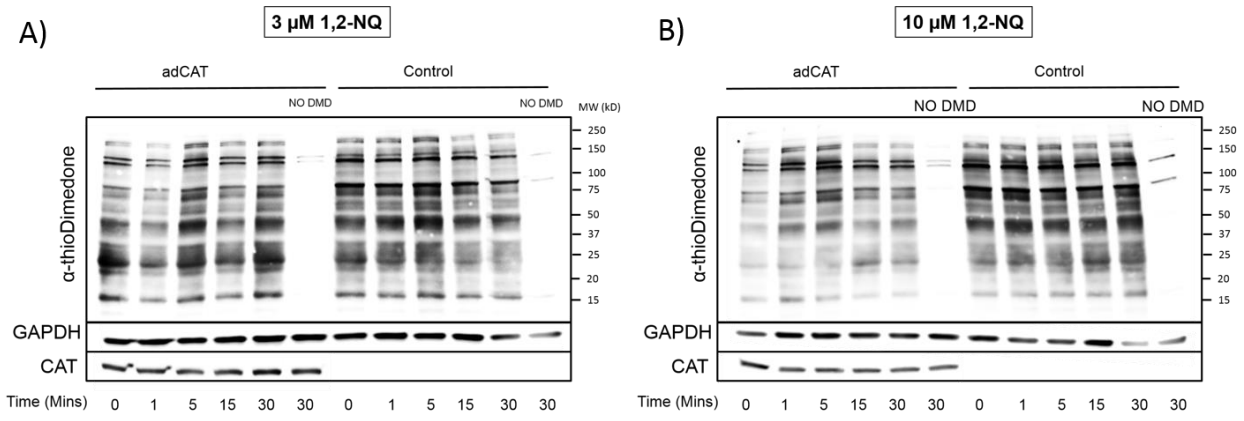

Figure 20: Catalase overexpression blunts 1,2-napthoquinone-induced protein sulfenylation. *Control BEAS-2B cells or BEAS-2B overexpressing catalase (adCAT) were exposed to 3 µM (A) or 10 µM (B) 1,2-NQ for the indicated time periods, labeled with dimedone and then harvested for immunoblotting. Lysates were blotted against thio-dimedone antibody, GAPDH, or catalase (CAT). Data shown are representative of three separate experiments, wherein the average densitometry of results are indicated in Figure 21 (Squares).*

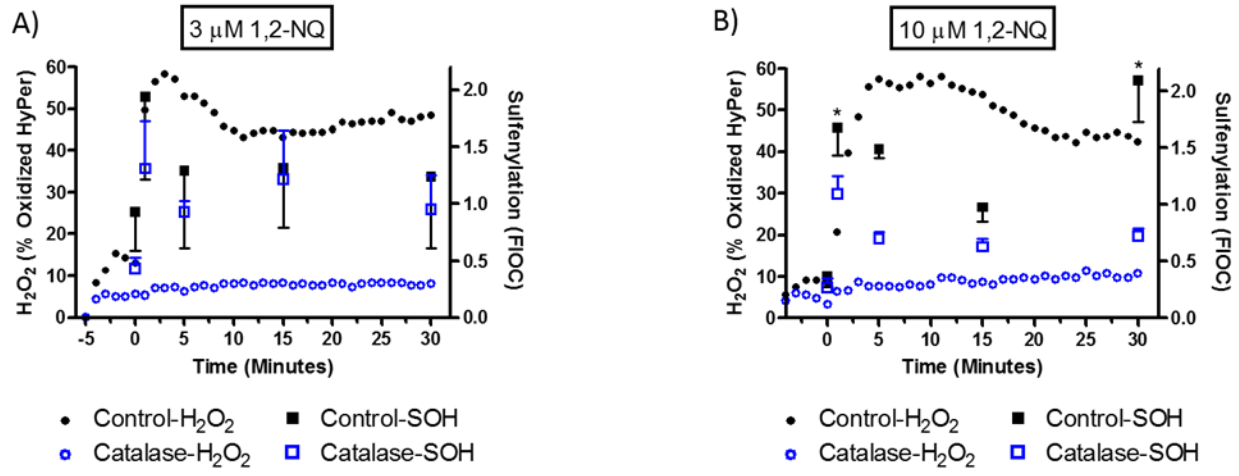

Figure 21: 1,2-naphthoquinone induced protein sulfenylation is H_2O_2 dependent. *Control BEAS-2B cells (black squares) and BEAS-2B cells overexpressing catalase (blue open squares) were exposed to 3 µM (A) or 10 µM (B) 1,2-NQ for the indicated time, labeled with dimedone and harvested for immunoblotting to detect protein sulfenylation (SOH). Values are presented as mean ± SEM (n=3), * indicates $p < 0.05$ compared to control. Cytosolic H_2O_2 was monitored in BEAS-2B cells expressing HyPer (black circles) exposed to 3 µM (A) or 10 µM (B) 1,2-NQ over a time course of 30 minutes. HyPer-expressing cells overexpressing catalase (blue open circles) were exposed in the same manner. Intracellular H_2O_2 levels are reported every minute as an average of the % maximum oxidation of HyPer induced by 1,2-NQ exposure (n=3, error bars omitted for clarity).*

3.3.3: 1,2-Naphthoquinone-Induces Sulfenylation of Regulatory Proteins

In order to examine the effect of 1,2-NQ on the sulfenylation of specific proteins of interest in BEAS-2B cells, we used a copper-catalyzed azide-alkyne cycloaddition (CuAAC) labeling strategy to biotinylate protein sulfenic acids, in order to gain the analytical sensitivity and specificity afforded by the strong avidin-biotin interaction (Figure 22A). CuAAC-based detection of protein sulfenylation in BEAS-2B showed elevations at concentrations as low as 1 µM 1,2-NQ for 10 minutes (Figure 22B-C). We next examined specific proteins sulfenylated as a result of 1,2-NQ exposure to BEAS-2B cells.

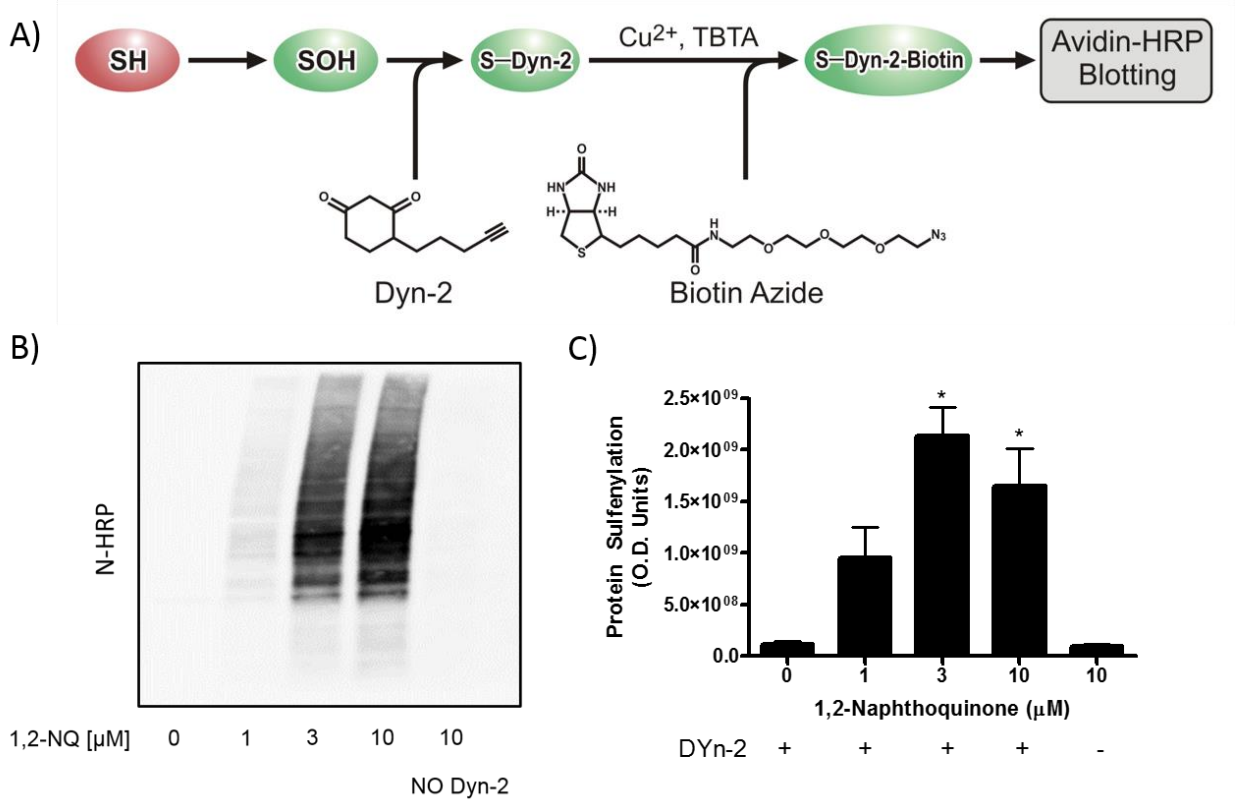

Figure 22: Detection of 1,2-naphthoquinone-induced protein sulfenylation through biotinylation in human airway epithelial cells*. (A) Biotynilation of protein sulfenic acids using copper catalyzed azide alkynyl cyclo-addition (CuAAC). (B) BEAS-2B cells were exposed to the indicated concentrations of 1,2-NQ for ten minutes followed by CuAAC and detected with neutravidin-HRP (N-HRP). (C) Shown is the average optical density relative to control of three separate experiments (X±SEM, *p < 0.05).*

We tested the effect of 1,2-NQ exposures on the sulfenylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) because of its integral role in cellular bioenergetics (226,227), and of Protein Tyrosine Phosphatase 1B (PTP1B) for its pivotal role in signaling processes (120,121). Levels of sulfenylation of GAPDH and PTP1B were measured in immunoprecipitates of the same protein extract samples prepared from BEAS-2B cells exposed to 10 μM 1,2-NQ using the CuAAC biotinylation protocol. BEAS-2B exposure to 1,2-NQ induced GAPDH sulfenylation that increased steadily over a 30-minute exposure time course (Figure 23A,C). In contrast, the level of sulfenylated PTP1B in the same samples attained a maximum at 1 minute of exposure and decreased rapidly thereafter (Figure 23B,D). The same

marked difference in the time-courses of 1,2-NQ-induced sulfenylation was observed when GAPDH and PTP1B were immunoblotted from the CuAAC-biotinylated protein fraction (Figure 24).

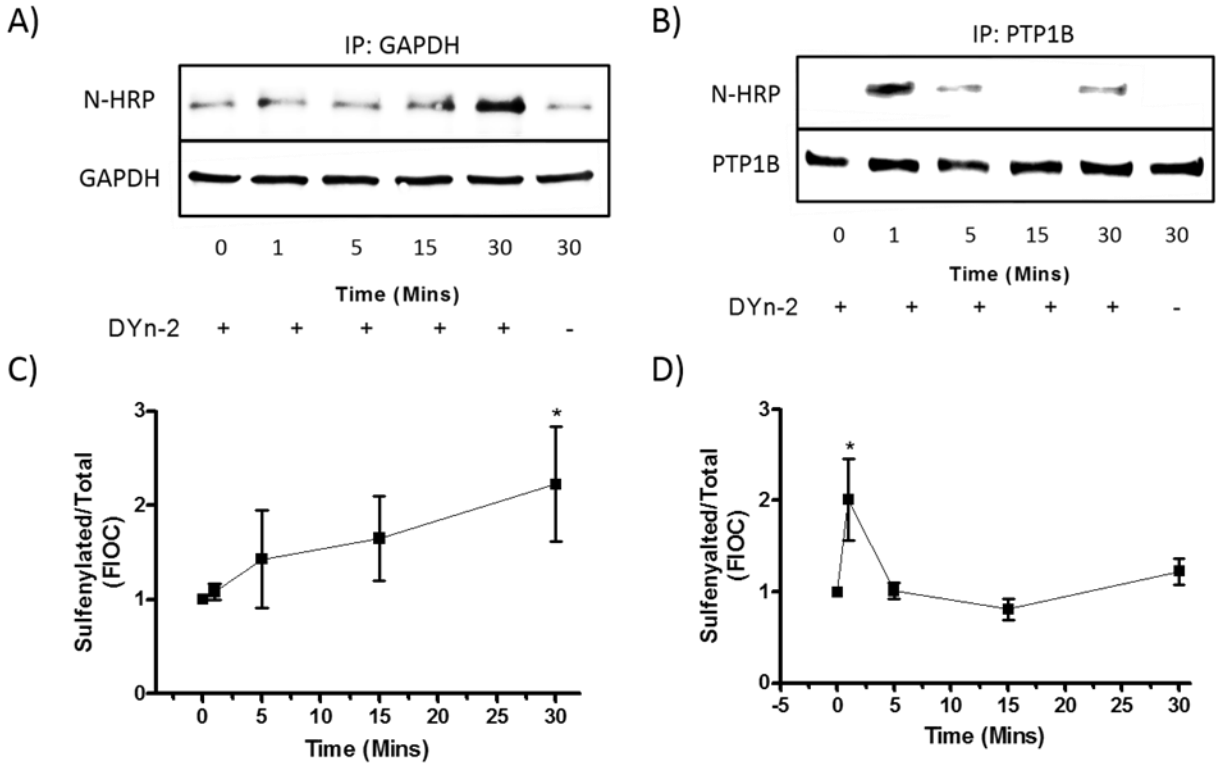

Figure 23: 1,2-naphthquinone-induces sulfenylation of regulatory proteins with differential kinetics. *BEAS-2B cells were exposed to 10 μM 1,2-NQ from 0-30 minutes followed by CuAAC. Lysates were subjected to immunoprecipitation using specific antibodies GAPDH (A, C) and PTP1B (B, D). Immunoblots were probed against neutravidin-HRP (N-HRP), then GAPDH (A) or PTP1B (B). Data are shown as a representative immunoblot of three experiments, quantified respectively as a ratio of the sulfenylated protein to total GAPDH and PTP1B, (C) N-HRP/GAPDH and (D) N-HRP/PTP1B, respectively. Values shown as mean ± SEM normalized to the unexposed control, * indicates $p < 0.05$ compared to control.*

