

**MYELOPEROXIDASE INDUCES ENDOTHELIAL DYSFUNCTION VIA
ACTIVATION OF THE CALCIUM DEPENDENT PROTEASE CALPAIN**

A Dissertation
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

by
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May, 2018

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ABSTRACT

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Doctor of Philosophy

Lewis Katz School of Medicine, Temple University 2018

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Cardiovascular disease and the associated endothelial dysfunction are characterized by leukocyte activation, decrease endothelial nitric oxide synthase (eNOS) activity, and increased endothelial cell adhesion molecules expression. This leads to the release of myeloperoxidase (MPO) by activated neutrophils and monocytes. MPO is a peroxidase enzyme that plays an important role in innate immune host defense, however it has been shown to play a pathogenic role in cardiovascular disease, mainly by causing endothelial dysfunction. The molecular mechanisms through which MPO induces endothelial damage are not fully understood. Calpains are a family of calcium-dependent proteases. Two calpain isoforms, μ - and m-calpain, are expressed in the vascular wall, including endothelial cells. Activation of calpains has been recently implicated in inflammatory disorders of the vasculature. The goal of this study was to test the hypothesis of a role for calpains in the molecular mechanism(s) through which MPO causes endothelial dysfunction and vascular inflammation.

To explore if MPO activates calpain and to identify the calpain isoform(s) involved, we first studied the effects of MPO treatment on calpain activity in mouse lung

microvascular endothelial cells (MMVEC). MMVECs were stimulated with 10 nmol/L MPO for 60, 120, 180, and 240 min. Using a fluorescent calpain activity assay, we found that MPO time dependently activates calpain in endothelial cells, with peak activity reached within 180 min. Using immunoblot analysis techniques we demonstrated that the calpain isoform targeted by MPO is μ -calpain. Interestingly, using a biotin switch assay, 10 nmol/L MPO appears to activate the μ -calpain isoform via denitrosylation of its C-terminus domain.

Using MMVECs, we studied the effects of the MPO/ μ -calpain signaling on endothelial dysfunction. MMVECs were stimulated with 10 nmol/L MPO for 180 min. Expression levels of Protein Phosphatase 2 (PP2A), total 5' AMP-activated protein kinase (AMPK), Thr¹⁷² phospho-AMPK, total endothelial nitric oxide synthase (eNOS), Ser¹¹⁷⁷ phospho-eNOS, total protein kinase B (AKT), Ser⁴⁷³ phospho AKT, Adiponectin receptor 1 (AdipoR1), and Adiponectin receptor 2 (AdipoR2), were measured by immunoblot analysis. Interestingly, MPO impaired Thr¹⁷²AMPK, Ser¹¹⁷⁷eNOS, but not Ser⁴⁷³ AKT phosphorylation in a calpain dependent manner. On the other hand, MPO significantly increased the expression levels of PP2A. Inhibition of PP2A with okadaic acid decreased the phosphorylation levels of AMPK, and eNOS, indicating that PP2A is a downstream target of the MPO/calpain system.

MPO treatment significantly increased the expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells. Pharmacological inhibition of calpain activity attenuated expression of VCAM-1. MPO also decreased protein levels of AdipoR1, and AdipoR2 in a calpain dependent manner. The treatment of MMVEC with adiponectin in the presence of MPO was not able to restore AdipoR2 expression levels. Using genetically

modified mice, we found evidence of reduced leukocyte adhesion to the aortic endothelium in response to MPO in μ -calpain deficient mice, compared to wild-type mice . These effects appears to be attributed to the endothelial calpain, since incubating wild type aortas with calpain deficient leukocytes did not reduce leukocyte adhesion to the endothelium.

The data in this study first establish a role for calpain in the endothelial dysfunction and vascular inflammation of MPO, with MPO activating the μ -calpain isoform via denitrosylation. Our data also report that increased calpain activity downregulates the expression of a number of signaling molecules important for endothelial cell function. This study may provide the MPO/calpain/PP2A signaling pathway as a novel pharmacological targets for the treatment of inflammation-driven vascular disorders.

DEDICATION

THIS DISSERTATION IS DEDICATED TO THE PEOPLE WHO SAW ME

THROUGH IT:

Ghasan, Najm, Diyaa, Ilyas, Mom, and Dad

ACKNOWLEDGMENTS

First of all I must express my gratitude to my advisor, Dr. Rosario Scalia, for his guidance and support throughout my doctoral studies. Dr. Scalia's knowledge of vascular physiology and metabolism paired with his rigorous and skeptical approach to science have provided inspiration on a daily basis. From him I have learned the way to think creatively and critically in doing research projects. He has also trained and enhanced my trouble shooting and problem solving skills.

I sincerely thank my committee members: Dr. Michael Autieri, Dr. Saruro Eguchi, and Dr. Diane Soprano, for their advice, suggestions and tremendous support during my time at Temple University. I must also extend special thanks to Dr. Lawrence E. Goldfinger, who has generously agreed to serve as my external reader on short notice. Thank you all. I would like to thank Scalia Lab members: Inna, Gavin, Christine, and Chiecko. The Temple University community especially Polina, Ariele, and Akruiti who have fostered a thoughtful and inviting atmosphere during my graduate school experience. It has been an honor to work with the CVRC scientists who provided such a friendly environment, I am grateful to have been a student here.

And last, I would like to thank my husband Ghasan, my kids, and my parents, who constantly supported and encouraged me, without them this would not be possible.

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LIST OF ABBREVIATIONS

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
AMPK	Adenosine monophosphate-activated protein kinase
CaMKK	Ca ²⁺ /calmodulin-dependent protein kinase kinase
EC	Endothelial cells
eCAMs	Endothelial cell adhesion molecules
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
Esel	E-selectin
FACS	Fluorescence-activated cell sorting
FFA	Free fatty acid
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
H ₂ O ₂	Hydrogen peroxide
HOCl	Hypochlorous acid
ICAM	Intercellular adhesion molecule
iNOS	Inducible nitric oxide synthase
IVM	Intravital microscopy
KH	Krebs-Henseleit
LA	Leukocyte adhering
LFA-1	Lymphocyte function-associated antigen-1
LKB1	Liver kinase B1

LMW	Low molecular weight
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
MPO ^{-/-}	Myeloperoxidase deficient
mTORC2	mammalian Target of Rapamycin Complex 2
NF- κ B	Nuclear factor-Kb
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO ₂	Nitrogen dioxide
NT	Nitrotyrosine
PAQR	progesterone and adiponectin Q receptor
PDPK1	Phosphoinositide Dependent Kinase 1
PP2A	Protein phosphatase 2A
PECAM-1	Platelet endothelial cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PPAR γ	Peroxisome proliferator-activated receptor γ
Psel	P-selectin
P-sel ^{-/-}	P-selectin deficient
PSGL-1	P-selectin glycoprotein ligand-1

PTEN	Phosphatase and Tensin homolog
ROS	Reactive oxygen species
TBW	Total body weight
TNF α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1
VAT	Visceral adipose tissue
WB	Western blot
WBC	White blood cell
WT	Wild-type

CHAPTER 1

INTRODUCTION

Cardiovascular Disease

Cardiovascular disease represents diseases that involve blood vessels and/ or the heart. Types of cardiovascular disease include coronary artery disease, heart failure, hypertension, atherosclerosis, and stroke. Cardiovascular disease is the number one cause of death worldwide with direct and indirect costs of up to 361 billion dollars. The underlying mechanisms vary depending on the disease in question [1].

Numerous epidemiological studies strongly associate white blood cells (WBC) and cardiovascular disease [2-4]. Leukocyte abundance influences heart failure after a myocardial infarction [5] and contributes to endothelial dysfunction and secondary cardiovascular events [6]. Preclinical studies have shown that circulating cells, specifically neutrophils, and monocytes, are critical for the initiation and progression of vascular disease [7]. Both neutrophils and monocytes are greatly associated with cardiovascular events in humans [8, 9]. Increased interaction between leukocytes and the endothelium is one of the main characteristics of endothelial dysfunction. Endothelial dysfunction, and especially the up-regulation of endothelial adhesion molecules, is also considered to be one of the important factors that lead to cardiovascular disease. One of the important components of neutrophils is a protein called myeloperoxidase (MPO).

Myeloperoxidase (MPO)

Myeloperoxidase (MPO) is a major neutrophil protein. Myeloperoxidase was first discovered by *Kjell Agner* in 1941 when he isolated an iron-containing protein that

exhibited peroxidase activity from patients with tuberculous empyema [10]. He named this protein verdoperoxidase due to its green color. However, subsequent studies found out it was limited to myeloid cells, and hence it was renamed myeloperoxidase (MPO). MPO is a peroxidase enzyme encoded by the MPO gene in humans. Current research has shown that it is abundantly expressed in neutrophils and stored within their azurophilic granules, accounting for ~5% of the cell's dry mass. It is also present in monocytes, where it comprises one-third of the MPO content found in neutrophils [11]. MPO gene expression in healthy individuals starts in the bone marrow at the promyelocyte stage and stops when cells mature and enter the circulation [12]. Similar to gene expression, production and packaging of MPO protein is completed in the bone marrow. MPO first undergoes proteolytic cleavage in the endoplasmic reticulum (ER), to produce an inactive apopro-MPO. Next, a process of glycosylation and insertion of a heme group in the ER results in an active, 90 kDa pro-MPO molecule; pro-MPO undergoes further proteolytic steps that result in 75 kDa protein consisting of a heavy and light chain [13, 14]. The resultant mature enzyme is directed to the azurophilic granules for final storage. Some active pro-MPO is constitutively secreted and is detectable in the circulation [14-16]. The main function of MPO is innate host defense through the killing of the invading microorganisms. In the presence of infection, a chemical signal will attract circulating neutrophils that become active. Then they interact with the endothelium where they migrate to the site of injury, accumulate, and phagocytose the invading pathogen. Parallel to phagocytosis, the activated neutrophil undergoes what is known as the respiratory burst, in which the activated neutrophils will produce reactive oxygen species; large amounts of superoxide that is

rapidly turned into hydrogen peroxide (H_2O_2) [17]. In the case of large organisms that cannot be phagocytosed the neutrophils release their content into the extracellular space; similar to sites of acute inflammation that was not induced by a pathogen. MPO introduces its antimicrobial effects through catalyzing a reaction between chloride and hydrogen peroxide to produce a very powerful oxidant known as hypochlorous acid (HOCl), the main chemical component of household bleach. HOCl is very reactive, with a short half-life that is capable of different reactions such as chlorination, and oxidation. Interestingly, even though chloride is the most likely substrate for MPO at physiological concentrations, MPO can generate other hypohalous acids using different substrates including bromide, iodide, and thiocyanate (**Figure 1**) [18]. HOCl enhances the immune response by making proteins more liable to proteolysis and increases their immunogenicity [19].

