# THE ROLE OF PEROXIREDOXINS AS MECHANOSENSITIVE ANTIOXIDANTS IN ENDOTHELIAL CELLS

A Dissertation
Presented to
The Academic Faculty

By

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In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy in the
Wallace H. Coulter Department of Biomedical Engineering at the
Georgia Institute of Technology and Emory University

Georgia Institute of Technology

August 2008

# THE ROLE OF PEROXIREDOXINS AS MECHANOSENSITIVE ANTIOXIDANTS IN ENDOTHELIAL CELLS

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Only the educated are free.

-Epictetus (55 AD - 135 AD), *Discourses*

To Mom and Dad for the opportunities and the motivation,
To Betsy, Cady, and Audrey for the laughter,
To Adam for the infinite friendship, support, and love

# ACKNOWLEDGEMENTS

The completion of this dissertation would not have been possible without the involvement of many exceptional individuals. I would first like to acknowledge my thesis advisor, Dr. Hanjoong Jo, for the opportunity to work on this project, to present my findings at many scientific conferences, and to contribute my talents to several collaborative manuscripts and review articles. I also extend my greatest appreciation to my thesis committee members, Drs. Ajit P. Yoganathan, W. Robert Taylor, Kathy K. Griendling, and Dean P. Jones, for supporting this work and my future career with new ideas, a positive outlook, and generous references. I thank Drs. Sang Won Kang, Sue Goo Rhee, and Hyun Ae Woo at Ewha Womans University and Dr. Xilin Chen at AtheroGenics, Inc. for collaborative support and providing essential reagents. I am also grateful to Johnafel Crowe in the IBB Core Lab for assistance with confocal microscopy, as well as to Beth Bullock Spencer, Sally Gerrish, Shannon Sullivan, Leita Young, and Sandra Wilson in the Georgia Tech and Emory BME Offices for their limitless help with financial and administrative matters. Furthermore, I would like to acknowledge my funding source, the American Heart Association Pre-Doctoral Fellowship.

During the course of this project, I have been privileged to work with several talented students and scientists in the Jo Lab. First, I would like to recognize Dr. George Sorescu for providing my initial training in the laboratory. In addition, I thank my undergraduate research mentees, Michael Taylor and April Kooy, for their hard work and for giving me the opportunity to mentor and teach them. I am very appreciative to Maria Aleman and Deb Smith for their efficient running of the lab, as well as for their humor and honesty. Credit is also due to Dr. Manu Platt, Dr. Hannah Song, Casey Holliday, Dr.

Hyuk Sang Kwon, Dr. Doug Nam, Michelle Sykes, and Dr. Won Jong Rhee for technical aid and for facilitating a truly collegial lab environment. I am indebted to Dr. Seth Brodie, Dr. Kyung Hwa Chang, and Chih-Wen Ni for scientific help and advise above and beyond their obligations. And lastly, I am especially grateful to Randy Ankeny, Dr. Sarah Tressel, and Dr. Mamta Patel. Over the last five years, they have provided vast technical assistance, and more importantly, immeasurable moral support, sincerity, and true friendship to last a lifetime.

Finally, I would like to thank my family for encouraging me in this endeavor. I am so appreciative to my father and mother, Keith and Teresa Holbrook, for giving me the opportunity to achieve any goal I could conceive, for setting high standards, and for showing me the benefits of both failure and success. I also thank my sisters, Betsy, Cady, and Audrey, for the endless laughter and for keeping me humble and true to my roots. I am grateful to my mother- and father-in-law, Drs. Erla and John Mowbray, for their empathy, optimism, and experience, as well as to my extended family, John, Erin, and David Mowbray, for their friendship and encouragement. And last, but certainly not least, I would not have been able to complete this undertaking without the infinite support of my husband, Adam Mowbray. I thank him from the bottom of my heart for being my best friend, my advocate, my counselor, and my partner.

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| | |
|---|---|
| Ad-GFP | Green fluorescent protein adenovirus |
| Ad-wt hNrf2 | Wild type human Nrf2 adenovirus |
| AP-1 | Activator protein-1 |
| ARE | Antioxidant response element |
| BAEC | Bovine aortic endothelial cells |
| $BH_4$ | Tetrahydrobiopterin |
| BMP4 | Bone morphogenic protein 4 |
| BSA | Bovine serum albumin |
| bZIP | Basic leucine zipper |
| CAT | Chloramphenicol acetyltransferase |
| Cdk | Cyclin-dependent kinase |
| CVD | Cardiovascular disease |
| Cys | Cysteine |
| *D* | Vessel diameter |
| DETA-NO | Diethylenetriamine NONOate |
| $\partial u/\partial r$ | Shear rate or velocity gradient |
| EC | Endothelial cells |
| EGF | Epidermal growth factor |
| eNOS | Endothelial nitric oxide synthase |
| EpRE | Electrophile response element |
| FBS | Fetal bovine serum |
| FTH | Ferritin heavy chains |
| GC | Greater curvature |

| | |
|---|---|
| GCLM | Glutamate-cysteine ligase modifier subunit |
| GFP | Green fluorescent protein |
| GPX | Glutathione peroxidase |
| GSH | Glutathione |
| GSR | Glutathione reductase |
| GST | Glutathione S-transferase |
| $H_2O_2$ | Hydrogen peroxide |
| HAEC | Human aortic endothelial cells |
| HO-1 | Heme oxygenase-1 |
| HUVEC | Human umbilical vein endothelial cells |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IL-1β | Interleukin-1β |
| Keap1 | Kelch-like ECH-associated protein 1 |
| KRP | Krebs Ringer Phosphate |
| LC | Lesser curvature |
| LDL | Low density lipoprotein |
| L-NAME | NG-nitro-L-arginine methyl ester |
| LOX-1 | Lectin-like oxidized low density lipoprotein receptor-1 |
| LS | Laminar shear stress |
| μ | Viscosity |
| MCP-1 | Monocyte chemoattractant protein-1 |
| NF-κB | Nuclear factor-κB |
| $NO^{\bullet}$ | Nitric oxide |
| NOS | Nitric oxide synthase |
| NQO1 | NADPH:quinone oxidoreductase-1 |

| | |
|---|---|
| Nrf2 | NF-E2-related factor 2 |
| $O_2^{\bullet-}$ | Superoxide |
| $ONOO^-$ | Peroxynitrite |
| OS | Oscillatory shear stress |
| Ox LDL | Oxidized low density lipoprotein |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PBS | Phosphate-buffered saline |
| PDGF | Platelet-derived growth factor |
| PECAM-1 | Platelet endothelial cell adhesion molecule-1 |
| PRX | Peroxiredoxin(s) |
| PTEN | Phosphatase and tensin homolog |
| PTP | Protein tyrosine phosphatases |
| *Q* | Flow rate |
| ROS | Reactive oxygen species |
| siRNA | Small interference RNA |
| SOD | Superoxide dismutase |
| ST | Static |
| $\tau$ | Shear stress |
| $\tau_{wall}$ | Wall shear stress |
| TGF-ß | Transforming growth factor-β |
| Thr | Threonine |
| TNF-α | Tumor necrosis factor-α |
| TPA | 12-O-tetradecanoylphorbol-13-acetate |
| TRX | Thioredoxin |
| TXNRD1 | Thioredoxin reductase1 |

| | |
|---|---|
| VCAM-1 | Vascular cell adhesion molecule-1 |
| wt | Wild type |
| XO | Xanthine oxidase |

# SUMMARY

In the United States alone, cardiovascular disease kills nearly one million people each year. The inflammatory disease atherosclerosis has been implicated in more than three-quarters of these deaths. Despite the global nature of most risk factors, atherosclerosis has been identified as a focal disease occurring in defined regions of the vasculature. Plaques have been shown to localize at bifurcations or curves in the arterial tree where disturbed blood flow patterns and low, oscillatory shear stress (OS) exist. In contrast, straight arteries exposed to high velocity, unidirectional laminar shear stress (LS) remain plaque free and "atheroprotected." In order to address the hemodynamic components of atherosclerosis development, recent work has focused on the mechanosensitive endothelium to establish new pathological mechanisms.

Endothelial cells (EC) exposed to oscillatory shear stress experience oxidative stress as a signature of atherosclerosis. Conversely, unidirectional laminar shear stress reduces reactive oxygen species (ROS) levels and subsequent inflammatory responses. Previous work by our group found that fluid flow stimulates these important functional changes through gene and protein expression regulation. In the following study, we proposed that mechanosensitive antioxidants play a considerable role in laminar shear-dependent redox balance and atheroprotection.

Recently, a new group of ubiquitous antioxidant proteins has been acknowledged in yeast, plant and animal cells species. The peroxiredoxins (PRX) are thiol specific-, non-selenium containing enzymes that use redox-active cysteines to reduce peroxides and eliminate peroxynitrite. However, they have yet to be investigated in response to shear

stress. As such**, the overall *objective* of this dissertation was to study the effects of atherogenic and atheroprotective fluid flow profiles on the mammalian antioxidants peroxiredoxins, and to identify mechanosensitive family members playing a role in redox regulation.** By understanding how peroxiredoxins are regulated and what roles they play physiologically, we will have better insight into endothelial cell (EC) biology and redox-sensitive mechanisms of vascular diseases. **The *central hypothesis* of this project was that *unidirectional laminar shear stress (LS), compared to disturbed oscillatory shear stress (OS), promotes increased expression of peroxiredoxins (PRX) via transcriptional regulation, which in turn influences the balance of reactive oxygen species in endothelial cells.*** This hypothesis was tested according to three specific aims using cellular, molecular, and biochemical studies of bovine aortic endothelial cells.

We first explored the effects of shear stress on expression of mammalian peroxiredoxin family members. Using an *in vitro* model, the cone-and-plate viscometer, we applied atheroprotective, high, unidirectional laminar shear stress or atherogenic, low, oscillatory shear stress to endothelial cells in culture. Here, we were able to identify all six PRX family members in bovine aortic endothelial cells (BAEC). Furthermore, through this work, we revealed for the first time that PRX are regulated by shear stress in endothelial cells. When compared to OS and static culture, exposure to chronic LS upregulated PRX 1 levels intracellularly. LS also increased expression of PRX 5 relative to static controls, but not OS. Consistent with reports in other cell types, PRX exhibited broad subcellular localization in BAEC, with distribution in the cytoplasm, Golgi, mitochondria, and intermediate filaments. However, these localization patterns did not appear to change in response to shear stress. Taken together, our results suggest that distribution of PRX in specific subcellular environments may provide protection against local ROS

production and facilitate ROS-mediated signal transduction. Simultaneously, widespread allocation of PRX might also be important for comprehensive management of the overall oxidative state of cells. Moreover, upregulation of PRX 1 by LS may act to reduce ROS levels in EC and prevent atherogenic oxidative stress.

To establish the functional importance of PRX 1 in shear stress-dependent redox balance, we next examined the role of PRX 1 in LS-mediated hydrogen peroxide regulation. Endothelial cells exposed to OS experience oxidative stress as a signature of atherosclerosis. Conversely, chronic LS reduces ROS levels. Here, we used Amplex Red assay as an independent method to measure ROS levels in BAEC. Consistent with previous reports, OS increased and LS decreased catalase-inhibitable hydrogen peroxide. Subsequently, we investigated whether PRX 1 was responsible for the decreased $H_2O_2$ levels in endothelial cells exposed to LS. Specific siRNAs were used to knockdown either PRX 1 or PRX 5 protein levels. Using isoform-specific PRX antibodies, we found that these siRNAs had no significant effect on other PRX, indicating that they exclusively targeted PRX 1 or 5, respectively, amongst all PRX family members. Depletion of PRX 1 using siRNA resulted in significantly higher ROS levels in BAEC exposed to LS, OS, and static conditions, while PRX 5 depletion did not. Altogether, these results indicate that chronic exposure to LS upregulates PRX 1 expression in order to keep ROS levels low in endothelial cells.

To identify the pathway by which atheroprotective laminar shear stress stimulates PRX 1 protein production, we undertook additional gene expression and siRNA depletion studies. Several reports have demonstrated that antioxidant genes are induced by laminar shear stress in endothelial cells. In line with this, we discovered that LS upregulates *Prdx1* gene in a time-dependent manner compared to OS or static culture.

However, this increase in expression was not due to shear stress-dependent stabilization of *Prdx1* mRNA. These findings lead us to believe that *Prdx1* was likely regulated at the transcriptional level. In addition, the antioxidant response element (ARE) has been shown to be conserved amongst several antioxidant genes, and studies showed it responds to chronic, steady laminar shear stress through its transcription factor Nrf2. Our analysis of the bovine *Prdx1* promoter revealed a Nrf2 binding site 160bp upstream of the gene. Furthermore, Nrf2 overexpression promoted basal PRX 1 protein production, while Nrf2 depletion reduced *Prdx1* mRNA following exposure to LS. Collectively, our work illustrates that laminar shear stress affects PRX 1 by inducing the *Prdx1* gene, in part via the transcription factor Nrf2.

In this dissertation, we have developed a comprehensive register of shear stress-dependent peroxiredoxin behavior and discovered a novel mechanosensitive antioxidant that plays an important functional role in redox regulation of endothelial cell biology. This work illustrates how physiologically relevant fluid flow affects reactive oxygen species, through the unique expression of antioxidants, in particular Peroxiredoxin 1. Identifying factors that play an important role in shear stress-dependent oxidative stress could help provide localized therapies at the site of endothelial cell dysfunction. Our findings are relevant to the inflammatory disease atherosclerosis, a major cause of cardiovascular disease. By understanding how PRX1 is regulated and what role it plays in physiology, we can better understand vascular diseases and develop effective preventative therapeutic approaches.

# CHAPTER 1

# INTRODUCTION

Cardiovascular disease (CVD) affects an estimated 80 million American adults (one in three) and has been the leading cause of mortality in the United States for more than a century. The inflammatory vessel disease atherosclerosis has been implicated in nearly three-quarters of these CVD deaths[1]. In an effort to develop effective treatments for this pervasive pathology, research is now focused on the mechanisms of atherogenesis. Growing evidence indicates that overproduction of reactive oxygen species contributes significantly to the pathogenesis of atherosclerosis. This dissertation will address the protective and pathological effects of arterial hemodynamics with respect to reactive oxygen species regulation. In particular, we will investigate the how fluid flow mediates oxidative stress in endothelial cells with a concentration on antioxidant gene and protein expression.

## Atherosclerosis

Atherosclerosis is responsible for more than half of reported deaths in the industrialized world[2]. It is an inflammatory disease of the large arteries and is characterized by the development of lipid-filled plaques that obstruct the vessel lumen. Lesion enlargement has been shown to progress over the lifetime of an individual, with plaque formation beginning decades before clinical manifestation[3]. The disease process begins as a protective physiological response-to-injury, whereby leukocytes and monocytes adhere to the endothelium as a result of insult to the vessel wall. These insults correspond to a number of behavioral and physiological risk factors including hypercholesterolemia,

smoking, hypertension, diabetes, genetic alterations, elevated homocysteine concentration, obesity, and infectious microorganisms[4, 5].

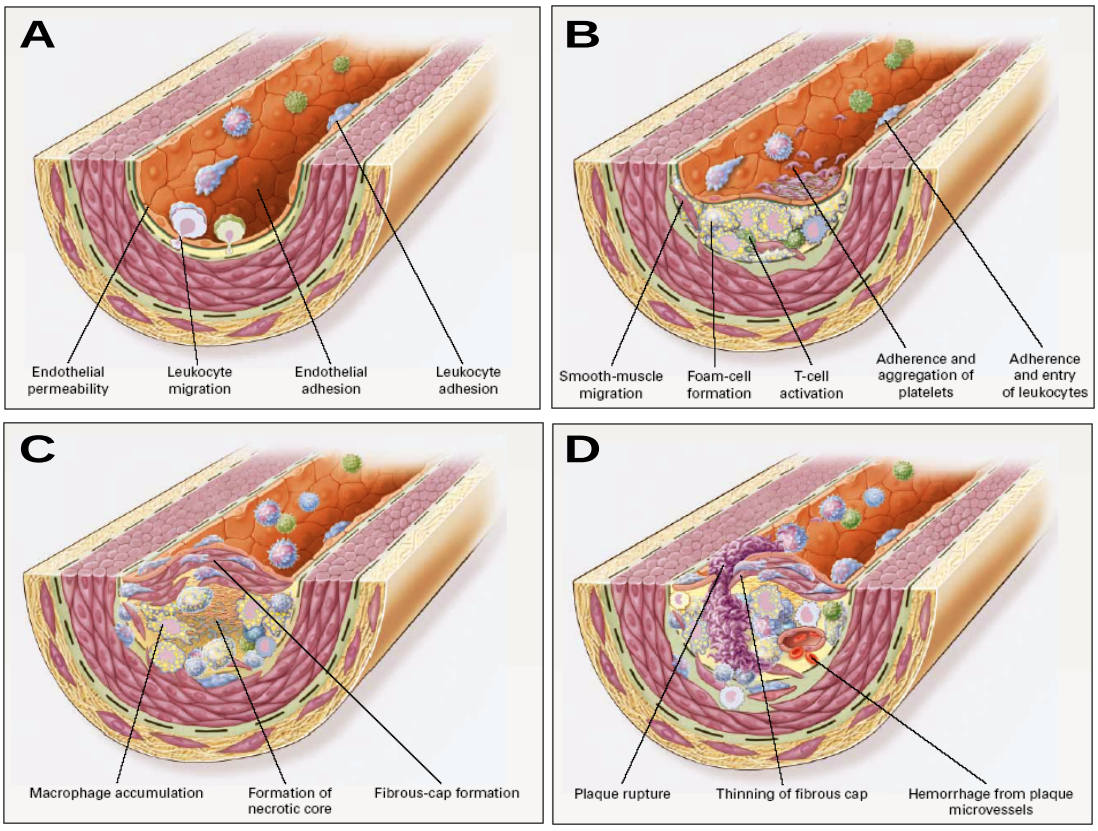


***Figure 1.1 Atherosclerosis is an inflammatory disease.*** *Endothelium dysfunction (A) results in fatty streak formation (B), advanced plaque development (C), and rupture (D). Figure reprinted with permission[4]. Copyright © 1999 Massachusetts Medical Society.*

Advanced atherosclerotic lesions result from a chronic inflammatory response and endothelial dysfunction due to continued and numerous insults (Figure 1.1). Early features in the pathogenesis of atherosclerosis include endothelial cell expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, followed by cell surface attachment of circulating monocytes[6-8]. Upon adhesion, monocytes migrate through the endothelium, localize subendothelially, and differentiate into macrophages. These macrophages take up oxidized LDL (ox LDL),

accumulate lipid, and subsequently become foam cells[2, 5]. In conjunction with T-cells and smooth muscle cells, these foam cells form a fatty streak that represents the earliest recognizable lesion[2]. Foam cells and the endothelium secrete cytokines, including tumor necrosis factor-α (TNF- α), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-ß), that promote endothelial cell apoptosis and initiate migration and proliferation of smooth muscle cells into the sub-intimal layer[5, 9]. A repetitive cycle of monocyte accumulation and smooth muscle cell proliferation results in an atherosclerotic plaque that occludes the vessel lumen severely and alters downstream blood flow[4].

Following plaque formation, metalloproteases, collagenases and elastases can induce degradation in an effort to remodel the vessel wall and open the occluded lumen. However, this action causes the lesion to weaken and become susceptible to rupture[5]. Myocardial infarction and stroke occur as a result of plaque erosion and subsequent thrombosis at, or downstream of, the rupture site.

**Atherosclerosis and Hemodynamics**

Despite the global nature of most atherosclerotic risk factors, atherosclerosis has been identified as a focal disease occurring in defined regions of the vasculature. Plaques have been shown to localize at bifurcations or curves in the arterial tree. As such, vessels including the carotid bifurcation, coronary vessels, aortic arch, and abdominal aorta are particularly prone to atheroma formation. Studies by Caro et al. in 1969 and Zarins et al. in 1983 showed that these areas of the vasculature coincide with regions of disturbed, low velocity blood flow[10-12]. In contrast, straight arteries exposed to high velocity, unidirectional laminar flow remain plaque free and “atheroprotected”[11, 13]. This phenomenon suggests that blood flow, and the forces it imparts on vessels, directly influence cellular function and play a critical role in vascular wall physiology.

**Hemodynamic Forces in the Vasculature**

As the heart beats, pushing blood through the vascular system, pulsatile pressure and flow are generated and act on the vessels via three forces[14, 15]. These hemodynamic stresses (forces per unit area) include compressive stress, circumferential stress, and shear stress (Figure 1.2). Compressive stress results from the hydrostatic pressure of blood acting normal to the vessel wall. Circumferential stress is caused by changes in vessel diameter due to the pulsatility of blood flow. And lastly, frictional wall shear stress acts tangentially as a result of blood flow over the vessel wall[14]. Although orders of magnitude smaller than blood pressure, this frictional stress has been identified as the critical force mediating flow-dependent atherogenesis or atheroprotection. Disturbed blood flow at vessel branches correlates to low, bidirectional shear stress, while unidirectional laminar flow results in high unidirectional shear stress[10, 11, 15].

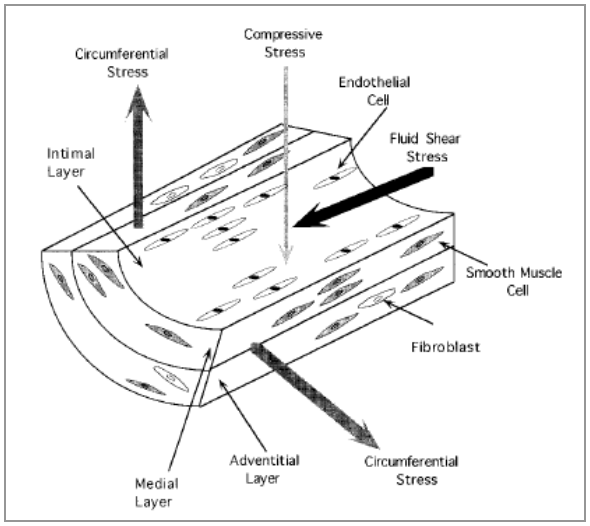


***Figure 1.2 Blood flow acts on the arterial wall via three hemodynamic forces.***
*Figure reprinted with permission[14]. Copyright © 1997 American Chemical Society and American Institute of Chemical Engineers.*

Fluid shear stress, $\tau$, is defined as the force produced by a moving viscous fluid over a solid body constraining its motion[16]. In the large arteries where atherosclerosis occurs,

vessel diameter is much larger than individual cells, and blood can be considered a Newtonian fluid. Newtonian fluids are those that possess a linear relationship between stress and strain rate, and their flow is governed by the Navier-Stokes equations. As such, blood shear stress is proportional to fluid viscosity, $\mu$, and the shear rate or velocity gradient, $\partial u/\partial r$, according to the following equation[17]:

$$\tau = \mu \frac{\partial u}{\partial r}$$

For the steady laminar flow in large vessels, wall shear stress, $\tau_{wall}$, can be described by:

$$\tau_{wall} = \frac{32 \mu Q}{\pi D^3}$$

where $Q$ is the flow rate and $D$ is the diameter of the vessel[18].

At the cellular level, the effects of blood shear stress have been correlated to the behavior of the exposed endothelium. Comparison of en face tissue staining from atheroprone and atheroprotected regions of the mouse aorta shows distinct differences in endothelial cell morphology, as well as expression of factors such as NFκB, ICAM-1, and VCAM-1[19]. In order to investigate these *in vivo* findings in a simplified setting, several *in vitro* systems have been developed that expose cultured cells to defined shear stresses[20].

**In Vitro Models of Shear Stress**

Devices such as the parallel plate flow chamber and cone-and-plate viscometer (shear apparatus) have allowed for controlled shear stress experiments on endothelial cells[20-24]. In the parallel plate system, cells are cultured on glass slides and positioned within a rectangular flow chamber. A roller pump is used to circulate medium through the chamber to provide uniform levels of shear stress. The dimensions of the chamber and

the pressure drop across it dictate the fluid flow rate and result in specific shear stress conditions[17].