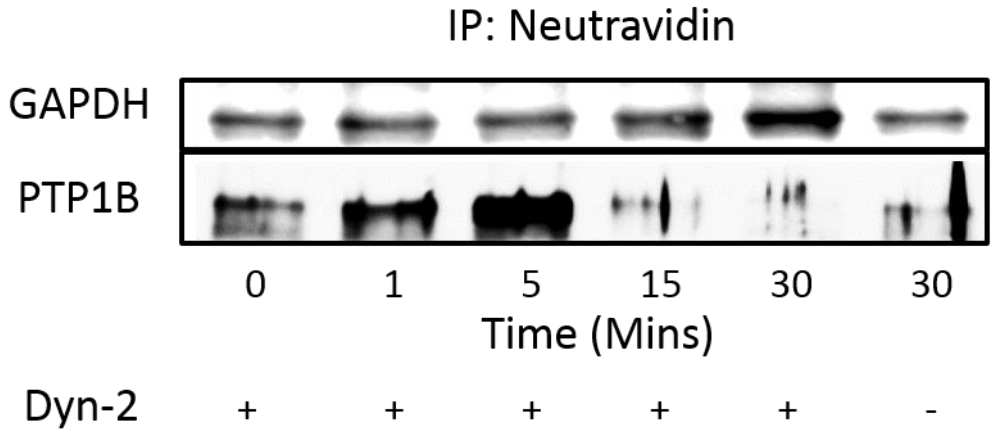

Figure 24: 1,2-naphthoquinone-induces sulfenylation of specific regulatory proteins with differential kinetics. *BEAS-2B cells were exposed to 10 μM 1,2-NQ from 0-30 minutes followed by CuAAC. Lysates were subjected to immunoprecipitation using neutravidin-agarose and then probed using specific antibodies against GAPDH and PTP1B.*

3.3.4: 1,2-Naphthoquinone Induces Sulfenylation of the Catalytic Cysteine (^{150}C) in GAPDH

In order to examine the site-specificity of 1,2-NQ-induced sulfenylation, we utilized an isotope labeling strategy devised by Seo and Carroll (224) to detect sulfenic groups in trypsin digests of recombinant GAPDH treated with 1,2-NQ *in vitro* (Figure 25A). The catalytic cysteine (^{150}C) of GAPDH serves as the peroxide-susceptible thiol that becomes sulfenylated upon oxidation, causing GAPDH to act through the glycolytic pathway (132). Mass spectrometric analysis of the isotopically coded, dimedone-labeled GAPDH peptides showed maximum sulfenylation of ^{150}C in GAPDH treated with 1.0 molar equivalents of 1,2-NQ. In contrast, H_2O_2 exposure induced increasing sulfenylation of ^{150}C with up to exposure to 2.0 molar equivalents (Figure 25B). We were able to confirm that even in cell free experiments 1,2-NQ was capable of generating H_2O_2 (Figure 26).

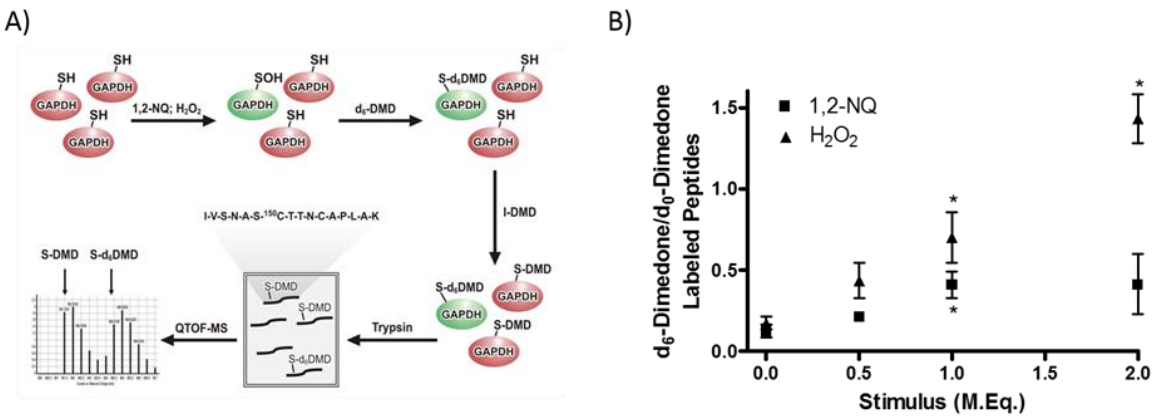

Figure 25: 1,2-naphthoquinone induces sulfenylation of the GAPDH catalytic cysteine. *(A) General scheme of isotope coded dimedone iodo-dimedone (ICDID) strategy for quantifying sulfenic acids relative to total thiols. Deuterated dimedone (d_6-DMD) labels all sulfenic acids, while subsequent treatment with iodo-dimedone (I-DMD) labels all remaining thiols (d_0-DMD). Samples are then trypsinized and then subjected to QTOF-MS analysis for the d_6/d_0 ratio of the indicated GAPDH peptide sequence (center) containing ^{150}C. (B) Recombinant GAPDH (25 µM) was incubated with either H_2O_2 or 1,2-NQ at indicated molar equivalencies and then subjected to ICDID and reported as a ratio of sulfenylated ^{150}C to reduced ^{150}C. Values are presented as mean ± SEM (n=3), * indicates $p < 0.05$ compared to control.*

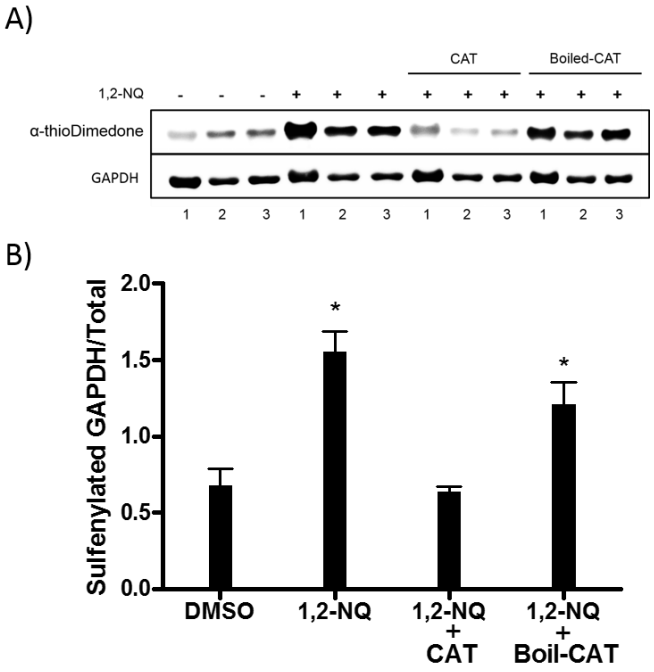

Figure 26: 1,2-naphthoquinone produces H_2O_2 in vitro to oxidize recombinant GAPDH. *(A) Recombinant GAPDH (25 µM) was incubated with either 1,2-NQ (50 µM) with active (CAT) or inactive catalase (Boiled-CAT) and then underwent dimedone labeling. Shown are three distinct experiments presented on the same immunoblot. (B) Results were then reported as a ratio of sulfenylated GAPDH to total (X ± SEM), * indicates $p < 0.05$ compared to control.*

3.4: Discussion

Toxicological studies have long equated oxidative stress with the production of ROS and damage to DNA, lipids, and proteins, leading to loss of function and cell death. However, there are now numerous examples of physiological redox reactions such as reversible cysteine sulfenylation that are involved in pivotal regulatory functions in the cell, from signaling to energy metabolism (84,219,221,228). These processes themselves represent potential targets of oxidant stress induced by xenobiotics. This study demonstrates that exposure to environmentally relevant concentrations of a ubiquitous redox-active environmental pollutant can induce H_2O_2-dependendent protein sulfenylation in a dose- and time-dependent manner.

Although 1,2-NQ toxicity has been thought to predominantly occur through covalent adduction (53), there is evidence to suggest that induction of oxidative stress is a major pathway of 1,2-NQ toxicity. Our previous study implicated 1,2-NQ-induced H_2O_2 generation in the induction of inflammatory gene expression, suggesting a mechanism of toxicity that does not require the adduction of proteins (56). The present study further supports the importance of 1,2-NQ induced H_2O_2, as it was shown to induce sulfenylation of thiols on regulatory proteins. Given the critical role of protein sulfenylation in many redox-dependent physiological processes (171,218,229), the inappropriate induction of sulfenylation by 1,2-NQ exposure represents a significant, previously unrecognized mechanism of cellular toxicity.

In general, redox-dependent signaling is associated with a classic negative-feedback process as the rapid activation of redox-dependent gene expression also leads to induction of expression of antioxidant mediators (63,97). Our finding of a biphasic response in total protein sulfenylation is in accord with the induction of redox-dependent gene expression observed in anticancer therapies such as ionizing radiation (230) and nitric oxide (231,232). The early

induction and subsequent decline of protein sulfenylation observed in this study may reflect the regulation of redox-dependent transcriptional activation via a biphasic response of signaling through protein sulfenylation. The second, later increase in protein sulfenylation resulting from 10 µM 1,2-NQ exposure may reflect loss of cell viability caused by prolonged exposure to 1,2-NQ.

GAPDH and PTP1B were selected in this study as examples of regulatory proteins whose activity are known to be controlled through cysteine thiol oxidation (118) and are also of toxicological importance as targets in cellular bioenergetics and inflammatory signaling, respectively. The differences in the time course of sulfenylation of GAPDH and PTP1B observed in this study were unexpected, as increasing H_2O_2 levels could be expected to oxidize GAPDH and PTP1B with similar efficacy. One possible explanation for the differential kinetics may be that some proteins become sulfenylated at different rates as a consequence of localized induction of intracellular H_2O_2 produced under different exposure conditions (57,233). Oxidants, including 1,2-NQ can also target cysteinyl thiols, most notably glutathione, that usually serve as a "first-line" of defense against ROS, allowing GAPDH and PTP1B to be more readily targeted (56,97). Thus more susceptible targets, including signaling regulators such as PTP1B that are subject to redox regulation in physiological contexts, may be oxidized more readily. By contrast, proteins like GAPDH that serve constitutive cellular functions such as the regulation of bioenergetics, may only respond to extreme concentrations of H_2O_2. Alternatively, these proteins may inherently have different susceptibilities to oxidation due to steric constraints or differences in the pKa of their cysteinyl thiols. In the case of tyrosine phosphatases such as PTP1B, the formation of the sulfenic acid is likely to be an intermediate step in the formation of the more stable sulfenamide (120) or to conjugation with glutathione (234). This would be supported by

the relatively short duration of detectable PTP1B sulfenylation, as the high specificity of dimedone for sulfenic acids would not detect the sulfenamides, gluthionyl-ethers or higher oxidation states such as the sulfinic (SO_2H) or sulfonic (SO_3H) acids (137,170,235). In contrast, sulfenylation of the GAPDH active site cysteine generates a more stable and long-lived species and as such would allow for labeling with dimedone (226,227,236). Additional studies will be needed to elucidate the mechanistic basis for the differential kinetics of GAPDH and PTP1B sulfenylation.

Our observation that, on a molar basis, 1,2-NQ appears to be 100 times more potent than H_2O_2 in inducing protein sulfenylation may be explained by differences in the targets with which each stimulus preferentially interacts. This would be consistent with reports that the pattern of EGF-stimulated protein sulfenylation is significantly different relative from that induced by H_2O_2 (84,94). H_2O_2 is subject to catabolism by multiple enzymes, including catalase, glutathione peroxidase and peroxiredoxin. In addition to 1,2-NQ generation of ROS through redox cycling (57), 1,2-NQ also generates H_2O_2 in mitochondria through uncoupling of the respiratory chain (56). Additionally our cell free studies demonstrate the interaction between GAPDH and 1,2-NQ, as proposed by Kumagai and his colleagues (53), is yet another non-enzymatic mechanism through which 1,2-NQ can lead to elevated ROS levels. Thus multiple sources of ROS may act synergistically to induce a sustained and localized elevation in H_2O_2 in cells exposed to 1,2-NQ at a rate that overcomes the capacity of antioxidant mechanisms. These considerations would suggest that 1,2-NQ exposure could be more effective in driving sulfenylation than addition of an exogenous bolus of H_2O_2.

3.5: Summary

Through the use of multiple analytical approaches, this study provides the first evidence that exposure of a lung epithelial cell line to environmental concentrations of a ubiquitous redox-active pollutant can induce cysteinyl sulfenylation of critical regulatory proteins, a novel biomarker of xenobiotic oxidant stress. Exposure to the ubiquitous environmental oxidant, 1,2-NQ induces H_2O_2–dependent sulfenylation of cysteine residues in proteins, including GAPDH and PTP1B, proteins involved in bioenergetic and signaling regulation, respectively. The work presented in this study shows that protein sulfenylation is a novel readout of oxidant stress induced by exposure to environmental agents.

CHAPTER 4: The Role of Glutathione in Redox Toxicology

4.1: Introduction

The biological basis of oxidative stress has recently been revisited. Many studies have implicated the deleterious nature of reactive oxygen species (ROS) (63,67); however, it is now apparent that ROS are integral in many cellular processes in normal physiology (87,94). Of particular importance is hydrogen peroxide (H_2O_2), which serves as a second messenger in signaling processes as well as an effector in many cellular regulatory processes including bioenergetics and cytoskeletal maintenance (79). H_2O_2 can oxidize the cysteine thiol of proteins to the sulfenic acid which is a functionally important posttranslational modification (PTM) that has been shown to both enhance (171) or inhibit (120) the activity of proteins. The biological effect of sulfenylation on proteins can be extended through the formation of a secondary PTMs of the sulfenic acid to the irreversiblely hyperoxidized sulfinic and sulfonic acid, or the reversible sulfenamide and mixed disulfide with glutathione (70). The formation of the mixed disulfide between proteins and glutathione or known more broadly has the PTM, S-gluathionylation, is linked to the regulation of protein function (237,238).

Glutathione is an important physiological tripeptide that is the predominant redox pair in cells based on concentration (239). Glutathione is credited as the primary antioxidant within the cells due to its substantial prevalence in all cells of all tissues. Not only can glutathione serve as an antioxidant for promiscuous ROS such as hydroxyl radicals ($HO^{\cdot}$), but it can also reduce H_2O_2 through enzymatic processes (92). Two reduced glutathione molecules (GSH) can serve as a reducing equivalent to a single H_2O_2 molecule through the action of glutathione peroxidase

(Gpx) resulting in two molecules of H_2O, a single molecule of glutathione disulfide (GSSG), the oxidized half of the glutathione redox pair. Then the protein, glutathione reductase (GR), can reduce GSSG to GSH at the expense of NADPH. The action of glutathione as an antioxidant process, which is regulated at many points, can serve as a means to fine tune intracellular concentrations of H_2O_2 in order for it to act on protein thiols.