Invading Pathogen

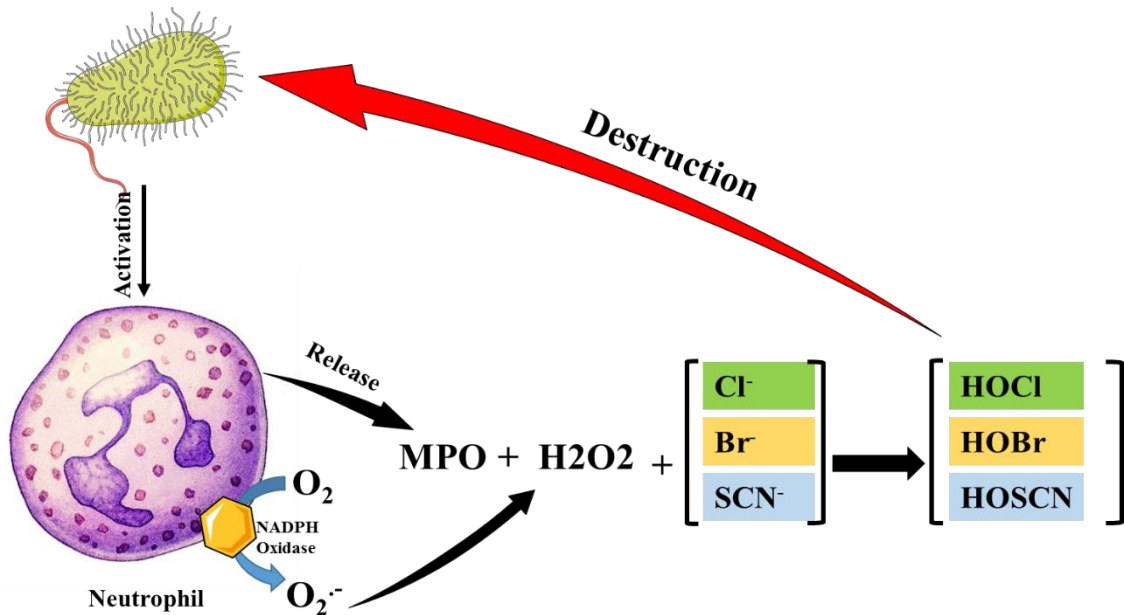


Figure 1: Neutrophil Respiratory Burst and the Actions of MPO. In the presence of an invading pathogen, neutrophils become active and release MPO that together with H₂O₂ and chloride (Cl⁻) will form hypochlorous acid HOCl that will destroy the pathogen.

MPO has the capability to nitrate both bound and free tyrosines by forming nitrogen dioxide (NO₂) in an H₂O₂ dependent manner [20]. The generation of reactive nitrogen species by MPO is believed to be linked to alteration in protein structure and function, nevertheless, it does not provide a major contribution to the antimicrobial properties of MPO [21].

Myeloperoxidase in Vascular Disease

Despite the host defense properties of MPO, it has been shown that in acute and chronic inflammatory conditions, there is an increased release of enzymatically active MPO into the circulation which is linked to endothelial dysfunction and cardiac disease progression [22-26].

Recent studies have discovered that MPO can be internalized with an increase in intracellular oxidant production *in vitro* [27] and *in vivo* [28]. And found that free MPO can interact with anionic endothelial cell-surface glycosaminoglycans [28]. MPO enters the endothelial cells and remains active in the subendothelial matrix through the process of transcytosis, independently of neutrophil transmigration [27, 28]. In 2007 Astern, J. *et al* discovered that MPO depends on cytokeratin 1; an endothelial protein to bind and internalize endothelial cells [29]. Others have found that MPO can form a complex with albumin. This MPO-albumin complex can then via cell surface albumin binding protein receptors, be internalized into caveoli; a site associated with increased calpain activity [30].

As mentioned above, one of the major characteristics of endothelial dysfunction is the loss of nitric oxide (NO) bioavailability [31]. MPO is well known as a nitric oxide oxidase. MPO catalytically consumes NO, oxidizing it to nitrite [32]. Hypochlorous acid, a byproduct of MPO, can chlorinate L-arginine, therefore, exhausting the substrate for eNOS [33].

The effects of MPO on NO bioavailability and the resultant endothelial dysfunction has been investigated *in vivo* in both mouse models and clinical studies. MPO null mice exposed to acute inflammation presented improved vascular function and increased NO availability compared to wild type mice [32].

MPO and its byproducts also have been shown to contribute to hypertension [25, 34]. MPO and HOCl are involved in atherosclerotic plaques rupture, through activation of matrix metalloproteinases (MMP) which are known to degrade matrix proteins by proteolytic activity, and by preventing MMP inhibition through decreasing Tissue Inhibitor of Metalloproteinase 1 (TIMP-1) their endogenous inhibitor [35]. MPO oxidizes both High Density Lipoprotein (HDL) and Low Density lipoprotein (LDL). This recruits macrophages to get rid of these oxidized lipoproteins resulting in foam cell formation and progression of the atherosclerotic plaque [36]. Plasma and serum levels of MPO independently predict endothelial dysfunction of large arteries in cardiovascular patients [25, 36], as individuals who have higher MPO levels are 15- to 20-fold more likely to demonstrate abnormal coronary angiograms [26]. Conversely, a promoter polymorphism associated with a twofold reduction in MPO expression protects from vascular disease and cardiac death [37].

Surprisingly, very little information is available in the scientific literature to explain the molecular mechanism(s) through which MPO impairs endothelial function, which limits therapeutic intervention to avert cardiovascular disease in the ever-growing population of patients with low-grade inflammatory disorders. It is important to note that in cases of endothelial dysfunction where MPO activity and plasma levels are increased there is a growing body of evidence showing an increase in the activity of a calcium-dependent proteases called calpains. However, to our knowledge no studies have shown any correlation between the two.

Endothelial Dysfunction

Normal Endothelium and Endothelial Dysfunction

The endothelium is a single layer of endothelial cells (EC) covering the vascular lumen. Endothelial cells are present in all tissues receiving blood flow; therefore the health of almost all tissues depends on proper endothelial cell function. Everything that requires transport into or out of the blood must traverse the endothelium. This single cell layer is known to be metabolically active with an important role in maintaining vascular homeostasis under physiological conditions [38, 39]. The vascular endothelium has many functions which include angiogenesis, vascular growth, and remodeling, as well as regulation of vessel integrity and permeability. Moreover, the endothelium plays a pivotal role in regulating vascular tone, controlling tissue blood flow and inflammatory responses [40-42].

It is important to differentiate normal endothelial activation from endothelial dysfunction. Normal endothelial activation is a physiological requirement for fighting

infections and healing wounds. Endothelial dysfunction is the loss of resting state endothelial processes, primarily due to diminished nitric oxide (NO) bioavailability [43]. Endothelial dysfunction happens when normal endothelium function becomes inadequate. This usually occurs in response to a pathologic stimulation. Endothelial dysfunction is characterized by reduced endothelium-mediated vasorelaxation, enhanced permeability of the endothelial cells, increased expression of adhesion molecules, a tendency to thrombosis, overproduction of growth factors, and excessive generation of reactive oxygen species (ROS), and increased oxidative stress, with impaired NO bioavailability being one of the hallmarks of endothelial dysfunction is. [44-46].

Leukocyte-Endothelium Interaction

The increased interaction between circulating leukocytes and endothelial cells is one of the most important characteristics of endothelial dysfunction. Leukocytes are divided into different polymorphonuclear cell types that include neutrophils, basophils, eosinophils, and mononuclear cells including lymphocytes and monocytes. With neutrophils being the most abundant cell component of leukocyte. In the case of inflammation, where a pro-inflammatory signal such as histamine or lipopolysaccharide (LPS) binds endothelial cells, leukocytes are recruited into the inflammatory sites in a process that is mediated by its interaction with endothelial cells [47].

Leukocyte-endothelium interaction occurs in three important steps, with each step involving a specific type of endothelial cell adhesion molecules (eCAM) expressed on the surface of the endothelial cell [48]. The first step is leukocyte rolling which is regulated by a family of adhesion molecules called selectins (i.e. P-selectin, E-selectin). P-selectin is

constitutively expressed and is stored in cytosolic Weibel-Palade Bodies, making it the fastest eCAM in response to stimuli. E-selectin, on the other hand, must be synthesized *de novo*, making it the slower one of the two endothelial selectins. P-selectin and E-selectin are transported to the luminal surface of vessels, extending into the blood. Both selectins tether leukocytes to the endothelium through the formation of reversible bonds with leukocyte expressed ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1). The simultaneous forming and breaking of selectin bonds with leukocyte ligands lead the leukocytes to roll along the vessel wall, hence the name leukocyte rolling. The next step, which involves a firm adhesion of leukocytes to the endothelium, is mediated by the immunoglobulin family of adhesion molecules: Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1). Finally, regulating proteins like platelet endothelial cell adhesion molecule (PECAM-1) leads to loosening of the tight junctions resulting in the opening of gaps between endothelial cells (**Figure 2**). [47, 48]. Leukocytes adhering to endothelial cells pass through these open junctions, through the basement membrane, and into extravascular tissues. Typically, after the pro-inflammatory stimulus is removed the endothelium returns to the resting state.

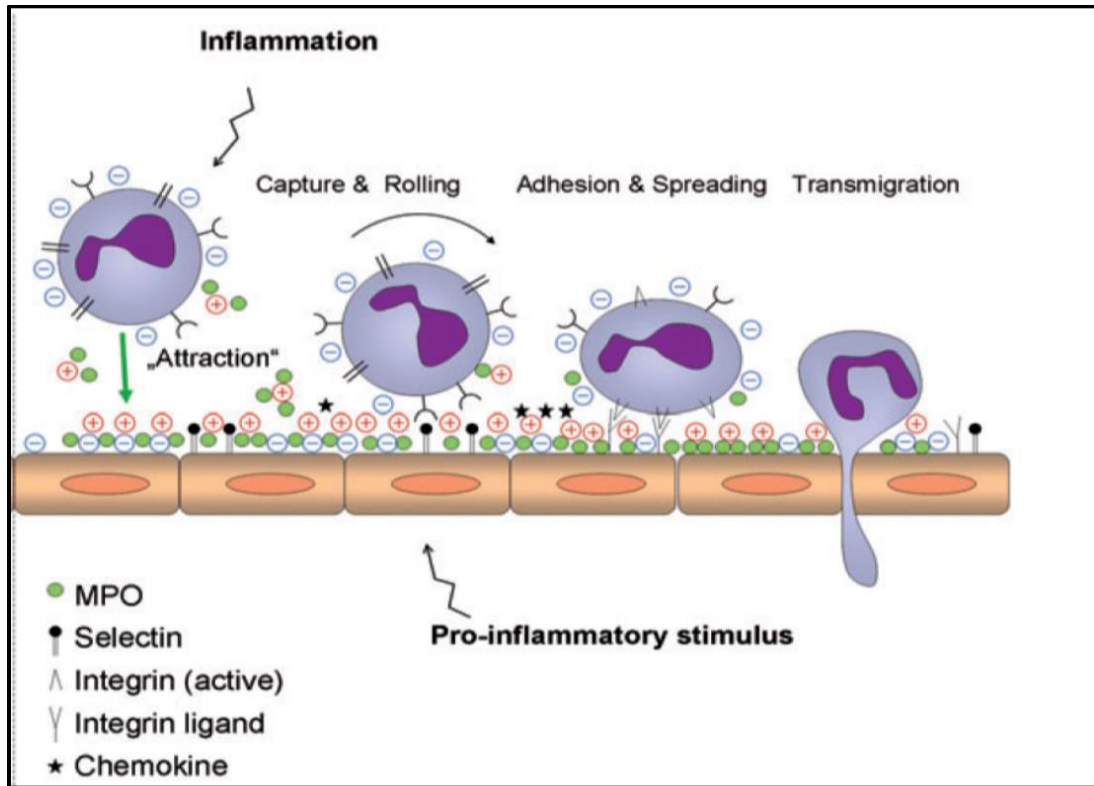


Figure 2: Leukocyte – Endothelial Interaction. When circulating leukocyte recognize signs of inflammation they migrate into the infected areas. Selectin-mediated rolling along endothelium (P-selectin and E-selectin), followed by integrin-mediated adhesion (ICAM-1 and VCAM-1), subsequently, the leukocyte traverses through the endothelium and arrives at the site of inflammation with the help of (PECAM-1) [49].