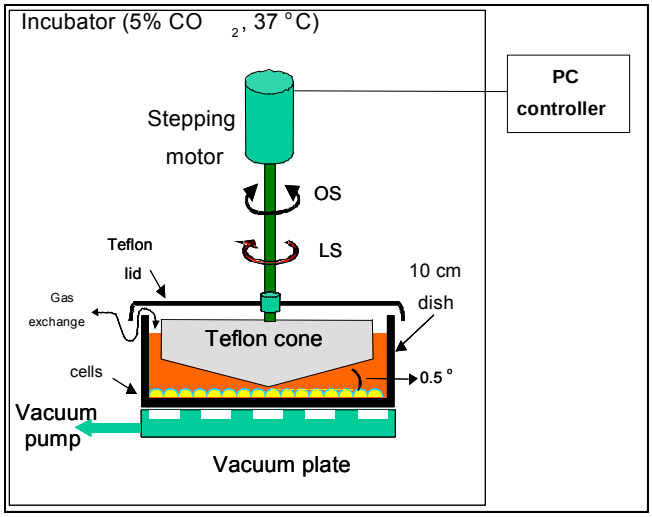


***Figure 1.3 Schematic of the cone-and-plate shear stress system***

In the cone-and-plate shear system, a Teflon cone is used to generate shear stress. The cone is placed in a circular culture dish containing adherent endothelial cells and medium. As illustrated in Figure 1.3, the angle of the cone is very small ($<1^0$). Rotation of the cone forces the fluid between the cone and plate to flow azimuthally and produce a shear stress on the stationary endothelial cell monolayer[16]. The Navier-Stokes equations can be used to accurately calculate the magnitude and direction of this shear stress and to determine the limits to which this system can produce laminar arterial flow profiles[16]. When compared to the parallel plate system, the cone-and-plate shear apparatus exhibits several disadvantages and advantages. For example, the low volume of media used in the cone-and-plate viscometer may permit secreted growth factors and cytokines to accumulate over time and produce non-physiological effects on cultured cells. In addition, this system results in a gradient of shear stress, and cells at the center of the culture dish, near the cone apex, may experience lower shear stress

than those at the perimeter. Alternatively, the cone-and-plate viscometer can expose a greater number of cells to shear stress when compared to the parallel plate system using small glass coverslips. This is critical in RNA and protein analysis of cells. The system can also be used to simulate reversal of flow by rotating the cone back and forth. Although secondary flow can occur at high rotational speeds, this is generally negligible to achieve physiologically relevant shear stresses[25]. As a result the cone-and-plate viscometer can expose cultured endothelial cells to both "atheroprone" oscillatory shear stress (OS) and "atheroprotective" unidirectional laminar shear stress (LS).

**Shear Stress and Endothelial Cell Biology**

*In vitro* studies utilizing the previously described flow models have established shear stress-dependent changes in endothelial cells similar to those observed *in vivo*. For examples, cells align and elongate in the direction of flow upon exposure to a threshold level of unidirectional laminar shear stress[17, 26]. Conversely, when cultured in the presence of low and oscillatory shear stress or static conditions, endothelial cells exhibit a cobblestone morphology with random orientation and do not align[22]. This cellular process of identifying different shear stress forces and converting them to physiological responses is called mechanotransduction[14, 27, 28].

As shown in Figure 1.4, several membrane components have been established as "mechanoreceptors" of shear stress including the glycocalyx, integrins, platelet endothelial cell adhesion molecule-1 (PECAM-1), vascular endothelial-cadherin, tyrosine kinase receptors, G proteins, primary cilium, and ion channels[14, 27-30]. Located at the surface of endothelial cells, they are poised to directly sense and transduce force across the membrane to the interior of cells[14, 27]. Mechanoreceptors can transmit their signals via the cytoskeleton through focal adhesions, cell-cell junctions, and the nuclear

membrane or via a biochemical cascade through the generation of second messengers and transcription factor activation[27, 28]. In addition to previously described morphological changes, mechanotransduction of different shear stresses can also elicit other structural, metabolic, and gene regulatory modifications[28].

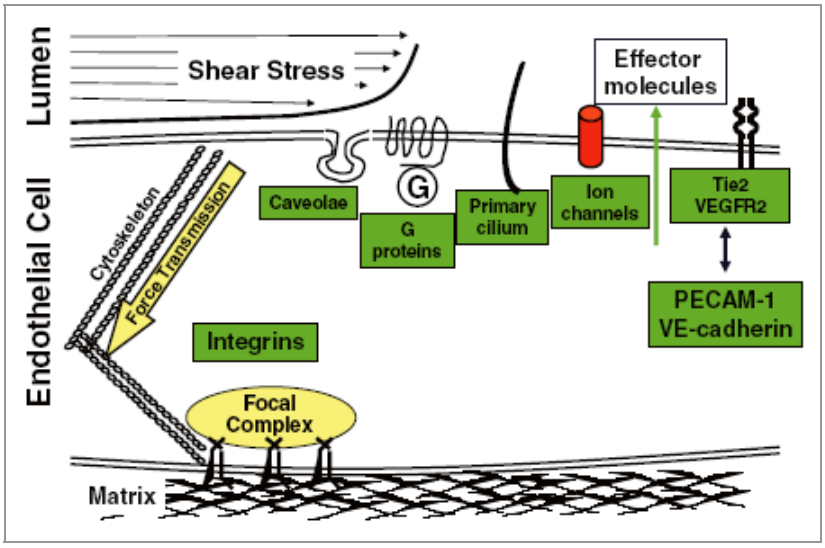


***Figure 1.4 Mechanoreceptors in endothelial cells exposed to shear stress***
*Figure reprinted with permission[27]. Copyright © 2008 Lippincott, Williams & Wilkins.*

In general, unidirectional laminar shear stress promotes an atheroprotective endothelial phenotype, while low, oscillatory shear stress stimulates atherogenic responses[27, 31, 32]. Steady laminar shear stress promotes the release of several factors that promote endothelial cell survival and prevent coagulation, migration of leukocytes, and smooth muscle cell and endothelial cell proliferation[32]. Vasoactive molecules, including nitric oxide ($NO^{\bullet}$) and prostacyclin, promote vessel dilation and increased blood flow[15, 17, 32]. In contrast, low and oscillatory shear stress induces the expression of surface molecules, including VCAM-1 and ICAM-1, as well as cytokines such as bone morphogenic protein-4 and monocyte chemoattractant protein-1 (MCP-1) that promote monocyte adhesion[17, 21, 32]. Secretion of PDGF, endothelin-1, and angiotensin II encourages vasoconstriction,

vascular smooth muscle cell proliferation, and reactive oxygen species production[15, 31, 32]. Such shear stress-dependent regulation of reactive oxygen species has been shown to play a critical role in the development of atherosclerosis.

**Reactive Oxygen Species**

Reactive oxygen species (ROS) are produced in all aerobic cells and form as a byproduct of the normal metabolism of oxygen[33]. Some are free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), nitric oxide ($NO^{\bullet}$), and lipid radicals, while others, like hydrogen peroxide ($H_2O_2$), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl), are not. Disproportionate generation of ROS, beyond the capacity of balancing antioxidant systems, results in oxidative stress through oxidative damage to DNA, protein, carbohydrates, and lipids[33, 34]. In fact, oxidative stress has been implicated in the development of numerous diseases including neurodegenerative diseases, premature aging, cancer, and vascular diseases[35].

Reactive oxygen species arise from many different cellular sources such as mitochondrial respiration, hemoproteins, peroxidases, lipoxygenase, cyclooxygenase, cytochrome P450, NADPH oxidase, xanthine oxidase (XO), and nitric oxide synthase (NOS). Of these, NADPH oxidase, xanthine oxidase, and NOS have been most extensively studied in the cardiovascular system[33, 34]. These enzymes use various substrates as electron sources to reduce molecular oxygen to superoxide or hydrogen peroxide. Reduction of oxygen by one electron results in superoxide, while a two electron reduction, or dismutation of $O_2^{\bullet-}$ by superoxide dismutase (SOD), produces $H_2O_2$[36]. Accumulating $O_2^{\bullet-}$ and $H_2O_2$ generated by these oxidases can further shift cellular redox balance by acting as a source of other ROS molecules. For example, endothelial NOS (eNOS) requires tetrahydrobiopterin ($BH_4$) and L-arginine to form $NO^{\bullet}$[37].

Without one or the other, eNOS becomes uncoupled and produces $O_2^{\bullet-}$ and $H_2O_2$[38, 39]. This dramatically reduces the bioavailability of the antioxidant $NO^{\bullet}$ and allows radicals it might normally react with to attack other targets. Furthermore, when eNOS is partially uncoupled, both $O_2^{\bullet-}$ and $NO^{\bullet}$ are produced, can react to generate the highly reactive $ONOO^-$, and lead to a dramatic increase in oxidative stress[33]. There is substantial evidence to suggest that these enzymes systems contribute significantly to such overproduction of ROS in the shear stress-dependent pathogenesis of atherosclerosis.

**ROS and Atherosclerosis**

ROS have been linked to atherosclerotic plaque formation in both animal models and human studies. In hypercholesterolemic rabbits, atherosclerosis resulting from diet was linked to XO-induced oxidative stress via the XO inhibitor oxypurinol[40]. Moreover, in diseased human coronary arteries, electron spin resonance experiments found activation of both NADPH oxidase and XO[41]. NADPH oxidases have also been found to be activated, with concomitant $O_2^{\bullet-}$ production, in the vessels of atherosclerotic rabbits[42]. Additionally, mice lacking a critical NADPH oxidase subunit, p47 phox, develop reduced plaque burden in the aorta compared to hypercholesterolemic control animals[43]. Peroxynitrite has also been implicated in atheroma formation. 3-nitrotyrosine, a marker of NO-mediated oxidative damage, was found in human atherosclerotic tissue, while LDL isolated from lesions was shown to be nitrated[44-46].

The pathological effects of ROS in the vasculature can be correlated to stress in endothelial, vascular smooth muscle, and adventitial cells. In endothelial cells, OS-dependent ROS can mediate inflammatory pathways via increased nuclear factor-κB (NF-κB) translocation, expression of adhesion molecules, and monocyte adhesion[47, 48]. In addition, ROS generated by the endothelium promote atherogenic signaling

mechanisms in the vascular wall. It has been shown that NADPH oxidase-dependent oxidative stress induces smooth muscle cell proliferation[49, 50]. Also, reduction of $NO^{\bullet}$ bioavailability results in hypertension by attenuating endothelium-dependent vessel dilation[51]. $O_2^{\bullet-}$ and the $NO^{\bullet}$ derivative $ONOO^-$ act to directly oxidize lipid proteins, specifically LDL to ox LDL[52, 53]. ox LDL can promote apoptosis, smooth muscle cell proliferation, and also act as powerful monocyte chemoattractant, upregulating proatherogenic molecules like MCP-1, IL-1β, LOX-1 and PAI-1[54-57].

Despite the pathological consequence of oxidative stress, transient subtoxic levels of ROS have recently been associated with several vital signaling pathways. Low levels of $H_2O_2$, produced in response to extracellular stimuli such as cytokines, neurotransmitters, peptide growth factors, and hormones, can affect protein function via oxidation of critical cysteine residues. This mechanism has been shown to influence the behavior of transcription factors, phospholipases, protein kinases and phosphatases, ion channels and G proteins[58-60]. As a result, hydrogen peroxide is considered a ubiquitous intracellular messenger at subtoxic concentrations. It has been shown to critically affect PDGF-, EGF-, and angiotensin II-dependent signaling and regulate targets such as protein tyrosine phosphatases (PTP) and lipid phosphatase PTEN[61-65]. This concept suggests that reactive oxygen species may play dual roles in the endothelium, contributing to the atheroprotective functions of laminar shear stress, as well as the inflammatory pathways induced by disturbed flow conditions[66-68].

**Shear Stress and ROS Production**

Laurindo et al. were the first to described flow-dependent generation of ROS in endothelial cells. In both *ex vivo* and *in vivo* experiments using rabbit aortas, they established that short-term application of unidirectional shear stress stimulated $O_2^{\bullet-}$

production[69]. *In vitro* shear systems have subsequently demonstrated that LS stimulates ROS production acutely, but transient elevation returns to basal levels within a few hours[66]. However, unlike LS, OS promotes sustained ROS production, maintaining elevated levels as long as cells are exposed to it [66, 70, 71].

Many of the previously described ROS sources have been implicated in OS-mediated ROS generation. Several groups have found that oscillatory shear stress is a potent stimulator of $O_2^{\bullet-}$ in endothelial cells via the NADPH oxidase[66, 70]. Moreover, McNally et al. showed that OS induces NADPH oxidase-dependent hydrogen peroxide, which shifts the balance of xanthine dehydrogenase and XO to promote additional $O_2^{\bullet-}$[72]. Work by Hsiai et al. also indicated that OS causes elevated peroxynitrite production[73]. These differences in LS- versus OS-dependent ROS production contribute greatly to pathological cellular behavior.

We and others have found that OS-stimulated ROS production leads to ICAM-1, VCAM-1, and E-selectin expression, and monocyte adhesion[8, 66, 71, 74]. Conversely, laminar shear stress acts to reduce ROS levels and subsequent monocyte attachment[75, 76]. The mechanism by which LS restricts such oxidative stress remains unclear. In the following study, we propose that mechanosensitive antioxidants play a considerable role in laminar shear-dependent redox balance and atheroprotection.

**Antioxidants and Laminar Shear Stress**

Antioxidant defense systems are critical to the protection of cellular macromolecules. They work to maintain a reductive cytosolic environment using both catalytic and nonenzymatic processes. In particular, it has been hypothesized that antioxidants are likely to regulate intracellular hydrogen peroxide in a localized manner. Production of

$H_2O_2$ occurs where needed for intracellular signaling, while hydrogen peroxide molecules diffusing away from the site of action are destroyed[77]. Numerous studies suggest that steady laminar flow promotes such a reducing environment in endothelial cells, decreasing both inflammation and apoptosis[27]. In fact, laminar shear stress has been shown to regulate several antioxidants including heme oxygenase-1 (HO-1), ferritin heavy chains (FTH), NADPH:quinone oxidoreductase-1 (NQO1), glutamate-cysteine ligase modifier subunit (GCLM), thioredoxin reductase1 (TXNRD1), glutathione reductase (GSR), endothelial nitric oxide synthase (eNOS), superoxide dismutase, glutathione (GSH) and thioredoxin (TRX)[27, 66, 75, 76, 78-80].

Many of these genes are regulated through a conserved antioxidant response element (ARE) or ARE-like transcriptional regulatory sequence in their promoter[75, 80, 81]. The ARE, also called the electrophile response element (EpRE), is a *cis*-acting DNA regulatory element with a core sequence of 5'-RTGACNNNGC-3'[81-83]. Transcription factor NF-E2-related factor 2 (Nrf2), a basic leucine zipper protein (bZIP), activates the ARE and its downstream genes by binding to the consensus sequence. Under homeostatic conditions, the cytoskeleton binding protein Kelch-like ECH-associated protein 1 (Keap1) binds Nrf2 in the cytoplasm and targets it for degradation via the ubiquitin-proteasome system. However, when cells are exposed to stress, Nrf2 is released and travels to the nucleus, where it forms a heterodimer with another bZIP, Maf, and binds to the ARE[81-85]. Interestingly, recent reports have shown that Nrf2 and the ARE are both sensitive to laminar shear stress[80, 82, 85, 86]. These findings suggest a mechanistic pathway for the atheroprotective effects of laminar shear stress and provide further experimental evidence for the important role of antioxidant systems in endothelial cells.

**Peroxiredoxins (PRX)**

Recently, a new group of ubiquitous antioxidant proteins has been acknowledged in yeast, plant and animal cells species. The peroxiredoxins (PRX) are thiol specific-, non-selenium containing enzymes that use redox-active cysteines to reduce peroxides and eliminate $ONOO^-$. They are produced at high levels in the cell, having been reported to comprise 100 nanograms to a few micrograms per milligram of soluble protein in mammalian cells[87, 88]. Based on conserved cysteine residues, six isoforms of peroxiredoxin (PRX 1-6) have been identified in mammalian systems.

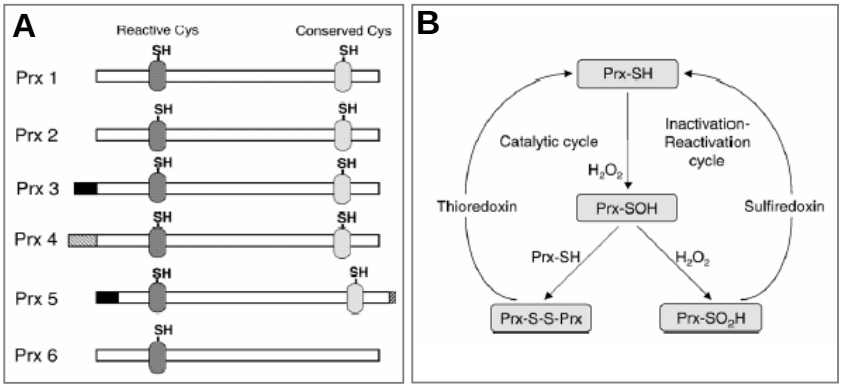


***Figure 1.5 Peroxiredoxin family members reduce hydrogen peroxide in cells.*** *(A) Mammalian PRX exist in three classes and contain either one or two conserved cysteine residues. Figure reprinted with permission[35]. Copyright © 2002 Maney Publishing. (B) Active PRX exist in a reduced state. Upon oxidation by hydrogen peroxide, they can either be inactivated by further oxidation or return to their active state with the help of an electron donor such as thioredoxin. Figure reprinted with permission[77]. Copyright © 2005 Elsevier.*

Peroxiredoxin isoforms can be divided into three subcategories (Figure 1.5). Typical 2-Cys PRX contain both the N-terminal and C-terminal conserved cysteine residues and require both of them for catalytic function. PRX 1, 2, 3, and 4 are typical 2-cys PRX and form homodimeric associations in a head-to-tail fashion upon oxidation[35, 88]. Atypical 2-Cys and 1-Cys PRX proteins possess only the N-terminal "peroxidatic" Cys, but atypical 2-Cys PRX requires an additional non-conserved cysteine for catalytic activity[77]. PRX 5

and 6 are atypical 2-Cys and 1-Cys PRX, respectively, and complete their catalytic cycle as monomers[35].

The peroxidase reaction for each subclass of PRX includes an identical first step where the peroxidatic cysteine attacks the peroxide substrate and is oxidized to a cysteine sulfenic acid. For typical 2-Cys peroxiredoxins, this intermediate can either be further oxidized and inactivated by hydrogen peroxide, or it can react with the C-terminal cysteine in the other subunit to form an intermolecular disulfide[35, 77, 88]. The catalytic cycle is completed when the sulfinylated (Cys-$SO_2H$) or dimeric PRX is reduced by sulfiredoxin or thioredoxin, respectively[88-91]. For PRX 5, the sulfenic acid intermediate reacts with the sulfhydryl group of the C-terminal cysteine on the same molecule and forms an intramolecular disulfide. As before, this disulfide is reduced via thioredoxin electron donation. The reaction scheme for the catalytic cycle of 1-Cys PRX proteins uses a reacting thiol as a reducing substrate. The disulfide bond is formed between the thiol and sulfinylated (Cys-$SO_2H$) PRX. Ground state is achieved by attack of a second thiol on the disulfide bridge[92]. Although the identity of this thiol donor remains controversial, GSH, lipoic acid, and cyclophilin A have been proposed as reductants[93, 94].

Peroxiredoxins are prime candidates for regulators of local hydrogen peroxide signaling in endothelial cells. They have been shown to be broadly distributed in the subcellular compartments of several mammalian cells types, and subsequently, have access to regional ROS production[87, 88]. In addition, 2-Cys PRX exhibit a high degree of affinity for hydrogen peroxide; a concentration of less then 20 micromolar $H_2O_2$ results in half maximal PRX activity[95]. Studies using siRNA and PRX-specific overexpression have shown that peroxiredoxin enzymes significantly affect the intracellular level of hydrogen peroxide.[77] In fact, overexpression of PRX 1 and 2 blunts PDGF- and EGF-dependent

$H_2O_2$ production and TNF-α-induced NF-κB transcriptional activity[96, 97]. PRX have also modulated signaling effects of these cytokines, affecting JNK, ERK, and p38 MAP kinase and blocking the induction of ceramide-dependent apoptosis[98, 99]. These results suggest that PRX serve as important members of the signaling cascade by specifically destroying hydrogen peroxide molecules.

Like many participants in intracellular signaling, peroxiredoxin proteins can be regulated at the transcriptional level and by post-translational modification. In 2000, Ishii et al. found that oxidative stress promotes PRX 1 gene expression via the Nrf2 transcription factor in macrophages[100]. Kim et al. also established Nrf2 as a regulator of PRX 1 in cancer cells exposed to hypoxia/reoxygenation[83]. However, additional work in macrophage and monocytic cell lines identified activator protein-1 (AP-1) as the important transcription factor in tumor promoter-dependent PRX 1 expression[101]. Furthermore, recent studies have revealed that FOXO3A induces PRX 3, NFR-1 and GABPA affect PRX5, and Pax5 regulates PRX 6 gene expression, in cardiac fibroblasts, HeLa cells, and mouse B cells, respectively[102-104].

Additionally, PRX have been shown to be both phosphorylated and sulfinylated[91, 105]. Phosphorylation of PRX 1 and 2 at $Thr^{90}$ by cyclin-dependent kinase (Cdk) results in a loss of peroxidase activity. These results suggest that accumulation of hydrogen peroxide may be critical during the normal progression of the cell cycle[77, 105]. Similarly, sulfinylation of PRX (conversion of Cys-SH to Cys-$SO_2H$) causes reduction in 2-Cys PRX activity that can be rescued by sulfiredoxin, but not thioredoxin[89]. This reversible inactivation is likely to accommodate the intracellular messenger function of hydrogen peroxide[106]. As previously mentioned, hydrogen peroxide is likely tightly regulated. Protection from destruction by the large pool of PRX allows $H_2O_2$ concentration to

increase rapidly above a certain threshold at the site of signaling. At the same time, the slow rate of PRX reactivation by sulfiredoxin allows enough time for hydrogen peroxide accumulation and signal cascade propagation[64, 77].

Recent studies have elucidated several functional roles for PRX. Work by the A.B. Fisher group has shown the importance of PRX 6 in heart recovery following ischemia reperfusion injury and in protection from phospholipid peroxidation-mediated membrane damage[107, 108]. PRX 2 has been found to regulate PDGF signaling and vascular remodeling, as well as cellular senescence and death[65, 109, 110]. In addition, PRX 1 affects NFκB signaling, PRX 4 is involved in acrosome formation during sperm development, and PRX 3 regulates mitochondrial apoptosis[102, 111-113]. However, no studies have yet investigated the role of shear stress on PRX regulation in endothelial cells. Based on their wide variety of physiological functions, we believe that peroxiredoxin antioxidants are likely involved in the molecular mechanisms that control shear stress-dependent redox state and atherosclerotic plaque development.

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# CHAPTER 2

# SPECIFIC AIMS

## Project Significance

The hemodynamic patterns generated by blood flow play an important role in the localized development of atherosclerosis. Straight regions of the vasculature are considered "protected" from atherogenesis by high levels of unidirectional laminar shear stress (LS). In contrast, plaque-prone areas in curves and bifurcations of the arterial tree correspond to locales exposed to low or unstable shear stress including oscillatory shear stress (OS)[1, 2]. Numerous studies have linked these local mechanical forces to the behavior of the exposed endothelium. Endothelial cells experiencing disturbed flow exhibit oxidative stress and subsequent inflammatory molecule expression and monocyte recruitment as early signatures of atherosclerosis[3, 4]. *In vitro* studies have established that OS increases reactive oxygen species (ROS) levels in endothelial cells, whereas LS blocks such responses. While OS-induced oxidative stress and inflammation have been shown to occur in an NADPH oxidase-dependent manner, the atheroprotective mechanisms of LS remain unidentified[5-7].