In addition to this antioxidant process that could regulate H_2O_2-dependent cellular processes by modulating intracellular H_2O_2 levels, glutathione can also serve as a cytoprotective molecule through the interaction of xenobiotics. The enzyme family, glutathione S-transferases (GST), conjugates glutathione to xenobiotics to facilitate their transport out of the cell (240). This is a critical step in the detoxification of many xenobiotics as the addition of glutathione to the xenobiotic can also quench the mechanism of toxicity. Recent reports have suggested that the GST enzymes are also important in the S-glutathionylation of proteins (241). Specifically, GST-facilitated glutathione conjugation of proteins targets sulfenic acids. This would suggest that the GST enzymes serve a regulatory role in the maintenance of protein sulfenylation (238).

The potential role of glutathione to regulate protein sulfenylation may in fact have great importance on the public health burden of air pollution. Epidemiological evidence has suggested that polymorphisms of the GST family can be considered a factor of susceptibility for an individual's exposure to ambient air pollution (24). Of particular interest are GSTM1-null individuals, which accounts for over 40% of the global population, which epidemiologically is associated with an increase risk to exposure to air pollution (26). This study aims to elucidate the mechanistic link between glutathione and protein sulfenylation, as well as determine whether GSTM1 status influences H_2O_2-induced PTMs in normal human bronchial epithelial cells

(NHBEC) exposed to the ambient air pollutant and potent oxidant, 1,2-naphthoquinone (1,2-NQ).

4.2: Materials and Methods

4.2.1: Reagents

Tissue culture media and supplements were purchased from Lonza (Walkersville, MD). Phenol red-free keratinocyte basal medium (KBM) was acquired from Cell Applications, Inc. (San Diego, CA). The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): hydrogen peroxide (H_2O_2), 1,2-naphthoquinone (1,2-NQ), DMSO, dimedone, DTT, EGF, BSO. Basic laboratory supplies were obtained from Fisher Scientific (Raleigh, NC).

4.2.2: Cell Culture

SV40 large T antigen-transformed HAEC (BEAS-2B, subclone S6) were cultured as previously described (201) in keratinocyte growth medium (KGM). BEAS-2B cells were transduced by lentiviral vector to stably express the genetically encoded redox sensors roGFP2 and HyPer as previously described (140). The plasmids for roGFP2 and HyPer were the generous gift of S.J. Remington (University of Oregon, Eugene, OR, USA) and purchased from Evrogen (Axxora, Farmingdale, NY, USA), respectively. Cells at 80% confluence were deprived of growth factors overnight by changing cell media to phenol-red free KBM.

NHBEC obtained through bronchial brush biopsies were initially stored under liquid nitrogen. Cells were grown through passage 3. Once passage 3 cells reached 80% confluence, NHBEC were deprived of growth factors overnight in bronchial epithelial basal media (BEBM). NHBEC were then exposed to BEBM containing EGF, H_2O_2 or and 1,2-NQ (dissolved in DMSO, final DMOS concentration <1:1000) at the indicated concentrations for ten min. NHBEC were

then quickly rinsed with PBS and then labeled in BEBM with 5 mM dimedone with DMSO at 1:200 dilution for one h.

4.2.3: Live Cell Imaging

BEAS-2B cells deprived of growth factors overnight with 500 µM BSO in KBM before live cell imaging analysis. Fluorescence in roGFP2 or HyPer expressing BEAS-2Bcells, were using a Nikon Eclipse C1si spectral confocal imaging system and 404 nm and 488 nm primary laser lines (Nikon Instruments Corporation, Melville, NY, USA; (140)). Sequential scans of each laser line were performed at a frequency of 60 secs with at least 10 cells expressing the biosensor in the field of view, with results calculated as a ratio of the respective 525/30 nm emission for the 404 nm and 488 nm excitation of each sensor. Baseline data points were collected 10 min prior to the addition of H_2O_2. HyPer expressing cells were treated with 50 µM H_2O_2 for 5 min followed by treatment with 1 mM H_2O_2 for 3 min and finally with DTT for 3 min, while roGFP2 expressing cells were treated with 1 mM H_2O_2 for 5 min followed by addition of 100 µM A-2 for 3 min and finally with DTT for 3 min to test the span of sensor responsiveness. Data were expressed normalized to the maximum sensor response (i.e., the maximum response elicited by H_2O_2) recorded during the experiment set as 100%, with the average starting baseline response set as 0%.

4.2.4: Immunoblotting of Intracellular Sulfenylation

NHBEC labeled with dimedone were washed three times with ice-cold PBS and lysed in a mild-detergent buffer—1% NP40, 150 mM tris-HCl, pH 7.4, 150 mM NaCl, 5 mM DTT supplemented with Calbiochem protease inhibitor cocktail set III (EMD Millipore, Temecula, CA)—for 20 min and then centrifuged at 4˚C, 12000g for 10 min. Supernatant was collected and

normalized for protein concentration via Bradford assay. Samples were prepared for Western Blotting with 4X Laemmli Sample Buffer and boiled for 10 min before loading into Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) alongside Precision Plus Protein Kaleidoscope Standards (Bio-Rad) and then gel electrophoresed for size separation. Gels were transferred using the Trans-Blot Turbo Transfer System onto nitrocellulose membranes (Bio-Rad). Membranes were then blocked with 5% Milk in TBST for one h at room temperature followed by incubation with primary antibody overnight at 4˚C, then secondary antibody incubation for one h at room temperature. The following antibodies were used: anti-cysteine sulfenic acid antibody (Millipore), and anti-GAPDH (6C5), goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP (all from Santa Cruz, Dallas, TX). After antibody incubation, membranes were set in Clarity Western ECL Blotting Substrate for 5 min followed by detection with LAS-3000 FujiFilm Imager.

4.3: Results

4.3.1: Glutathione depletion perturbs the dynamic nature of redox homeostasis

Cells treated with BSO when challenged with 50 μM H_2O_2 reached a lower maximum increase in E_{GSH} (Figure 27A) as reported by the fluorogenic sensor roGFP2. Additionally, cells with lower glutathione levels challenged with 50 μM H_2O_2 are unable to maintain intracellular H_2O_2 levels as compared to cells not treated with BSO, as they have elevated H_2O_2 levels after exposure (Figure 27B). However, it should be noted that extreme levels of H_2O_2 (fully oxidizing) and DTT (fully reducing) led to identical sensor responses suggesting that both sensors utilized are fully functional.

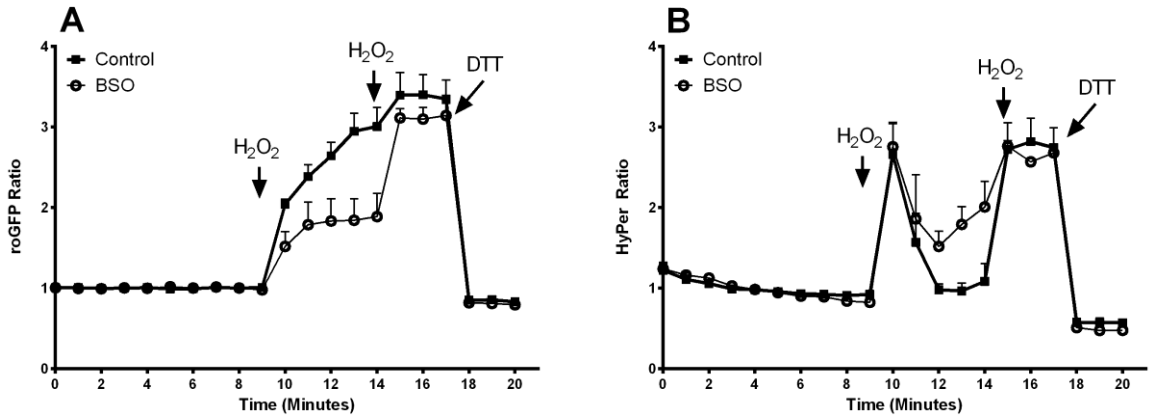

Figure 27: Treatment with BSO leads to perturbed response to H_2O_2. *BEAS-2B cells expressing roGFP2 (A) or HyPer (B) were treated with 500 μM BSO for 24 hours. Cells were then challenged with 50 μM (min 10), 1 mM H_2O_2 (min 15), and 2 mM DTT (min 28). Shown are ratiometric values calculated from the fluorescent 525/30 emission of the 404 nm and 488 nm laser excitation normalized to the average values obtained from the baseline. Values shown are mean ± SE (n = 3, where each n consists of an averaging of 10 distinct cells' response).*

4.3.2: EGF-induced changes in protein sulfenylation are not impacted by GSTM1 status

Upon demonstrating a relationship between glutathione availability and intracellular H_2O_2 levels, we next tested whether GSTM1 played a role in other H_2O_2-induced cellular effects, such as protein sulfenylation. We obtained NHBEC that were either null or sufficient for the glutathione-transferring enzyme GSTM1. These NHBEC were challenged with 300 μM H_2O_2 or 100 ng/mL EGF for 10 minutes and then assayed for protein sulfenylation by labeling with dimedone followed by immunoblot detection using an anti- dimedone-thioether antibody. As with previous reports (171), both H_2O_2 and EGF demonstrated an increase, although insignificant, in protein sulfenylation over control untreated NHBEC (Figure 28). Both agents had similar effects on protein sulfenylation despite GSTM1 status.

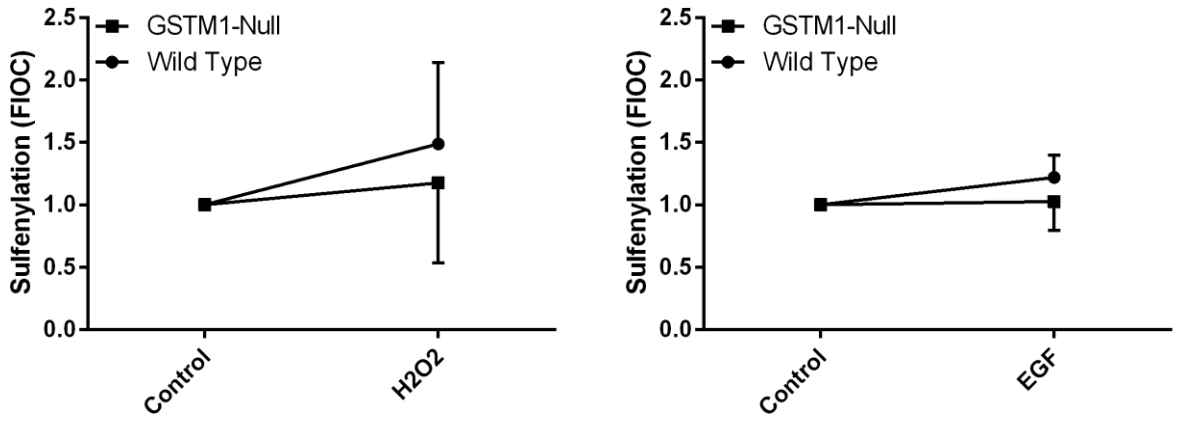

Figure 28: GSTM1-status Does Not Determine Protein Sulfenylation in Response to Physiological Stimuli*. Primary human bronchial epithelial cells were treated with either 300 μM H_2O_2 or 100 ng/mL EGF for ten minutes to induce protein sulfenylation. Cells were then labeled with 5 mM dimedone for one hour to capture all sulfenic acids. Global protein sulfenylation is shown as fold increase over GAPDH was normalized to background levels of protein sulfenylation of unstimulated cells from the same donor. Data is shown as mean ± SEM (n=3).*

4.3.3: GSTM1-null cells show more pronounced protein sulfenylation in response to exposure to 1,2-Naphthoquinone

We next challenged the NHBEC to the environmental oxidant 1,2-NQ, which we previously reported is a potent inducer of protein sulfenylation (Chapter 3) in the human bronchial epithelial cell line, BEAS-2B. GSTM1-sufficient cells appeared to have no significant increase in protein sulfenylation following exposure to 10 μM 1,2-NQ. In contrast, NHBEC obtained from GSTM1-null individuals demonstrate a 2 fold higher level of protein sulfenylation compared to untreated NHBEC from the same individual when exposed to 10 μM 1,2-NQ for ten minutes (Figure 29).

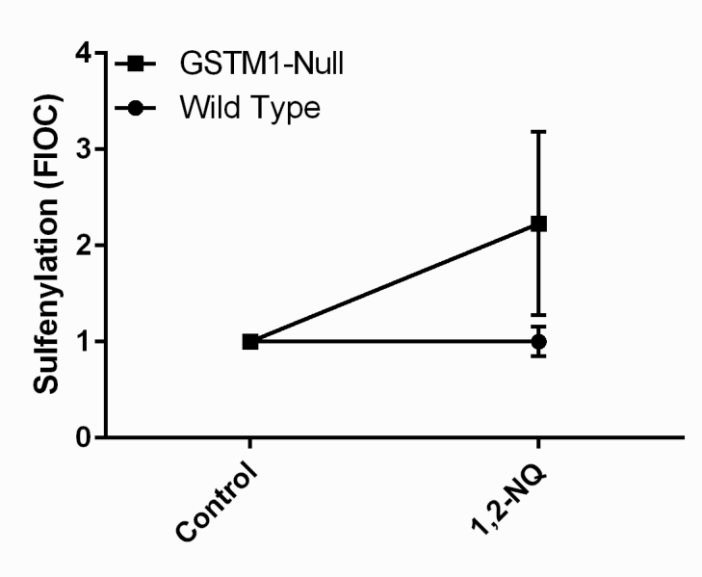

Figure 29: 1,2-Naphthoquinone-Induced Protein Sulfenylation is Enhanced in GSTM1-Null Cells*. Primary human bronchial epithelial cells were treated with either 10 μM 1,2-NQ for ten minutes to induce protein sulfenylation. Cells were then labeled with 5 mM dimedone for one hour to capture all sulfenic acids. Global protein sulfenylation is shown as fold increase over GAPDH was normalized to background levels of protein sulfenylation of unstimulated cells from the same donor. Data is shown as mean ± SEM (n=3).*

4.4: Discussion

The major finding of this study is the clear relationship between glutathione availability and readouts of redox toxicology. First, we demonstrated that depleting the pool of glutathione in BEAS-2B cells impacts the cellular response to oxidative stress with a lower E_{GSH} and elevated intracellular H_2O_2 levels. The lower maximum E_{GSH} attained by these cells may be indicative of a higher basal GSSG to GSH ratio, which correlates well with elevated intracellular H_2O_2 levels, as there is less reduced GSH to enable the glutathione-dependent metabolism of H_2O_2. Additionally, we also observed that NHBEC deficient in the enzyme GST, which facilitates the biological function of glutathione, are substantially more affected by environmental oxidant exposure as they have increased protein sulfenylation. Furthermore, the finding that compromising integral glutathione conjugation results in a substantial impact on protein

sulfenylation. This would suggest glutathione participates as an active regulator of intracellular protein sulfenylation through enzymatic interaction during physiological processes. Given that protein sulfenylation is integral in many redox-dependent cellular processes, this demonstrates that glutathione system enzymes potentially have indirect influence over those same redox-dependent cellular processes.