Cell Adhesion Molecules

As mentioned above, E-selectin, P-selectin, and their glycoprotein ligands are the first two adhesion molecules that become up-regulated during inflammation. Selectins are required for the tethering, rolling, and weak adhesion of leukocytes. Afterward, the immunoglobulin superfamily of cell adhesion molecules including Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) react with

integrins such as the constitutively expressed leukocytic integrins lymphocyte function-associated antigen-1 (LFA-1), for firm adhesion and downstream signal transduction, which eventually leads to leukocyte extravasation. The leukocytes are then guided with the help of chemo-attractants to reach inflammatory site [50, 51]. It is important to know that this step comes later in the leukocyte –endothelium interaction due to the fact that the up-regulation of ICAM-1, VCAM-1 is much slower, their *de novo* synthesis takes several hours to happen [51].

All endothelial cells synthesize nitric oxide (NO) [52]. Different functions of the resting endothelium are impaired when NO activity is reduced. NO bioavailability can be compromised by diminished NO production in endothelial cells due to a decrease in endothelial nitric oxide synthase (eNOS), and inactivation of NO by reactive oxygen species (ROS) [53].

Endothelial nitric oxide (eNO) is a major inhibitor of Weibel-Palade body exocytosis, which further influences the surface expression of P-selectin [54]. Therefore, a decrease in the physiologic levels of eNO leads to up-regulation of P-selectin, this results in increased leukocyte-endothelium interaction [55-57].

Endothelial Nitric Oxide Synthase

Nitric oxide synthases (NOSs) are a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine. This family has three known isoforms: inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial nitric oxide synthase (eNOS) [58]. Normal NO production by eNOS appears to be a very important factor in maintaining the physiological function of the vasculature endothelium.

In endothelial cells, the phosphorylation of certain tyrosine (Tyr), serine (Ser), and threonine (Thr) sites of eNOS by protein kinases is important for its enzymatic activity [59]. Phosphorylation sites for eNOS have been identified. They are Tyr⁸¹, Ser¹¹⁴, Thr⁴⁹⁵, Ser⁶¹⁵, Ser⁶³³, and Ser¹¹⁷⁷ [60]. From these sites, Ser⁶³³ and Ser¹¹⁷⁷ are functioning as phosphorylation sites that stimulate NO production [61-63]. A decrease in NO bioavailability has been strongly associated with endothelial dysfunction [53]. Loss of NO, as well as a decrease in eNOS phosphorylation, leads to increased expression levels of cell adhesion molecules which cause increased leukocyte-endothelium interactions [64].

eNOS phosphorylation at Ser¹¹⁷⁷ has been shown in numerous studies to be critical for eNOS activation in response to several stimuli such as: adiponectin (through the adiponectin receptors 1, and 2), and shear stress by activating multiple protein kinases such as AMP-activated protein kinase (AMPK) [65-67]. During endothelial dysfunction levels of eNOS phosphorylation at Ser¹¹⁷⁷ are significantly decreased which leads to decrease in NO bioavailability [68]. eNOS inhibition has been found to accelerate atherosclerosis, and phosphorylation of eNOS at Ser¹¹⁷⁷ is needed for NO production during coronary artery disease [68].

AMP-Activated Protein Kinase

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase consisting of a catalytic subunit (α) and 2 regulatory subunits (β and γ). It is activated by increased intracellular concentrations of AMP. Phosphorylation at Thr¹⁷² located in the activation loop of AMPK is required for AMPK activation [69, 70]. In the literature tumor suppressor gene product Liver Kinase B (LKB1) has been identified as the major AMPK

kinase, however, it is believed that AMPK could also be phosphorylated by the Ca²⁺/calmodulin-dependent protein kinase kinase (CaMKK) [69, 70]. AMPK has a very important role in maintaining vascular function. Inhibition of AMPK activity has been associated with endothelial dysfunction [40]. There are numerous reports of attenuated Nuclear factor- κ B (NF- κ B) activation, as well as endoplasmic reticulum (ER) stress, and ROS following AMPK activation in different cell types, including endothelial cells [71-74]. AMPK has been shown to regulate eNOS activity. AMPK activates eNOS by phosphorylating serine at either 1177 site or 633 site, inducing the eNOS-derived NO production. Furthermore, studies have found that nutrient overload acutely reduces AMPK activity in endothelial cells [75]. Recent studies propose an increase in protein phosphatase 2A (PP2A) is responsible for decreased AMPK activity by dephosphorylation [75]. Conversely, stimulation of AMPK activity attenuates activation of pro-inflammatory pathways and increases NO release in human endothelial cells exposed to nutrient overload [76]. A number of physiological and pharmacological stimuli that can activate AMPK include adiponectin, shear stress, and HMG-CoA reductase inhibitors [62, 77-79]. Interestingly eNOS is also stimulated by adiponectin [65]. Adiponectin in endothelial cells exerts its functions through adiponectin receptors [80].

Adiponectin Receptors

Adiponectin receptors are part of the progesterone and adiponectin Q receptor (PAQR) family. There are two types of adiponectin receptor with different binding affinities for full-length or globular adiponectin: AdipoR1 and AdipoR2. The molecular structure of these receptors is very interesting, with an internal NH₂-terminus and external

COOH-terminus domain [81]. AdipoR1 is found in many tissues, being particularly abundant in skeletal muscle, whereas AdipoR2 is most commonly found in the liver and the vasculature. Many studies have confirmed that AdipoR1 and AdipoR2 are the major adiponectin receptors *in vivo* [82]. AdipoR1 and AdipoR2 help to regulate normal glucose metabolism and insulin sensitivity. These effects have been confirmed with AdipoR1, AdipoR2 double knockout mice presenting glucose intolerant and insulin resistance [83]. Other studies have also shown that in cases of obesity, which has long been associated with inflammation and endothelial dysfunction, the expression levels of AdipoR1 and AdipoR2 are significantly reduced [84]. It is important to note that the actions of adiponectin require the activation of AMPK. This is confirmed when AMPK activation is blocked both fatty-acid combustion, and glucose utilization are also inhibited [80, 83].

The level of circulating adiponectin is negatively correlated with endothelial dysfunction. Globular adiponectin has been shown as a potent anti-inflammatory molecule by significantly inhibiting the expression of adhesion molecules, including ICAM-1, VCAM-1, and E-selectin in endothelial cells [81]. However, studies also suggest that adiponectin signaling is dampened in obesity, referred to as adiponectin resistance [85, 86], and even adiponectin administration failed to draw its signaling activation [87]. This focuses the light more on the importance of adiponectin receptors. Overexpression of AdipoR1, AdipoR2 enhanced the anti-inflammatory effect of globular adiponectin in endothelial cells both *in vitro* and *in vivo* [88]. Therefore, AdipoR1 and 2 serve as major receptors for adiponectin *in vivo* and play a pivotal role in regulating glucose and lipid metabolism, inflammation and oxidative stress *in vivo* [82].

Low molecular weight (LMW) adiponectin is negatively correlated with myeloperoxidase (MPO) in diabetic patients [89]. Preliminary data from our lab show reduced adiponectin mRNA levels in the mesenteric visceral adipose tissue (VAT) of mice fed a high fat diet, a phenomenon not occurring in MPO deficient mice (MPO^{-/-}). MPO is also associated with increased risk of cardiovascular diseases and endothelial dysfunction [89]. However, to our knowledge, no studies have investigated the direct effects of MPO on adiponectin and its receptors.

Calpain

The calpain family of proteases is mainly divided into three groups named typical, atypical, and calpastatin. Typical calpains are defined as calpains that have similar structure to the large subunit of μ - and m calpains, and exhibit an EF-hand in their domain IV. Atypical calpains lack the calmodulin like EF-hand in their domain IV and some of them completely lack a domain IV [90]. The typical group also known as ubiquitous, or conventional calpains consists of calpains (1, 2, 3, 8, 9, 11, 12, and 14), CALPA, CALPB, and CALPC. The second group, the atypical calpain also known as novel calpains were recently identified and consists of calpain (5, 6, 7, 10, 13, and 15). Calpastatin is a protein that specifically inhibits μ - and m calpains only and no other proteases tested [91]. In this study, we are focusing on calpain 1, and 2 also known as μ - and m calpains respectively. In 1989 the terms μ -calpain and m-calpain were first used to refer to the Ca^{2+} concentration required by μ -calpain (micromolar Ca^{2+}), and m-calpain (millimolar Ca^{2+}) [92, 93], respectively. From then onwards it has been recommended that these names be used to distinguish these two calpains [93].

μ -and m-Calpains

There is a great deal of information known about the properties of these two calpains. Both μ - and m-calpain are heterodimers. They both have a large subunit with a mass of 80-KDa (this subunit shares 55-65% sequence homology between both proteases), and an identical small subunit with a mass of 28-KDa. Both μ - and m-calpain are Ca^{2+} dependent cysteine proteases, with μ -calpain requiring (3-50 μM), and m-calpain requiring (400-800 μM) Ca^{2+} concentration for half maximum activity [91]. These calpains are ubiquitously expressed and have been found in all vertebrate cells tested. They appear to be located intracellularly and primarily associated with subcellular organelles [91]. In unstimulated cells, calpain appears in the cytoplasm and predominantly around the nucleus, however when cells are stimulated with Ca^{2+} , calpains translocate to be around plasma membranes [91].

The amino acid sequence of these mammalian calpains is highly conserved with 99% homology between mammals with no evidence of alternative splicing. Based on the amino acid sequence these calpains consist of six domains: a) Domain I: this domain represents the NH_2 -domain which consists of 17-19 amino acids. b) Domain II: known as the catalytic domain and consists of the active cysteine (Cys) residue, histidine (His) residue, and asparagine (Asn) residues. These residues form together what is known as the catalytic triad that is characteristic of cysteine proteases. c) Domain III: a phospholipid binding domain that is also a linking domain that links the catalytic domain to the calcium binding domain. d) Domain IV: a COOH-terminal which is a calcium binding domain that has five EF-hands for calcium binding. e) Domain V: the NH_2 -terminal domain of the small

subunit similar to Domain I. f) Domain VI: the COOH-terminal domain of the small subunit which is similar to domain IV in that it has five EF-hands that have calcium binding sites and is slightly similar to a calmodulin domain (**Figure 3**) [91].

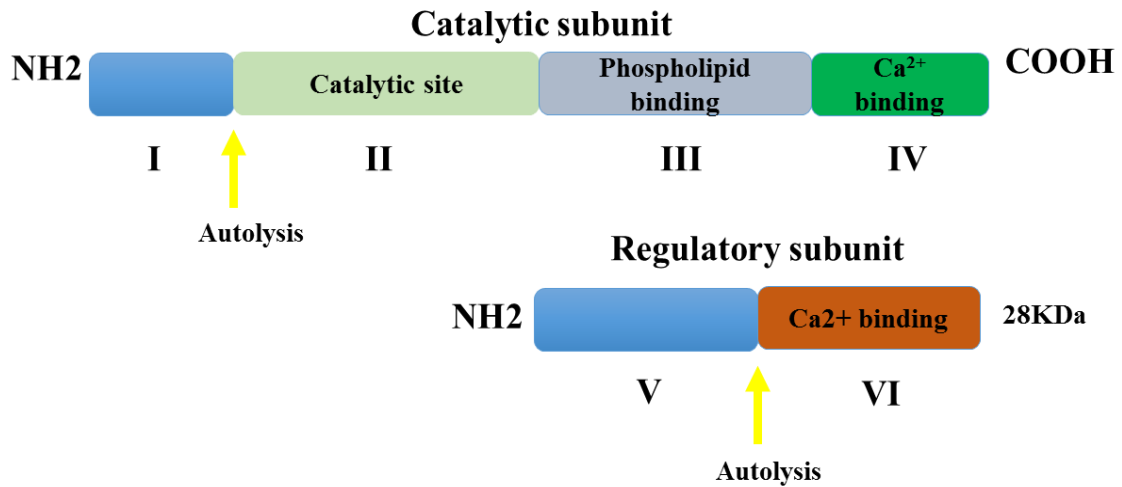


Figure 3: Calpain Structure. The protease calpain is a heterodimer. The amino-terminal ends of domain I and domain V are autolyzed upon calpain activation. Yellow arrows depict autolysis.