Antioxidant defense systems are likely to play an important part in this LS-dependent balance of ROS. As antioxidant proteins, all six mammalian peroxiredoxins (PRX) reduce harmful hydrogen peroxide and have been linked to the prevention of oxidative stress, apoptosis, and inflammation[8, 9]. However, PRX have been overlooked in endothelial cells and the oxidative pathways involved in plaque development. In the following study, we investigate the expression of all PRX family members in response to

physiologically relevant hemodynamic patterns and identify PRX 1 as an important regulator of endothelial cell redox balance.

**Project Objective**

The goal of this project is to investigate how atherogenic and atheroprotective fluid flow profiles regulate peroxiredoxin proteins and to identify how mechanosensitive antioxidants, specifically PRX 1, play a role in redox regulation. An understanding of how PRX 1 is regulated and what role it plays physiologically may contribute important insights into endothelial cell biology and vascular diseases.

**Overall Hypothesis**

***Unidirectional laminar shear stress (LS), compared to disturbed oscillatory shear stress (OS), promotes increased expression of peroxiredoxins via transcriptional regulation, which in turn influences the balance of reactive oxygen species in endothelial cells.*** This hypothesis was tested according to three specific aims using cellular, molecular, and biochemical studies of bovine aortic endothelial cells (BAEC). Bovine aortic endothelial cells were chosen as an *in vitro* model to represent the arterial endothelial cells exposed to physiologically significant flow patterns in an *in vivo* setting.

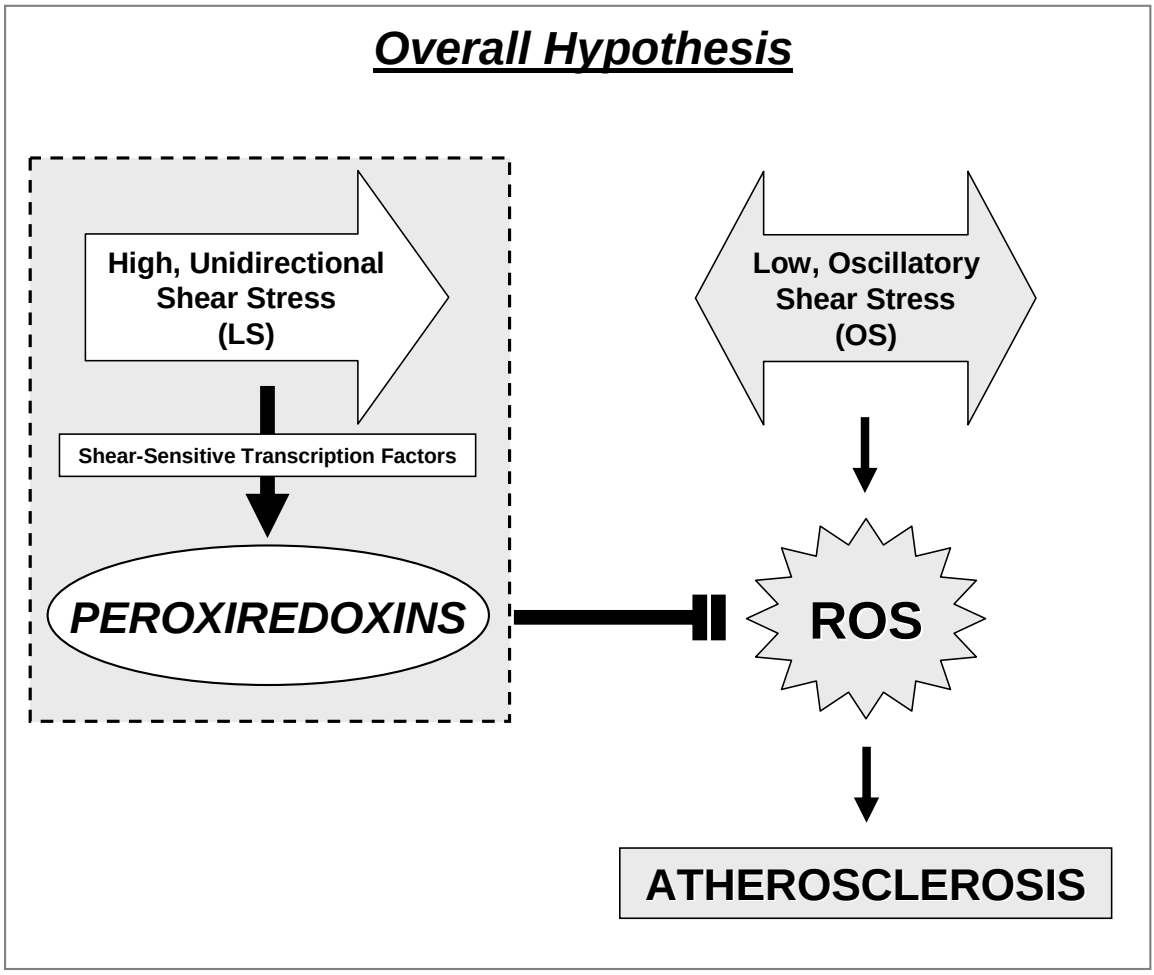


***Figure 2.1 Overall Hypothesis*** *Atheroprotective laminar shear stress reduces reactive oxygen species levels in endothelial cells by inducing peroxiredoxin expression.*

**Specific Aim 1**

**Investigate shear stress-dependent expression and subcellular localization of peroxiredoxin family members in aortic endothelial cells.**

*Hypothesis:* Laminar shear stress, compared to oscillatory shear stress, promotes increased expression of peroxiredoxins and modifies subcellular localization of individual PRX.

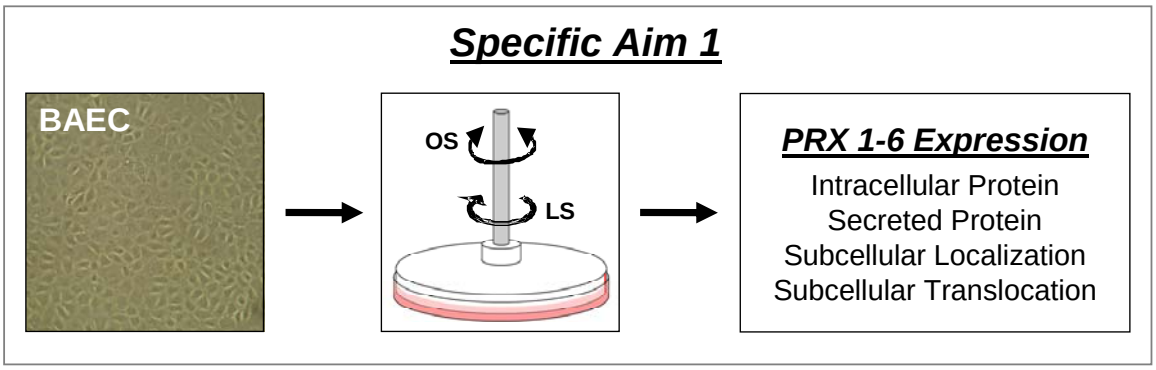


***Figure 2.2 Experimental layout for Specific Aim 1***

To determine the effect of different hemodynamic profiles on peroxiredoxin family members, we examined overall protein expression and protein localization in response to shear stress (Figure 2.2). An *in vitro* shear system, the modified cone-and-plate viscometer, was used to expose bovine aortic endothelial cells to chronic unidirectional laminar shear stress or oscillatory shear stress. Immunoblot and immunofluorescence staining were employed to characterize shear stress-dependent expression patterns and establish a clear picture of the individual behavior of all six mammalian peroxiredoxins.

**Specific Aim 2**

**Identify the role of PRX 1 in shear stress-dependent reactive oxygen species regulation.**

*Hypothesis:* Laminar shear stress maintains low levels of reactive oxygen species in endothelial cells via the expression of PRX 1.

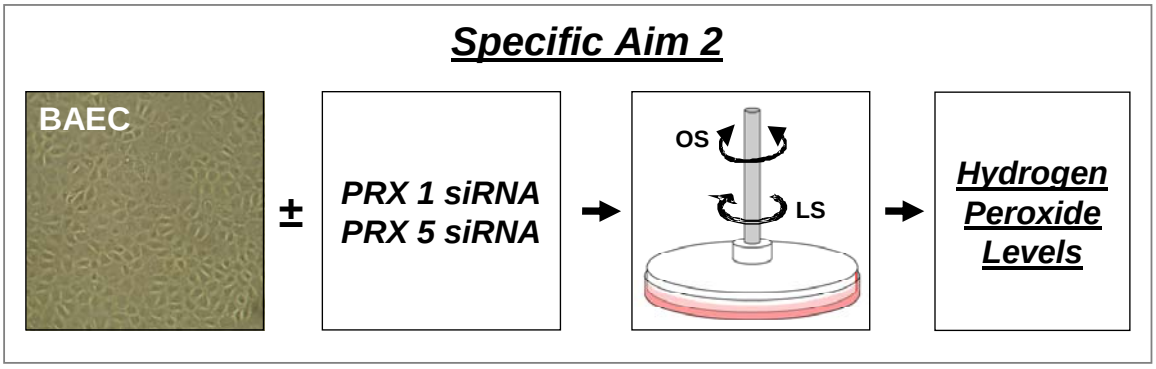


***Figure 2.3 Experimental layout for Specific Aim 2***

Characterization data from Specific Aim 1 demonstrated that intracellular PRX 1 is upregulated by chronic laminar shear stress and is strategically located to access reactive oxygen species from membrane-bound oxidases. To establish the physiological importance of PRX 1 in shear stress-dependent redox balance, we examined the role of PRX 1 in LS-mediated ROS regulation (Figure 2.3). The depletion of individual PRX from cellular systems provided a useful tool to study the functional role of each PRX isoform. In these studies, specific siRNAs were used to knockdown PRX 1 expression in endothelial cells. Subsequently, shear stress-induced hydrogen peroxide levels were assessed via Amplex Red fluorescence assay. Additional experiments using PRX 5 siRNAs further established the functional importance of PRX 1, amongst other PRX family members, in endothelial cell redox regulation.

**Specific Aim 3**

**Investigate the mechanism of shear stress-dependent PRX 1 expression in aortic endothelial cells.**

*Hypothesis:* Laminar shear stress induces PRX 1 protein expression via transcriptional regulation of the *Prdx1* gene.

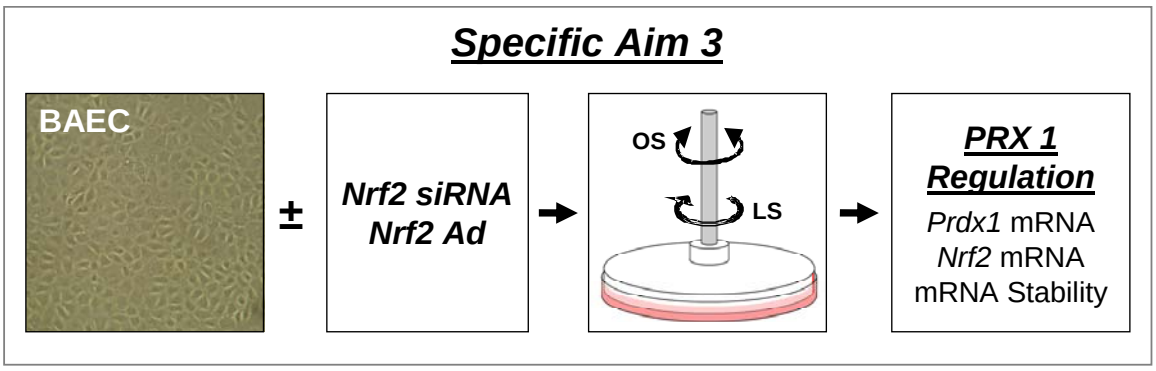


***Figure 2.4 Experimental layout for Specific Aim 3***

In Specific Aims 1 and 2, PRX 1 was found to be a novel mechanosensitive antioxidant playing an important role in shear stress-dependent regulation of endothelial cell biology. To identify the mechanism by which laminar shear stress promotes protein expression of PRX 1, we investigated the regulation of the *Prdx1* gene (Figure 2.4). Here, *Prdx1* gene expression was assessed in response to unidirectional laminar shear stress or oscillatory shear stress, as well as a possible transcription factor (Nrf2). mRNA degradation and *Prdx1* promoter studies further elucidated the critical regulation points in LS-dependent PRX 1 expression and provided potential targets for therapeutic intervention.

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# CHAPTER 3

# LAMINAR SHEAR STRESS UPREGULATES PEROXIREDOXINS (PRX) IN ENDOTHELIAL CELLS – PRX 1 AS A MECHANOSENSITIVE ANTIOXIDANT

The overall objective of this thesis study is to examine the regulation of peroxiredoxin (PRX) family members in endothelial cells (EC) and to identify their role in shear stress-mediated redox control. Via Specific Aim 1, we sought to investigate protein expression and subcellular localization of peroxiredoxins in response to physiologically relevant flow. Subsequently, the goal of Specific Aim 2 was to establish the functional importance of any mechanosensitive PRX in reactive oxygen species (ROS) management. To achieve these aims, an *in vitro* shear system was employed to generate atheroprotective or atherogenic shear stress patterns. For the first time, we have characterized the mechanosensitive nature of PRX antioxidants, able to locally and globally regulate hydrogen peroxide levels in endothelial cells.

**Summary**

Shear stress plays a significant role in endothelial cell biology and atherosclerosis development. Previous work by our group has shown that fluid flow stimulates important functional changes in cells through protein expression regulation. Peroxiredoxins are a novel family of antioxidant enzymes, but have yet to be investigated in response to shear stress. Studies have shown that oscillatory shear stress (OS) increases reactive oxygen species concentration in endothelial cells, whereas laminar shear stress (LS) blocks this response. We hypothesized that PRX are responsible for the anti-oxidative effect of LS. To test this hypothesis, bovine aortic endothelial cells (BAEC) were subjected to LS (15 dyn/cm$^2$), OS (± 5 dyn/cm$^2$, 1 Hz) or static (ST) conditions for 24 hours. Using Western

blot and immunofluorescence staining, all six isoforms of PRX were identified in BAEC. When compared to OS and ST, exposure to chronic LS upregulated PRX 1 levels intracellularly. LS also increased expression of PRX 5 relative to static controls, but not OS. PRX exhibited broad subcellular localization, with distribution in the cytoplasm, Golgi, mitochondria, and intermediate filaments. In addition, PRX 1-knockdown, using specific small interference RNA (siRNA), attenuated LS-dependent ROS reduction in BAEC. However, PRX 5 depletion did not. Together, these results suggest that PRX 1 is a novel mechanosensitive antioxidant, playing an important role in shear-dependent regulation of endothelial biology and atherosclerosis.

**Introduction**

Shear stress acting on the blood vessel wall plays an important role in the development of atherosclerosis. Straight regions of the arterial tree are considered "protected" from atherogenesis by high levels of unidirectional laminar shear stress[1, 2]. In contrast, plaque-prone areas in curves and bifurcations of the vasculature correspond to locales exposed to low or unstable shear stress including oscillatory shear stress[2-4]. These local mechanical forces have been correlated to the behavior of the exposed endothelium[5-7].

Endothelial cells exposed to disturbed flow experience oxidative stress, inflammatory molecule expression, and monocyte recruitment as early signatures of atherosclerosis[8-12]. *In vitro* studies have established that OS is a potent stimulator of reactive oxygen species production in EC, and quantitative measurements by our group showed a significant increase in both OS-dependent superoxide ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$) production[12-14]. We found that OS-stimulated ROS occurs in an NADPH oxidase-dependent manner and leads to inflammatory responses (ICAM-1 expression and monocyte adhesion)[13, 15]. Conversely, LS acts to reduce ROS production and

subsequent inflammatory response[13, 16, 17]. Nevertheless, the mechanism by which LS restricts oxidative stress remains unclear.

Antioxidant defense systems are critical to the protection of cellular macromolecules. They work to maintain a reductive cytosolic environment using both catalytic and non-enzymatic processes[18]. In particular, it has been hypothesized that antioxidants are likely to regulate intracellular hydrogen peroxide in a localized manner. Production of $H_2O_2$ occurs where needed for intracellular signaling, while hydrogen peroxide molecules diffusing away from the site of action are destroyed[19]. Recently, a new group of ubiquitous antioxidant proteins has been acknowledged in yeast, plant, and animal cells. The peroxiredoxins (PRX) are thiol specific-, non-selenium containing enzymes that use redox-active cysteines to reduce peroxides and eliminate $ONOO^-$. They are produced at high levels in the cell, having been reported to comprise 0.1-1% of soluble protein in mammalian cells[20]. Based on conserved cysteine residues, six isoforms of peroxiredoxins (PRX 1-6) have been identified in mammalian systems, and a variety of investigations have described their functional roles in vascular remodeling, cancer and pulmonary and neurodegenerative diseases[21-24]. However, no study has yet explored the role of shear stress on PRX regulation in endothelial cells.

In this study, we tested the hypothesis that shear stress alters PRX function by regulating protein expression and localization, which in turn affect the redox status of endothelial cells. Our studies show that all six forms of PRX are abundantly expressed in bovine aortic endothelial cells (BAEC) and that atheroprotective LS increased intracellular PRX 1 levels, compared to atherogenic OS. In addition, PRX 1 knockdown experiments implicated PRX 1, but not PRX 5, as an important regulator of shear-dependent cellular redox state.

## Materials and Methods

*Endothelial Cell Culture*

Bovine aortic endothelial cells were obtained from Cell Applications Inc. Cells were maintained in a standard humidified incubator (37°C, 5% $CO_2$) in Dulbecco's minimum Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), heparin sodium (American Pharmaceutical Partners), endothelial cell growth supplement (isolated by us), and minimum non-essential amino acids (Gibco). BAEC from passage 8-15 were used in the following experimental protocols.

*Shear Stress Studies*

BAEC were grown to confluent monolayers in 100 mm tissue culture dishes (Falcon) and were subsequently exposed to static (ST) culture conditions or arterial levels of shear stress via cone-and-plate shear apparatus. Atheroprotective LS at 15 dynes/cm$^2$ was simulated by rotating a Teflon cone (0.5$^o$ cone angle) unidirectionally in medium as previously described by us[25]. To mimic unstable atherogenic OS, the cone was rotated bidirectionally in medium using a stepping motor (Servo Motor) and computer program (DC Motor Company). Endothelial cells were exposed to OS at ±5 dynes/cm$^2$ with directional changes of flow at 1 Hz frequency [10]. All shear stress studies were performed in low serum (0.5% FBS) growth medium for 24 hours.

*Preparation of Whole Cell Lysate*

Following experimental treatment, cells were washed three times with ice cold phosphate buffered saline (PBS) and lysed with 600 µL lysis buffer (50 mM Tris-HCl, pH 7.4 at 4$^o$C, 1% Nonidet P40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 30 mM NaF, 40 mM ß-glycerophosphate, 10 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$, 1 mM PMSF,

0.1% SDS). The lysate was further homogenized by sonication. The protein content of each sample was determined by Bio-Rad DC assay.

*Immunoblotting*

Aliquots of cell lysate (20-40 µg of protein each) were resolved by size on 12.5 or 15% SDS polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with primary antibody overnight at 4 °C, followed by incubation with an alkaline phosphatase-conjugated secondary antibody for 1 hour at room temperature. Protein expression was detected by a chemiluminescence method and the intensities of immunoreactive bands were determined via densitometry using the NIH Image program [26]. Primary antibodies specific for PRX 1, 2, 3, 4, 5, and 6 (Lab Frontier), phospho-eNOS (Ser1177), (Cell Signaling Technology), total eNOS (BD Biosciences Pharmingen) and actin (Santa Cruz Biotechnology) were used.

*Immunocytochemistry*

Following shear stress exposure or static culture, BAEC in 100 mm tissue culture dishes were washed three times with PBS. Cells were fixed with 2% paraformaldehyde and permeabilized in 0.2% Triton-X 100. Primary antibody in 3% bovine serum albumin (BSA) was applied overnight at $4^0$C, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 or 568 (Molecular Probes) for 1 hour at room temperature. Nuclei were labeled with Hoechst stain in 3% BSA for 15 minutes at room temperature. To identify mitochondria, cells were incubated with 100 nM Mitotracker Red CMXRos (Molecular Probes) in growth medium for 15 minutes at $37^0$C prior to fixation. All cells were mounted using the Prolong Antifade Kit (Molecular Probes) and fluorescence images were collected via confocal microscope (Zeiss LSM 510). Primary

antibodies specific for PRX 1, 2, 3, 4, 5, and 6 (Lab Frontier), the Golgi marker GM 130 (Transduction Laboratories), and the intermediate filament marker Vimentin (Sigma) were used.

*Small Interfering RNA (siRNA)*

Annealed siRNA duplexes and Oligofectamine (Invitrogen) transfection agent were applied to BAEC for 48-72 hours per manufacturer's recommendation. Control non-silencing siRNA [sense: 5'-UUCUCCGAACGUGUCACGUtt, antisense: 5'-ACGUGACACGUUCGGAGAAtt] (Qiagen), Alexa Fluor 546-labeled control non-silencing siRNA (Qiagen), bovine PRX 1 siRNA [sense: 5'-GUGCUUCUGUGGAUUCUCAtt, anti-sense: 5'-PhoUGAGAAUCCACAGAAGCACtt] (MWG), and bovine PRX 5 siRNA [sense; 5'-GUGGCAUGUCUGACCGUUAtt, antisense: 5'-PhoUAACGGUCAGACAUGCCACtt] (MWG) were used.

*Hydrogen Peroxide Detection*

Using a horseradish peroxidase-linked Amplex Red fluorescence assay, intracellular hydrogen peroxide production was measured via extracellular leakage of $H_2O_2$ from conditioned BAEC as previously described[14]. Briefly, cells were exposed to either static culture conditions or shear stress in low serum culture medium. After 24 hours, cells were washed twice with Krebs Ringer Phosphate (KRP) buffer and incubated with 5 µM Amplex UltraRed (Molecular Probes) and 0.1 U/ml horseradish peroxidase type II (Sigma-Aldrich) in KRP for 40 minutes. To identify the hydrogen peroxide-specific signal, control samples were co-incubated with 500 U/ml catalase. Triplicate reading were taken in a 96-well plate using 100 µl samples of media, and fluorescence was detected via plate reader at excitation and emission of 530 nm and 580 nm, respectively. Hydrogen peroxide levels were calculated in terms of catalase-inhibitable signal and

were normalized to cellular protein as measured by the Bio-Rad DC assay. $H_2O_2$ concentrations were estimated using a standard curve.

*Statistical Analysis*

For all quantitative data collected, statistical analysis was assessed by Student's *t* test using Microcal Origin statistical package. A significant difference between control and treatment groups was defined as $p<0.05$ for three or more independent experiments.