Another key observation is that stimulation withEGF, which produces endogenous ROS through the interaction with epidermal growth factor receptor (EGFR) (242), did not have a GSTM1-dependent effect while the toxicological agent, 1,2-NQ did. This is of particular interest because both EGF and 1,2-NQ have previously been shown to induce protein sulfenylation via H_2O_2 (56,171)(Chapter 3). These findings suggest that GSTM1 status dictates 1,2-NQ-induced protein sulfenylation through H_2O_2-independent mechanisms. Thus it could by hypothesized that GSTM1 activity may be compensated by other GST isoforms during EGF stimulation. However, when cells are exposed to 1,2-NQ they are influenced by one or a combination of three different outcomes: 1) 1,2-NQ induces relatively more H_2O_2 than EGF, 2) 1,2-NQ electrophilic activity impacts the utilization of glutathione to regulate protein sulfenylation, and/or 3) GSTM1 has a detoxifying role in 1,2-NQ metabolism.

The high variability observed in levels of protein sulfenylation of NHBEC is worh hghlighting. It will be important to expand the number of distinct individuals assayed to fully understand the magnitude of the difference between EGF- and 1,2-NQ-induced protein sulfenylation in NHBEC. Additionally, it would be enlightening to interrogate how glutathione impacts oxidant-induced protein sulfenylation by inhibiting key steps in glutathione synthesis and glutathione reducing enzymes using BSO and 2-AAPA, respectively.

Whatever the mechanistic reason driving the GSTM1-influenced effect of 1,2-NQ-induced protein sulfenylation, the finding that GSTM1 modulates enhanced environmental oxidant exposure is informative. This specific effect may in part explain mechanistically why GSTM1-null individuals are susceptible to air pollution. It is possible that early responses to exposure are more important in explaining epidemiological results, as opposed to most other toxicological readouts which are reported following longer exposure (gene expression) or days (inflammatory infiltration). These immediate changes in cellular redox homeostasis are corrected downstream during a toxicological study. Yet, during real world scenarios where multiple exposures and different doses are accounted for during a lifetime, these redox effects may accumulate. The accumulation of these redox events may prime the individual to an environmental exposure, which leads to the adverse health outcome.

4.5: Summary

Many studies have implicated glutathione conjugation as a marker of toxicology. This study defines a role of glutathione in regulating intracellular H_2O_2 concentrations as well as a mechanistic force in cellular processes by regulating protein sulfenylation in response to the environmental oxidant, 1,2-NQ. Furthermore, this study is one of the first attempts to characterize protein sulfenylation in NHBEC. The preliminary findings presented in this study suggest that protein sulfenylation may provide a mechanistic explanation for the observed genetic susceptibility of air pollution to GSTM1-null individuals.

CHAPTER 5: Ranking Asbestos Fiber Toxicity Using Live Cell Imaging of E_{GSH}

5.1: Introduction

There is a growing appreciation that xenobiotic-induced oxidant events may serve as important mechanistic factors in the progression of their toxicity (102,103). Whether the oxidant event is a mechanistic factor in the development of environmental-associated disease or is merely a side effect of an exposure, the oxidant event is a promising candidate as a biomarker to prioritize and rank the toxicity of xenobiotics for further assesment. The use of these events as biomarkers for prioritization of xenobiotics could be especially important in the regulation and remediation of regulatory agencies where the mechanism of toxicity is unknown. For instance, exposure to asbestos and asbestos-like fibers, such as erionite, has been linked to malignant mesothelioma (243,244); however, the mechanism leading to this cancer has yet to be elucidated (245).

It is possible that these asbestiform fibers cause oxidative stress, and this oxidative stress may inform not only the mechanistic basis of exposure, but could rank the cytotoxicity of asbestos types (246,247). The glutathione redox potential (E_{GSH}) is an important marker of redox metabolism, which is effected by oxidant events including the elevation of intracellular H_2O_2 (90,239)(Chapter 2). E_{GSH} is a quantitative measure of the glutathione redox pair as described by the Nernst equation (133). E_{GSH} is influenced by the concentration of reduced (GSH) and oxidized glutathione (GSSG) pair. This means that a change in E_{GSH} reflects the intracellular presence of oxidants and electrophiles, as well as the general health of the glutathione redox

relay which regulates the synthesis glutathione and reduction of GSSG (140). For these reasons the E_{GSH} serves as a good marker of the general redox metabolism to inform general toxicity.

Here, we rank the impact of a variety of asbestiform fibers on the E_{GSH} of human airway epithelial cells utilizing the genetically encoded fluorescent sensor roGFP2-Grx1 (164). This study tested eight different asbestiform fibers including Crocidolite, Chrysotile, and Amosite. We also compared asbestiform fiber-induced E_{GSH} to asbestos fibers collected from the Libby Montana Superfund site. Asbestos from Libby Montana has been linked to a variety of adverse health outcomes and has imposed a significant negative impact on the local population (248-252). This study demonstrated that $PM_{2.5}$ Libby Montana Amphibole fibers as well as Crocidolite fibers elevated E_{GSH} in the human airway epithelial cell line, BEAS-2B.

5.2: Materials and Methods

5.2.1: Fiber Preparation

Asbestos and asbestos fibers [UICC Crocidolite, UICC Chrysotile, Swift Creek Chrysotile, Libby Amphibole $PM_{2.5}$, Libby Amphibole PM_{10}, Erionite $PM_{2.5}$, Wollastonite $PM_{2.5}$, RTI Amosite] were provided by Drs. Andrew Ghio and Stephen Gavett. Fibers were freshly prepared by measuring 6-21 mgs into a 15 mL conical tube. The tube was briefly centrifuged, before addition of sufficient deiozined distilled water to give a concentration of 5 mg/mL. The suspension was then sonicated at 80% power for 45 sec vortexed briefly and sonicated an additional 45 sec using a Model 500 Sonic Dismembrator (Fisher Scientific, Raleigh, NC). All fibers were handled and disposed of according to EPA safety regulations.

5.2.3: Cell Culture

BEAS-2B cells, an immortalized bronchial epithelial cell line, (passage 60-73) were plated on 35 mm culture dishes with 14 mm coverslips at 200K cells per dish in serum-free keratinocyte growth media (Lonza, Walkersville, MD). After 24 hours cells were transfected with roGFP2-Grx1 using X-tremeGENE 9 DNA Transfection Reagent (Roche, Indianapolis, IN) according to manufacturer's protocol, and incubated for another 24 hours. After 48 hours post initial plating cells were deprived of growth factors using keratinocyte basal media (Lonza).

5.2.4: Confocal Analysis

Confocal microscopy analyses were conducted using a C1Si system equipped with an Eclipse Ti microscope (Nikon, Melville, NY). Green fluorescence was derived from excitations at 404 and 488 nm, and emission was detected using a band-pass filter of 525/530 nm (Chroma, Bellows Falls, VT). The results were calculated as ratios of the emissions excited by 488 nm and 404 nm lasers sequentially with a scanning frequency of 60 seconds for 61 rounds. Transmissible light was monitored along with the 404 and 488 nm excitations in order to examine the presence of fibers and general shape of cells.

Two hours prior to exposure cells were deprived of glucose, to enhance roGFP2-Grx1 signal, by changing media to Locke Solution. Cells were then transferred to the above-stage chamber on the confocal microscope that maintains normal CO_2, humidity, and temperature as under normal incubation times. Multi-time point analysis was established with excitations as previously described with z-plane maintained between 2300-2450 nm. Forty μg (or as indicated in results) of fibers or eight microliters of water was added to the cells after 5 minutes. In order to assess for redox sensitive response cells were exposed to 100 μM H_2O_2 after 51 minutes and 1

mM dithiothreitol after 56 minutes for maximal and minimal oxidative response. Each challenge to fibers was repeated three times alongside one or two challenges to water as a vehicle control; except for Erionite $PM_{2.5}$, which was challenged only twice.

5.2.5: Statistical Analysis

Imaging data were collected with Nikon EZ-C1 software. All cells in view were collected as regions of interest as long as asbestos was visually present in same field of view. Outlier cells were removed from analysis if they failed the z-score test at >3/4 of all time points analyzed between exposure 6 and 51. The region of interests associated with each cell was then averaged at each time point to establish a single exposure, which was then expressed as S.E.M. (n=2-3) that was then compared to the average of all vehicle exposures collected (n=9). Two-way ANOVA using Bonferroni post-test, with $p<0.05$ as significant, was used to assess statistical significance between each average fiber exposure to the collected vehicle exposure average (GraphPad Prism, La Jolla, CA).

5.3: Results

5.3.1: Exposure to Asbestos Does Not Compromise Cell Viability Acutely

Most techniques that assess cell viability are ineffective in the presence of asbestos and asbestos-like fibers, as the fibers act as a sink for both proteins (Lactate Dehydrogenase (LDH)) and chemicals (calcein-AM) (253). However, it is known that chronic exposure to asbestos can be cytotoxic, as indicative in the classical presentation of frustrated phagocytosis when macrophages attempt to remove asbestos fibers (254). Thus we relied on visual verification for viability of cells expressing the fluorogenic sensor roGFP2-grx1.

BEAS-2B cells overexpressing roGFP2-Grx1 were monitored with both fluorescent and transmissible light. As demonstrated in Figure 30, despite the direct application of fibers (initial exposure) no overt cytotoxicity was observed throughout the 45 minute exposure. Sensor responsivity was assessed with the application of 100 μM H_2O_2 and 1 mM DTT, which further demonstrated that the cell integrity was maintained throughout the exposure.

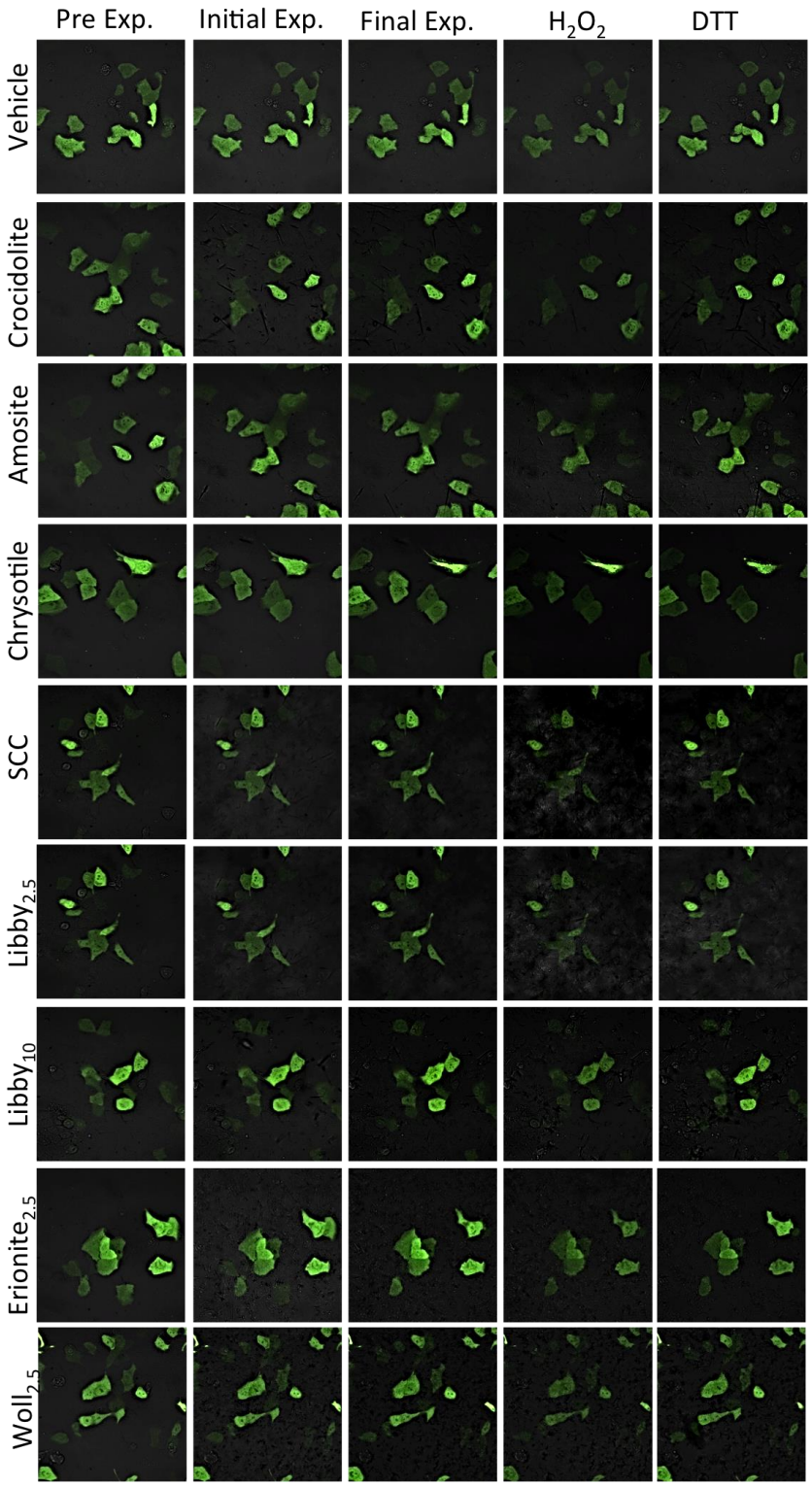

Figure 30: Cells Remain Visibly Viable throughout Direct, Acute Exposure to Asbestiform Fibers*. The emission from the 488 nm excitation is presented for each of the experimental exposures of the fibers at 2 minutes (Pre. Exp.), 6 minutes (Initial Exp.), 45 minutes (Final Exp.), and most prominent response from 100 μM hydrogen peroxide (H_2O_2) and 1 mM DTT (DTT) as they were the oxidizing and reducing controls, respectively, to assess the responsiveness of the roGFP2-Grx1 sensor*

5.3.2: Asbestiform Fibers are Relatively Weak Environmental Oxidants

Eight asbestos and asbestos-like fibers were assessed for changes in E_{GSH} via live cell imaging using the fluorogenic sensor roGFP2-Grx1. Preliminary studies indicated minimal changes, therefore BEAS-2B cells were sensitized to redox changes by depleting NADPH levels using glucose starvation (140). The final analysis is expressed as the percent oxidized sensor, with only UICC Crocidolite and Libby Amphibole$_{2.5}$ showing consistent oxidized state above the vehicle control, while the remaining six fibers were either equal to or below the E_{GSH} state associated with the exposure to the vehicle control (Table 1, Figure 31).