Studies have found that upon Ca²⁺ binding to domains IV and VI, a conformational change results in the autolysis of the NH₂ domain where about 14-18 amino acids are removed and the calpain becomes proteolytically active. Autolysis appears to occur consistently during events where calpain is proteolytically active [94]. Therefore, autolysis can be used as a potential sign that calpain is active. However, it is important to know that a recent group of studies have pointed to the possibility of having a proteolytically active calpain that is unautolyzed [94]. Hence, absence of autolysis of calpains does not assure they are not proteolytically active.

This raised the possibility that autolysis and proteolytic activity are two separate events. Nevertheless, more research needs to be done to clarify the significance of μ - and m-calpain autolysis properties. Ca^{2+} binding is required for calpain activation. However, the concentrations of Ca^{2+} mentioned in the literature is higher than physiological concentrations in cells *in vivo*. Consequently, there have been numerous attempts to find mechanisms that could reduce the Ca^{2+} requirements for both calpains *in vivo*. One example mechanism shows that phospholipids, especially phosphatidylinositol 4,5-bisphosphate (PIP2), lowers the Ca^{2+} concentration required for activation by μ - and m- calpain [95, 96]. Another area that has been gaining attention is the possibility of the presence of certain molecules that interact with or alter the calpains in such a way that would reduce their Ca^{2+} requirements [97]. One study by *Ma, H. et al* in 2001 suggested the presence of a heat stable polypeptide that is believed to reduce the Ca^{2+} concentration required by μ - and m-calpain [98]. Another study shows a negative effect of NO on calpain activity where NO increases the levels of calpain nitrosylation resulting in a decreased activity. This effect was reversed by adding NOS inhibitors [99].

The physiological functions of the calpain system are not well understood due to the fact that most studies used calpain inhibitors to distinguish its physiological functions and these inhibitors had other effects in the cell making it difficult to specify calpains functions. Nevertheless, calpains have shown to have different functions within the cells which include: substrate degradation in certain apoptotic pathways, gene expression regulation, and enzyme degradation during cell cycle, remodeling during cell fusion and motility, and proteolytic changes to molecules during signaling pathways. *In vivo* and *in*

vitro studies have shown that calpains have a number of substrates that include kinases and phosphatases, receptors and ion channels, transcription factors, and cytoskeletal proteins involved in plasma membrane interactions [91].

The proteolytic activity of both μ - and m-calpain is controlled by the indigenous inhibitor, calpastatin, first named by Takashi Murachi in 1979 [100]. This inhibitor is heat stable, and resistant to urea, trichloroacetic acid, and sodium dodecyl sulfate (SDS) [101, 102]. Calpastatin is specific to calpains and does not inhibit other proteases including the cysteine proteases, bromelin, papain, or cathepsin B [91]. Calpastatin consists of five domains with domains I, II, III, and IV all having the ability to inhibit calpains. Therefore, one calpastatin molecule has the ability to inhibit up to four calpains [103, 104]. For calpastatin to bind and inhibit calpain it requires Ca^{2+} . However, the Ca^{2+} concentration needed is significantly lower than that needed by calpains to initiate their proteolytic activity [105]. Calpastatin binds calpain at domains IV and VI, as well as domain II [106]. It is suggested that the binding of calpastatin to calpain prevents translocation and the binding of calpains to the cell membrane [107].

Calpain in Vascular disease

The two major calpain isoforms, namely μ - and m- calpain, are expressed in endothelial cells[108] where they have been shown to impact nitric oxide generation[109] and endothelial adhesiveness to leukocytes through increasing the expression of certain cell adhesion molecules such as VCAM-1 [110]. Moreover, in acute events such as hemorrhagic shock, there is a significant increase in μ -calpain activity associated with multiple organ dysfunction as well as endothelial dysfunction. And, that the use of a μ -

calpain inhibitor attenuates endothelial dysfunction in acute hemorrhagic shock in rats [111]. In chronic cases such as diabetes where cardiovascular complications are the leading cause of morbidity and mortality, studies have found that the Ca^{2+} dependent protease calpain causes vascular inflammation and endothelial dysfunction in the microcirculation as well as impaired endothelial NO and eNOS activity in diabetic rats. There was also evidence of increase leukocyte recruitment into the microcirculation of these rats [112]. Inhibition of calpain activity significantly reduced leukocyte-endothelium interactions in the vasculature of these rats as well as the expression levels of cell adhesion molecules [91] [111, 112]. Calpain inhibition prevented leukocyte recruitment in the heart after ischemia/reperfusion injury (a condition that exhibits elevated plasma MPO levels), via a reduction in adhesion molecules expressed on microvessels [107]. Similarly, in glomerulonephritis a case associated with severe inflammatory responses, calpain activity were significantly elevated in mice. Interestingly, the plasma MPO levels in these mice was increased as well [113].

Accordingly, we hypothesized that MPO induces endothelial dysfunction in part via activation of the endothelial Ca^{2+} - dependent protease calpain. This hypothesis can be further divided into three specific hypotheses:

- **Hypothesis 1:** We hypothesized that MPO activates calpains in endothelial cells.

Aims

- a) To study if MPO is capable activating calpain in endothelial cells in vitro. This was possible by conducting a calpain activity assay as well as immunoblot analysis.

- b) To identify which calpain isoform is being activated by MPO. This was accomplished using specific antibodies against the NH₂-terminus for the two calpains known to be in endothelial cells μ - and m- calpain.
- c) To identify the mechanism(s) through which MPO activates calpain in endothelial cells. In order to accomplish this, we studied the effects of MPO on calpastatin protein levels, whole cell chlorination, and nitrosylation of μ -calpain in endothelial cells *in vitro*.
- **Hypothesis 2:** We hypothesized a role of calpain in the mechanism through which MPO downregulates AMPK/eNOS signaling and increases leukocytes adhesiveness to the endothelium.

Aims

- a) To determine if MPO/Calpain system play a role in AMPK/eNOS signaling in endothelial cells, and the mechanisms through which it does that. We studied the effects of MPO treatment on the phosphorylation levels of AMPK, and eNOS in the presence and absence of the specific calpain inhibitor ZLLal. As well as MPO's effects on the upstream kinases (LKB1, and PI3K) and phosphatases (PP2A).
- b) To identify if calpains are involved in the MPO-induced Increased Endothelial Adhesiveness to Leukocytes. Using genetically modified mice we studied the effect of MPO treatment on leukocyte adhesiveness in wild type mice compared to μ -calpain deficient mice.
- **Hypothesis 3:** We hypothesized a role of calpain in the mechanism through which MPO decreases adiponectin signaling in endothelial cells.

Aims

- a) To study if MPO decreases AdipoR expression in endothelial cells *in vitro*. To identify if inhibition of calpain will abolish MPO's effects on AdipoR *in vitro*. We investigated the effects of MPO treatment on AdipoR expression levels in the presence and absence of the specific calpain inhibitor ZLLal
- b) To explore if pharmacological doses of adiponectin is capable of abolishing MPO effects on AdipoR2 expression levels in endothelial cells *in vitro*. We studied the effects of MPO treatment on AdipoR expression levels in the presence and absence of adiponectin.

CHAPTER 2

MATERIAL AND METHODS

This study was performed in accordance with the NIH and Temple University IACUC guidelines for the use of experimental animals. Eight to twelve-week-old wild-type C57BL/6J mice (Stock #002682, Jackson Laboratory, Ann Arbor MI), and mice deficient in μ -calpain (Capn1^{-/-}; stock number EM: 02362; European Mouse Mutant Archive (<http://www.emmanet.org/strains.php>) were used. All mice were male with an average body weight of 22 grams. Mice were used to study the effect of MPO/calpain signaling on leukocyte-endothelium interaction in the aorta, according to a previously published method [114]. Systemic activation of circulating leukocytes occurs in the microcirculation where, following interaction with endothelial cells, leukocyte become primed for degranulation [115]. Accordingly, we used mouse lung microvascular endothelial cells (MMVEC). MMVEC were obtained as cryopreserved secondary culture from Cell Biologics (cat# C57-6011), and cultured in Endothelial Cell Medium (ECM) (Cell Biologics, cat# M1168), with 5% Fetal Bovine Serum (FBS) (Gibco). All experiments were conducted in confluent MMVEC after overnight incubation in serum free ECM. MMVEC were stimulated with 10 nmol/L MPO for 180 min. Expression levels of m- and μ -calpain, Protein Phosphatase 2 (PP2A), total 5' AMP-activated protein kinase (AMPK), Thr¹⁷² phospho-AMPK, total endothelial nitric oxide synthase (eNOS), Ser¹¹⁷⁷ phospho-eNOS, total protein kinase B (AKT), Ser⁴⁷³ phospho AKT, Adiponectin receptor 1 (AdipoR1), and Adiponectin receptor 2 (AdipoR2), were measured by immunoblot

analysis. Calpain activity in MMVEC was measured by immunofluorescent assay with the highly sensitive fluorescent calpain substrate Succ-LLVY-AMC (Sigma Aldrich, cat# S6510), as previously described [116]. Calpain activity was calculated as the delta in fluorescence intensity between fluorescence reading after MPO treatment (I_f) and baseline fluorescence reading (control)(I_0) according to the equation $\Delta I = I_f - I_0$; b) Immunoblot analysis of μ -calpain and m-calpain NH₂-terminus domain cleavage was also used to identify the endothelial calpain isoform(s) activated by MPO, using a previously described approach[116].

Chemicals. Human myeloperoxidase was purchased from Millipore Sigma, (cat# 475911). Human adiponectin (Sigma, cat# SRP4901). The calpain inhibitor Aldehyde benzyloxycarbonyl-leucyl-leucinal (ZLLal) (Biomol Research Laboratories, cat# BML-PI116) was used because it has the highest selectivity for calpains over other proteosomal enzymes [117], and because of its efficacy in preventing activation of μ -calpain in the vascular endothelium [116]. The PP2A inhibitor okadaic acid (Sigma, cat# O9381) was used because of its highest selectivity for PP2A [118]. Solutions of ZLLal and okadaic acid (OA) were prepared in DMSO. DMSO was used at the final concentration of 0.007% and 0.005%, respectively. In control experiments, 0.007% DMSO did not affect endothelial cell signaling (data not shown).