**Results**

*LS upregulates PRX 1 expression in cell lysates.*

Our previous work established that fluid shear stress critically affects endothelial cell function by regulating protein expression patterns. The PRX family is undeniably significant to cellular physiology and pathology, but remains understudied in the fluid flow field. This led us to investigate whether shear stress regulates the expression of mammalian PRX in BAEC. To this end, total BAEC lysates were collected after 24 hours of static culture, LS (15dynes/cm$^2$), or OS (±5dynes/cm$^2$) and analyzed by Western blot using PRX-specific antibodies. In these studies, static conditions (ST), cells cultured under no shear stress, were examined as a control for shear stress exposure. As previously described, physiologically "normal" arterial endothelial cells do not encounter chronic static conditions, but rather experience continuous fluid flow. Therefore, LS is a more relevant control, representing a healthy state, which we will compare to OS, the disease state. As such, endothelial cell alignment, phosphorylated endothelial nitric oxide synthase (eNOS), and total eNOS were also assessed as internal LS-dependent controls (Figure 3.1). With this in mind, all six PRX were detected in BAEC and densitometric analysis indicated the level of PRX 1 was significantly increased by LS compared to OS and static controls. In addition, PRX 5 expression was

significantly increased by LS with respect to static conditions, but was not statistically different from OS (Figure 3.1). Secreted forms of PRX 2, 4, and 6 were also detected in conditioned media following chronic LS (Figure A.1). However, we could not exclude cellular damage as a contributing factor in these findings. Therefore, our data suggest that PRX 1 is mechanosensitive and likely to play an important role in shear-dependent cell biology.

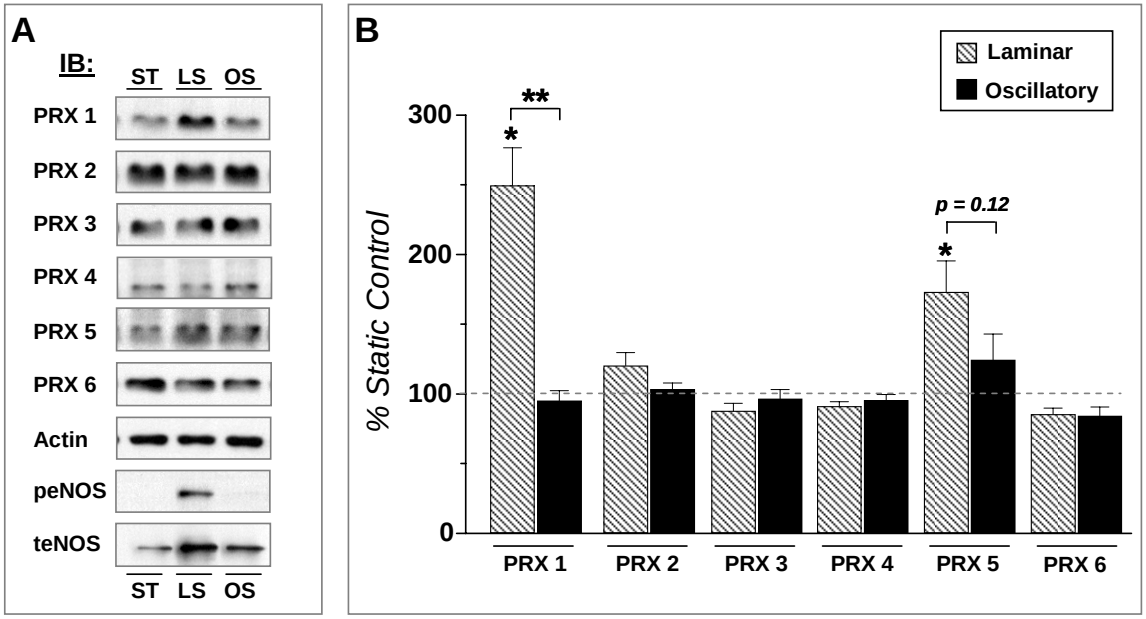


***Figure 3.1 PRX 1 is upregulated by LS.*** *Confluent BAEC were exposed to LS, OS or static conditions for 1 day, and cell lysates were obtained. (A) Equal aliquots of protein (20-40μg) were analyzed by Western blot using antibodies specific to PRX 1-6. peNOS, teNOS, and actin blots were used as shear stress controls and internal loading controls, respectively. (B) Densitometric analysis was used to quantify the intensity of each band and the average values (mean ± SEM, n=12) are shown in bar graphs as % of static control. * p<0.05 indicates significance compared to static control (ST). ** p<0.05 indicates significance compared to OS.*

*PRX exist in various subcellular locations throughout BAEC.*

Via Western blots, we have thus far found that shear stress regulates PRX 1 and 5 expression in BAEC. Based on the large number of PRX family members, we hypothesized that individual PRX likely play important roles in specific subcellular

compartments. To investigate the intracellular distribution of PRX, confocal immunofluorescence staining studies were performed. This study revealed data consistent with Western blot analysis of shear-induced protein expression in BAEC. Image analysis of staining intensities indicated that PRX 1, increased after 24 hours of LS compared to OS and static conditions (Figure 3.2A).

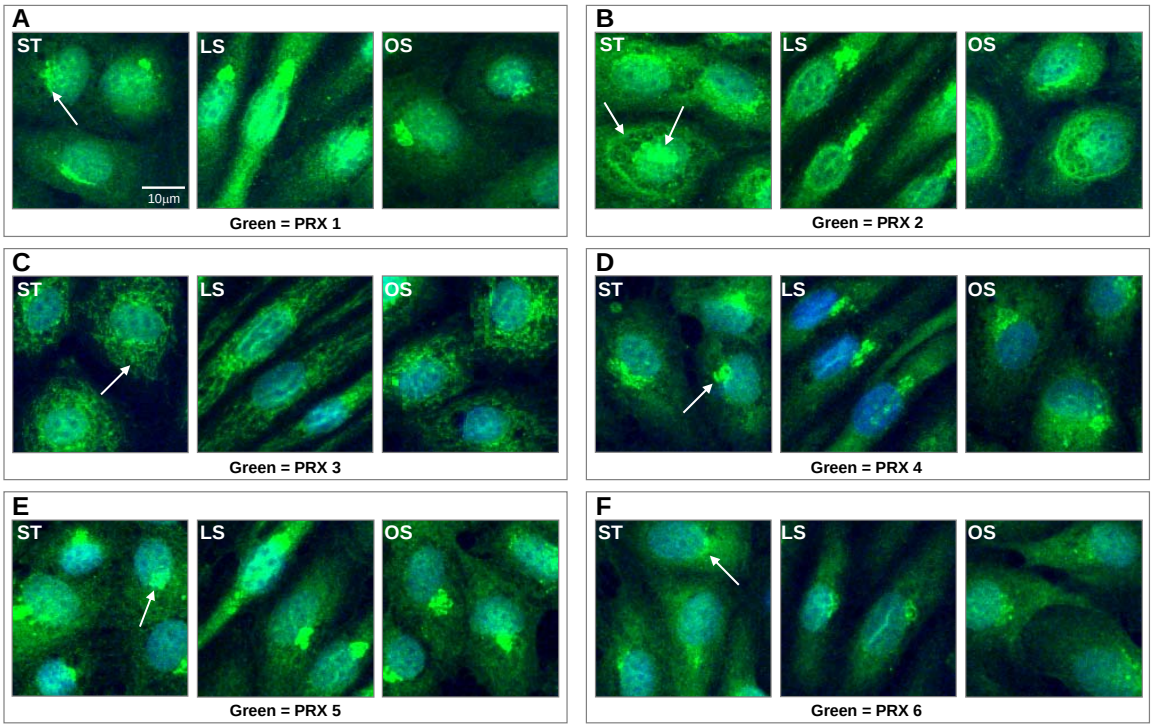


***Figure 3.2 LS stimulated PRX 1 expression in BAEC.*** *Confluent BAEC were exposed to LS, OS or static conditions for 1 day as in Figure 3.1. Cells were stained using antibodies specific to PRX 1-6. Secondary antibodies conjugated to Alexa Fluor 488 (green) were imaged by confocal microscopy. Nuclei were counter-stained with Hoechst dye (blue). Arrows indicate unique subcellular staining patterns of each PRX.*

In addition to the shear-dependency, Figure 3.2 also showed that PRX members exhibited assorted staining patterns. This result raised two interesting questions: 1) Are PRX located in specific subcellular locations and 2) does shear stress alter this subcellular localization? In order to clearly characterize the location of each PRX within specific subcellular compartments, colocalization staining was performed in static BAEC

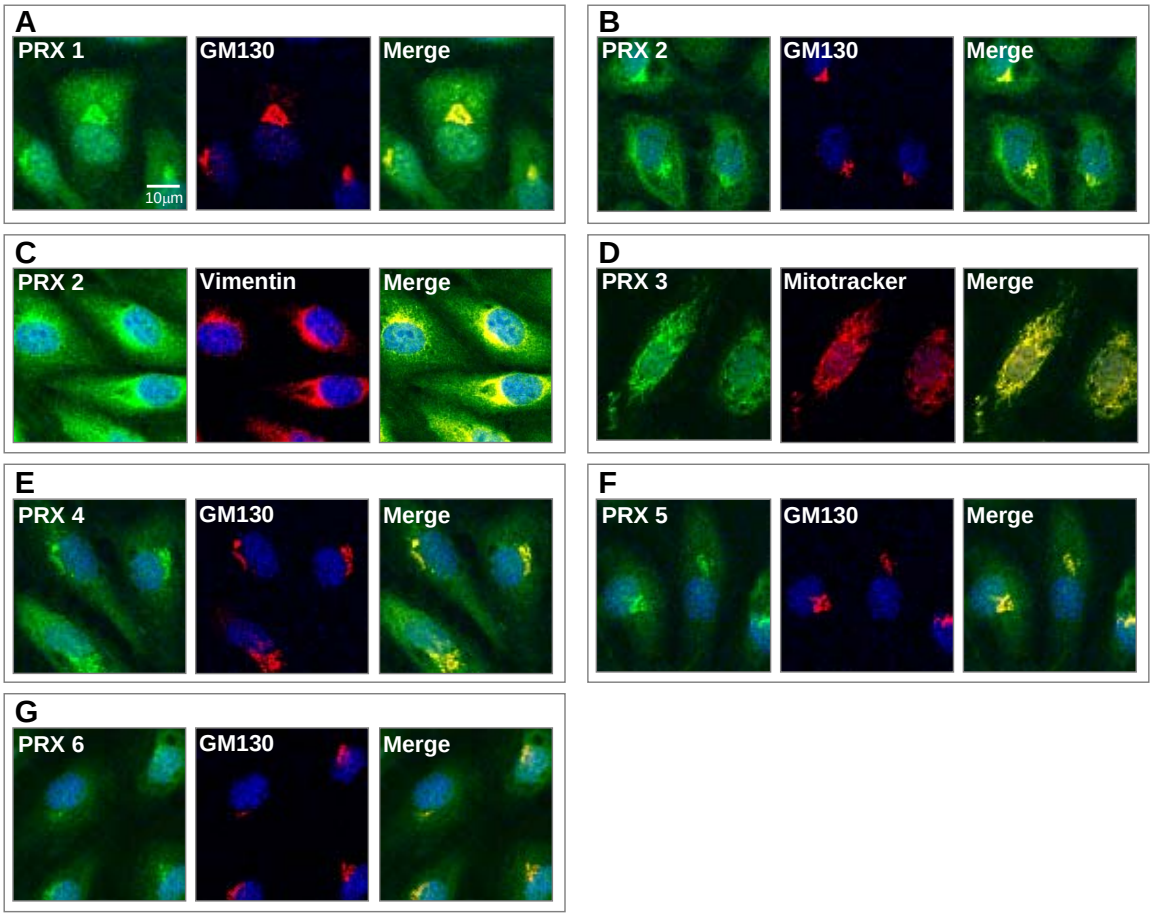


***Figure 3.3 PRX are located in various subcellular organelles in BAEC.*** *Static BAEC were co-stained for PRX 1-6 and subcellular organelles using PRX-specific antibodies (green), as in Figure 3.2, and organelle-specific markers (red). Nuclei were counterstained with Hoechst dye (blue). Center panels: Mitochondria, Golgi, and intermediate filaments are stained with Mitotracker CMXRos, GM 130 antibody and Vimentin antibody, respectively. GM 130 and Vimentin were visualized by secondary antibodies conjugated to Alexa Fluor 568 (Red). Merged images are shown in the right panel. Yellow staining indicates colocalization.*

using PRX-specific antibodies and organelle-specific markers for Golgi (GM 130), endoplasmic reticulum (KDEL receptor, data not shown), lysosome (cathepsin S, data not shown), intermediate filament (Vimentin) and mitochondria (Mitotracker). PRX 1 staining (green) overlapped with the Golgi marker staining (red), shown as yellow in the merged image (Figure 3.3A), suggesting that PRX 1 exists in the Golgi apparatus. In addition, PRX 2, 4, 5 and 6 also appeared to be found in the Golgi (Figure 3.3B, E, F,

and G). The PRX 3 staining pattern was distinctly different from other PRX, showing clear colocalization with the mitochondria marker (Figure 3.3D). Interestingly, PRX 2 staining revealed colocalization with the intermediate filament marker (Figure 3.3C). In addition to these subcellular localizations, PRX 1, 2, 4, 5 and 6 were also expressed in the cytosol (Figure 3.3A, B, C, E, F and G). Next, we examined whether shear stress stimulated expression of PRX members in other subcellular locations. Subcellular location did not appear to change in response to shear stress, although Golgi were located upstream of the direction of flow after chronic LS, consistent with previous reports (Figure 3.2A, B, D, E and F). Taken together, these results clearly indicate that PRX are abundantly expressed throughout the subcellular organelles of endothelial cells.

*PRX 1 prevents oxidative stress in endothelial cells exposed to LS.*

PRX 1 is a prominent antioxidant and our data indicates its expression is highly upregulated by LS [27]. Consequently, we investigated whether PRX 1 was responsible for the decreased ROS levels in endothelial cells exposed to LS. The depletion of individual PRX from cellular systems provides a useful tool to study the functional role of each PRX isoform. Here, specific siRNAs were used to knockdown either PRX 1 or PRX 5 protein levels in order to investigate PRX-dependent ROS accumulation. Western blots with isoform-specific PRX antibodies were used to determine the efficacy and specificity of these siRNAs. Compared to non-silencing siRNA and Alexa Fluor 546-labeled non-silencing siRNA (50 nM each), 48 hour-treatment of BAEC with PRX 1 siRNA dramatically reduced (by 75% of non-silencing control) expression of PRX 1, at a concentration as low as 10nM (Figure 3.4A). As shown in Figure 3.4B, 10nM PRX 5 siRNA also effectively reduced PRX 5 expression (by 90% of non-silencing control). Via Western Blots, the specificity of PRX 1 and 5 siRNAs were assessed by examining the

expression of all other PRX family members (Figure 3.4A and B). Using isoform-specific PRX antibodies, we found that these siRNAs had no significant effect on other PRX, indicating that they exclusively targeted PRX 1 or 5, respectively, amongst all PRX family members (Figure 3.4A and B). Therefore, PRX 1 and 5 siRNAs were confidently used at 10nM concentration in subsequent functional studies.

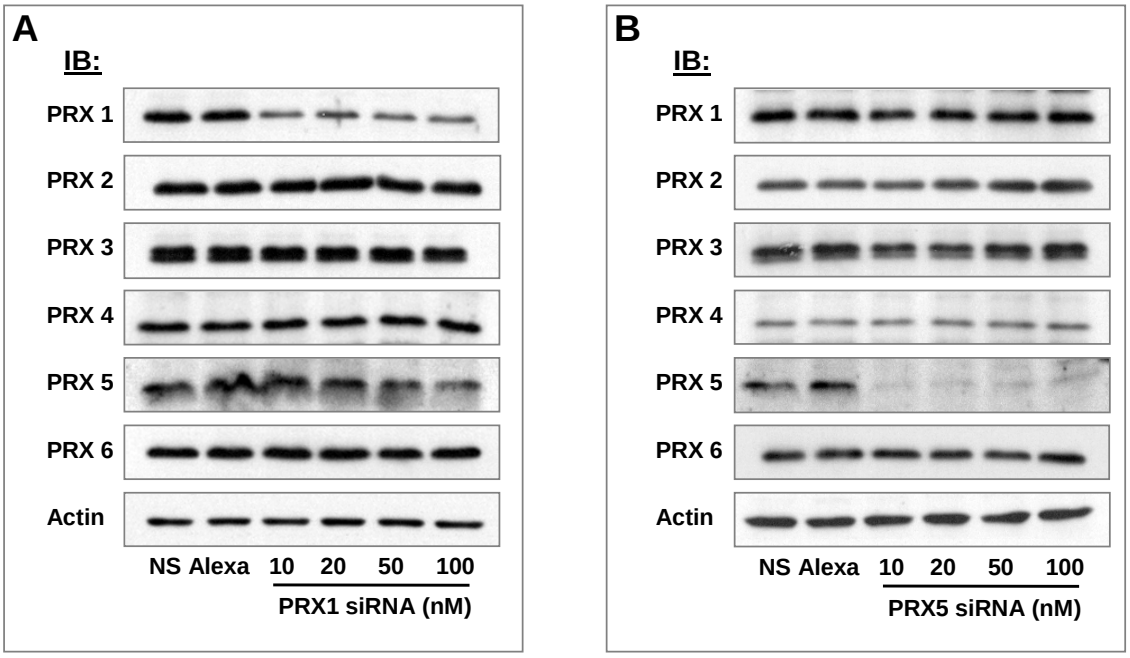


***Figure 3.4 PRX 1 and PRX 5 siRNAs specifically reduce PRX 1 and PRX 5 protein expression, respectively.*** *BAEC were transfected with either 50nM non-silencing siRNA (NS), 50nM Alexa Fluor 546-labeled siRNA (Alexa), or PRX 1 (A) or PRX 5 (B) siRNAs (10, 20, 50 and 100nM) for 48 hours. Cell lysates were analyzed by Western Blot with PRX-specific antibodies, as indicated. actin was used as an internal control.*

It has been well established that chronic exposure of endothelial cells to OS stimulates, while LS inhibits, ROS production[13, 14, 17, 28, 29]. Utilizing an Amplex Red assay in the presence or absence of catalase, we verified that hydrogen peroxide levels were 87% *less* in BAEC exposed to LS compared to those treated with OS (Figure 3.5A). Using a standard hydrogen peroxide dose curve, we also found that the relative hydrogen peroxide levels following static culture, LS, and OS were consistent with expected

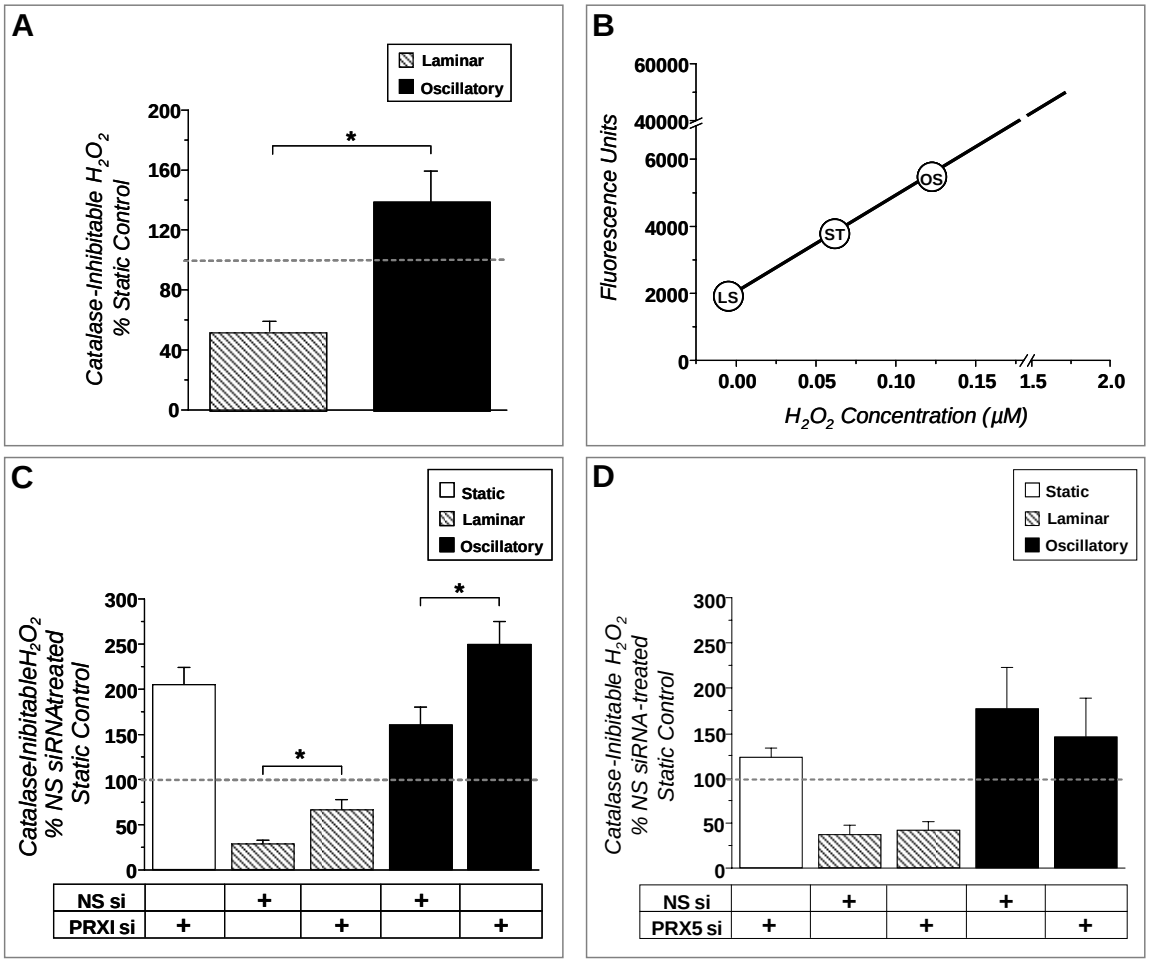


***Figure 3.5 PRX 1 knockdown increases $H_2O_2$ production in BAEC, while PRX 5 knockdown does not.*** *Catalase-inhibitable hydrogen peroxide levels were assessed via Amplex Red Assay and average values (mean ± SEM, n=6-12) are shown in bar graphs as % of non-silencing (NS) siRNA-treated static controls. (A) Confluent BAEC were exposed to ST, LS, or OS for 24 hours prior to assay. (B) A hydrogen peroxide dose curve was used to estimate relative hydrogen peroxide concentrations in cells conditioned with shear stress. (C) BAEC were transfected with either non-silencing or PRX 1 siRNA (10nM) for 48 hours and then exposed to ST, LS, or OS for 24 hours, prior to assay. (D) BAEC were transfected with either non-silencing or PRX 5 siRNA (10nM) for 48 hours and then exposed to ST, LS, or OS for 24 hours, prior to assay. * p<0.05 designate significance between indicated groups.*

cellular concentrations (Figure 3.5B). To determine whether PRX 1 was responsible for the LS-dependent decrease in ROS levels in BAEC, we knocked down PRX 1 using PRX 1 siRNA as indicated above (Figure 3.4A). PRX 1 depletion significantly increased catalase-inhibitable hydrogen peroxide by two fold above non-silencing controls, in

static- LS-, and OS-treated BAEC (Figure 3.5C). To investigate whether this was a global effect of mechanosensitive PRX family members, we also knocked down PRX 5 using PRX 5-specific siRNA. However, when compared to non-silencing controls, PRX 5 depletion had no significant effect on hydrogen peroxide levels in any group (Figure 3.5D). Taken together, these data suggest that PRX 1 is a critically important regulator of ROS levels in both a basal and shear-dependent manner.

**Discussion**

Through protein expression analysis and subsequent functional studies, we have discovered PRX 1 as a mechanosensitive antioxidant. Data to support this concept include: (1) PRX 1 is upregulated intracellularly by chronic LS compared to OS; (2) PRX exhibit broad staining patterns in BAEC and localize in important cellular structures; (3) ROS production is significantly reduced in cells exposed to chronic LS, and (4) this effect can be attenuated by PRX 1 depletion, but not PRX 5 depletion.