Fiber	**Maximum % Oxidized**
UICC Crocidolite	3.1
Libby Amphibole$_{2.5}$	2.3
UICC Chrysotile	0.6
Libby Amphibole$_{10}$	0.3
Wollastonite$_{2.5}$	0.1
RTI Amosite	-0.6
Erionite$_{2.5}$	-0.7
Swiftcreek Chrysotile	-0.8

Table 1: Ranking of Asbestiform Fiber Toxicity by Induced E_{GSH}. *Maximum % of oxidized sensor was calculated using maximum response (100 μM H_2O_2) and minimum sensor response (1 mM DTT). Values are presented as the mean of 2 or 3 individual time-course analyses.*

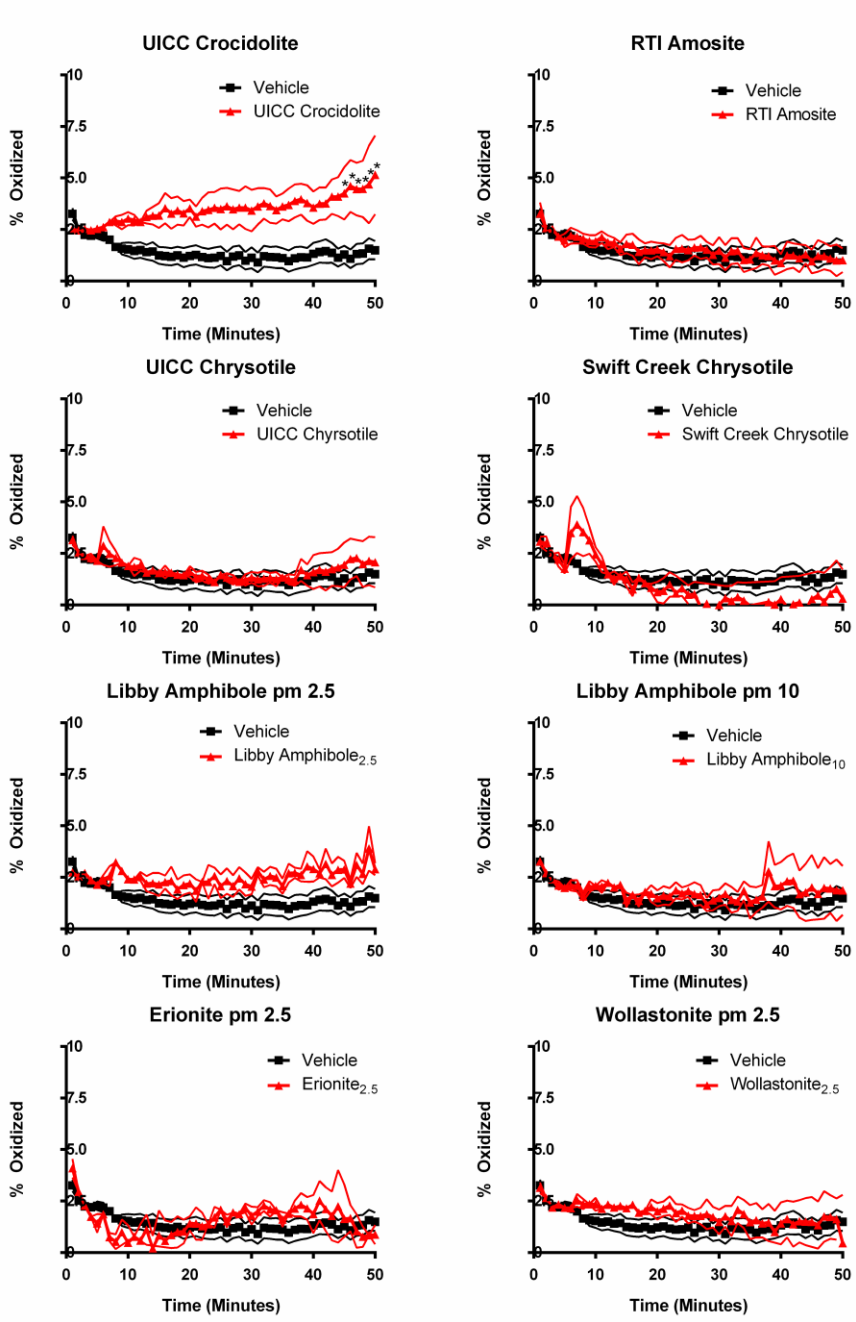

Figure 31: BEAS-2B Cells Respond Variably to Asbestiform Fiber Exposure*. Data is presented as percent oxidized of the roGFP2-Grx1 sensor with each exposure to a fiber is shown as the average of three separate experiments, with each experiment quantified as an average of >6 cells (red triangles); the erionite$_{2.5}$ is the exception as it is representative of an average of two separate experiments. The vehicle control (black squares) is shown as an average of nine separate experiments, with each experiment quantified as an average of >10 cells. Bold lines indicate mean +/- standard error (n=2, 3, 9; where n is an average of response from all visible cells in a single experiment); * indicates p-value<0.05.*

5.3.3: Crocidolite Induces Cytosolic E_{GSH}

Since crocidolite was the most potent oxidant in this screen, we further characterized its effects to validate its effects on E_{GSH}. The results showed that at the highest concentration of UICC Crocidolite tested (40 μg or $63x10^6$ $\mu m^2/cm^2$) there was a distinctive increase in E_{GSH} when cells were exposed to the asbestos compared to that of the vehicle control. Furthermore, there was a clear dose-response effect observed using a range of concentrations ($12.6x10^6$ $\mu m^2/cm^2$ - $63x10^6$ $\mu m^2/cm^2$) of UICC Crocidolite (Figure 32).

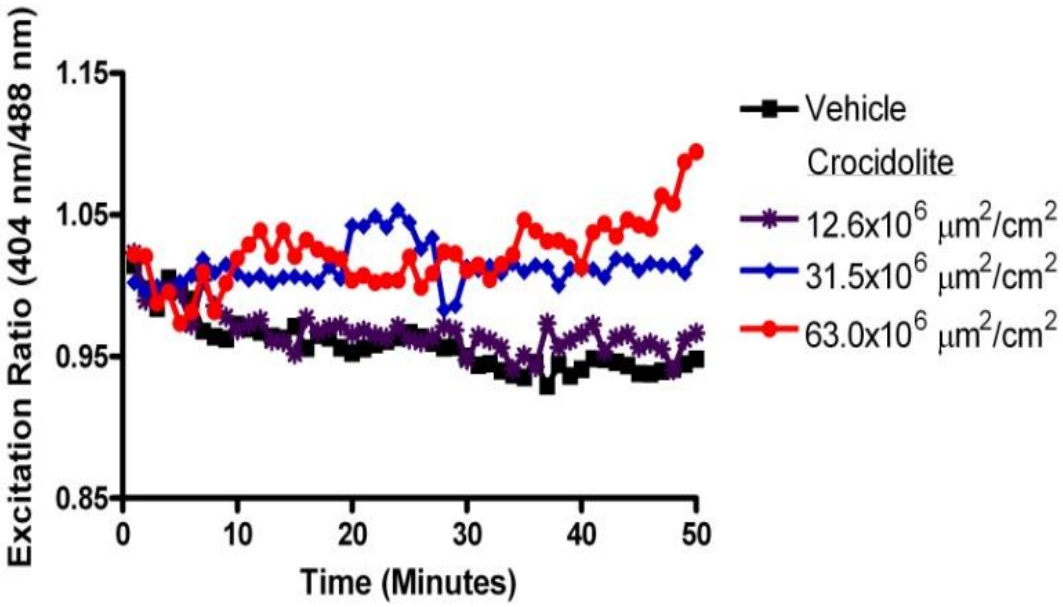

Figure 32: Cells Respond to Acute Exposures to UICC Crocidolite in a Dose Dependent Manner. *Exposure is expressed as the excitation ratio of the grx-roGFP2 sensor as an average of two, in the case of the $12.6X10^6$ and $31.5X10^6$ conditions, or three separate experiments, in the case of the vehicle control and the $63.0X10^6$ conditions, over 50 minutes. At the end of the exposure cell viability and response was assessed with the addition of 100 μM H_2O_2 and 1 mM DTT, data not shown. Each condition is an average of n=2, 3; where each n is calculated as an average of response from 10 cells in a single experiment.*

5.4: Discussion

In an attempt to better understand the relationship between asbestos exposure and respiratory disease, a method to assess the intracellular redox state of living cells exposed to fibers was developed. The endpoint of this method was a quantitative measurement of the oxidized glutathione pool (GSSG), which is one half of the primary intracellular antioxidant system along with the reduced form (GSH). This was accomplished using the fluorescent sensor roGFP2-Grx1, which can be measured at two separate wavelengths 404 nm and 488 nm that increase in emission fluorescence with increasing presence of GSH and GSSG, respectively (164). This project implemented live-cell imaging techniques for high resolution to reduce signal-to-noise false positives. Furthermore roGFP2-Grx1 reactivity was increased by depleting glucose levels prior to exposure to further sensitize the sensor by removing the available NADPH pool to reduce GSSG(140).

There are multiple factors that need to be addressed to fully appreciate these results. First, the observation that these fibers produce a minimal change in E_{GSH}, with the maximal response observed being 6% oxidized, which is substantially less than those observed by zinc (196)(Chapter 2) and ozone (140). On the other hand, it may be argued that these changes are too small to have any significant biological effect, while the responses observed are reproducible These experiments did not assess the production of individual reactive oxygen species such as H_2O_2, which could reveal another aspect of how asbestos fibers could produce acute toxicity via oxidant stress.

In addition to the factors previously addressed, time of exposure is another major limitation of the current method. Although the use of the confocal microscope increases sensitivity and makes it possible to observe the intracellular redox state with high spatiotemporal

resolution, it is restricted to only observing single exposures of cells. We took precautions to avoid variance between exposures such as running multiple exposures as well a vehicle control each day and establishing a regimented cell culture method. However, even with those precautions, the exposure scenario implemented in this study may have been biased towards toxicity from UICC Crocidolite.

The results of this study are promising as they demonstrate a novel approach to rank the toxicity of xenobiotics. Especially since asbestos-induced disease is complex and progressive (255), this approach may assist with approaching public health problems. Crocidolite is frequently regarded as the most toxic asbestos fiber (256), and this is supported with this data, suggesting E_{GSH} may be a valid biomarker of toxicity. Exposure to fibers at different concentrations or for different durations could have provided enhanced toxicity. Based on the most recently available methods to assess oxidative stress endpoints in cells, it is suggested the majority of asbestos and asbestos-like fibers do not induce a large adverse response after a direct, acute exposure to the fibers; however, the continuing development of the sensors used in this experiment as well as others could provide a better understanding of the effect these fibers have on cells in the future.

5.5: Summary

The results presented demonstrate the utility of assessing redox-dependent processes as a means to inform risk of toxicity of environmental materials. Although only slight changes were observed, a clear and consistent outcome demonstrated that both UICC Crocidolite and Libby Amphibole $PM_{2.5}$ were relatively more potent than the other fibers analyzed. This is of particular relevance in lieu of the adverse effects of the Libby Montana Superfund site on the surrounding population. This study suggests that oxidant events could be an important marker for toxicity and could be utilized as a biomarker.

CHAPTER 6: Summary Discussion

6.1: Redefining Oxidative Stress in Environmental Exposures

The research within this dissertation suggests an increasing appreciation of the causative role of oxidative stress in the toxicological basis of disease. The traditional view of oxidative stress in toxicology focused on the irreversible damage of biological molecules such as proteins, lipids, and nucleic acids. In this view the irreversible damage leads to overt cytotoxicity by compromising physiological function at a gross level (67,105). However, many studies have clearly demonstrated a more complex role of oxidant species and byproducts in cellular processes (81,134,257,258) and toxicological exposures (56,140,196), leading "oxidative stress" to be an even poorer term to characterize and define redox toxicology. In many cases the term oxidative stress in the field of toxicology has lost significant mechanistic meaning, precisely at a time when our appreciation for the role of redox-dependent processes in physiology is growing.

6.1.1: Defining Redox Toxicology

The revelation that redox-dependent cellular processes are essential in normal physiology not only upturned the traditional role of oxidative stress, but also suggested new mechanistic targets of environmental exposures. Thus oxidative stress is but one outcome of an environmental exposure, suggesting that a new term is needed to be used in these studies. Redox toxicology is the impact of a xenobiotic on any redox-dependent process up to and including the induction of gross cellular stress. Redox toxicology at a cellular level exists at two extremes that

result in cytotoxicity: reductive stress and oxidative stress. Between these two extremes is a spectrum of conditions associated with a range of readouts of redox status as depicted in Figure 33 (concentration of ROS, redox potential of molecules, oxidation status of macromolecules, etc.).