Immunoblot Analysis. Cell lysates were centrifuged and protein concentration of the supernatant measured using a ThermoFisher Scientific kit, (cat# 23225) according to standard methods [119]. Protein extracts were then separated on a 4-20%SDS-polyacrylamide gel, and then transferred onto a PVDF membrane (Bio-Rad, cat# 456-

1094) by electrophoresis. PVDF membranes were incubated with primary antibodies, followed by species-matched secondary antibody, and developed using Western Lightning chemiluminescence reagent (SRX-101A) (GE Healthcare Life Sciences CAT# RPN2109). Polyclonal antibodies were used to measure Ser¹¹⁷⁷ eNOS phosphorylation (Cell Signaling, cat# 9571), total Endothelial Nitric Oxide (eNOS) (Cell Signaling, cat# 9572), Thr¹⁷² AMPK phosphorylation (Cell Signaling, cat# 2535), total 5' AMP Activated Protein Kinase (AMPK) (Cell Signaling, cat# 2532), Liver kinase B1 (LKB1) (Abcam, cat# ab63473), Protein Phosphatase 2 (PP2A) (Abcam, cat# ab32141), Vascular Cell Adhesion Molecule 1 (VCAM-1) (Santa Cruz Biotechnology cat# cs-1504), Ser⁴⁷⁶ AKT phosphorylation (Cell Signaling, cat# 9271), total 5' AMP Activated Protein Kinase (AKT) (Cell Signaling, cat# 9272), Phosphoinositide 3-kinase (PI3K) (Abcam, cat#ab189403), Adiponectin receptor 1 AdpoR1 (Abcam, cat#ab126611), Adiponectin receptor 2 AdipoR2 (Thermo Fisher, cat#PA1-1071), and β -actin (Cell Signaling, cat# 3700). Secondary antibodies used were horseradish conjugated Goat anti rabbit (Santa Cruz, cat#sc-2030). All dilutions for primary antibodies were 1:1000, and for secondary are 1:5000.

RNA Extraction and Quantitative RT-PCR. Total RNAs were prepared using commercially available Mini RNase Kit (Qiagen, cat# 74104). Reverse transcription was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems), and the reaction mix was subjected to quantitative real-time PCR to detect Calpain, Calpastatin, and GAPDH mRNA expression. Real time PCR was performed using 1 μ L/well of reverse transcription products in 10 μ L of TaqMan PCR master mix (Thermo Scientific, cat# K0231) with primers at 300 nmol/L and probe at 200 nmol/L. PCR was

performed at 95°C for 5 minutes for AmpliTaq Gold activation, and then run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Relative gene expression is reported as gene/housekeeping gene ratio in the same sample. Primers were purchased from (Invitrogen, Carlsbad, USA).

Calpain Activity. We used a twofold approach to measure calpain activity. **Immunofluorescent assay.** We have previously described this method to measure calpain activity in live MMVEC [116]. Briefly, cells were cultured in 24 well plates until confluent, then were incubated with 10 nmol MPO for 60, 120, 180, and 240 minutes in serum free, phenol red free DMEM (Gibco) at 37°C. Calpain activity was determined with the highly sensitive fluorescent calpain substrate Succ-LLVY-AMC (Sigma Aldrich, cat# S6510). Mean fluorescence signals were measured using a Bio-Tek FLx800 microplate fluorescence reader (Bio-Tek, Winooski, VT; excitation $\lambda = 360$ nmol, emission $\lambda = 460$ nmol). Calpain activity was calculated as the change in fluorescence intensity from the fluorescence reading after main treatment (If) to the fluorescence reading without treatment (control)(I0) according to the equation $\Delta I = I_f - I_0$. Specificity of the Succ-LLVY-AMC for calpain was previously confirmed by pharmacological inhibition [112]. **Immunoblot analysis of calpain NH₂-Terminus domain cleavage.** Polyclonal antibodies were used against either the NH₂ terminus domain (Abcam, cat# ab28257, triple point biologics) or domain IV (Abcam cat# ab39170, cat# ab39165) of the large subunit of m- and μ -calpain, respectively. Once activated, m- and μ -calpains autolytically cleave their NH₂-terminal ends, respectively, resulting in the loss of NH₂-terminus antibody recognition, which can

be used as a measure of calpain activation. Quantification of the stable domain IV was also used to measure total m- and μ -calpain content.

Evaluation of Chlorination by Flow Cytometry. Chlorination level in MMVEC cells treated with 10 nM MPO, or PBS as control was performed by FACS analysis using an anti-chlorotyrosine (HP5002 Hycult Biotech PA, USA) or control isotype IgG according to standard procedure previously reported [120]. Briefly, antibodies were added overnight at 4°C to fixed and permeabilized cells to detect intracellular chlorination. Cells were then washed and incubated with Alexa f488 conjugated secondary antibody (Thermo Fisher Scientific, USA) for 60 minutes at room temperature. Data were then analyzed by FlowJo software (Three Star Inc. Ashland, OR).

Detection of Calpain S-nitrosylation by Biotin Switch Assay. We followed a previously described method[121]. Briefly, MMVEC cells were treated with 10 nmol/L MPO for 180 min and then lysed using M-PER buffer (Thermo Scientific cat# 78501) containing 1:100 protease inhibitor cocktail (Thermo Scientific cat# 78430). Samples were diluted to 0.6 $\mu\text{g}/\mu\text{l}$ with HEN buffer containing in mmol/L, 250 HEPES (pH 7.4), 1 EDTA and 0.2 neocuproine (Sigma, cat# N1501). Free thiol residues in cell lysates were then blocked by adding 0.1% Methyl methanethiosulfonate (MMTS) (Fluka, cat# 64306) for 30 min at 50°C with frequent vortexing. Next, the protein S-nitrosocysteine were reduced with L-ascorbate (30 mmol/L) (Fluka, cat# 11140) and labeled with N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide (0.2 mmol/L) (Pierce, cat# 21341). Biotinylated proteins were then precipitated using streptavidin-agarose beads (Fluka, cat# 85881). In control studies, L-ascorbate was replaced by NaCl during the biotin labeling step. Calpain

was detected in the streptavidin-purified mixture using the standard immunoblot techniques described above. A schematic explanation of the Biotin switch assay is available in (Figure 4).

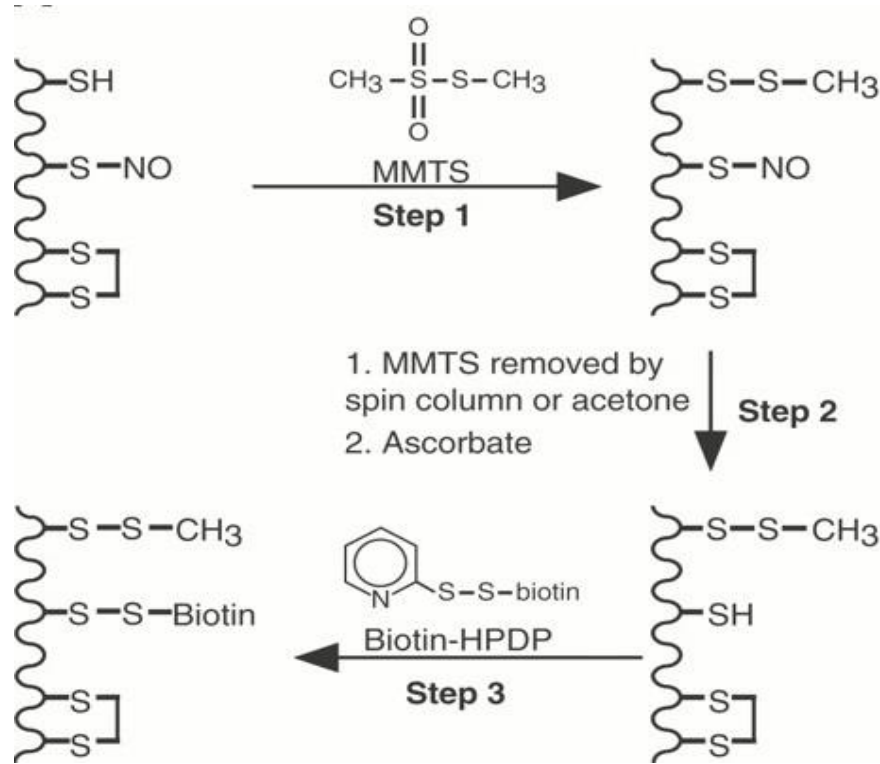


Figure 4: Biotin Switch Method. This method is used to detect the –nitrosylation levels of proteins [122].

Adhesion of Leukocytes to the Mouse Aorta. Leukocyte-endothelium interactions were studied in wild-type C57BL/6J mice (Stock cat#002682, Jackson Laboratory, Ann Arbor MI), and mice deficient in μ -calpain (Capn1^{-/-}; stock number EM: 02362; European Mouse Mutant Archive (<http://www.emmanet.org/strains.php>)). Eight to twelve-week-old male mice with an average body weight of 22 grams were used in these studies. We used a well-established *ex vivo* adhesion assay previously described by our laboratory [114].

Briefly, the thoracic aortas and blood resident leukocytes were isolated from anesthetized donor control C57BLK mice, and *Capn1*^{-/-} mice. Following midline thoracotomy, the aortas were quickly removed, placed in cold, oxygenated Krebs-Henseleit Buffer, and carefully freed of the adventitia with the help of a dissecting microscope (PZMIV, WPI, and Sarasota, FL). The aortas were then opened longitudinally and pinned in silicon elastomer coated culture dishes containing 1 ml oxygenated K-H solution with their endothelial surface facing up. Ten nmol/L MPO was added to the aortas and incubated at 37°C for 180 minutes. In the cases where we used a pharmacological inhibitor ZLLal the aortas were pretreated for 30min with 100 µmol/L ZLLal. Whole blood was obtained from anesthetized mice by apex puncture of the heart. Leukocytes were isolated by Percol gradient centrifugation. Isolated leukocytes were then fluorescently labeled using PKH26GL staining kit (Sigma, cat#PKH26GL), as previously described [116]. Labeled leukocytes were incubated with the aortic segments at a concentration 10⁵ cells/aortic segment in incubation wells for 60 min at room temperature in an orbital shaker platform. The aortic segments were then removed, gently washed in fresh K-H buffer, and placed lumen side up on microscope slides. Slides were treated with a drop of immersion oil followed by a glass cover slip. The number of leukocytes adhering to the endothelial surface in three separate microscopic fields was counted under epifluorescent microscopy at a magnification of 200x. Three mice were studied in each group. Three aortic segments were studied in each mouse. Results are expressed as total number of cells/microscopic field.

Blood Pressure monitoring using Telemetry Method. 8-12 week control C57BLK mice, and Capn1^{-/-} mice were used. Under anesthesia, a carotid catheter (PA-C10) attached to a radiotelemetry transmitter (DSI equipped with ADInstrument 6 software) was inserted in the abdominal aorta via the femoral artery. The transmitter was placed subcutaneously. Mice were maintained on a 12:12-h light-dark cycle. Blood pressure and heart rate were evaluated in conscious mice 4 days after telemetry implantation as previously described [123]. Data are mean±SEM of 3 mice per group.

Statistical Analysis. Data are presented as the means ± SE. One, and two-way ANOVA with post hoc analysis by Newman-Keuls's corrected t test was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com". Probabilities of ≤0.05 were considered statistically significant.

CHAPTER 3

RESULTS

MPO Activates Calpain.

In order to know the MPO concentration that will be used for our studies and based on the range of MPO plasma levels found in humans with coronary artery disease[124], we conducted a dose-response curve. MMVEC cells were treated with different concentrations of MPO for 180 min. Cleavage of the μ -calpain NH₂-terminus domain, an indicator of calpain activation. Our results found that 10nmol/L MPO was the lowest concentration capable of activating calpain (**Figure 5**).