Through this work, we reveal for the first time that PRX are regulated by shear stress in endothelial cells. Previous studies have shown that other antioxidants are also controlled by shear stress. LS has been shown to regulate antioxidant genes including eNOS, Cu/Zn superoxide dismutase (SOD), Mn SOD, glutathione peroxidase (GPX), glutathione (GSH) and thioredoxin (TRX)[6, 13, 17, 30-34]. In addition, Chen et al. observed that many genes protective against oxidative stress are induced by exposure to prolonged LS. They have also noted that such genes are regulated through a conserved, shear-sensitive antioxidant response element (ARE)[35, 36]. In support of our finding that PRX 1 expression is LS-dependent, recent work has shown that PRX 1 is a target gene of nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2), a key transcription factor which binds to ARE[37]. Collectively, these studies indicate that cells

possess an elaborate system of shear-responsive antioxidants and that each may play an independent role to mediate oxidative stress and modulate redox-sensitive signaling pathways.

The ubiquitous nature of the PRX family itself exemplifies this concept. Immunofluorescence microscopy revealed PRX throughout the cellular milieu of BAEC, colocalizing with the cytoplasm, Golgi apparatus, mitochondria, and intermediate filaments. These observations were consistent with previously reported localization studies in other cell types, but we are the first to report an apparent PRX 2 colocalization with vimentin[38, 39]. This costaining of PRX 2 with vimentin suggests that it may be located in the intermediate filament, but further studies will be necessary to confirm this finding. Though detection of PRX in the Golgi body likely reflects protein processing or packaging, localization within other organelles indicates that PRX may act both globally and in a site-specific manner to regulate ROS in endothelial cells. In the endothelium, ROS, such as $O_2^{\bullet-}$ and $H_2O_2$, arise from several sources including NADPH oxidase, xanthine oxidase, mitochondrial oxidase, cytochrome P450 and uncoupled nitric oxide synthase[40, 41]. At relatively low concentrations, ROS play critical roles in redox signaling and normal cell function. However, higher concentrations of ROS induce oxidative damage of DNA, proteins, carbohydrates and lipids [42-44]. This damage has been shown to critically affect cellular function and apoptosis when it occurs in mitochondria, lysosomes, and nuclei[45-47]. In addition, cytosolic proteins modified by ROS have been shown to affect local cell signaling and, collectively, the redox status of the cell[48, 49]. Ubiquitous distribution of PRX in BAEC may reflect diverse sources of ROS throughout the cells and provide protection for important macromolecules and structures against local ROS production. In addition, widespread allocation of PRX may also be important for comprehensive management of the overall oxidative state of cells.

Several studies have shown that oxidative stress is regulated by shear stress in endothelial cells[13, 14]. We have previously published that both LS and OS stimulate ROS production acutely, but the ROS transiently elevated by LS returns to basal levels within a few hours[13]. However, unlike LS, OS continues to increase ROS production, maintaining elevated levels as long as cells are exposed to OS [13, 29]. The mechanism by which ROS levels are lowered in cells exposed to chronic LS is undefined. This study demonstrates that endothelial cells exposed to chronic LS express much more PRX 1 compared to OS and static conditions. These findings suggest that PRX 1 is upregulated by LS and that this may be responsible for LS-mediated decrease in ROS levels.

As previously determined by electron spin resonance spectrometry and dichlorofluorescein-diacetate methods, endothelial cells exposed to OS produce significantly more superoxide and hydrogen peroxide than those in static culture[14]. In contrast, endothelial cells treated with chronic, unidirectional high shear generate considerably less $O_2^{\bullet-}$. Here, we used Amplex Red assay as an independent method to measure ROS levels in BAEC. Consistent with our previous reports, OS increased and LS decreased ROS production (Figure 3.5A). The ROS measured by this assay was inhibitable by catalase, indicating that $H_2O_2$ is the primary ROS component.

PRX 1 is the most abundant and ubiquitously distributed member of mammalian PRX[27]. Our current study demonstrated that PRX 1 is dramatically upregulated by chronic LS compared to OS and is located in the cytoplasm and Golgi. Knockdown of PRX 1 using siRNA resulted in significantly higher ROS levels in BAEC exposed to LS, OS, and static conditions, while PRX 5 depletion did not. Although PRX 1 knockdown did not fully

abolish the antioxidative outcome of LS, its significant effect was somewhat surprising considering the presence of other PRX family members and additional mechanosensitive antioxidant pathways. In addition, PRX 5 depletion studies provide further evidence that PRX 1 is crucial to shear-dependent ROS regulation. Altogether, these results indicate that chronic exposure to LS upregulates PRX 1 expression in order to keep ROS levels low in endothelial cells.

In summary, we have shown that shear stress regulates expression of the PRX family and that PRX 1 plays a critical role in regulating ROS levels in endothelial cells. Furthermore, this discovery of PRX 1 as a mechanosensitive antioxidant may contribute important insights into endothelial cell biology and vascular diseases.

**Acknowledgments**

This work was supported by funding from National Institute of Health grants HL75209, HL87012, and HL80711 (HJ) and 21C Frontier Functional Proteomics Project (FPR05C2-510) from Korean Ministry of Science & Technology (DHK and SWK), as well as an American Heart Association pre-doctoral fellowship (ALM). We would like to thank Kyung Hwa Chang, Hannah Song, Mamta Patel, Chih Wen Ni, and Sarah Tressel at Georgia Institute of Technology and Emory University for helpful comments during these studies.

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# CHAPTER 4

# *PRDX1* GENE IS UPREGULATED BY LAMINAR SHEAR STRESS IN PART BY NRF2 TRANSCRIPTION FACTOR

In Chapter 3, we identified Peroxiredoxin 1 as a novel mechanosensitive antioxidant that plays an important role in shear stress-dependent regulation of endothelial biology. In turn, the goal of Specific Aim 3 was to examine the pathway by which laminar shear stress, but not oscillatory shear stress, promotes the expression of PRX1 protein. To achieve this aim, we investigated the regulation of bovine *Prdx1*, the gene encoding PRX 1, in response to different shear stress patterns and a potential transcription factor, nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2).

**Summary**

Endothelial cells (EC) exposed to oscillatory shear stress (OS) experience oxidative stress as a signature of atherosclerosis. Conversely, unidirectional laminar shear stress (LS) reduces reactive oxygen species (ROS) levels. Previous studies by our group have found that fluid flow stimulates these important functional changes through gene and protein expression regulation. More specifically, others have shown that LS induces many protective genes via a shear-sensitive antioxidant response element (ARE). Peroxiredoxins (PRX) are a family of antioxidant enzymes that remain understudied in the field of endothelial cell biology. Recently, we showed that the protein Peroxiredoxin 1 (PRX 1) is upregulated intracellularly by chronic LS, compared to OS and static (ST) culture. In addition, PRX 1-knockdown attenuated LS-dependent ROS reduction in EC. Therefore, in this study, we hypothesized that shear-dependent PRX 1 expression may be regulated by the ARE through its transcription factor nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2). Quantitative, real-time PCR indicated that LS increases *Prdx1*

mRNA in a time-dependent manner, compared to OS and ST. Immunoblots also showed increased PRX 1 protein expression in response to Nrf2 adenovirus infection. In addition, depletion of Nrf2 using specific siRNAs reduced *Prdx1* expression both under static culture conditions and in response to LS. These results indicate that shear-dependent PRX 1 expression is regulated at the transcriptional level, and Nrf2 may play an important role in this mechanism.

**Introduction**

Shear stress produced by blood flow plays a critical role in the focal development of atherosclerosis. At the cellular level, the exposed endothelium acts as sensor of this force, translating it into physiological or pathophysiological functions[1]. In general, unidirectional laminar shear stress promotes an atheroprotective endothelial phenotype, while low, oscillatory shear stress stimulates atherogenic responses[2-4]. It is well established that OS induces oxidative stress and inflammation via production of reactive oxygen species (ROS) like superoxide ($O_2^{\bullet-}$) and hydrogen peroxide ($H_2O_2$)[2, 5, 6]. In contrast, steady LS prevents inflammation and macromolecule damage by maintaining low ROS levels in endothelial cells[7, 8]. Our group has demonstrated that this intracellular redox balance is critically dependent on shear stress-mediated gene and protein regulation. In previous publications, we have shown that OS increases $O_2^{\bullet-}$ and $H_2O_2$ through gene and protein expression of the cytokine Bone Morphogenic Protein 4 (BMP4)[9, 10]. Similarly, numerous studies suggest that steady laminar flow promotes a reducing environment in endothelial cells through the generation of antioxidants[3].

Antioxidant defense systems are crucial to the modulation of intracellular levels of ROS. They can directly scavenge ROS and disrupt the oxidative signaling cascades of pathological events such as inflammation, proliferation, and apoptosis[11, 12]. The

peroxiredoxins (PRX) are a newly identified family of antioxidant enzymes that use redox-active cysteines to reduce peroxides and eliminate $ONOO^-$. Based on conserved cysteine residues, six isoforms of peroxiredoxin (PRX 1-6) have been identified in mammalian systems[13, 14]. Recently, we examined the expression of all six PRX family members in bovine aortic endothelial cells (BAEC) exposed to shear stress. Our data revealed that PRX 1 expression was elevated in response to LS, but not OS, and that this increase significantly influenced hydrogen peroxide levels[15]. However, we have yet to identify the pathway by which atheroprotective laminar shear stress stimulates PRX 1 protein production.

Interestingly, laminar shear stress has been found to influence other antioxidants including heme oxygenase-1 (HO-1), ferritin heavy chains (FTH), NADPH:quinone oxidoreductase-1 (NQO1), glutamate-cysteine ligase modifier subunit (GCLM), thioredoxin reductase1 (TXNRD1), glutathione reductase (GSR), endothelial nitric oxide synthase (eNOS), superoxide dismutase (SOD), glutathione (GSH) and thioredoxin (TRX)[3, 6-8, 16-18]. Many of these genes are regulated through a conserved antioxidant response element (ARE) or ARE-like transcriptional regulatory sequence in their promoter[7, 11, 18]. The ARE, also known as the electrophile response element (EpRE), is a *cis*-acting DNA regulatory element with a core sequence of 5'-RTGACNNNGC-3'[11, 19, 20]. Transcription factor NF-E2-related factor 2 (Nrf2), a basic leucine zipper protein (bZIP), activates the ARE and its downstream genes by binding to this consensus sequence. Expectedly, several reports have shown that the ARE itself is sensitive to laminar shear stress, suggesting a conserved mechanism for the induction of cytoprotective, mechanosensitive factors[19, 21].

As such, we tested the hypothesis that laminar shear stress promotes the expression of PRX 1 protein via transcriptional regulation of the *Prdx1* gene, through an ARE-dependent pathway. Our studies found that the bovine *Prdx1* is upregulated by LS in a temporal manner compared to OS and static controls, and that *Prdx1* mRNA stability is not affected by shear. In addition, overexpression and knockdown of Nrf2 significantly induced and blunted *Prdx1* gene expression, respectively.

## Materials and Methods

### *Endothelial Cell Culture*

Bovine aortic endothelial cells were obtained from Cell Applications Inc. Cells were maintained in a standard humidified incubator (37°C, 5% $CO_2$) in Dulbecco's minimum Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), heparin sodium (American Pharmaceutical Partners), endothelial cell growth supplement (isolated by us), and minimum non-essential amino acids (Gibco). BAEC from passage 6-15 were used in the following experimental protocols.

### *Shear Stress Studies*

BAEC were grown to confluent monolayers in 100 mm tissue culture dishes (Falcon) and were subsequently exposed to static (ST) culture conditions or arterial levels of shear stress via cone-and-plate shear apparatus. Atheroprotective LS at 15 dynes/cm$^2$ was simulated by rotating a Teflon cone (0.5$^o$ cone angle) unidirectionally in medium as previously described by us[22]. To mimic unstable atherogenic OS, the cone was rotated bidirectionally in medium using a stepping motor (Servo Motor) and computer program (DC Motor Company). Endothelial cells were exposed to OS at ±5 dynes/cm$^2$ with directional changes of flow at 1 Hz frequency[9]. All shear stress studies were performed

in low serum (0.5% FBS) growth medium, and shear stress exposure time was varied from 2 to 24 hours.

*Reverse Transcription and Quantitative, Real Time Polymerase Chain Reaction (PCR)*

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) and reverse transcribed with random primers and a Superscript-II kit (Invitrogen) to synthesize first-strand cDNA. The purified cDNA was amplified via quantitative, real-time PCR with a LightCycler (Roche Applied Science). Reactions were performed in glass capillaries (Roche Applied Science) containing PCR buffer (20mM Tris-Cl, pH 8.4), 3mM $MgCl_2$, 250µg/ml bovine serum albumin, 200µM deoxynucleotides, SYBR green (1:84,000 dilution), 0.05unit/µl Taq DNA polymerase (Denville), and Taq Start antibody (Clontech, 1:100 dilution). The following primer pairs (5'to 3') and reaction conditions (primer concentration, annealing temperature, extension time, and base pair yield, respectively) were used to amplify bovine *Prdx1*- forward: GACCCATGAACATTCCCTTG and reverse: GCTCTTCTGGACATCAGGCTTGAT (150nM, 62$^{o}$C, 12 seconds, 283bp) and bovine *Nrf2*- forward: TGCCACTGCTGTTTTAGACG and reverse: ACTGGCTGGAGTCTTCAGTGGAAA (250nM, 60$^{o}$C, 12 seconds, 280bp). Copy numbers were determined based on standard curves generated with bovine *PRX1, Nrf2,* and *18S* templates. *PRX1* and *Nrf2* mRNA copy numbers were normalized to *18S* mRNA copy numbers, and gene products were verified by size via agarose gel electrophoresis.

*Prdx1 mRNA Stability*

Following exposure to chronic shear stress, conditioned BAEC were treated with the RNA polymerase II inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (10µM DRB,

Calbiochem) to halt RNA synthesis. Copy number of *Prdx1* mRNA was assessed via quantitative, real time PCR at 0, 4, 8, and 12 hours after treatment.

*Plasmid Construction*

A 1,350 base pair fragment of the bovine *Prdx1* promoter was isolated via PCR. An *XhoI* site was added to the 5' primer and a *HindIII* site was added to the 3' primer- forward: CGGCTCGAGTGGGCTCCTGTTTCCTATTC and reverse: CGGAAGCTTAATCACCGCTTTCTGCTTGT. The PCR product was digested with *XhoI/HindIII*, gel-isolated, and subcloned into either pGL3-Basic (Promega) upstream of the luciferase reporter or β-CAT-pSP72 (Promega) upstream of the chloramphenicol acetyltransferase (CAT) reporter. The sequences off all constructs were verified by DNA sequencing in both directions (Agencourt).

*Transfection and Reporter Gene Assay*

Transient luciferase reporter assays were performed using standard transfection methods. Briefly, plasmid constructs (4.5μg of either pGL3 control or *Prdx1* promoter-pGL3 and 1.5 μg of β-galactosidase normalizing vecotor) were transfected into 90% confluent BAEC using Metafectene Pro (Biontex) in reduced-serum Opti-MEM (GIbco) according to manufacturer's recommendation. After 4 hours, media was replaced with growth media containing 10% FBS. Cells were allowed to recover up to 48 hours and then were subjected an additional 6 hours of shear stress. Luciferase activities were measured using the luciferase assay system (Promega), and β-galactosidase activities were assessed via plate reader (420nm). Luciferase activities were normalized to β-galactosidase activities.

*Adenoviral Infection*

BAEC were infected with either control green fluorescent protein (Ad-GFP, 20 MOI) or wild type human Nrf2 adenovirus (Ad-wt hNrf2, GFP-tagged, 20, 40, 80 MOI) in serum-free Dulbecco's minimum Eagle's medium. After 6 hours, media was removed and replaced with 10% FBS growth media. Cells were allowed to recover up to 48 hours and then were lysed for protein collection.

*Preparation of Whole Cell Lysate*

Following experimental treatment, cells were washed three times with ice cold phosphate buffered saline (PBS) and lysed with 600 µL lysis buffer (50 mM Tris-HCl, pH 7.4 at 4$^{o}$C, 1% Nonidet P40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 30 mM NaF, 40 mM ß-glycerophosphate, 10 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$, 1 mM PMSF, 0.1% SDS). The lysate was further homogenized by sonication. The protein content of each sample was determined by Bio-Rad DC assay.

*Immunoblotting*

Aliquots of cell lysate (20-40 µg of protein each) were resolved by size on 12.5% SDS polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with primary antibody overnight at 4°C, followed by incubation with an alkaline phosphatase-conjugated secondary antibody for 1 hour at room temperature. Protein expression was detected by a chemiluminescence method[23]. Primary antibodies specific for PRX 1 (Lab Frontier) and Nrf2 (C-20, Santa Cruz Biotechnology) were used.

*Small Interfering RNA (siRNA)*

Annealed siRNA duplexes and Oligofectamine (Invitrogen) transfection agent were applied to BAEC per manufacturer's recommendation. In brief, 70-80% confluent BAEC were transfected with control or specific siRNA in reduced-serum Opti-MEM (GIbco). After 6 hours, media was supplemented to 10% FBS. Cells were allowed to recover up to 48 hours, then were lysed, or subjected an additional 24 hours of shear stress. Control non-silencing siRNA sense: 5'-UUCUCCGAACGUGUCACGUtt, antisense: 5'-ACGUGACACGUUCGGAGAAtt (Qiagen), Alexa Fluor 546-labeled control non-silencing siRNA (Qiagen), and bovine Nrf2 siRNA sense: 5'- CCAGACCACUCAGUGGAAUtt, anti-sense: 5'- AUUCCACUGAGUGGUCUGGtt (MWG) were used.

*Statistical Analysis*

For all quantitative data collected, statistical analysis was performed by Student's *t* test. Significance is defined as $p<0.05$ for three or more independent experiments.

**Results**

*Prdx1 gene is upregulated by LS in a time-dependent manner.*

Recent work by our group linked the atheroprotective effects of laminar shear stress to the antioxidant protein PRX 1. In previous studies, we showed that intracellular PRX 1 is significantly increased by LS compared to OS and mediates hydrogen peroxide levels in endothelial cells[15]. To further investigate these findings, we sought to examine the pathway by which different shear stress patterns regulate PRX 1 protein expression. As such, *in vitro* shear stress studies were carried out, and bovine *Prdx1*, the gene encoding PRX 1 protein, was assessed. Total RNA was collected from BAEC following static culture, or 2, 4, 8 or 24 hours of LS (15dynes/cm$^2$) or OS (±5dynes/cm$^2$). Quantitative, real time PCR was used to determine *Prdx1* mRNA copy number relative to

the internal control 18S. Here, we found that *Prdx1* increased in a time-dependent manner in response to laminar shear stress (Figure 4.1). With as little as 2 hours of LS exposure, *Prdx1* levels were elevated compared to static control. Additionally, LS-induced expression was statistically different from OS beginning at 4 hours and peaked at 24 hours with a 4.5 fold increase. This shear stress-mediated regulation of *Prdx1* mRNA and the temporal nature of expression led us to believe that PRX 1 protein was likely regulated at the transcriptional level (Figure 4.1).

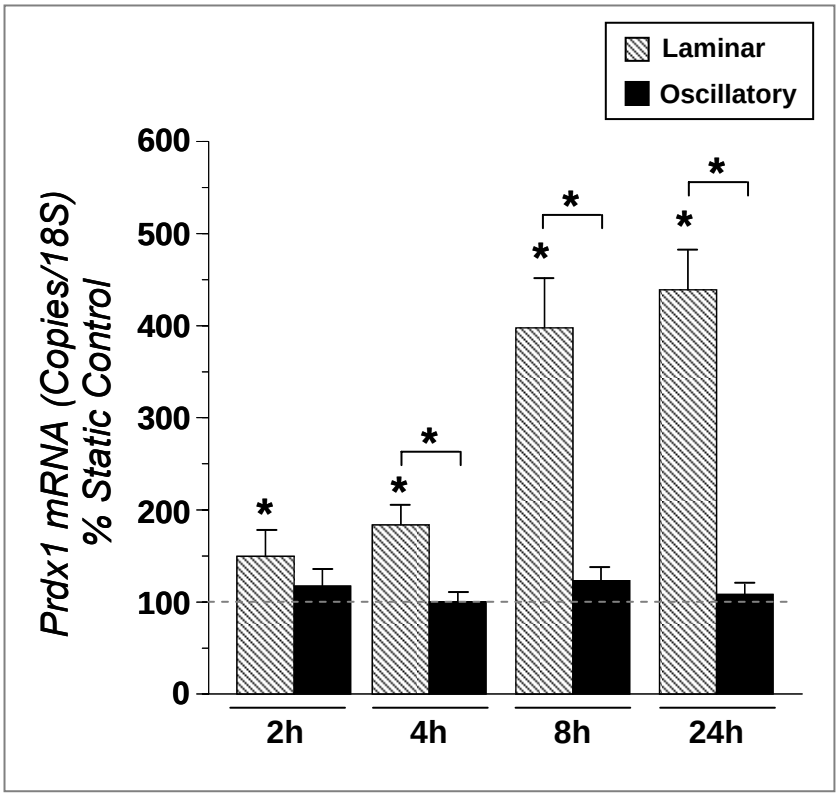


***Figure 4.1 Prdx1 mRNA is upregulated by LS compared to OS in a time-dependent manner.*** *Confluent BAEC were exposed to LS, OS or static conditions for 2, 4, 8, or 24h. Total RNA was collected and reverse-transcribed to synthesize first-strand cDNA, which was amplified via real-time, quantitative PCR. mRNA copy numbers were determined based on standard curves generated with bovine Prdx1 and 18S templates. Prdx1 mRNA copy numbers were normalized to 18S mRNA copy numbers. Prdx1 mRNA levels (mean ± SEM, n=3-15) are shown in bar graphs as % of static control. * p<0.05 designate significance between indicated groups.*

*LS does not affect the mRNA stability of Prdx1.*

To verify that increased *Prdx1* levels were due to active transcription, as opposed to mRNA stabilization, we evaluated the degradation rate of *Prdx1* mRNA following *in vitro* shear stress. In these experiments, BAEC were conditioned for 1 day with static culture, LS, or OS. Transcription was then halted using the RNA polymerase II inhibitor DRB. mRNA levels were examined at time points between 0 and 12 hours following DRB treatment using quantitative, real time PCR. Under static culture, *Prdx1* mRNA levels fell to roughly 60% after 8 hours (Figure 4.2). Interestingly, conditioning with neither LS, nor OS, affected this rate of mRNA degradation. These results suggested that LS promotes production of the *Prdx1* transcript, rather than protection of mRNA by polyadnenylation or other factors.