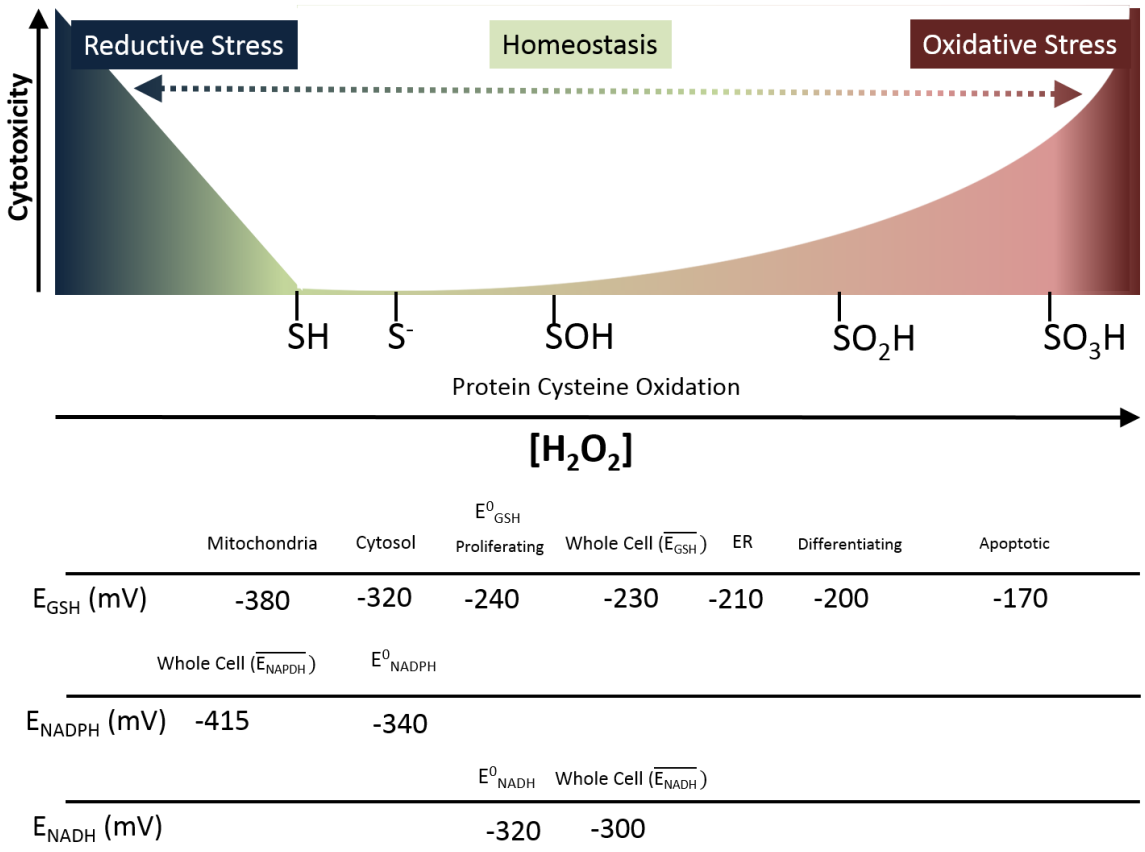

Figure 33: Biomarkers of Redox Biology Related to Cytotoxicity*. Oxidative stress is influenced by multiple redox factors linked to cytotoxicity on a spectrum of cellular health. These redox factors include, but is not limited to concentrations of reactive oxygen species such as H_2O_2, the ratio of redox pairs including glutathione (GSH), NADPH, and NADH, and oxidative posttranslational modifications for example cysteine oxidation. This spectrum is dynamically balanced between two extremes (oxidative and reductive stress) that are indicative of cytotoxicity. Xenobiotic exposure can perturb these homeostatic processes towards one of these extreme redox stresses, which may lead to cytotoxicity.*

6.1.1.1: H_2O_2

The link between an increase in H_2O_2 and an adverse outcome has been demonstrated in many studies (162,259,260), including the work discussed in this dissertation dealing with zinc-induced adaptive changes (Chapter 2) and 1,2-NQ-induced protein oxidation (Chapter 3). The accumulation of H_2O_2 could eventually lead to cytotoxicity, as an increase in intracellular H_2O_2 induces an imbalance of redox status in the cell towards oxidation. On the other extreme, the depletion of H_2O_2 could similarly result in a cytotoxic event indicative of reductive stress. In fact, there is mounting evidence a lower threshold concentration of H_2O_2 is required for normal cellular physiology (81,82). The requirement for this ROS is due to the function of H_2O_2 as a second messenger in signaling processes as well as a regulator of other cellular functions including bioenergetics and cell signaling (79). In this context, environmental exposures able to reduce or induce intracellular H_2O_2 at any level have the potential to cause toxicity. Thus levels of intracellular H_2O_2 concentration reflect a spectrum spanning from cell health to cell death. This may also be applicable to other ROS although their relative cytotoxic potential are likely different.

6.1.1.2: Glutathione Redox Potential

As described earlier in this document, the glutathione redox potential (E_{GSH}), as well as its related measurements [GSH], [GSSG], and ratio of GSH to GSSG, can be used to describe the redox metabolism and antioxidant capacity of the cell in response to an environmental exposure. The E_{GSH} is a suitable readout for the effect of an environmental exposure on the health of the cell, as reduced glutathione serves as the primary antioxidant due to its relatively high concentration in the cell (90). Under physiological conditions the E_{GSH} of resting cells is

approximately -230 mV. When the E_{GSH} rises to ~-170 mV the cell undergoes apoptosis or necrosis (Figure 33). Although the change in E_{GSH} in itself is not believed to effect cellular responses (91), it can be used as a quantitative marker that can be associated with the health or activity of the cell. For instance cells that reach an E_{GSH} of -200 mV frequently are observed to be undergoing differentiation (133).

While the E_{GSH} should not, by itself, be used as a readout that informs the general redox state of the cell, it can be used as an informative readout of redox metabolism resulting from an exposure to an environmental oxidant as shown in Chapter 5 of this document. However, the E_{GSH} should be defined for each cellular organelle as each individual subcellular compartment maintains a distinct E_{GSH} (133). This means that when a cell is lysed and the E_{GSH} is calculated from the [GSH] and [GSSG] present in a homogenized mixture of all subcellular compartments, at best it describes the average E_{GSH} over the entire cell. That being noted, even this average E_{GSH} can inform the effect of an environmental exposure if it is dramatically altered, since it is likely other redox-dependent processes will have been impacted.

6.1.1.3: Protein Sulfenylation

Protein oxidation is another oxidant effect that has been traditionally been associated with the cytotoxicity of oxidative stress (261). The oxidation of protein residues occurs after translation of the protein by a variety of ROS, including H_2O_2. Although any nucleophilic amino acid, such as methionine and lysine, can be targeted by ROS, cysteine is the most notable in terms of consequences for cellular physiology (84,168)(Chapter 3, 4). Cysteinyl thiols confer structural and catalytic functions to proteins and can be the target of both oxidants and electrophiles (53,171). The cysteine thiolate, the ionized from of the thiol, can be reversibly

oxidized by H_2O_2 to the sulfenic acid. The sulfenic acid can be hyperoxidized to the sulfinic and sulfonic acid, which are the traditional markers for oxidative stress as these modifications are irreversible and direct the protein to proteosomal degradation (262). Although, sulfenic acids serve as the "gatekeeper" to irreversible modifications, the formation of the sulfenic acid is also essential in cellular physiology (97). Proteins that utilize the sulfenic acid as a means to regulate function are referred to as redox switches, as the protein will be "on" or "off" based on the redox status of the thiol moiety (96). The need for this dynamic protein function means that if the formation of sulfenic acids is inhibited it could lead to a reductive stress, while elevated rates of formation of the sulfenic acid increases the likelihood for the existence of sulfinic and sulfonic acids, leading to frank oxidative stress.

6.1.1.4: Energy Equivalents (NADPH and NADH)

The transfer of electrons is the most fundamental process in biology. In terms of adverse effects of chemicals on living systems, redox toxicology can be described through the action of NADPH and NADH. These two molecules are the most basic sources of reducing equivalents for energetic and synthetic processes in the cell. They are also the two most immediate regulators of redox biology by means of transfer of electrons through the oxidation or reduction of molecules. NADPH serves as the cell's primary reducing agent by providing electrons to oxidized molecules including Trx and Grx (263). NADH intracellular concentrations are much lower than its redox pair NAD^+, and as such serves as the cell's primary oxidizing agent for the citric acid cycle. These two redox pairs are regulated separately, just as all redox pairs including GSH and GSSG. However, E_{NADPH} and E_{NADH} can provide important information regarding the general

redox status of the cell as a high E_{NADPH} can indicate oxidative stress while a lower E_{NADH} suggests reductive stress (Figure 33).

6.1.2: Environmental Exposure-Induced Redox Toxicology

Contrasting the redox toxicology of zinc and 1,2-NQ illustrates differences in oxidative stress mechanisms. Many studies have implicated zinc-induced toxicity through its electrophilic affinity for thiols (45). It has been shown that through zinc's action on protein thiols, kinase activity can be enhanced and mitochondrial respiration inhibited (41). However, work within this dissertation clearly demonstrated an integral role of H_2O_2 in zinc-induced adaptive responses (Chapter 2). Yet, it is also apparent that zinc's electrophilic activity is equally important as the pro-oxidant activity in the zinc-induced HO-1 gene expression (115). It is likely that both effects contribute to zinc-induced toxicity. This is in contrast to 1,2-NQ-induced toxicity, where the results presented in this dissertation (Chapter 3), as well as other studies, indicate that 1,2-NQ pro-oxidant activity, specifically the generation of H_2O_2, is a primary mechanism of action leading to an inflammatory response (56).

6.1.3: Oxidative Stress or Electrophilic Stress

The work described in this dissertation can also provide insight on another key distinction that has developed within the role of redox biology in toxicology: the general association of oxidative stress with electrophiles. Although oxidative attack of nucleophiles is technically electrophilic in chemical terms, the mechanistic role of oxidation and electrophilic adduction can be distinct. This can be best exemplified by examining the antioxidant response element (ARE) (264). The ARE has been demonstrated to be an important element in the promoter region of

many antioxidant genes, and activated in response to increased levels of intracellular oxidants (109). However, one of the master regulators of Nrf2-binding to ARE is the protein KEAP1, which recently has been shown to only be effective at Nrf2-induced activation via the ARE through the action of electrophiles (115). In fact H_2O_2, as an oxidant, is a poor inducer of the ARE response. Many of the genes induced via the KEAP1/Nrf2 signaling axis are better attuned to reducing the negative impact of electrophiles, among which are pro-oxidative effects such as ROS generation. Thus the ARE could better be described as the electrophilic response element.

This argument may at first seem semantic, but it actually has practical meaning with regards to understanding the mechanism of action of chemical-induced toxicity. Quinones, including 1,2-NQ, can be described as both pro-oxidants and electrophiles. This is because quinones can redox cycle leading to the generation of ROS and they can also directly target nucleophiles through adduction by Michael addition (53). Therefore, quinone toxicity should not simply be attributed to either mode of toxicity, but the primary mode of toxicity defined for each quinone in order to predict its effects. For example 1,4-benzoquinone is not efficient at generating ROS, but is a potent electrophile, while the addition of one benzene ring to form 1,4-naphthoquinone leads to a chemical that is equally active as an electrophile and ROS generator (265,266). Despite the similar structure and both classically referred to as electrophiles, one (1,4-benzoquinone) causes electrophilic stress and the other (1,4-naphthoquinone) causes both oxidative and electrophilic stress.

Furthermore, the question that arises beyond the proper classification of the mechanism of toxicity is the relative contribution of each mechanism to a chemical's toxicity if it can be considered both an oxidant and electrophile. Included in this discussion would be 1,4-naphthoquinone and 1,2-NQ, which are both prominent air pollutants (49). Both of these

quinones are not only capable of targeting nucleophiles within the cell, but also generate ROS through redox cycling that target those same nucleophilic residues. The question thus arises, is there a relevant toxicological consequence between the competitions between these two different mechanisms of toxicity? Even though many nucleophiles exist, including glutathione, which can serve as a protective barrier to electrophiles, it is still possible for the toxicant to interact with essential molecules as an electrophile to cause toxicity. The findings presented in this document demonstrate that molecules that have both pro-oxidant and electrophilic properties (Zinc and 1,2-NQ) are able to induce toxicity through both mechanisms, but the relative contribution of each has yet to be fully determined (Chapter 2 & 3).

6.2: The Thin Line Separating Redox Biology and Redox Toxicology

Although oxidative stress is an essential cellular process, both constitutively active and inducible processes tightly regulate the many redox-dependent functions of the cell (63,85,258). All of these regulatory processes exist in harmony, yet are individually maintained allowing for multiple targets for deregulation. Despite the redundant pathways maintaining redox homeostasis, toxicological exposures frequently compromise many of these targets simultaneously. This lends redox biology to be a susceptible target for not only environmental exposures, but also any xenobiotic exposure. It is likely for this reason that the roles of redox-dependent effects have been demonstrated to be just as, or even more, important in the normal functions of the cell (120,171,237,238). Thus the threshold at which redox biology turns to redox toxicology is not difficult to cross, especially when compared to most other biological processes including protein phosphorylation or even synaptic excitation.

6.2.1: Redox Second Messengers

Unlike many other cellular processes, the second messengers in redox-dependent signaling, such as H_2O_2 or $NO^{\cdot}$, can continue to impact its target even after its initial effect (63). In contrast, other second messengers such as Ca^{2+} or cAMP have their initial effect on its target, and that effect is maintained for as long as the second messenger is present (267), while H_2O_2 can have not only its initial effect on its target but with continued presence hyperoxidation and irreversible damage (70). Thus, what separates redox-dependent cellular processes and many other cellular processes is that the effectors have the ability to contribute both dynamic and permanent effects on their target. For this reason the second messengers needed to initiate most redox-dependent processes are constitutively targeted for catabolism by many processes including enzymatic degradation (82). If these processes are affected by a xenobiotic leading to either the degradation or induction of these messengers it could have deleterious effects on the cell.

Another characteristic of these redox second messengers is that although they are largely specific with regards to their respective cellular effects, they at times can have competing interactions. Specifically, $NO^{\cdot}$ and H_2O_2 are both capable of targeting nucleophilic residues, and many times the same residue (68). Even though the cell can compartmentalize the potential competition of these effects through differing concentrations of enzymes that target each ROS, this can be complicated by the generation of other ROS (64,70). For instance, $O_2^{\cdot -}$ is frequently elevated along with H_2O_2, but H_2O_2 is a much better ROS to serve as a second messenger kinetically. However, $O_2^{\cdot -}$ can interact directly and with high specificity with $NO^{\cdot}$ to form peroxynitrite ($ONOO^-$), which has nearly identical reactivity and specificity as H_2O_2, and is not readily metabolized by peroxidatic enzymes including catalase (142). This means that if $O_2^{\cdot -}$ and

$NO^{\cdot}$ are both elevated it can result in the generation of a species that mimics H_2O_2, which could initiate peroxide-dependent processes or ROS-induced damage without constitutive antioxidant processes available to efficiently regulate those peroxide-dependent cellular processes.

The selective nature of redox-dependent processes can be trumped through the induction of other ROS that can serve similar purposes, but are rarely present under normal physiology. For this reason a xenobiotic exposure to alter intracellular ROS levels is a critical contributor to redox toxicology. Some xenobiotics are directly capable of elevating ROS, as in the case of metals and quinone redox cycling (268). However, a more intricate mechanism of toxicity is the induction of intracellular ROS through endogenous cellular processes (53), such is the case with redox inert metals like zinc and cadmium (41). It would also be possible for xenobiotics to accelerate the protein activity of Nox or NOs to induce H_2O_2 or $NO^{\cdot}$, respectively, similar to pharmacological targeting (269-272). Another common mechanistic feature of xenobiotics to induce oxidative stress, and, therefore, redox toxicology is the perturbation of mitochondrial respiration.

6.2.2: Mitochondria

The mitochondria and endoplasmic reticulum are the two primary sources of intracellular ROS under normal conditions. A handful of studies have manipulated mitochondrial-derived ROS through the altered expression of antioxidant enzymes, which have had substantial effects on cellular growth and induction of gene expression (273,274). This suggests that the mitochondrial generation of ROS is a necessary part of redox biology, including cell cycle and growth. As shown in this dissertation, xenobiotic exposure can specifically target the

mitochondria, leading to elevated ROS to induce a toxicological response (Chapter 2). Thus the health of the mitochondria is an important consideration in redox biology and redox toxicology.