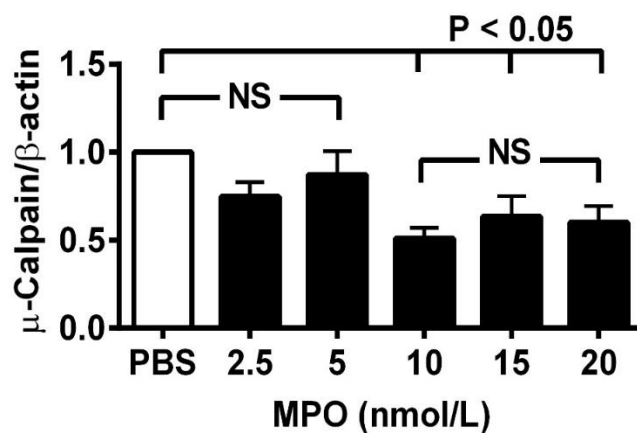
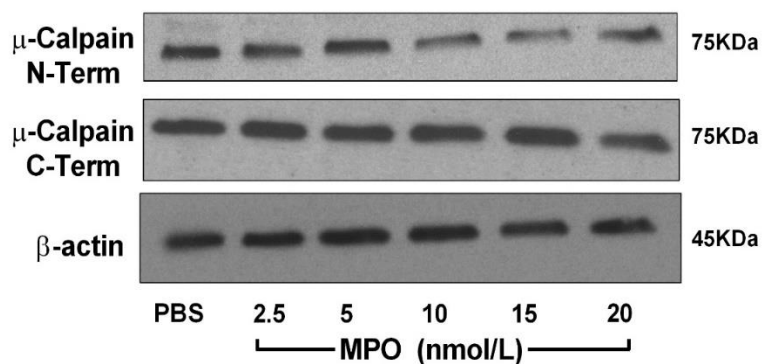


Figure 5: MPO Dose Dependently Activates μ -Calpain. MMVEC cells were treated with 2.5, 5, 10, 15, and 20 nmol/L MPO for 180 min. Cleavage of the μ -calpain NH₂-terminus domain, an indicator of calpain activation, was measured as described in the Methods. A primary antibody against the C-terminus domain of μ -calpain large subunit was also used to quantify total μ -calpain expression. Bar graph show quantification of NH₂-terminus domain cleavage at increasing MPO concentrations. No difference in total μ -calpain expression levels was observed under these experimental conditions, as demonstrated by lack of changes in C-terminus domain expression. Data are mean \pm SEM of 3 independent experiments.

In order to see if the MPO activated calpain is capable of proteolytically cleaving target substrates we conducted a calpain activity assay using a fluorogenic substrate of calpain. Data shown in **(Figure 6)** clearly demonstrate that exposure of MMVEC to 10 nmol/L MPO causes a time-dependent increase in calpain activity demonstrated by cleavage of the substrate and emission of a fluorescent signal, with peak activity reached after 180 minutes.

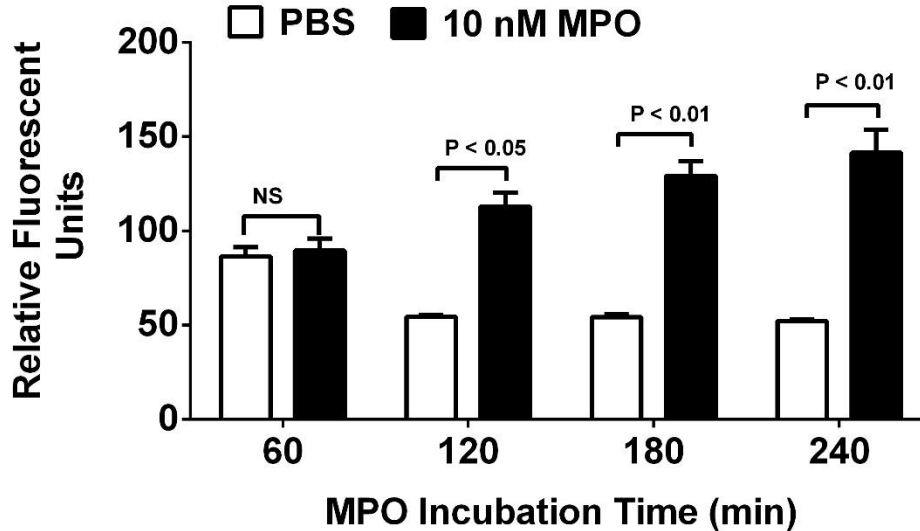


Figure 6: MPO Activates Calpain in Endothelial Cells. Serum starved MMVEC were incubated with 10 nmol/L MPO and calpain activity was measured at 60, 120, 180, and 240 min using the calpain specific fluorogenic substrate Succ-LLVYAMC.

MPO Activates The μ -Calpain Isoform.

To identify the calpain isoforms activated by MPO, we then measured expression levels of the NH₂-terminus domain of the 80 kDa subunit of both m- and μ -calpain. Upon activation, calpains undergo autoproteolysis with removal of the 9-15 amino acids of the NH₂-terminus domain. Cleavage of the NH₂-terminus domain was detected by immunoblot analyses as loss of NH₂-terminus domain recognition (see also method section). We found evidence demonstrating that MMVEC exposed to MPO experience cleavage of the NH₂-terminus domain of μ -calpain (**Figure 7, upper panel**), but not that of m-calpain (**Figure 7, lower panel**). To rule out the occurrence of nonspecific degradation of μ -calpain by MPO, we quantified total μ -calpain expression using a primary antibody against domain IV of the large subunit, which recognizes both unautolyzed and

autolyzed μ -calpain. As shown in (Figure 7, upper panel), MPO did not cause significant changes in μ -calpain expression level under our experimental conditions. Similarly, μ -calpain mRNA levels were not significantly changed by MPO (Figure 8).

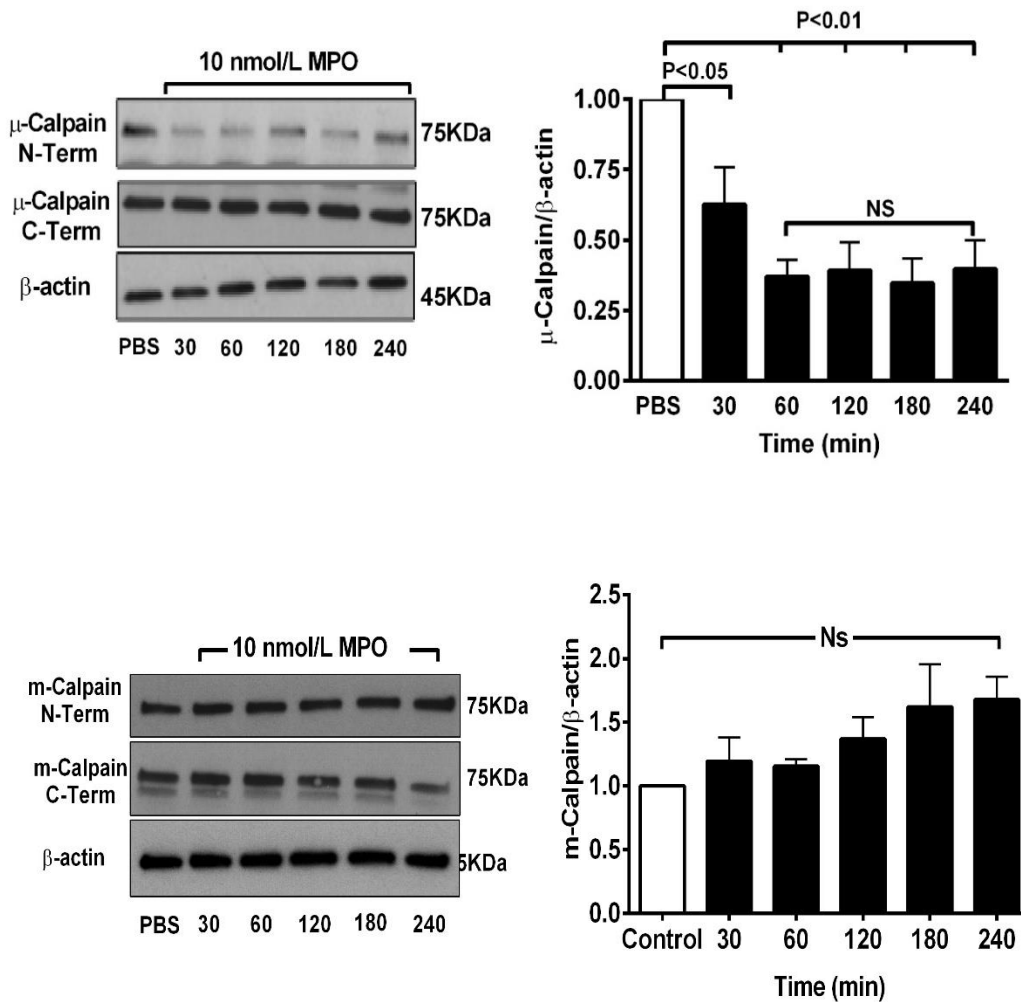


Figure 7: MPO Activates μ -Calpain but not m-Calpain in Endothelial Cells. MMVEC

cells were treated with 10 nmol/L MPO for different time points. Cleavage of the μ - and m-calpain NH₂-terminus domain, an indicator of calpain activation, was measured at 30, 60, 120, 180, and 240 min as described in the Methods. A primary antibody against the C-terminus domain of μ - and m-calpain large subunit was also used to quantify total μ - and

m-calpain expression. Bar graph show quantification of NH₂-terminus domain at all time points. (Upper panel) Representative Immunoblot demonstrate cleavage of MMVEC μ -calpain NH₂-terminus domain following incubation with 10 nmol/L MPO at all time points, indicating that MPO activates μ -calpain. (Lower panel) No difference in NH₂-terminus domain as well total m-calpain expression levels were observed under these experimental conditions, which demonstrate that MPO does not affect m-calpain activity. Data are mean \pm SEM of 4, and 3 independent experiments.

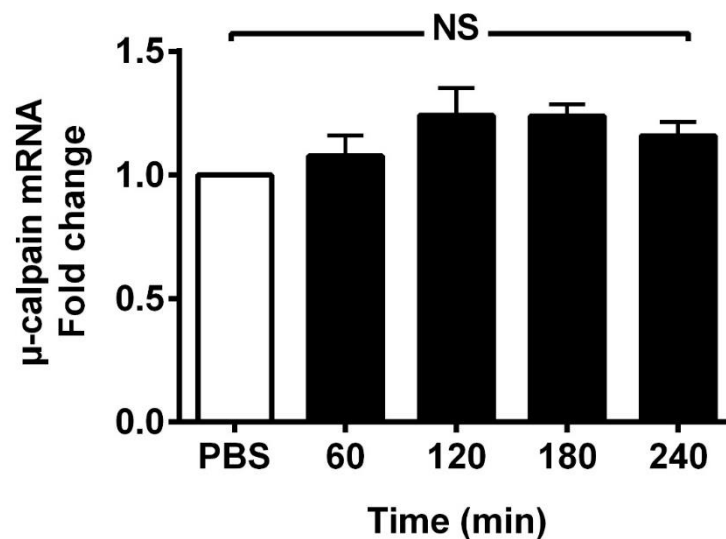


Figure 8: MPO Does not Change mRNA Levels of μ -Calpain. MMVEC cells were treated with 10 nmol/L MPO for 60, 120, 180, and 240 min. mRNA levels of μ -calpain were measured using TaqMan. Relative gene expression was calculated as ratio of target genes over housekeeping gene in the same sample. Data are mean \pm SEM of 3 independent experiments.

In parallel experiments, the activating effect of MPO on μ -calpain was blocked by pretreatment of MMVEC with 100 μ mol/L ZLLal, a selective calpain inhibitor[112]. Data shown in **(Figure 9)** demonstrate that ZLLal effectively prevented calpain activation in response to MPO. Taken together, these data demonstrate that MPO activates μ -calpain in endothelial cells, and that this process is amenable to pharmacological inhibition.