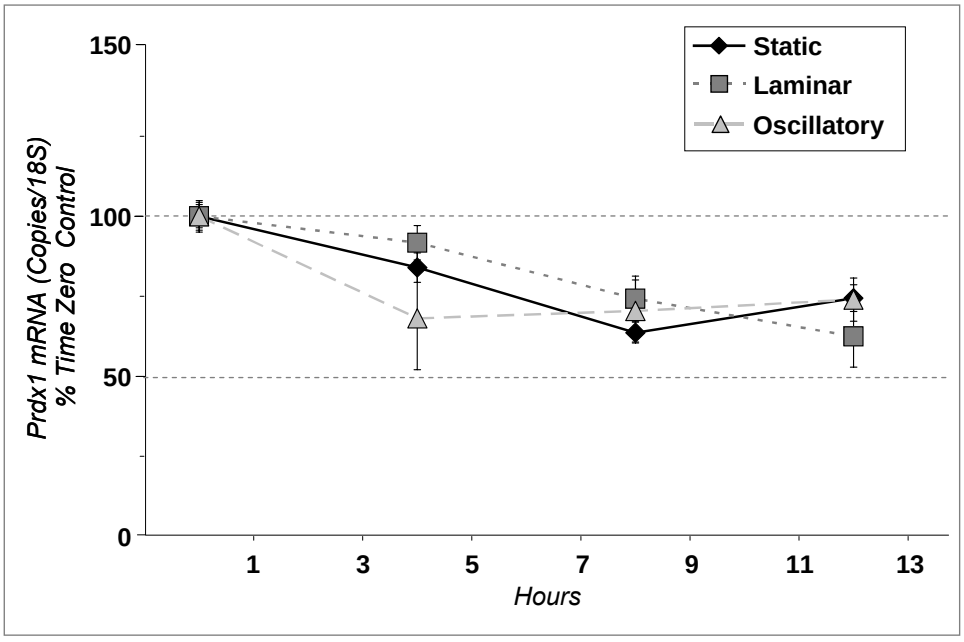


***Figure 4.2 Prdx1 mRNA stability is not affected by shear stress.*** *Confluent BAEC were exposed to LS, OS or static conditions for 24h. Following shear stress, cells were treated with the RNA polymerase II inhibitor DRB (10μM) to stop transcription. Total RNA was collected and purified cDNA was amplified via real-time, quantitative PCR as in Figure 4.1. Prdx1 mRNA copy numbers were normalized to 18S mRNA copy numbers. Prdx1 mRNA levels (mean ± SEM, n=3-15) are shown in line graphs as % of time 0 hour for each group.*

*Bovine Prdx1 promoter is functional in BAEC.*

Thus far, we demonstrated that *Prdx1* mRNA is upregulated in response to LS, but that this increase is not a product of mRNA stabilization. Together, these results suggested that the *Prdx1* expression mediated by shear stress is controlled at that transcriptional level. To further investigate this hypothesis, the 5' 1.4-kb region upstream of the bovine *Prdx1* gene was cloned into either luciferase or CAT reporter constructs. In validation studies, *Prdx1* promoter-luciferase vector was transfected into BAEC, and luciferase activities were assessed following 6 hours of ST, LS, or OS. Cells containing *Prdx1* promoter possessed significantly more luciferase activity then those with empty vector controls. In addition, LS appeared to decrease activity in promoter groups, inconsistent with our previous findings (Figure 4.3). However, this phenomenon occurred in control groups as well, and it has been widely reported that nitric oxide (NO) inhibits luciferase activity by decreasing luciferase mRNA stability[24]. LS is a potent stimulator of NO suggesting that atheroprotective shear itself reduces luciferase activity, independent of promoter activation[25]. Taken together, these data indicated that our promoter construct is functional and can best be used in further assays with a CAT reporter system.

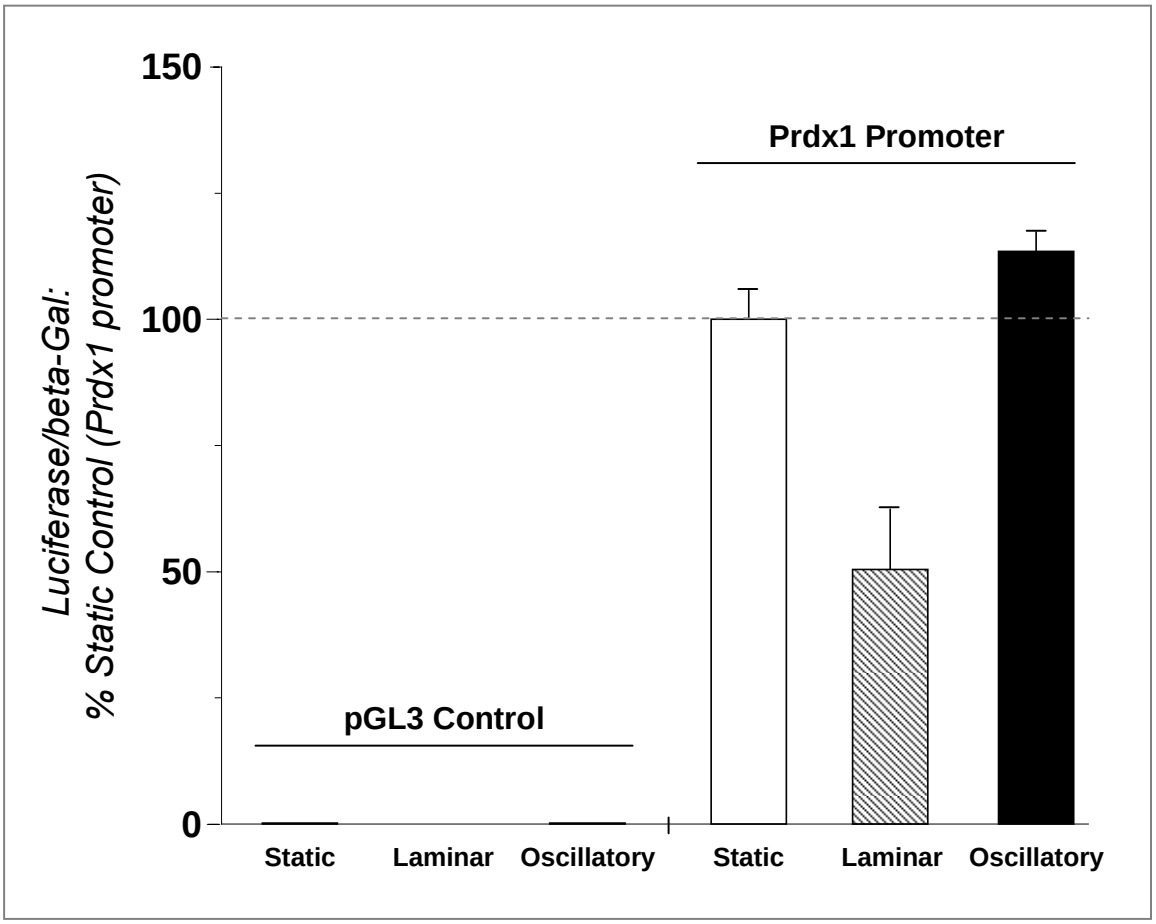


***Figure 4.3 Prdx1 promoter is functional in BAEC.*** *BAEC were transfected with either control pGL3 or Prdx1 promoter-pGL3 for 48 hours and then exposed to ST, LS, or OS for 6 hours. Luciferase activity was measured, normalized to β-galactosidase internal control, and is represented in bar graphs as % of static control transfected with Prdx1 promoter.*

*The bovine Prdx1 promoter region contains a Nrf2 binding site.*

Due to the limited information on transcriptional regulation of bovine *Prdx1*, we also carried out computer-based sequence analysis to identify potential transcription factors in the promoter region of this gene. Approximately 2 kb of genomic sequence, upstream of bovine *Prdx1*, were retrieved in *FASTA* format from the UCSC Genome Browser (http://genome.ucsc.edu)[26, 27]. This sequence was analyzed using TFSEARCH (Version 1.3) software for highly correlated sequence fragments in the TFMATRIX transcription factor binding site profile database (http://www.cbrc.jp/research/db/TFSEARCH.html)[28, 29].

Employing a 95 point scoring threshold for vertebrate matrices, we discovered a single NF-E2-related factor 2 binding site located 160bp upstream of the transcription start site (Figure 4.4)[30]. Based on these findings and previous studies of the ARE binding site, we hypothesized that Nrf2 might be an important regulator of *Prdx1* in endothelial cells.

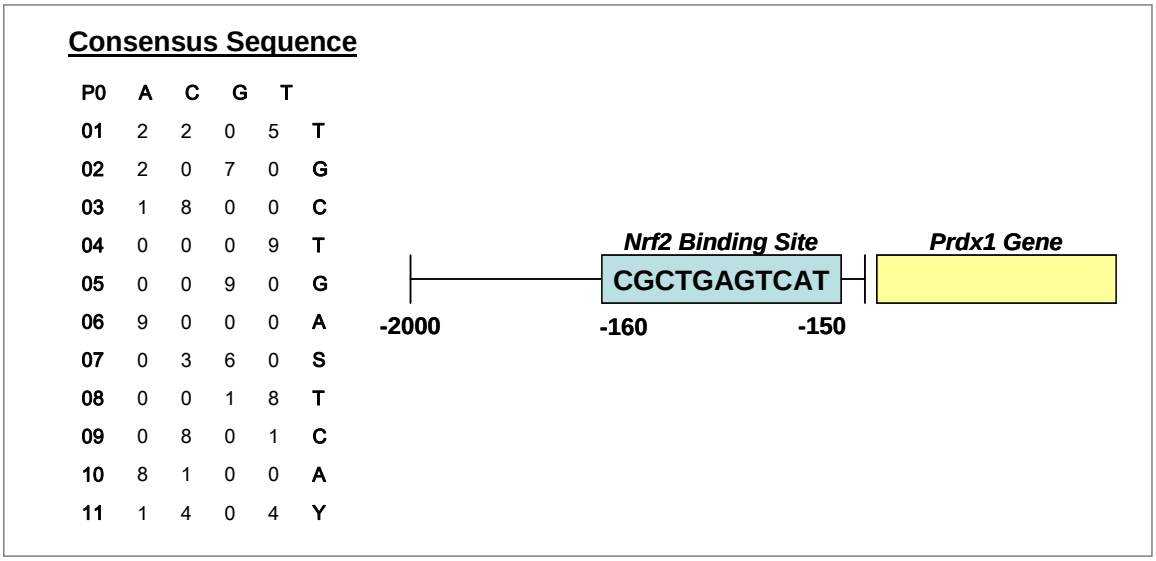


***Figure 4.4 The promoter region of bovine Prdx1 gene contains a Nrf2 binding site.*** *On the left, the DNA consensus sequence for the Nrf2 transcription factor is shown[30]. This binding site is found 160bp upstream of the bovine Prdx1 gene.*

*Nrf2 induces PRX 1 expression under static culture conditions.*

Our work has shown that LS, but not OS or ST, upregulates both *Prdx1* gene and its product PRX 1 protein[15]. To assess Nrf2's capability to stimulate this event, we first attempted to induce PRX 1 production during static culture via exogenous Nrf2 protein expression. In these studies, static BAEC were infected with either GFP adenovirus as a control or wild type human Nrf2 (GFP-tagged) adenovirus to overexpress Nrf2 protein. Immunofluorescence imaging of GFP was used to examine infection efficiency, and Western blot with Nrf2- and PRX 1-specific antibodies revealed adenovirus effectiveness and PRX 1 protein expression. Compared to Ad-GFP (20 MOI), Ad-wt hNrf2 increased

both Nrf2 and PRX 1 in a dose-dependent manner in endothelial cells (Figure 4.5). This data indicated that the transcription factor Nrf2 is able to promote PRX 1 protein expression under basal conditions and may contribute to shear stress-dependent transcription of *Prdx1*. However, further work was necessary to establish whether Nrf2 is required for LS-induced upregulation of bovine *Prdx1* gene.

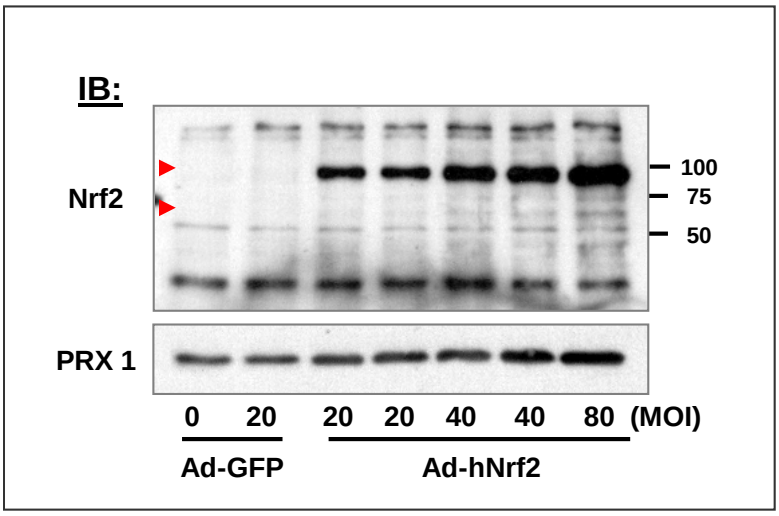


***Figure 4.5. Nrf2 stimulated PRX 1 expression in static cultured cells.*** *BAEC were infected with either GFP or hNrf2 Adenovirus (Ad) at indicated concentrations for 48 hours. Intracellular protein was analyzed by Western blot using antibodies specific to Nrf2 and PRX 1.*

*Nrf2 contributes in part to LS-induced Prdx1 expression.*

The depletion of Nrf2 from cellular systems provides a useful tool to study its functional role in endothelial cells. In the following experiments, specific siRNA was used to knockdown Nrf2 in order to investigate *Prdx1* expression in BAEC. Quantitative, real-time PCR with *Nrf2*- and *Prdx1*-specific primers was used to determine the efficacy of this siRNA, as well as the effect on basal *Prdx1* levels. Compared to non-silencing siRNA and Alexa Fluor 546-labeled non-silencing siRNA (50 nM each), 48 hour-treatment of BAEC with Nrf2 siRNA dramatically reduced (by 70% of non-silencing control) expression of *Nrf2* mRNA, at a concentration as low as 20nM (Figure 4.6A). In

addition, Nrf2 siRNA also decreased *Prdx1* mRNA by 40-50% of controls at all doses (Figure 4.6B). In conjunction with data from adenovirus experiments, these findings provided further support for Nrf2's role in PRX 1 regulation and suggested that Nrf2 may be important to *Prdx1* expression even under static conditions. Furthermore, we were able to perform subsequent studies using Nrf2 siRNA at 20nM concentration with confidence.

To determine whether Nrf2 is responsible for the LS-dependent increase in *Prdx1* levels in BAEC, we knocked down Nrf2 using Nrf2 siRNA as indicated above (Figure 4.6A). Nrf2 depletion significantly decreased *Prdx1* mRNA in both LS- and OS-treated cells (Figure 4.7A). However, after 72 hours of treatment (48 hours transfection, followed by 24 hours of shear stress), *Nrf2* levels were reduced only 40-50% (Figure 4.7B). Despite this reduced knockdown of Nrf2, the results of these studies indicated that Nrf2 contributes in part to both the basal and LS-mediated expression of *Prdx1*.

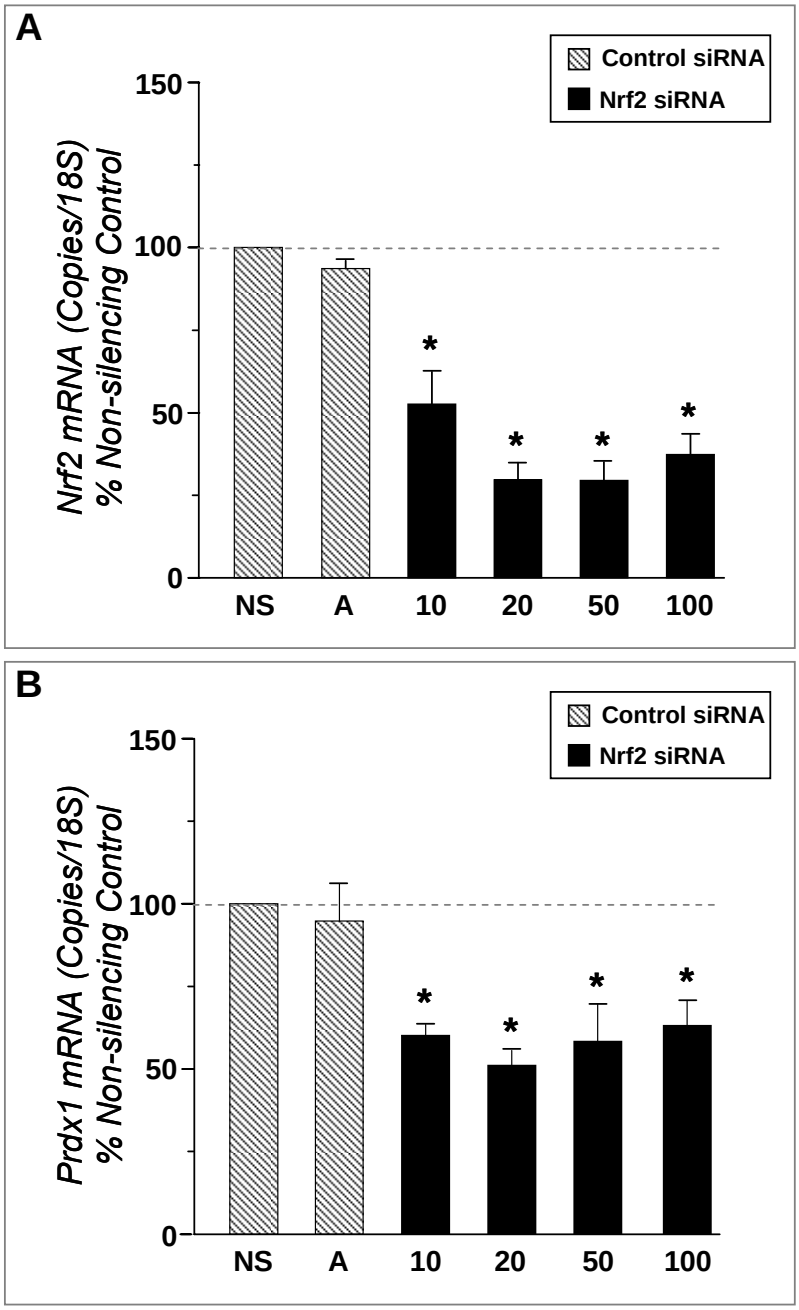


***Figure 4.6 Nrf2 siRNA inhibits Nrf2 mRNA and reduces expression of Prdx1 gene in static cultured cells.*** *BAEC were transfected with either non-silencing (NS) , Alexa-labeled non-silencing (A) or Nrf2 siRNA at indicated concentrations for 48 hours. Total RNA was collected and reverse-transcribed to synthesize first-strand cDNA, which was amplified via real-time, quantitative PCR. mRNA copy numbers were determined based on standard curves generated with bovine Prdx1, bovine Nrf2 and 18S templates. Nrf2 and Prdx1 mRNA copy numbers were normalized to 18S mRNA copy numbers. Nrf2 and Prdx1 mRNA levels (mean ± SEM, n=12) are shown in bar graphs as % of non-silencing siRNA-treated control. * $p<0.05$ designate significance between indicated groups.*

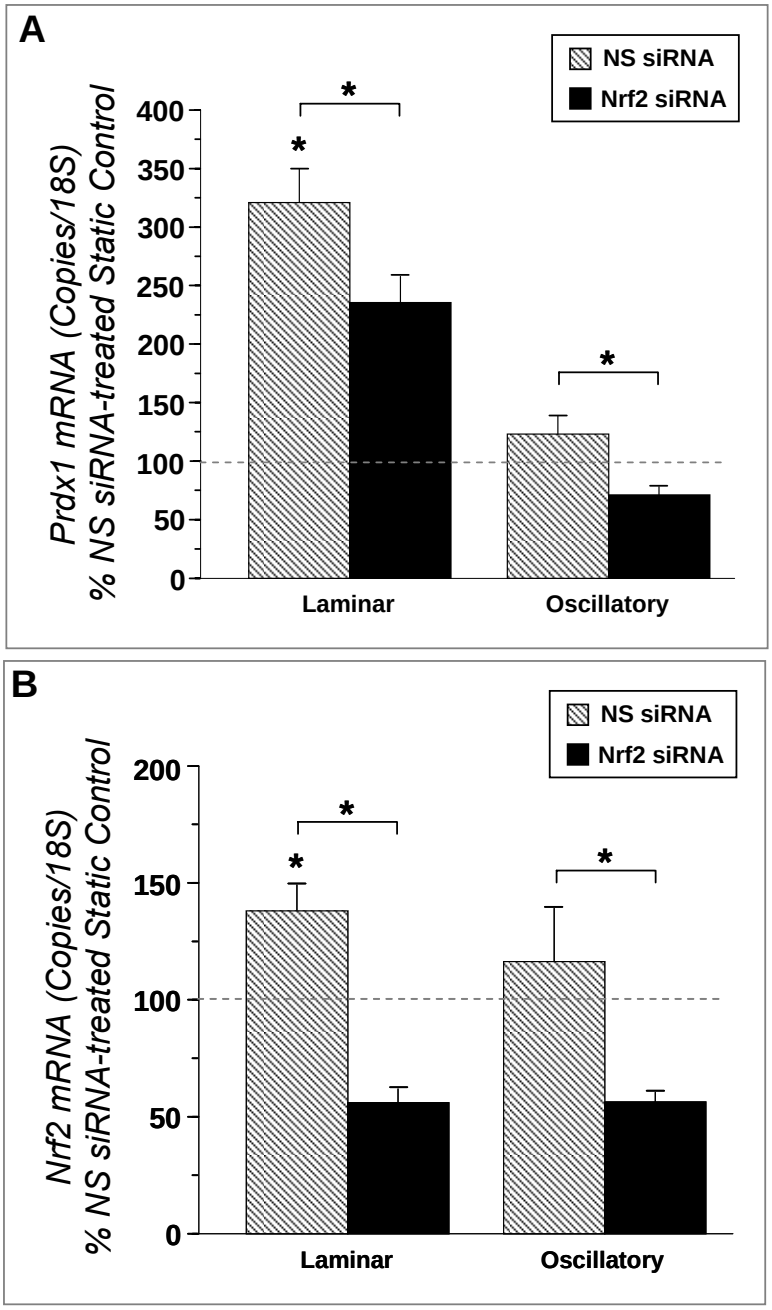


***Figure 4.7 Nrf2 knockdown reduces Prdx1 expression in response to LS.*** *BAEC were transfected with either non-silencing (NS) or Nrf2 siRNA (20nM) for 48 hours and then exposed to ST, LS, or OS for 24 hours. Total RNA was collected and cDNA was amplified via real-time, quantitative PCR as in Figure 4.5. Nrf2 and Prdx1 mRNA copy numbers were normalized to 18S mRNA copy numbers. Nrf2 and Prdx1 mRNA levels (mean ± SEM, n=12) are shown in bar graphs as % of non-silencing siRNA-treated static control. * p<0.05 designate significance between indicated groups.*

## Discussion

Through gene expression and siRNA depletion studies, we have discovered that: (1) LS upregulates *Prdx1* gene in a time-dependent manner compared to both OS and static culture conditions, but (2) this increase in expression is not due to stabilization of *Prdx1* mRNA; (3) The bovine *Prdx1* promoter region contains a Nrf2 binding site, and (4) Nrf2 overexpression promotes basal PRX 1 protein production, while (5) Nrf2 knockdown reduces *Prdx1* mRNA following exposure to LS. Collectively, these results suggest that laminar shear stress affects PRX 1 by inducing the *Prdx1* gene, in part via the transcription factor Nrf2.

Recent findings by our group showed that intracellular PRX 1 protein is upregulated by chronic laminar shear stress in BAEC and plays a crucial part in shear stress-dependent hydrogen peroxide diminution[15]. Through the current study, we sought to investigate the pathway by which LS promotes PRX 1 expression. The observed rise in protein generation could occur via several mechanisms, including increased transcription, mRNA stabilization, and augmented translation. As illustrated in Figure 4.1, the elevation of *Prdx1* mRNA levels suggested that PRX 1 expression is transcriptionally regulated. The time-dependent nature of this response and the lack of mRNA stabilization by shear stress indicated that mRNA is being produced in response to LS over the course of hours (Figure 4.2).

Several reports have demonstrated that other cytoprotective genes are also induced by laminar shear stress in endothelial cells. Chen et al. discovered NQO1, HO-1, microsomal epoxide hydrolase, glutathione *S*-transferase (GST), ferritin, and γ-glutamylcysteine synthase as targets of laminar flow in human aortic endothelial cells and human microvascular endothelial cells[19]. Work by Dai et al. also identified NQO1,

HO-1, and ferritin, as well as GSR, GCLM, and TXNRD1, as genes promoted by atheroprotective, but not atheroprone, flow in human umbilical vein endothelial cells[21]. The common thread amongst these phase II detoxification and antioxidant genes is the presence of an antioxidant response element (ARE) in their 5' flanking regions[31-33]. This ARE is activated by cellular exposure to insults that induce oxidative stress, including hydrogen peroxide, ionizing radiation, and a variety of chemical compounds[34]. Recently, it has also been shown to respond to chronic, steady laminar shear stress through its transcription factor Nrf2[19, 21, 35].