Although mitochondria are a source of endogenous ROS, its health can also be impacted by those same ROS potentially leading to a toxicologically induced positive feedback loop. The mitochondrial compartment is well-defended from ROS through the activities of SOD and peroxidases (47); however, the mitochondrion is less resistant to electrophilic attack. Additionally, the mitochondria are enriched with many nucleophilic residues. When these cysteine residues are targeted by electrophiles, such as zinc ions, the result is an inhibition of mitochondrial respiration that leads to an elevation of ROS (41). Those ROS can then damage cellular components including lipids that result in the formation of additional reactive electrophiles such as 4-hydroxynonenal (4-HNE). Those lipids then serve as endogenous electrophiles that can disrupt mitochondrial respiration, leading to further ROS accumulation in the cell (275,276). The ultimate outcome of this toxicological-induced perturbation of mitochondrial function can be cytotoxicity unless the electrophilic attack is halted through an anti-electrophilic mechanism such as the generation of non-target thiols that serve as a sink for reactive electrophiles.

The final role of mitochondria in maintaining the balance between redox biology and redox toxicology is its essential function as a supplier of cellular energy. Individual mitochondria provide the cell with energy through aerobic metabolism. Even though the primary energy product from mitochondrial respiration is ATP, mitochondria are also the site for side reactions that also provide energy (128). When the flow of energy from aerobic metabolism is disturbed it compromises the cell's ability to maintain normal physiology. Redox-dependent processes would be one of the initial targets affected by mitochondrial perturbed energy production, since both

NADPH and NADH pools are essential for maintaining the dynamic nature of redox biology (129). The perturbed energy pools would compromise nearly all redox enzymes including Trx and Grx. Thus, not only does perturbing mitochondrial function lead to the accumulation of ROS, it also compromises the cells ability to provide reducing equivalents to actively mitigate the elevation in ROS.

6.2.3: Extrapolating the Redox Basis of Disease to Redox Toxicology

The inherent fragility of redox-dependent processes would also suggest that they are also involved in the development of a variety of pathologies. Not only is redox biology important in the progression of many acute diseases, but it has also been implicated in the development of progressive diseases such as cancer, neurodegeneration, and diabetes (65). Furthermore, increased oxidative stress is a hallmark in many complex multifactorial pathologies such as hypertension and obesity (277). Understanding the specific role of redox biology in the progression of a predisposing factor to outright disease could contribute to the elucidation of the consequences of xenobiotic exposure-induced adverse health outcomes. For example, redox biology as a target may provide explanations as to the role of low-dose acute and chronic environmental exposures to disease.

6.2.3.1: Redox Theory of Asbestos-Induced Mesothelioma

Despite the clear, and largely definitive, link between asbestos exposure and the development of malignant mesothelioma (243,244), the mechanism through which a single environmental exposure takes nearly two decades to present with disease is unknown (245). This is alarming due to the length of time between exposure and diagnosis of disease. Understanding

this mechanism would allow for interventions to mitigate or potentially prevent the negative and lethal effects of asbestos since there is substantial time to provide therapy. When one explores the redox biology of carcinogenesis, one can begin to hypothesize how a single exposure to asbestos could lead to cancer.

First, carcinogenic tissues and cells present with increased oxidative activity and decreased antioxidant capabilities (179). Cells leading to a cancer phenotype have elevated ROS levels, particularly in the mitochondria. This leads to an increase in mitochondrial DNA mutations that over time cause a sustained elevation of ROS through the perturbation of mitochondrial function (278). The elevated ROS are hypothesized to accelerate the cell cycle leading to a shorter time for DNA repair (279). The sustained level of oxidative stress along with decreased time for DNA repair develops into increased genomic mutations, many sensitive genomic targets for mutation are those linked to cell cycle control including p53 (280). This ultimately leads to a selection for cells that are characteristic for the development of tumors. Although perturbed redox biology is only a single contributor to the development of cancer, it can be seen as a key driver in carcinogenesis.

The role of redox biology in the progression of cellular carcinogenesis can be used to frame a mechanism through which a single asbestos exposure causes mesothelioma. Asbestos fibers are inefficiently removed from the respiratory tract as they are thin enough to reach the deep lung, which lack cilia and macrophages (281). However, respiratory action eventually "pushes" the fibers to the outer portion of the lung primarily through mechanical force. This does provide a physical protection, as the fiber is unable to significantly interfere with alveolar function; however, once it reaches the mesothelium of the lung, the fibers are permanently

placed. Once at this location, the fiber initiates its potential redox toxicology as described in Chapter 5 and in other studies (282), which may contribute to the development of mesothelioma.

The presence of fibers on the physical surface of the mesothelium stresses the cells and serves to continually recruit inflammatory cells to the area (283). The inflammatory cells, primarily neutrophils and macrophages, recognize the fiber as a foreign body and utilize both oxidative burst attacks and frustrated phagocytosis to modify the fiber, but leads to a further elevation of ROS levels in the mesothelium (247,284). Thus, the mesothelium is already under an increased level of oxidative stress through physiological mechanisms. In addition the asbestos fibers themselves have metals and other components that are capable of redox cycling, adding to the physiological elevation of oxidative stress (246). Further, since, the fiber remains present through the duration of the exposure, the increased oxidative stressors ultimately causes the cells to adapt to the elevated oxidative activity, similar to the initiation of redox-associated carcinogenesis.

Another relevant consideration of asbestos-related health effects is its ability to serve as a substrate for many proteins (253). The same consideration that makes the use of cell integrity assays futile when using asbestiform fiber exposures is also pertinent with regards to cell signaling molecules. The same molecules and proteins used to communicate between cells, both mesothelial and inflammatory cells, can interact with the fiber surface. This compromises the communication between cells, causing the mesothelium to be ineffective at mounting a coordinated response to the pathologic response (285,286). This, too, allows for the progression of a carcinogenic phenotype.

Despite the potential of this mechanism of toxicity to complicate and perturb cellular function, the fundamental concern with asbestiform fibers is their prolonged longevity in the

tissue. Therefore both of these mechanisms, the induction of oxidative stress and disruption of cell-cell communication may not sufficiently induce an effect; however, over many years the toxicity may accumulate to cause disease. Even though this exposure is representative of a single exposure potentially leading to a chronic effect, it is possible to extrapolate this explanation to a low-dose chronic exposure. Wherein a frequent and consistent exposure, such as daily exposure to traffic related air pollutants including zinc and 1,2-NQ, induce an effect that ultimately causes cells to adapt to higher oxidant activity. These cellular adaptations over a chronic exposure accumulate to cause diseases such as cancer. In these scenarios it would be argued that the xenobiotic-induced ROS is leading to a higher number of mutations. However, in the scenario of a genetic-based disease, such as cancer, occurs absent of a toxicological exposure it would be expected that the mutation would likely occur first driving the increased oxidant capacity of the tissue leading to further mutations. The rationale of the development of low-dose chronic exposure to environmental contaminants could also account for the role of air pollution in other progressive disorders such as neurodegeneration and diabetes.

6.3: Redox Toxicology as a Translational Science

An important characteristic to consider within redox toxicology is that many redox-dependent processes can be targeted separately without a clear cause-and-effect interaction between them being apparent (140). For instance, an increase in the E_{GSH} and increase in intracellular H_2O_2 levels can both indicate cytotoxicity, but that does not necessarily mean that the increase in E_{GSH} led to the increase in H_2O_2 or vice-versa (Chapter 2). Establishing this distinction between such events is desirable from a translational perspective, as it could lead to specific therapies to target one redox-dependent process without affecting others. Additionally,

when describing how a xenobiotic exposure leads to redox toxicology, it is also necessary to determine the temporal relationship of these redox changes as they relate to a biological or toxicological outcome. Thus, there are two distinct oxidant events that could result after exposure to an environmental toxicant: 1) those that are easily detectable (extreme changes in redox biology) and 2) those with a causal impact (temporal changes in redox biology). The potential exists to implement these findings in translational science by utilizing the extreme changes as biomarkers, in order to diagnose the pathology and toxicological exposure, while the causal effects could be targeted therapeutically to either mitigate or prevent an adverse response.

6.3.1: Biomarkers of Redox Toxicology

Stable readouts of oxidative stress, including oxidized lipids and DNA, are currently used in toxicological studies. Serum levels of 4-HNE, a byproduct of lipid peroxidation, have been associated with the progression of diseases with varying degrees of success (287-289). A more reliable biomarker to predict and diagnose severity of disease is the oxidized DNA product 8-hydroxyguanosine (8-OHdG). Many studies have demonstrated a clear relationship with adverse health outcomes and increased levels of 8-OHdG (290-293). In addition to these terminally oxidized biological molecules, relative levels of ROS including H_2O_2 and $NO^{\cdot}$ have been measured in exhaled breath condensate as a marker of disease (294,295). However, it should be noted that the measurement of ROS in exhaled breath condensate has only recently been implemented in human studies in the clinic and still requires optimization before they it can reliably predict severity or outcome of a disease, let alone in response to an environmental exposure.

Based on many studies, including the ones discussed in this document (Chapter 3, 4), it is suggested that the concentration of H_2O_2 is a useful biomarker since it is predictive of multiple outcomes including gene expression (56). Although it is currently possible to detect H_2O_2 in exhaled breath condensate, the current methods are difficult to reproduce and the technique lacks specificity (296). The best method in current use is a chemiluminescent approach to detect H_2O_2 in exhaled breath condensate, which affords substantial sensitivity (pM levels); however, it relies on a direct interaction of H_2O_2 with luminol, which is not specific as other ROS are able to react with luminol (297,298). Thus it is important to implement appropriate controls and interventions, such as the use of exogenous catalase to establish the relative contribution of H_2O_2 to total ROS. Furthermore, for clinical studies the samples must be sufficiently large and fresh to provide informative data, which is limiting. That being noted, direct detection of specific ROS in clinical samples would be difficult with the currently available methods.

In lieu of directly measuring H_2O_2 as a biomarker, indirect measures of H_2O_2 levels would be the next most useful biomarker. These indirect measures are considerably more functional as a biomarker. First, the stability of indirect effects of H_2O_2 may be increased allowing for improved technical detection providing enhanced reproducibility. Also, with a relatively increased half-life of this biomarker it may be possible to use other biological matrices that are easier to handle and utilize, such as serum. One potential indirect biomarker of H_2O_2 to be implemented in future studies is sulfenylation.

The results reported in this dissertation (Chapter 3, 4) suggest that protein sulfenylation could be an informative biomarker to predict severity as well as the outcome of a toxicological exposure. First, detection of a change in sulfenylation reports a change in redox homeostasis that could represent an adverse effect. Secondly, protein sulfenylation occurs through a two-electron

transfer of an electrophile to the nucleophilic cysteine, which under physiological conditions would suggest that the effector of the change is H_2O_2 (171,299,300). It is possible another species, such as $ONOO^-$, could provide the two-electron transfer, so this would need to be accounted for (94). The third piece of information that can be gleaned from protein sulfenylation is the identity of the affected target. With this information one can reliably predict what downstream effects could occur in response to an exposure. Thus, sulfenylation as a single biomarker could provide information regarding both severity of toxicity, as the change in sulfenylation would be relative to the amount of ROS present as well as provide a basis to predict the outcomes of an exposure by interrogating the targets of sulfenylation.

6.3.2: Clinical implications of redox toxicology

Intracellular oxidative stress has been a target of therapies to combat the progression of diseases, most notably cancer. Both oxidant ($NO^{\cdot}$, UV radiation) and antioxidant (N-acetylcysteine, NAC) therapies have been implemented with varying success in the treatment of various cancers (231,232). Most cancer cells characteristically have higher oxidant levels, so the current implemented therapeutic approaches is to further modify the ROS levels. The use of therapies that induce oxidant stress is targeted to the tumor or tumor-like area to cause cell death of that cancerous tissue by further elevating ROS levels (301). However, introducing elevated ROS systemically could be harmful as it can impose elevated oxidant stress to other tissues to increase the likelihood of disease, so these therapeutic approaches must be localized and even then still present with some danger. The use of antioxidant therapies to lower the elevated ROS levels theoretically should prevent or at least resist the transformation of cells to cancer. Although promising, most in vivo studies and human trials using antioxidants show no

therapeutic value, and in certain cases actually accelerate the progression of the cancer (302-304).

The targeting of oxidative stress haphazardly by modifying all ROS levels in view of the intricate nature of redox biology in fact would be expected to cause redox toxicology. In fact the objective of oxidant therapies such as UV radiation is targeted redox toxicity to the cancerous tissue by elevating ROS. However, it should be possible to develop an efficacious therapy that targets ROS, but the therapy needs to specifically target the ROS that is involved in the development of the pathogenesis of disease. Not only will the therapy need to be specific to the ROS, but it also may be tissue or cell specific (133,134,165). For instance the role of H_2O_2 as an intercellular communicator or intracellular second messenger may need to be specifically utilized, not to mention that H_2O_2 may serve a different function in lung cells compared to neurons, and as such the therapy should be targeted to a specific tissue (67,68). Furthermore, even within the cell the therapy may need to be specific at a subcellular level as H_2O_2 may have distinct roles in the cytosol and mitochondria. For these reasons, targeting either the elevation or removal of ROS directly as a therapeutic measure is daunting, but theoretically possible.

A potentially more feasible option to develop therapeutic approaches would be to mitigate or prevent redox toxicology is manipulation of the enzymes involved in redox biology to rescue a normal phenotype. In light of the developing role of redox-dependent cellular processes including bioenergetics, signaling, and cytoskeletal maintenance it may be possible to target these processes rather than passively targeting the ROS mediators. For instance, cytosolic H_2O_2 is a necessary mediator in 1,2-NQ induced inflammatory responses (56), so instead of targeting the H_2O_2 directly it may be more efficacious to induce the ability to reverse the H_2O_2-induced protein sulfenylation. Theoretically one could develop a therapy to increase NADPH

production or reduce the catalytic need of Trx or Grx to reverse the protein cysteine to the thiol, it would negate the pro-oxidant effect of the xenobiotic to halt any further toxicity induced by the oxidant stress. Although promising, there is still relatively little known about the regulation of redox-dependent cellular processes so the mechanisms that could be clinically targeted are widely unknown.