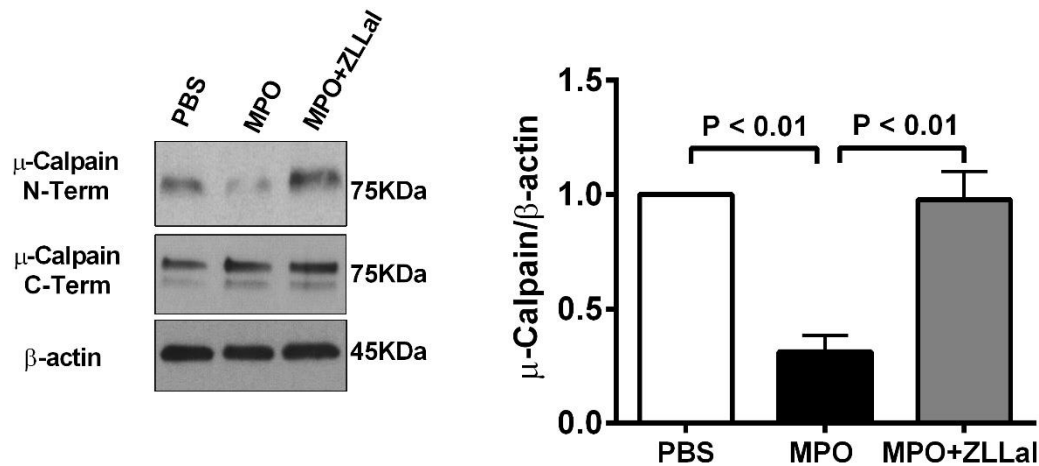


Figure 9: MPO Activation of μ -Calpain in Endothelial Cells is Susceptible to Pharmacological Inhibition. MMVEC were then incubated with either vehicle (8 μ L PBS) or 10 nmol/L MPO for 180 min with or without ZLLal (100 μ mol/L - 30 min pretreatment). The activity of the μ -calpain isoform was assessed by immunoblot analysis using a primary antibody that recognizes cleavage of the NH₂-terminus domain of μ -calpain large subunit. Loss of NH₂-terminus domain indicates calpain activation. An antibody against μ -calpain large subunit C-terminus domain was used to quantify total μ -calpain expression. β -Actin detection was used as a loading control. Representative immunoblot demonstrate cleavage of MMVEC μ -calpain NH₂-terminus domain following incubation with 10 nmol/L MPO

for 180 min. Bar graph summarizes densitometry quantification of μ -calpain NH₂-terminus domain. Total μ -calpain expression level was not changed by MPO, as demonstrated C-terminus domain immunoblot. Data are mean \pm SEM of 6 to 8 independent experiments.

The Mechanism(s) Through Which MPO Activates μ -Calpain.

To investigate the mechanism(s) responsible for μ -calpain activation, we first measured the effect of MPO on calpastatin, which is the endogenous inhibitor of calpain. **(Figure 10)** shows reduced calpastatin abundance in MMVEC exposed to 10 nmol/L MPO. At first, this result suggested that degradation of calpastatin by MPO could be the mechanism potentially responsible for activation of μ -calpain.

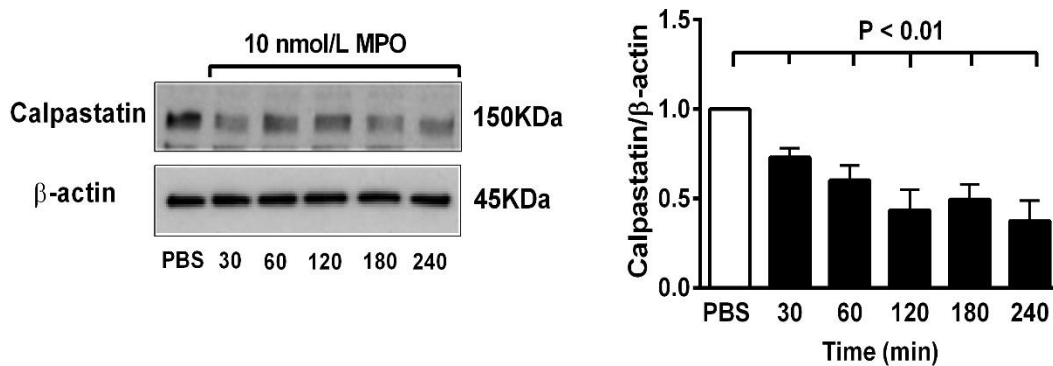


Figure 10: MPO Causes Degradation of Calpastatin. Serum starved MMVEC were incubated with vehicle (8 μ l PBS) or 10 nmol/L MPO for 30, 60, 120,180, and 240 min. The expression of the endogenous calpain inhibitor calpastatin was measured by immunoblot analysis and quantified by densitometry. Beta actin detection was used as a loading control. Data are mean \pm SEM of 7 independent experiments.

Data in the literature though have reported loss of calpastatin in the presence of active calpains, due to the fact that calpastatin itself is a calpain substrate[91]. Accordingly, we further studied whether the loss of calpastatin in MPO treated MMVEC was secondary to calpain activation. Indeed, MPO failed to reduce calpastatin levels in MMVEC pretreated with the calpain inhibitor ZLLal (**Figure 11**), thus confirming that, under our experimental conditions, loss of calpastatin is secondary to and due to μ -calpain activation.

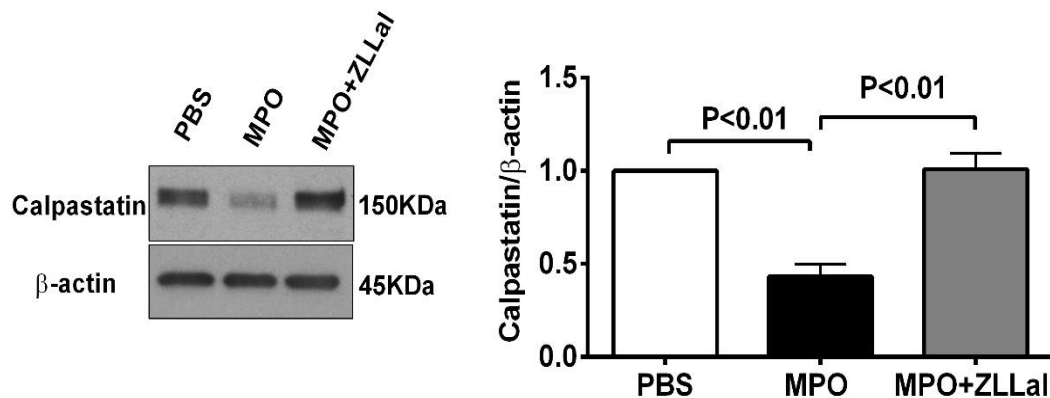


Figure 11: MPO Causes Degradation of Calpastatin via Calpain Activation. Serum starved MMVEC were incubated with vehicle (8 μ l PBS) or 10 nmol/L MPO for 180 min with or without ZLLal (100 μ mol/L, 30 min) pretreatment. The expression of the endogenous calpain inhibitor calpastatin was measured by immunoblot analysis and quantified by densitometry. Beta actin detection was used as a loading control. Data are mean \pm SEM of 6 to 7 independent experiments.

Consistent with these results calpastatin mRNA expression levels were also not significantly changed (**Figure 12**). These data indicate that MPO alters the

calpain/calpastatin balance in endothelial cells, by causing sustained activation of μ -calpain with subsequent degradation of calpastatin.

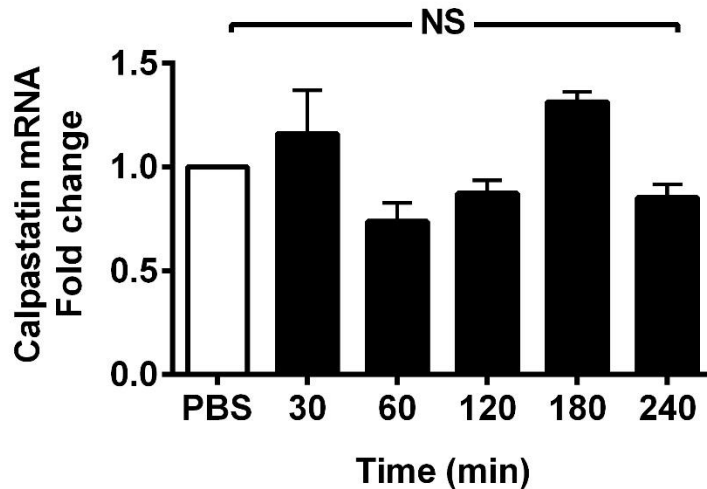


Figure 12: MPO Does Not Change mRNA Levels Calpastatin. MMVEC cells were treated with 10 nmol/L MPO for 30, 60, 120, 180, and 240 min. mRNA levels of calpastatin were measured using TaqMan. Relative gene expression was calculated as ratio of target genes over housekeeping gene in the same sample. Data are mean \pm SEM of 3 independent experiments.

In the presence of hydrogen peroxide, MPO forms hypochlorous acid (HOCl), which has been shown to affect protein activity via chlorination of tyrosine residues [125]. Data have also associated HOCl with increased calpain activity in monocytes [125]. Thus, we measured chlorination level in MMVEC exposed to MPO using flow cytometry and a monoclonal antibody against 3-chlorotyrosin. No significant increase in whole cell chlorination levels were detected under our experimental conditions (**Figure 13**).

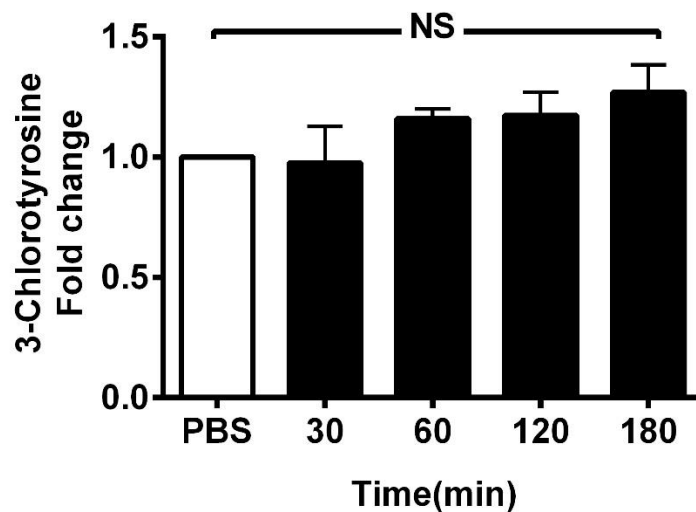


Figure 13: MPO Does not Activates μ -Calpain by Chlorination. To understand the possible mechanism by which MPO activates μ -calpain, we assessed the protein levels of cell chlorination. Detection of chlorination using flow cytometry were done in MMVEC cells treated with 10nM MPO for (30, 60, 120, and 180 minutes). Cells were then permeabilized using 0.1% triton and incubated with anti-3 chlorotyrosin. Levels of chlorination was not significant between control and treatment. Data are mean \pm SEM of 3 independent experiments.

We then tested whether MPO activates calpain via a nitrosylation mediated mechanism. Studies have shown that nitric oxide inactivates calpains by nitrosylation[99], and that denitrosylation increases calpain activity [126]. Therefore, we measured μ -calpain nitrosylation levels in MMVEC exposed to MPO using a previously described biotin switch assay [121]. As shown in **(Figure 14)** we found definitive evidence that MPO reduces μ -calpain nitrosylation levels in endothelial cells. These data suggested that

perturbation in the eNOS/NO biosynthetic machinery were a probable mechanism through which MPO activates the endothelial calpain system.