Although the mechanism of shear stress-mediated Nrf2 induction remains controversial, the conserved nature of the ARE/Nrf2 sequence in cytoprotective, mechanosensitive genes suggested that it may also be important to the regulation of PRX 1 (Figure B.1)[21, 35]. Interestingly, analysis of the bovine *Prdx1* promoter revealed a Nrf2 binding region 160bp upstream of the transcription start site. Further investigation also found that overexpression of Nrf2 stimulated the production of intracellular PRX 1 protein, while Nrf2 depletion significantly reduced transcription of *Prdx1* mRNA in response to LS, as well as static conditions and OS. Together these results suggested that Nrf2 is an important contributor to both basal and shear stress-mediate PRX 1 production.

In support of our findings, studies in other cell types have also linked the ARE/Nrf2 system to PRX 1 expression. Ishii et al. showed PRX 1 to be induced by Nrf2 via oxidative stress. In peritoneal macrophages from Nrf2-deficient mice, PRX 1 generation was blunted in response to stress agents such as paraquat and $CdCl_2$[36]. Additionally, other work found nuclear translocation of Nrf2 promoted human *Prdx1* gene expression in cancer cells exposed hypoxia/reoxygenation[20].

However, in the current study, Nrf2 knockdown did not fully abolish the LS-mediated upregulation of *Prdx1* mRNA (Figure 4.7A). These findings may be a product of reduced Nrf2 siRNA efficacy (less than 50% reduction at 3 days), or these data may imply that Nrf2 is not the only factor relevant to *Prdx1* transcription. Tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) have been found to activate transcription factor AP-1 sites in the rat *prx1* promoter of macrophages and monocytes[37]. Our analysis of genomic sequence upstream of bovine *Prdx1* also identified several AP-1 binding sequences in its promoter region. Consistent with the action of PRX 1 in BAEC, AP-1-regulated genes allow cells to respond oxidative stress[38]. Additional studies with the bovine *Prdx1* promoter-CAT construct will allow for further investigation of Nrf2, AP-1, and other transcription factor binding sites. In addition, recent work by Diet et al. showed that PRX 1 and 6 are controlled by endogenously-produced NO in macrophages[39]. It is well established that endothelial cells generate NO in response to LS[25, 40]. This may represent a supplementary pathway for shear stress-dependent PRX 1 expression in BAEC.

In summary, we have shown that laminar shear stress promotes the expression of bovine *Prdx1,* and that transcription factor Nrf2 may contribute to this important pathway. Furthermore, this discovery of *Prdx1* gene as a mechanosensitive transcript may contribute critical insights into endothelial cell biology and therapeutics for vascular diseases.

**Acknowledgments**

This work was supported by funding from National Institute of Health grants HL75209, HL87012, and HL80711 (HJ), as well as an American Heart Association pre-doctoral fellowship (ALM). Wild type human Nrf2 adenovirus was a generous gift from Dr. Xi-Lin

Chen at AtheroGenics, Inc. We would also like to thank Dr. Seth Brodie, Dr. Kyung Hwa Chang, Dr. Hannah Song, Dr. Mamta Patel, Chih Wen Ni, and Dr. Sarah Tressel at Georgia Institute of Technology and Emory University for helpful comments during these studies.

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# CHAPTER 5

# DISCUSSION

## Summary and Conclusions

In the United States alone, cardiovascular disease kills nearly 2600 people each day; its primary cause, atherosclerosis, affects millions of Americans each year[1]. Despite the localized nature of plaque formation in the arterial tree, preventative pharmaceutical treatments remain limited to drugs targeting systemic risk factors. In order to address the hemodynamic components of atherosclerosis development, recent work has focused on the mechanosensitive endothelium to establish new pathological mechanisms. Understanding how unidirectional, laminar blood flow protects vessels from atherogenesis, while disturbed, oscillatory blood flow promotes it, will provide enormous insight into direct therapeutic approaches for cardiovascular disease intervention.

The overall *objective* of this dissertation was to study the effects of atherogenic and atheroprotective fluid flow profiles on the mammalian antioxidants peroxiredoxins, and to identify mechanosensitive family members playing a role in redox regulation. By understanding how peroxiredoxins are regulated and what roles they play physiologically, we will have better insight into endothelial cell (EC) biology and redox-sensitive mechanisms of vascular diseases. The *central hypothesis* of this project was that *unidirectional laminar shear stress (LS), compared to disturbed oscillatory shear stress (OS), promotes increased expression of peroxiredoxins (PRX) via transcriptional regulation, which in turn influences the balance of reactive oxygen species in endothelial cells.* This hypothesis was tested according to three specific aims using cellular, molecular, and biochemical studies of bovine aortic endothelial cells:

- ***Specific Aim 1: Investigate shear stress-dependent expression and subcellular localization of peroxiredoxin family members in aortic endothelial cells.***

- ***Specific Aim 2: Identify the role of PRX 1 in shear stress-dependent reactive oxygen species regulation.***

- ***Specific Aim 3: Investigate the mechanism of shear stress-dependent PRX 1 expression in aortic endothelial cells.***

To achieve these specific aims, we first explored the effects of shear stress on expression of mammalian peroxiredoxin family members. Using an *in vitro* model, the cone-and-plate viscometer, we applied atheroprotective, high, unidirectional laminar shear stress or atherogenic, low, oscillatory shear stress to endothelial cells in culture. Previous studies established that reactive oxygen species (ROS) play a critical role in atheroma formation[2-5]. Peroxiredoxins have the ability to regulate ROS by reducing peroxides and eliminating peroxynitrite, but are understudied endothelial cell biology[6, 7]. Here, we were able to identify all six PRX family members in bovine aortic endothelial cells (BAEC). Furthermore, through this work, we revealed for the first time that PRX are regulated by shear stress in endothelial cells. When compared to OS and static culture, exposure to chronic LS upregulated PRX 1 levels intracellularly. LS also increased expression of PRX 5 relative to static controls, but not OS. Consistent with reports in other cell types, PRX exhibited broad subcellular localization in BAEC, with distribution in the cytoplasm, Golgi, mitochondria, and intermediate filaments[8, 9]. However, these localization patterns did not appear to change in response to shear

stress. Taken together, our results suggest that distribution of PRX in specific subcellular environments may provide protection against local ROS production and facilitate ROS-mediated signal transduction. Simultaneously, widespread allocation of PRX might also be important for comprehensive management of the overall oxidative state of cells. Moreover, upregulation of PRX 1 by LS may act to reduce ROS levels in EC and prevent atherogenic oxidative stress.

To establish the functional importance of PRX 1 in shear stress-dependent redox balance, we next examined the role of PRX 1 in LS-mediated hydrogen peroxide regulation. Endothelial cells exposed to OS experience oxidative stress as a signature of atherosclerosis. Conversely, chronic LS reduces ROS levels[2, 4, 5]. Here, we used Amplex Red assay as an independent method to measure ROS levels in BAEC. Consistent with previous reports, OS increased and LS decreased catalase-inhibitable hydrogen peroxide. Subsequently, we investigated whether PRX 1 was responsible for the decreased $H_2O_2$ levels in endothelial cells exposed to LS. Specific siRNAs were used to knockdown either PRX 1 or PRX 5 protein levels. Using isoform-specific PRX antibodies, we found that these siRNAs had no significant effect on other PRX, indicating that they exclusively targeted PRX 1 or 5, respectively, amongst all PRX family members. Depletion of PRX 1 using siRNA resulted in significantly higher ROS levels in BAEC exposed to LS, OS, and static conditions, while PRX 5 depletion did not. Altogether, these results indicate that chronic exposure to LS upregulates PRX 1 expression in order to keep ROS levels low in endothelial cells.

To identify the pathway by which atheroprotective laminar shear stress stimulates PRX 1 protein production, we undertook additional gene expression and siRNA depletion studies. Several reports have demonstrated that antioxidant genes are induced by

laminar shear stress in endothelial cells[10, 11]. In line with this, we discovered that LS upregulates *Prdx1* gene in a time-dependent manner compared to OS or static culture. However, this increase in expression was not due to shear stress-dependent stabilization of *Prdx1* mRNA. These findings lead us to believe that *Prdx1* was likely regulated at the transcriptional level. In addition, the antioxidant response element (ARE) has been shown to be conserved amongst several antioxidant genes, and studies showed it responds to chronic, steady laminar shear stress through its transcription factor Nrf2[10-14]. Our analysis of the bovine *Prdx1* promoter revealed a Nrf2 binding site 160bp upstream of the gene. Furthermore, Nrf2 overexpression promoted basal PRX 1 protein production, while Nrf2 depletion reduced *Prdx1* mRNA following exposure to LS. Collectively, our work illustrates that laminar shear stress affects PRX 1 by inducing the *Prdx1* gene, in part via the transcription factor Nrf2.

By addressing the three specific aims proposed, this project has developed a comprehensive register of shear stress-dependent peroxiredoxin behavior and discovered a novel mechanosensitive antioxidant that plays an important functional role in redox regulation of endothelial cell biology. This dissertation illustrates how physiologically relevant fluid flow affects reactive oxygen species, through the unique expression of antioxidants, in particular Peroxiredoxin 1 (Figure 5.1). Identifying factors that play an important role in shear stress-dependent oxidative stress could help provide localized therapies at the site of endothelial cell dysfunction. Our findings are relevant to the inflammatory disease atherosclerosis, a major cause of cardiovascular disease. By understanding how PRX1 is regulated and what role it plays in physiology, we can better understand vascular diseases and develop effective preventative therapeutic approaches.

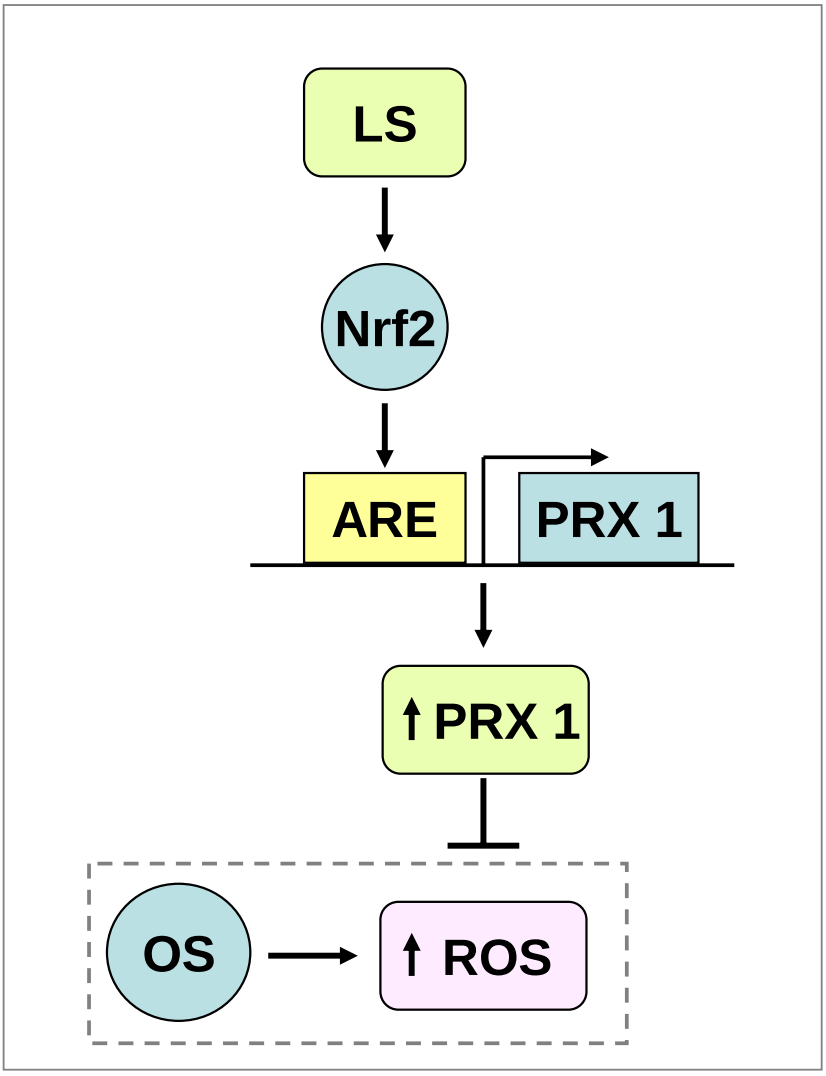


***Figure 5.1  Overall summary of project findings***

**Limitations**

There are several limitations to the work performed in this project including, but not restricted to, the following points:

1. Use of cultured cells
2. Use of bovine aortic endothelial cells
3. Use of simulated blood flow conditions
4. Assessment of ROS limited to global levels
5. Investigation of one potential transcription factor
6. *In vivo* studies limited to future work

In this dissertation, studies were performed solely with an *in vitro* model. To simplify the experimental layout, endothelial cells alone were examined. Unfortunately, cells cultured in a dish likely behave differently from those in body. In the vasculature, endothelial cells are in close proximity to several other cells types, including smooth muscle cells, fibroblasts, and leukocytes. Studies have previously shown that shear stress promotes the release of factors from the endothelium that directly affects smooth muscle cells and leukocytes[15]. Thus, it probable that communication with other cell types equally affects endothelial cell behavior[16].

Here, bovine aortic endothelial cells were chosen to represent the arterial endothelial cells exposed to physiologically significant flow patterns *in vivo*. To directly relate our findings to human pathologies, it would be more appropriate to employ a human endothelial cell line. Many groups have used human umbilical vein endothelial cells in studies similar to ours[17]. However, endothelial cells in veins do not typically experience the shear stress profiles found in large arteries, and as a result, may be conditioned to respond differently. Although human aortic endothelial cells are available, BAEC offer

considerable ease of culture and should experience somewhat similar fluid flow patterns *in vivo*.

To evaluate shear stress-dependent endothelial cell behavior, we utilized a cone-and-plate viscometer. As previously described, this system provides a simplified, well-defined simulation of hemodynamic environments. In contrast, the fluid flow endothelial cells experience *in vivo* is much more complex. This *in vitro* model fails to account for the pulsatility of blood flow generated by the heart, as well as normal pressure and cyclic stretch EC might perceive in an *in vivo* mechanical environment[18]. Although many others have shown that *in vitro* shear systems convincingly recapitulate *in vivo* findings, it remains possible that some responses may be different[19, 20].

Beyond the *in vitro* aspect of this work, other limitations also constrained our findings. In Chapter 3, we discovered that PRX family members were located throughout the cellular milieu of BAEC. This indicated they may provide protection against local sources of ROS. In subsequent functional experiments, we were only able to assess the overall oxidative state of cells in response to PRX depletion. Currently available techniques do not allow for examination of ROS at specific intracellular organelles, but such studies may be particularly informing with respect to the many PRX family members. In addition, this project explored only a single possible transcription factor for the mechanosensitive *Prdx1* gene. As discussed in Chapter 4, several other candidates exist and should be addressed in further work. Finally, this dissertation did not examine the *in vivo* implications of *in vitro* findings. Future experiments should validate these results in relevant animal models of disease.

## Future Directions

The sheer number of peroxiredoxins, and their poor representation in the mechanobiology field, signifies many new opportunities for atherosclerosis research. The findings described in this dissertation have laid the groundwork for additional future studies of Peroxiredoxin 1 in endothelial cells exposed to shear stress. Advancement of this work may focus on both *in vitro* and *in vivo* experimental approaches.

### *Signaling pathways*

Our finding that laminar shear stress upregulates PRX 1 may be relevant to several signal transduction pathways. Shear stress has been shown to influence a number of important signaling cascades in endothelial cells. MAP kinases including ERK 1/2, JNK, and p38 are differentially affected by fluid flow profiles and affect downstream targets such as eNOS and Akt[21, 22]. In addition, LS inhibits, while OS promotes, the inflammatory pathway of NF-κB. Previous work has established that PRX 1 is an important regulator of p38 in macrophages exposed to oxidative stress[23]. Similarly, PRX 1 affects NF-κB, suppressing NF-κB transcriptional activity, JNK activity and apoptosis induced by TNF-α[9, 24]. Experiments using PRX 1 siRNA would reveal if LS-dependent PRX 1 expression regulates any of these critical pathways in response to shear stress.

### *In vitro physiological roles*

In Chapter 3, we showed that PRX 1 significantly affects the redox state of endothelial cells exposed to shear stress. However, we did not explore the functional endpoint of this role. Reactive oxygen species play an important part in many physiological functions relevant to atherosclerosis, including apoptosis and inflammation. Both of these events are inhibited in endothelial cells exposed to atheroprotective laminar shear stress, and apoptosis has been linked to PRX 1 in cancer cells[15, 25]. As such, further

work should focus on the shear stress-dependent role of PRX 1 in apoptosis and inflammation of endothelial cells. Analysis of *in vitro* markers such as Annexin V and monocyte attachment, in conjunction with PRX 1 depletion, would provide valuable mechanistic insight into these cellular behaviors.

*Transcription factors*

Studies in Chapter 4 indicated that fluid flow regulates PRX 1 at the transcriptional level, and Nrf2 contributes in part to the mechanism. Additionally, this work suggested that Nrf2 may not be the only factor promoting *Prdx1* expression. As described above, both NO and AP-1 elevate *Prdx1* expression in monocytes and macrophages[26, 27]. Endothelial cells generate NO in response to atheroprotective shear stress[16, 28]. Valuable experiments might employ the nitric oxide donor diethylenetriamine NONOate (DETA-NO) to induce *Prdx1* expression in static cells or those exposed to OS. In contrast, the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) could be used to block LS-dependent transcription. Furthermore, the transcription factor AP-1 is also shear stress responsive and has been implicated in cellular response to oxidative stress[29, 30]. As previously mentioned, our analysis of genomic sequence upstream of *Prdx1* identified several AP-1 binding sequences in its promoter region. We have recently cloned the bovine *Prdx1* promoter into both luciferase and chloramphenicol acetyltransferase (CAT) reporter vectors. These constructs could be transfected into endothelial cells exposed to LS, OS or static culture. Truncation and mutation studies may reveal the importance of many transcription factor binding sites, including AP-1.

*In vivo implications*

Ultimately, *in vivo* research must be performed to validate the *in vitro* findings of this project. Preliminary studies in wild type C57BL6 mice suggest that PRX 1 may also be

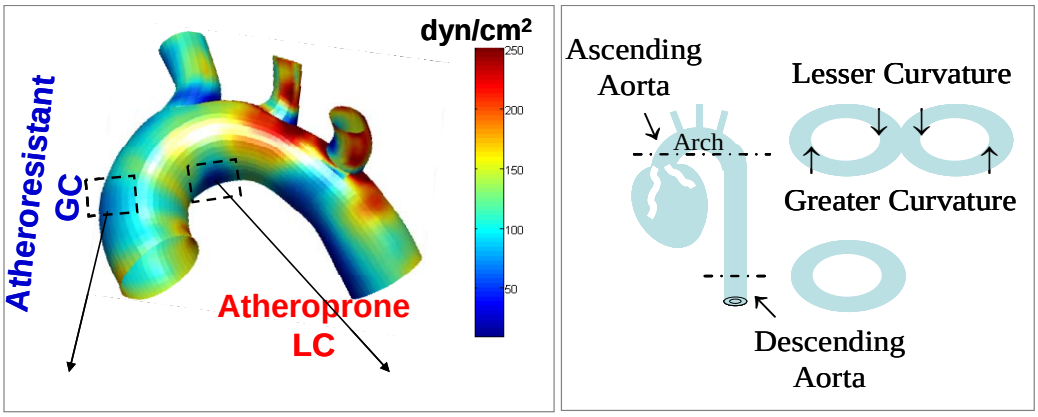


***Figure 5.2 The mouse aortic arch is a model for both LS- and OS-like flow.*** *Following isolation, the arch is embedded, frozen and sectioned as depicted on the right. This allows for examination of both the atheroresistant GC and atheroprone LC regions in the same cross-section. Figure on left reprinted with permission[31]. Copyright © 2007 Lippincott, Williams & Wilkins.*

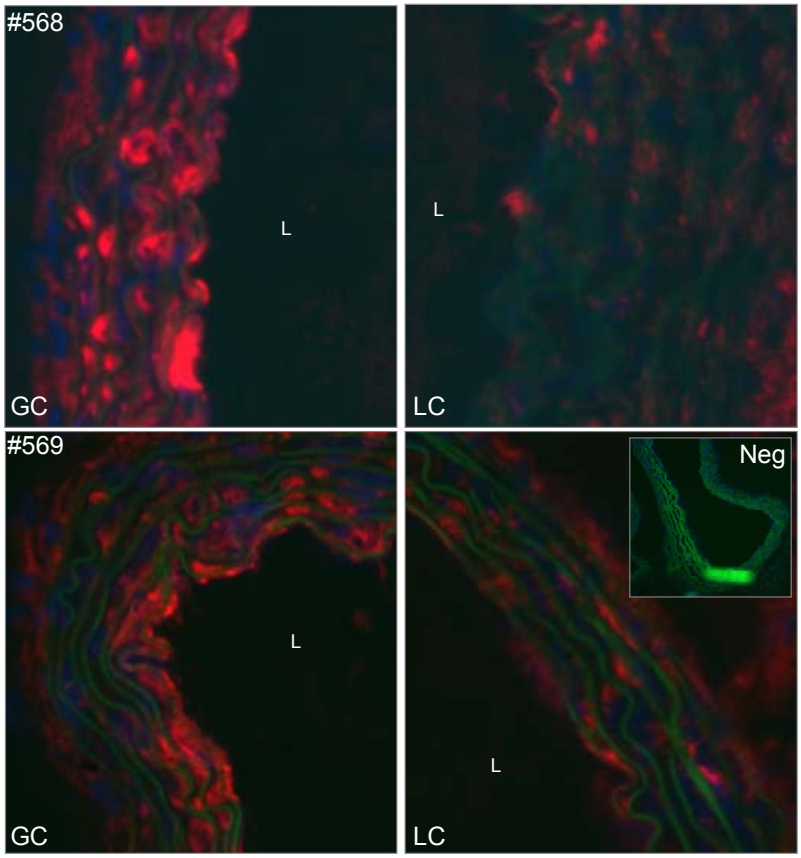


***Figure 5.3 PRX 1 expression is elevated in the LS-like GC of the aortic arch.*** *Frozen sections of aortic arches from wt C57BL6 mice were stained with PRX 1-specific antibodies and Alexa Fluor 568-conjugated secondary (red). Nuclei were counter-stained with Hoechst (blue) and elastic lamina autofluorescence is green. L = vessel lumen.*

regulated by shear stress in the aorta. As determined by computational fluid dynamics, the mouse aortic arch serves as an excellent model for both unidirectional laminar shear stress and disturbed, oscillatory shear stress (Figure 5.2)[31]. The greater curvature (GC) experiences flow much like *in vitro* LS, while the lesser curvature (LC) is exposed to OS-like conditions. Here, frozen cross-sections of the arch were stained with PRX 1-specific antibodies. Immunofluorescent images revealed elevated PRX 1 expression in the GC region, consistent with our *in vitro* findings in Chapter 3 (Figure 5.3). En face immunostaining will be necessary to confirm these results and verify that PRX 1 staining is endothelial cell specific.

Further studies in animal models will be required to establish the physiological relevance of PRX 1 in atherosclerosis progression. To determine whether endothelial PRX 1 expression correlated with atherosclerotic lesion development, we stained human coronary arteries with various stages of disease. Serial sections of human coronary arteries were stained with both PRX 1- and PECAM-1-specifc antibodies. As illustrated in Figure 5.4, PECAM-1 was used to verify an intact endothelial cell layer in all sections. In low-magnification images (Figure 5.5), PRX 1 was easily detected in the endothelium and medial layer regardless of disease stage. However, expression in the intimal smooth muscle layer tended to decrease as disease progressed. Examination of high-magnification images (Figure 5.6) further supported this phenomenon. Although these findings do not implicate endothelial-derived PRX 1 in plaque development, they are consistent with previous studies of PRX. Work by Choi et al. found that PRX 2 prevents PDGF-dependent smooth muscle cell proliferation and migration in neointimal thickening[32]. Additional assessment of PRX 1 in this model may reveal a similar function role in smooth muscle cells.