6.3.3: Redox toxicology use in susceptible populations

One of the most pertinent topics in the broader field of toxicology is the characterization of factors that confer susceptibility to the adverse effects of toxic exposures. What in particular leads one individual or a group of individuals to be at a higher risk to an environmental exposure than the average person or group? This is vitally important with regards to air pollution in the developed world as certain people are still effected by levels of air pollution at ambient levels, which based on the available research should be safe (5). These susceptible populations are those that have preexisting disease, the elderly, and in some cases genetic polymorphisms. Despite these risk factors, it could be argued that susceptibility is inherently founded in redox biology.

6.3.3.1: Preexisting Disease

Of the most relevant preexisting conditions that are classified as a risk factor to air pollution is cardiovascular disease and asthma (19,20). Although both of these conditions are multifactorial, they have been demonstrated to elevate oxidant stress. The role of whether this oxidant stress is a byproduct or a causative mechanism is largely contested (305,306). For this reason the use of this outcome as either a biomarker or a target for therapy is still unknown, but as an explanation for susceptibility it is an immediate factor. In this context the preexisting

condition causes an elevated oxidant stress, which may prime the redox-dependent cellular mechanisms to be more sensitive to an environmental exposure. In other words the preexisting disease leads to a systemic or tissue specific level of redox biology that is closer to the threshold of a maladaptive response or possibly overt cytotoxicity (107).

6.3.3.2: Genetic Polymorphisms

At the epidemiological level there are many genetic polymorphisms that have been identified as a risk factor to the adverse health effects of air pollution (24). Of these polymorphisms the most studied are those that lead to a compromised or null phenotype in the family of GSTs. The GSTs physiological role is the conjugation of glutathione to xenobiotics to make the xenobiotic more soluble for cellular export, and more recently the glutathionylation of protein targets which can afford cyto-protective function (307,308). Despite the epidemiological evidence, there is at present little mechanistic explanation as to how a GST-null phenotype could enhance air pollutant-induced cytotoxicity. It is possible that the redundancy of GSTs within the cell would compensate for the loss of a single GST at the total cellular level. However, the role of GST to utilize GSH in both its function as well as a thiol donor to glutathionylate a cysteine suggests that this protein is intimately involved in redox-dependent processes, and in this dissertation has been shown to be a potential factor in enhancing the pro-oxidant effects of 1,2-NQ (Chapter 4). Moreover, the GST-null phenotype, like a preexisting disease or increased age, may not itself be the functional target of the air pollutant, but it primes the biology towards redox toxicology.

6.4: The Future of Redox Toxicology

The better understanding of redox biology in so many physiological and pathological processes has been instrumental in improving the biochemical basis of many diseases (47,84,92,123,258). These improved theories and technologies that drove those findings can now be applied to the field of toxicology to better understand the mechanistic basis of environmental exposure-induced toxicity (70,133,309). Although currently in its infancy, it has the potential to revolutionize not only how to predict safety but may suggest novel approaches to the global public health problem of air pollution. There are many promising avenues of research to effectively utilize redox toxicology in order to further characterize the mechanism of action of xenobiotics towards an improvement of public health.

6.4.1: Reconciling redox chemistry with redox biology

Despite evidence reported in this dissertation (Chapter 2,3) and elsewhere that H_2O_2 is an essential mediator of not only normal physiology (120,132), but also of the toxicity for environmental exposures (56), there is uncertainty as to how H_2O_2 serves as a second messenger effectively. There are two major concerns driving this uncertainty. The first is that there is a large quantity of peroxidases, including catalase, in both the extracellular and intracellular spaces to effectively eliminate most if not all H_2O_2 in the intracellular compartments (81). This would mean H_2O_2 could be an effective local mediator, but in the case of membrane generation of H_2O_2 by Nox enzymes, the H_2O_2 would likely encounter too many peroxidases in the cytosol to have any relevant, or consistent, impact on distant targets. The second concern is that the chemical nature of H_2O_2 is not conducive to react readily with most nucleophilic amino acids in cellular pH, and in most cases the residue must have a unique microenvironment, as in the case with

protein tyrosine phosphatases that under physiological conditions exist in the thiolate form (87,94). This may in part explain the selectivity of H_2O_2's biological effects, but consequently this also means that there would only be a few targets for H_2O_2, which contradicts many reports of H_2O_2's ability to have pleiotropic effects on cell biology (84). Two explanations exist that attempt to reconcile the chemical nature of H_2O_2 and its role in redox biology: the flood-gate hypothesis and the chaperone theory. Not only will these two explanations need to be verified, but they also serve as targets for xenobiotic perturbation of cellular processes.

6.4.1.1: Flood-Gate Hypothesis of Redox Regulation

Due to the high concentration of peroxidases within the cell it is likely that H_2O_2 can exert its effects only within a limited radius of the site where it is produced. On the other hand, it is also acknowledged that most of the peroxidases are inactivated upon interaction with H_2O_2 or other ROS. Thus, it is possible that elevation of H_2O_2 and other ROS can inhibit the function of peroxidases, which can provide the physiological means to introduce an elevated level of H_2O_2 in intracellular compartments (310). The elevation of ROS, and in this case specifically H_2O_2, to the point where all available enzymes to serve as metabolic enzymes are occupied leaving excess H_2O_2 to act on signaling mediators is referred to as the flood-gate hypothesis (311).

This hypothesis has gained favor from some researchers, as it accounts for both the peroxidatic capability of the cell as well as the potential for H_2O_2 to act as a second messenger in redox-dependent cellular processes. Briefly, this hypothesis suggests that H_2O_2 needs to reach a defined intracellular level to overwhelm the peroxidatic functions, or the gates holding back the "floods" of ROS, allowing for H_2O_2 to serve as a signaling mediator (312). This view is supported by the fact that many of the H_2O_2-induced signaling effects lead to the induction of

H_2O_2-metabolizing enzymes (68,81). Therefore, when H_2O_2 is able to break through the "flood-gates" not only does it serve important cellular processes, but it also leads to the transcription and translation of proteins such as catalase that effectively target H_2O_2. This provides temporal regulation of H_2O_2 signaling, as once H_2O_2 concentrations exceed levels the constitutive factors the cell can handle, they induce signaling to regulate not only physiological responses, but also induce mechanisms that regulate its own concentration.

This process could easily be perturbed by xenobiotic exposures, as chemicals could either elevate H_2O_2 levels that exceed the "flood-gates" or the chemical could directly impact the function of the "flood-gate" enzymes to inactivate them. Both of these xenobiotic-induced effects could theoretically occur. However, based on the data presented in this dissertation (Chapter 2, 3) the elevation of H_2O_2 is observed within minutes of the initial exposure and remains elevated throughout the exposure. This suggests that the enzymes are not directly targeted, as this would likely require more time to lead to signaling activation, as opposed to the overwhelming presence of relatively large quantities of ROS, including H_2O_2. Although, as it is beyond the scope of these studies, it could be argued that these chemicals either through exogenous or endogenous generation of H_2O_2 to levels that exceed the metabolic capabilities of the cell allow H_2O_2 to serve its second messaging function to induce adaptive gene expression (Chapter 2) or increase sulfenylation of regulatory proteins (Chapter 3).

6.4.1.2: Chaperone Theory of Redox Regulation

Even though the "flood-gate hypothesis" is a compelling theory to establish how redox-dependent cellular processes are regulated, it is not able to explain how H_2O_2 can interact with proteins that are not readily nucleophilic. To account for this, researchers have proposed that

specific proteins may be able to serve as chaperones to carry the H_2O_2-induced signal to regulatory proteins (81). This is an attractive theory as it accounts for the high peroxidatic capacity of the cell, but it also rationalizes how a ROS-dependent signal could be carried to less nucleophilic proteins. The basis of this theory is that proteins that have a high capacity to react with H_2O_2 are modified by the species and then are directed in an active and specific manner to a specific cellular target without interference from peroxidases, as they would no longer be able to exert their effect on H_2O_2 (313). These chaperones would likely be proteins that already serve a peroxidatic function.

A protein of particular interest in the field of redox biology, and a likely candidate to serve as a chaperone to shuttle the H_2O_2-induced cellular signal is peroxiredoxin (Prx) (314,315). There are six different mammalian Prx that are separated into three different classes the typical 2-cys, atypical 2-cys, and 1-cys. Prx has a high affinity to metabolize H_2O_2 and exists in high concentrations within the cell, and as such serves as the primary cellular peroxidase (315). Specifically, the typical Prxs initial function is to metabolize H_2O_2 to H_2O leaving the peroxidatic cysteine as a sulfenic acid. The sulfenic acid then interacts with a resolving cysteine on another Prx to form a dimer, which is reduced through the interaction of Trx. However, the peroxidatic cysteine can be hyperoxidized to the sulfinic resulting in a gain of function allowing it to act as a protein chaperone that inhibits protein aggregation. Additionally, the sulfinic cysteine of Prx is the only known target of sulfiredoxin (Srx), which, through the expense of ATP, reduces the hyperoxidized product back to the sulfenic acid (95,316). The unique characteristic of Prx of having two distinct functions (as a thiol it is a peroxidase and as a sulfinic it is a chaperone) in addition to its prevalence within the cell, suggests that the Prx family may be a key regulator in H_2O_2 signaling.

Additionally, recent reports have contributed an additional cellular role of Prx as it can interact with other proteins. This finding led to the hypothesis that Prx facilitates the messaging function of H_2O_2 within the cell (317). The finding demonstrated that sulfenylated Prx is able to interact with transcription factors (STAT3) and other regulatory proteins (ER) to induce a cellular response (318,319). This finding demonstrated the potential for Prx to be functional during all three of its redox states, and since the cell can regulate the redox state of Prx through Trx and Srx, this further reveaed the integral role of Prx in H_2O_2 signaling. In short, it is currently thought that Prx can modulate H_2O_2 levels, serve as a mediator in H_2O_2-induced signaling, and provide cytoprotection. Although there is a need to validate these studies, as well as confirm the specific function of each member of Prx family, this protein serves as a candidate target for redox toxicology that has yet to be thoroughly explored. For instance it is possible that the accumulation of H_2O_2 observed in the studies presented here (Chapter 2, 3) and the subsequent increase in protein sulfenylation, may in fact be a direct oxidant or electrophilic attack on the Prxs leading to perturbed H_2O_2-induced signaling.

6.4.2: Emerging Technologies and Needs

As with all biomedical sciences, toxicology and, specifically, this subtopic redox toxicology, is limited by the available techniques to provide novel findings. There is a need to expand the tools to directly detect other ROS including $NO^{\cdot}$ and $O_2^{\cdot -}$ as well as other oxPTMs such as the sulfinic and sulfonic acids. This section will specifically explore the immediate needs to explore the role of H_2O_2 and its downstream effects in environmental exposures.

6.4.2.1: Detecting H_2O_2

HyPer is currently the best tool to detect H_2O_2 due to its high specificity and fluorescent readouts (147,320). It is also powerful because it can be targeted to subcellular compartments (Chapter 2), but a current limitation is its lack of functionality in highly oxidized subcellular regions such as the ER. Thus an immediate need to detect H_2O_2 is the development of a sensor that can be utilized in the ER, especially since it is one of the most important sources of endogenous ROS for the cell. As mentioned previously HyPer fluorescence changes must be controlled for by assessing pH, but this concern is already addressed by the sensor roGFP-Orp1 (133,165), which is pH insensitive at physiological conditions. Another important distinction between HyPer and roGFP-Orp1 is that they are reduced through two different enzymatic processes, Grx and Trx, respectively (133). So, future studies could utilize this characteristic to assess the effect of environmental exposures on these critical reducing pathways.

One final concern with regards to HyPer is that its recombinant form is unstable and cannot be utilized in validation experiments (147). This is of particular importance in environmental exposures as it can be desirable to assess the direct effects of the exposure on the sensor. Even though it would be useful to have a sensor that is stable when purified, this can be accomplished through other means, such as testing sensor responsivity in the presence of excess catalase. In line with this concern is that, while fluorogenic sensors are powerful because their expression can be controlled and allow for high spatial resolution, it is currently not feasible to implement them to study clinical samples. Thus it will be useful to develop a reliable sensor for the analysis of H_2O_2 concentrations in clinical samples (serum, BAL).

6.4.2.2: Detecting Protein Sulfenylation

As mentioned previously and demonstrated in this dissertation (Chapter 4), protein sulfenic acids could be utilized as a powerful biomarker as an indirect measure of H_2O_2 and predict the outcomes of an environmental exposure. However, the detection of sulfenic acids currently requires an alkenyl attack of the sulfenic residue to form a thio-ether complex, such as dimedone or its various analogs. This sulfenic labeling with dimedone is a relatively slow reaction requiring nearly an hour to obtain any significant signal (235). Thus it would be desirable to develop a label with increased reactivity to complete the reaction quicker; however, this would likely come at the expense of reduced selectivity.

Additionally, the detection of sulfenic acids is limited by challenges that are similar to those involved in the detection of H_2O_2 in clinical samples. Sulfenylation can be readily detected in these samples as it only requires a substrate to be labeled with a dimedone-like molecule to trap the sulfenics (174,309). However, this form of detection requires forethought in the experimental design, and it is likely that banked samples that have not been treated with dimedone immediately before collection cannot be assessed for sulfenic acids. Additionally, due to the short half-life of sulfenic acids, any relevant sulfenic acids will likely be lost in samples that are not labeled immediately after collection. Many biomarkers and tests require fresh samples, and this is by no means a reason to stop exploring the utility of protein sulfenylation as a readout in environmental exposures. This simply implies that the techniques utilized to detect protein sulfenylation need to be optimized for detection sensitivity so that minimal sample is required to detect any changes to obtain full utility in clinical studies.

6.5: Conclusions

The data presented in this dissertation along with other studies discussed in this document suggest that oxidative stress could be a unifying characteristic of environmental exposure to air pollutants. Although this association is generally accepted, the new evidence described within this document supports an intimate relationship between air pollution-associated disease and oxidative stress. For instance, the mechanistic involvement of air pollutant induced H_2O_2 in perturbations of cellular processes as well as the novel finding in these studies that protein sulfenylation is disturbed by exposure to air pollution demonstrates a role of oxidative stress in the progression of disease linked to air pollution. Thus it is reasonable to suggest that xenobiotic-induced perturbations of redox biology, or redox toxicology, are a unifying mechanistic feature of air pollutant-induced adverse health outcomes.

The growing evidence that many of the diseases linked to air pollution exposure involve elevated oxidant stress in non-xenobiotic-induced pathogenesis further supports this theory. This observation coupled with the fact that many air pollutants are redox active or induce redox changes, there is a mechanistic basis to link air pollutant-induced oxidative stress in the development and cause of its related toxicology. If in fact redox toxicology is a unifying characteristic of air pollution-induced toxicology, the continuing elucidation of its specific role and development of better monitoring of oxidant events could lead to improved public health either through better therapeutic approaches or improved regulatory efforts.

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