Finally, PRX 1 knockout mice also are available, and erythrocytes and embryonic fibroblasts from these animals contain higher levels of ROS than their wild type counterparts[33]. Crossing these mice with ApoE-/- mice, and feeding them high fat diet, will generate a model that is both hyperlipidemic and oxidatively stressed. Atherosclerosis development could be evaluated in these animals using traditional assessment of plaque formation via en face Oil Red O staining, as well as in a shear stress models such as partial carotid ligation or aortic coarctation.

In summary, the future directions of this project should examine the *in vivo* consequences of shear stress and peroxiredoxins in atherosclerosis. By understanding the mechanisms behind plaque formation, important therapies can be developed. Cardiovascular disease is the primary cause of death in the United States. Clarifying the role of antioxidants such as PRX 1 in its progression could provide insight into direct therapeutic approaches for disease intervention.

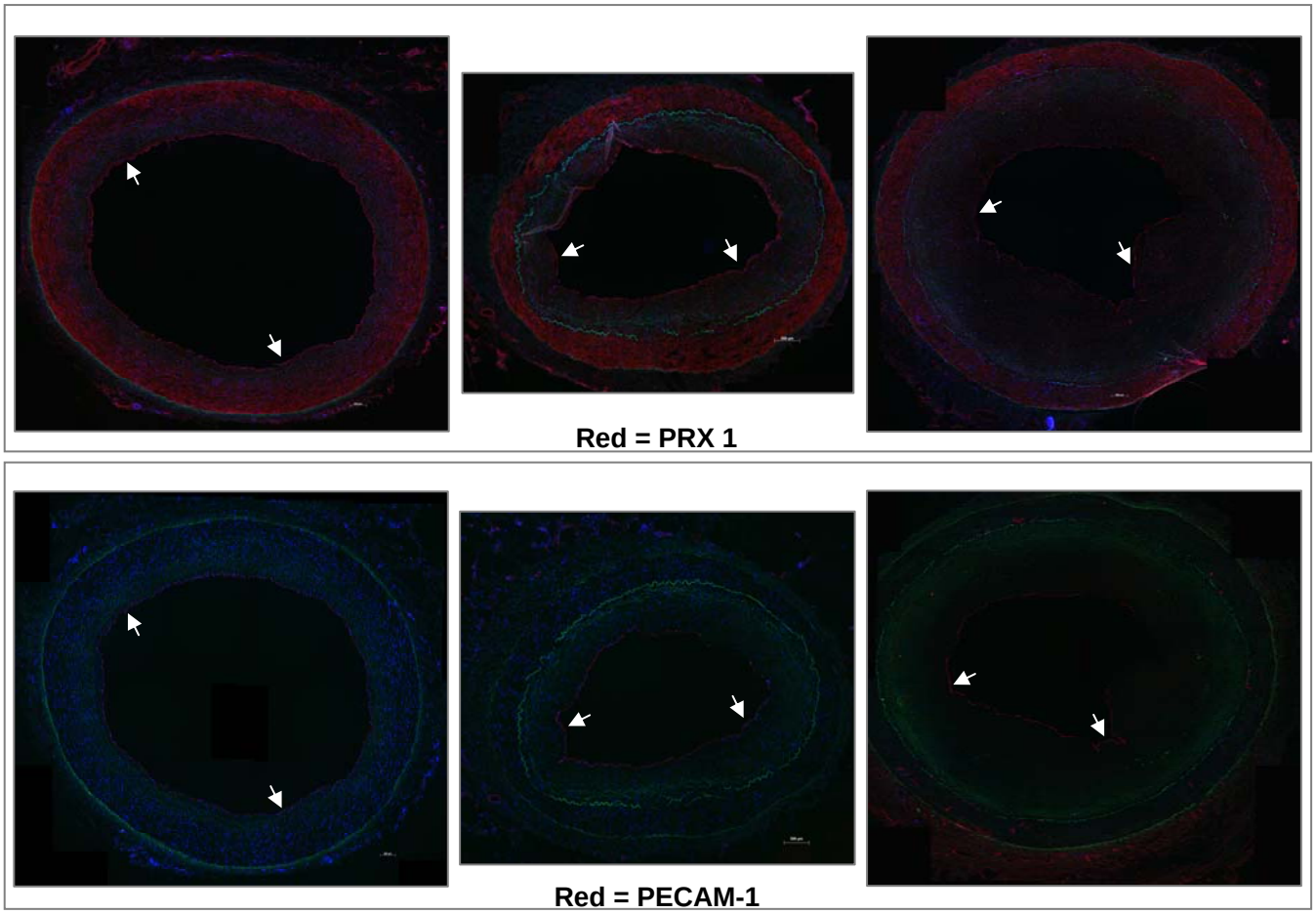


***Figure 5.4 PECAM-1 verifies an intact endothelium in human coronary arteries.*** *Frozen sections from human coronary arteries with various stages of disease were stained with either PRX 1- or PECAM-1- specific antibodies and Alexa Fluor 568-conjugated secondary (red). Nuclei were counter-stained with Hoechst dye (blue) and elastic lamina autofluorescence is green.*

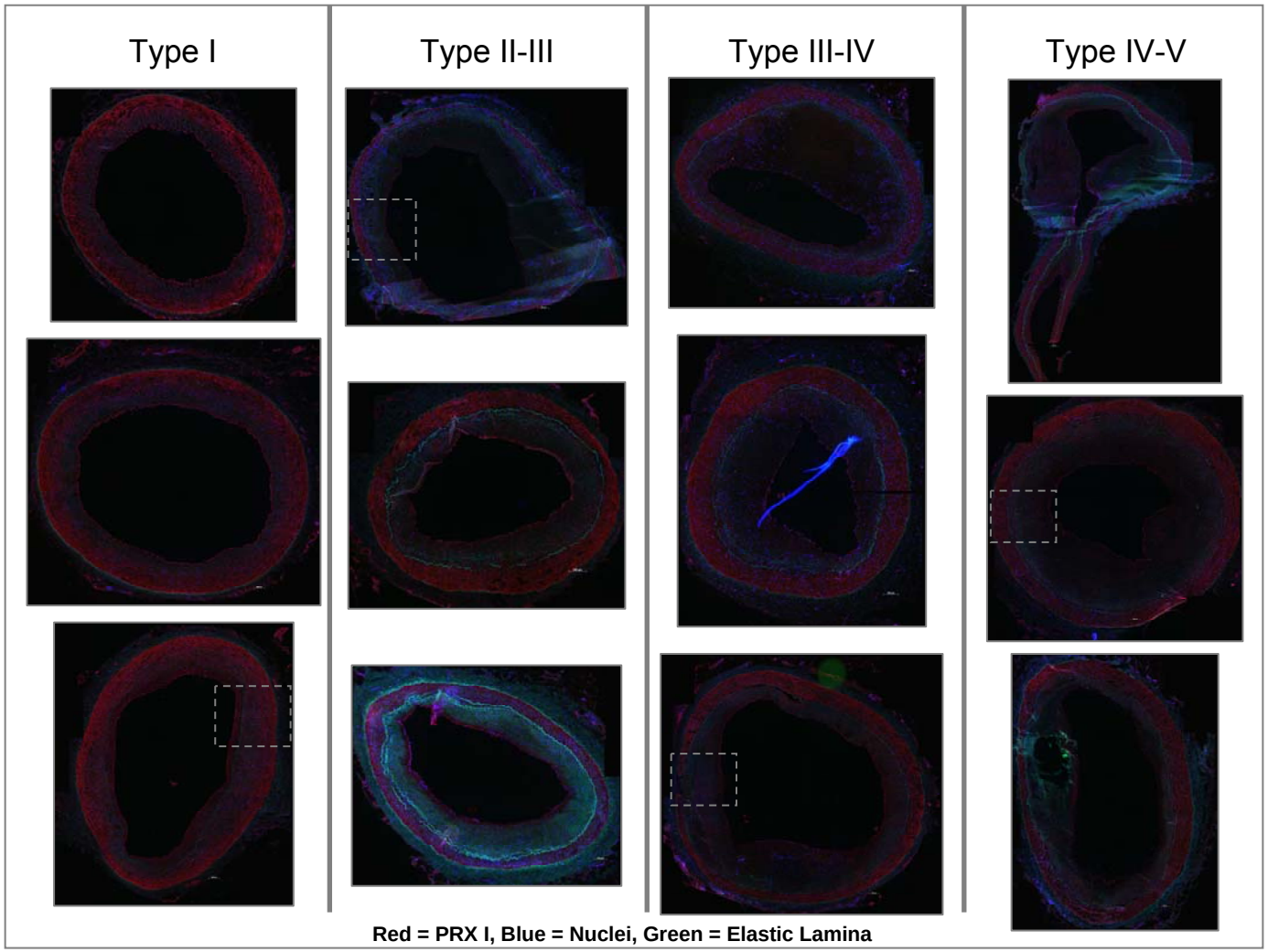


***Figure 5.5 PRX 1 expression did not change in the endothelium as disease progressed.*** *Frozen sections from human coronary arteries were stained for PRX 1 (red) as described in Figure 5.5. Nuclei were counter-stained with Hoechst dye (blue) and elastic lamina autofluorescence is green. Hatched boxes are shown at higher magnification in Figure 5.6.*

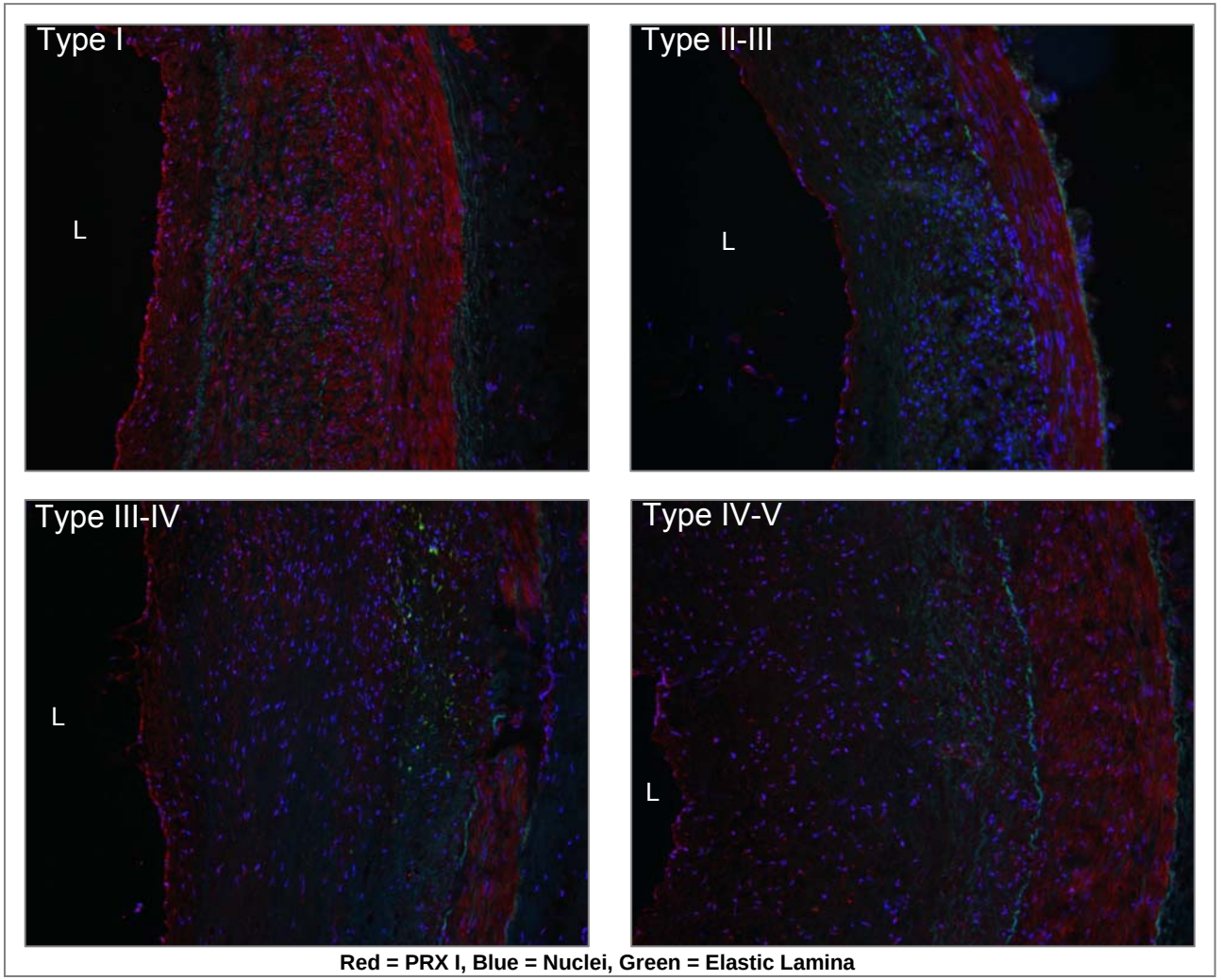


***Figure 5.6 PRX 1 expression decreased in* the intimal smooth muscle with increasing disease progression.** *Frozen sections from human coronary arteries were stained as described in Figure 5.6. Higher magnification images are shown here according to American Heart Association classification. L = vessel lumen.*

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# APPENDIX A

# CHAPTER 3 SUPPLEMENTARY DATA

**Analysis of Secreted PRX in Response to Shear Stress**

In Chapter 3, we investigated the intracellular expression of all six peroxiredoxin family members. Our results showed that PRX 1 was induced by chronic laminar shear stress (LS), compared to oscillatory shear stress (OS) and static (ST) control. Further studies were conducted to assess whether extracellular PRX were also regulated by shear stress. As previously described, bovine aortic endothelial cells (BAEC) were exposed to static culture, LS at 15 dynes/cm$^2$, or OS at ±5 dynes/cm$^2$ for 24 hours. Following experimental treatment, conditioned medium was collected from culture dishes. To prepare secreted proteins, this media was centrifuged at 1,000 x *g* for 10 minutes to remove cell debris, and the supernatant was concentrated via spin column (Vivaspin) at 4$^0$C. The protein content of each sample was determined by Bio-Rad DC assay, and immunoblots were performed as before for PRX 1, 2, 3, 4, 5, and 6.

We were able to detect PRX 2, 4 and 6, but not PRX 1, 3 or 5, in concentrated media taken from BAEC. Levels of PRX 2 and 6 were significantly elevated in response to LS, while PRX 4 was unaffected by shear (Figure A.1). Previous studies have shown that PRX 4 contains a secretion signal peptide at its amino terminus and is secreted extracellularly[1]. In contrast, PRX 2 and 6 are recognized as cytosolic proteins. The presence of PRX 2, 4, and 6 in conditioned media taken from static control cells supported the concept that these PRX are secreted from BAEC under basal conditions. In addition, the absence of PRX 1, 3, and 5 implied that not all cytosolic proteins are released. However, the suggestion that shear stress promotes increased secretion, independent of cellular damage, required further confirmation.

To address this concern, we probed conditioned media for the non-exporting proteins, actin and lactate dehydrogenase (LDH). Both actin and LDH were increased by LS,

despite modification to our cone-and-plate shear stress apparatus and the well-known protective effect of laminar shear stress on endothelial cells. As such, we were unable to exclude the possibility that PRX found in the conditioned media was a result of cell membrane damage during shear stress exposure (Figure A.1). However, it is most likely that a few senescent or weak endothelial cells are detached from the substrate by shear stress, and subsequently release actin and LDH into the media.

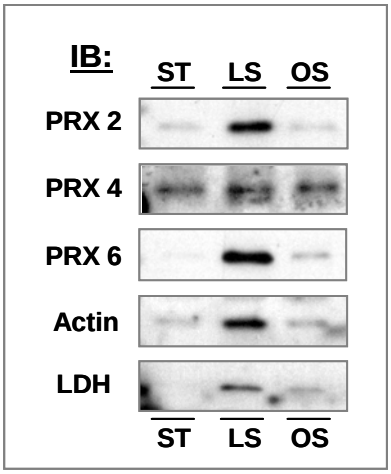


***Figure A.1 PRX 2, 4, and 6 are present in conditioned media taken from BAEC.*** *Confluent BAEC were exposed to LS, OS or static conditions for 1 day, and the conditioned media was obtained. Equal aliquots of media (20 µg of protein each) were analyzed by immunoblot using antibodies specific to PRX 1-6, actin, or LDH.*

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# APPENDIX B

# CHAPTER 4 SUPPLEMENTARY DATA

**Analysis of *Nrf2* mRNA in Response to Shear Stress**

In Chapter 4, we investigated the antioxidant response element (ARE) as a potential mediator of laminar shear (LS)-induced *Prdx1* gene expression. Previous studies have found that the ARE is conserved amongst several mechanosensitive, cytoprotective genes and responds to chronic laminar shear stress through the transcription factor NF-E2-related factor 2 (Nrf2)[1]. However, the exact mechanism by which Nrf2 responds to shear stress remains controversial.

Transcription factor Nrf2, a basic leucine zipper protein (bZIP), activates the ARE and its downstream genes by binding to ARE consensus sequence. Under homeostatic conditions, the cytoskeleton binding protein Kelch-like ECH-associated protein 1 (Keap1) binds Nrf2 in the cytoplasm and targets it for degradation via the ubiquitin-proteasome system. However, when cells are exposed to stress, Nrf2 is released and travels to the nucleus, where it forms a heterodimer with another bZIP, Maf, and binds to the ARE[1-5]. In human aortic endothelial cells (HAEC) and human microvascular endothelial cells, Chen et al. first showed that anti-sense Nrf2, dominant negative Nrf2, and overexpression of Keap1 could all block laminar flow-induced activation of ARE-mediated genes[1]. Later work in both HAEC and human umbilical vein endothelial cells (HUVEC) corroborated these findings using Nrf2-specific siRNA[4, 6, 7]. However, several discrepancies exist in these recent studies concerning the mechanism of regulation of Nrf2 by shear.

In 2005, Hosoya et al. reported that both LS and OS induce nuclear accumulation of Nrf2 in HAEC. Nonetheless, OS prevents Nrf2 binding to the ARE, and subsequent gene transcription, through an unknown nuclear factor[4]. On the other hand, Dai et al. showed in HUVEC that “atheroprotective” shear stress, compared to “atherprone” shear

stress, increased nuclear accumulation of Nrf2, but did not affect cytoplasmic protein levels. Using *en face* immunohistochemical staining, they found a similar Nrf2 expression pattern in atherosclerosis-resistant regions of the mouse aorta[6]. Finally, Warabi et al. established that intracellular protein levels of Nrf2 were markedly increased in HUVEC, although the mRNA levels of Nrf2 were unchanged. They demonstrated that shear stress stabilizes Nrf2 protein via an ROS-dependent pathway[7].

To examine how LS might promote *Prdx1* expression through Nrf2 in bovine aortic endothelial cells (BAEC), we assessed Nrf2 gene expression in response to shear stress. Total RNA was collected from BAEC following static culture (ST), or 2, 4, 8 or 24 hours of LS (15dynes/cm$^2$) or oscillatory shear stress (OS, ±5dynes/cm$^2$). Quantitative, real time PCR was used to determine *Nrf2* mRNA copy number relative to the internal control 18S. As illustrated in Figure B.1, we found that *Nrf2* expression increased in response to laminar shear stress after 8 hours and by 24 hours was significantly different from both ST and OS. Though this temporal pattern did not exactly match the regulation of *Prdx1* by LS, it did suggest that chronic LS regulates *Nrf2* gene in BAEC. As implied by work in other cell types, gene regulation of Nrf2 may not be the only mechanism for regulation. It is possible that LS stabilizes Nrf2 protein or affects the nuclear translocation of Nrf2. However, in conjunction with previous findings, our studies suggest that Nrf2 may be uniquely regulated by LS in endothelial cells of different origin and species. Similarly, the magnitude of shear stress and the *in vitro* model system employed could account for diverse experimental outcomes.

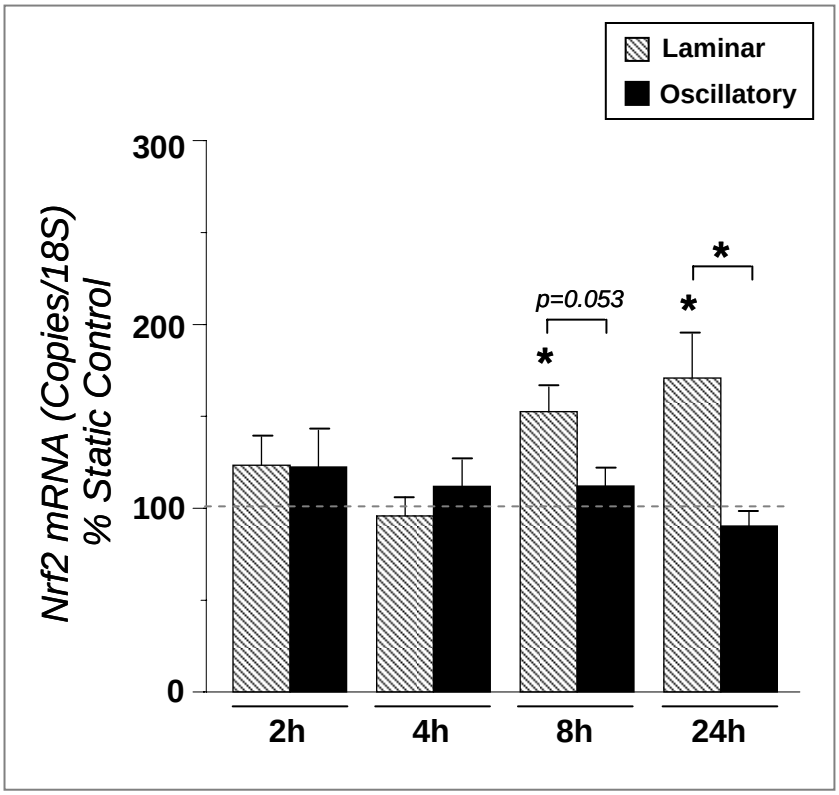


***Figure B.1 Nrf2 mRNA is upregulated by LS compared to OS.*** *Confluent BAEC were exposed to LS, OS or static conditions for 2, 4, 8, or 24h. Total RNA was collected and reverse-transcribed to synthesize first-strand cDNA, which was amplified via real-time, quantitative PCR. mRNA copy numbers were determined based on standard curves generated with bovine Nrf2 and 18S templates. Nrf2 mRNA copy numbers were normalized to 18S mRNA copy numbers. Nrf2 mRNA levels (mean ± SEM, n=3-15) are shown in bar graphs as % of static control. * p<0.05 designate significance between indicated groups.*

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# VITA

## AMY LEIGH MOWBRAY

Amy Leigh Mowbray was born Amy Leigh Holbrook to Keith and Teresa Holbrook on January 28, 1980 in Kettering, Ohio. She spent her childhood in Beavercreek, Ohio, growing up with her sisters Betsy, Cady, and Audrey. After graduating from Beavercreek High School in 1998, Amy attended the University of Kentucky and studied chemical engineering. In 2002, she graduated with honors from UK and married Adam Andrew Mowbray. Amy worked for one year with Procter & Gamble Pharmaceuticals before beginning graduate school at the Georgia Institute of Technology and Emory University in 2003. In her free time, Amy enjoys spending time with her husband and visiting family. She loves to travel, especially to the lake or the beach, read fictional literature, run in organized half marathons, and take dance or Pilates lessons.