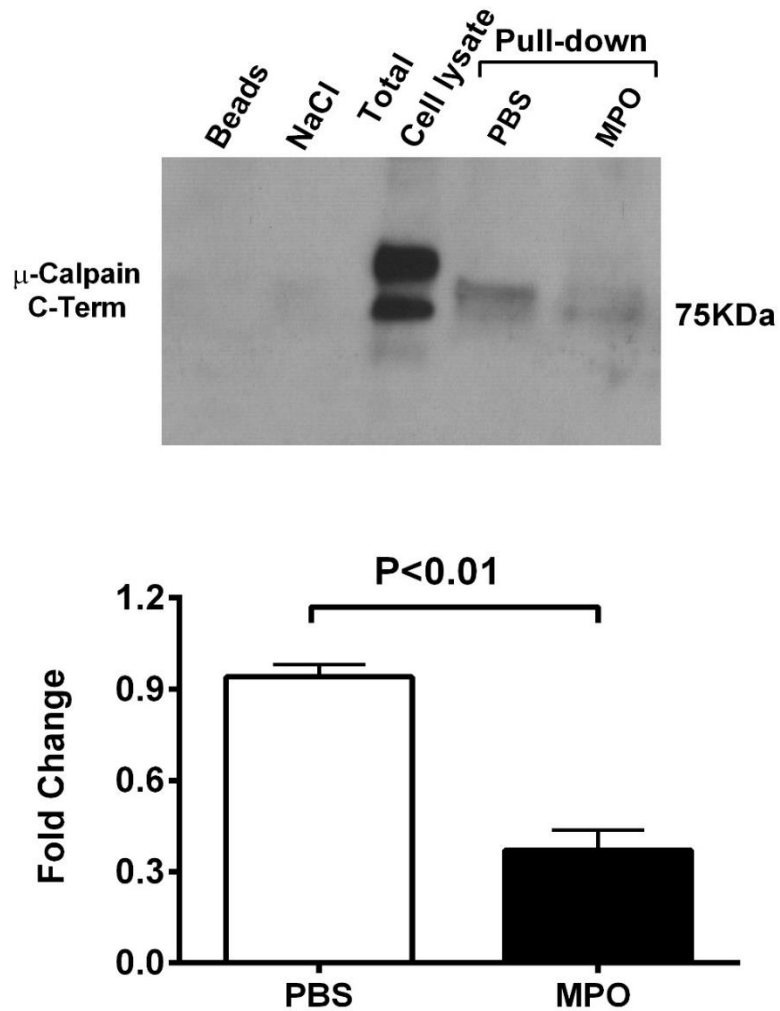


Figure 14: MPO Induces Denitrosylation of μ -Calpain. Serum starved MMVEC were incubated with vehicle (8 μ L PBS) or 10 nmol/L MPO for 180 min. Nitrosylation levels of μ -calpain in MMVEC was assessed by biotin switch assay. μ -Calpain was detected in the streptavidin-purified mixture using standard immunoblot techniques. Data are mean \pm SEM of 5 independent experiments.

μ -Calpain plays a role in the mechanism through which MPO Decreases AMPK/eNOS Phosphorylation.

Phosphorylation of NOS at Ser¹¹⁷⁷ is required for maintaining physiological levels of nitric oxide in the vascular endothelium [62]. Thus, we tested the hypothesis that MPO impairs eNOS phosphorylation thereby reducing calpain nitrosylation. Data shown in the upper panel of **(Figure 15)**, demonstrate that 10 nmol/L MPO significantly decreases Ser¹¹⁷⁷ eNOS phosphorylation in MMVEC in the absence of significant changes in total eNOS expression. Studies have demonstrated that in endothelial cells the energy sensing kinase AMPK phosphorylates eNOS at the Ser¹¹⁷⁷ activation site [127]. Interestingly, studies have shown that calpain can affect AMPK expression and signaling [128]. Data shown in **(Figure 16)** provide clear evidence of a significant decrease in the phosphorylation of Thr¹⁷² activation site of AMPK in lysates from MMVEC exposed to 10 nmol/L MPO. We also studied the effect of calpain inhibition on eNOS Ser¹¹⁷⁷, and AMPK Thr¹⁷² phosphorylation in MMVEC exposed to MPO. Treatment of MMVEC with 100 μ mol/L ZLLal restored AMPK and eNOS phosphorylation in the face of MPO **(Figure 15, Lower panel, and Figure 16)**, which suggests the existence of a feed-forward control mechanism through which in response to MPO active calpains maintain a denitrosylated status by sustaining downregulation of AMPK/eNOS signaling. To rule out the occurrence of nonspecific degradation of μ -calpain by MPO, we quantified total eNOS, and AMPK. As shown in **(Figure 15, and Figure 16)** MPO did not cause significant changes in both total eNOS, and AMPK expression level under our experimental conditions. Similarly, eNOS, and AMPK mRNA levels were not significantly changed by MPO **(Figure 17)**.

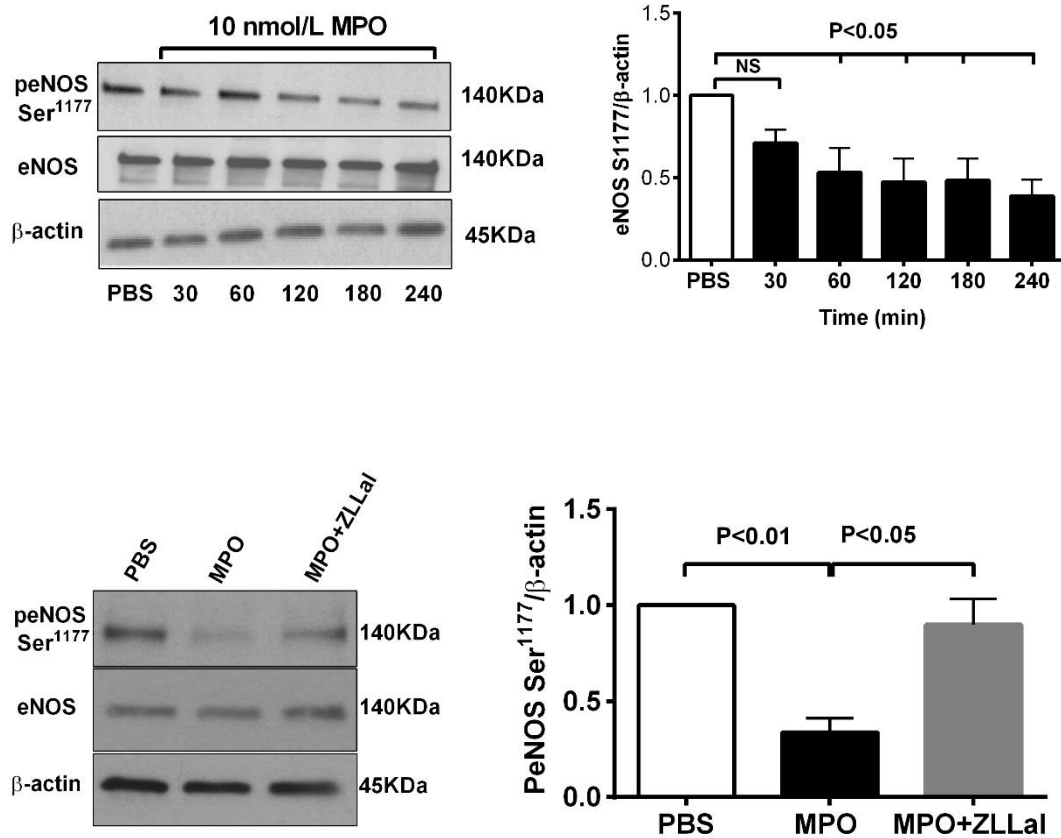


Figure 15: MPO/Calpain Signaling Downregulates Ser¹¹⁷⁷ eNOS Phosphorylation.

Upper panel Serum starved MMVEC were incubated with vehicle (8 μl PBS) or 10 nmol/L MPO for 30, 60, 120, 180, and 240 min. **Lower panel** serum starved MMVEC were incubated with vehicle (8 μL PBS) or 10 nmol/L MPO for 180 min with or without ZLLal (100 μmol/L, 30 min) pretreatment. Phosphorylation levels of Ser¹¹⁷⁷ eNOS in MMVEC were assessed by immunoblot analysis. Data are mean±SEM of 6 independent experiments.

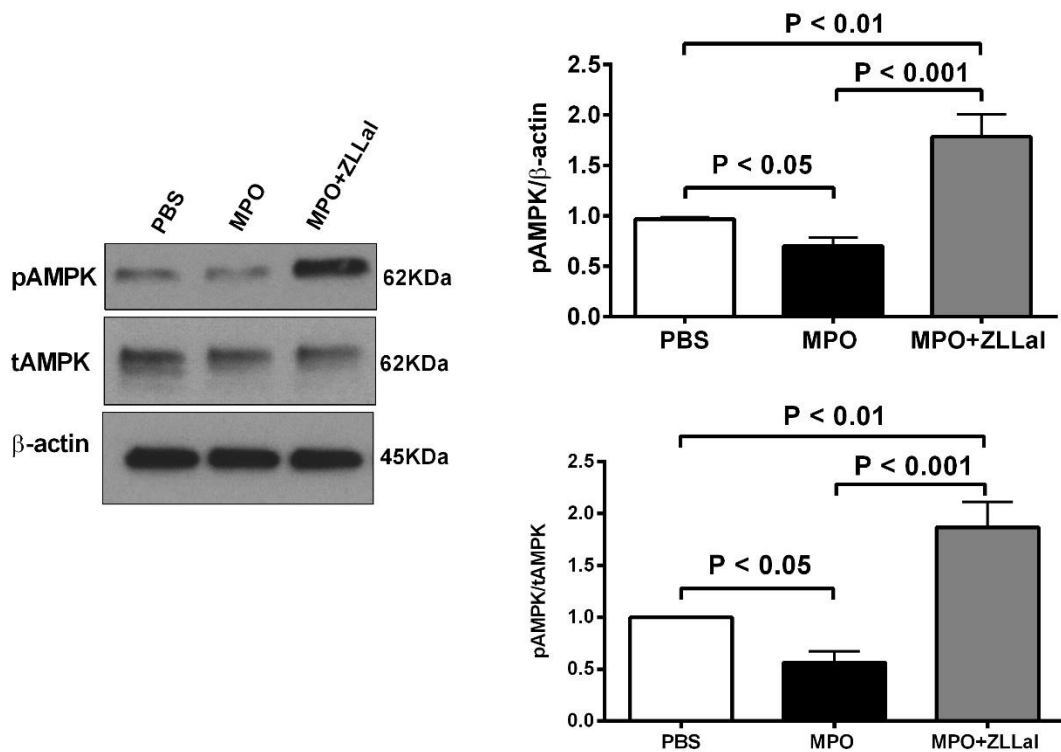


Figure 16: MPO/Calpain Signaling Downregulates Thr¹⁷² AMPK Phosphorylation.

Serum starved MMVEC were incubated with vehicle (8 μL PBS) or 10 nmol/L MPO for 180 min with or without ZLLal (100 μmol/L, 30 min) pretreatment. Phosphorylation levels of Thr¹⁷² AMPK, in MMVEC were assessed by immunoblot analysis. Data are mean±SEM of 6 to 8 independent experiments.

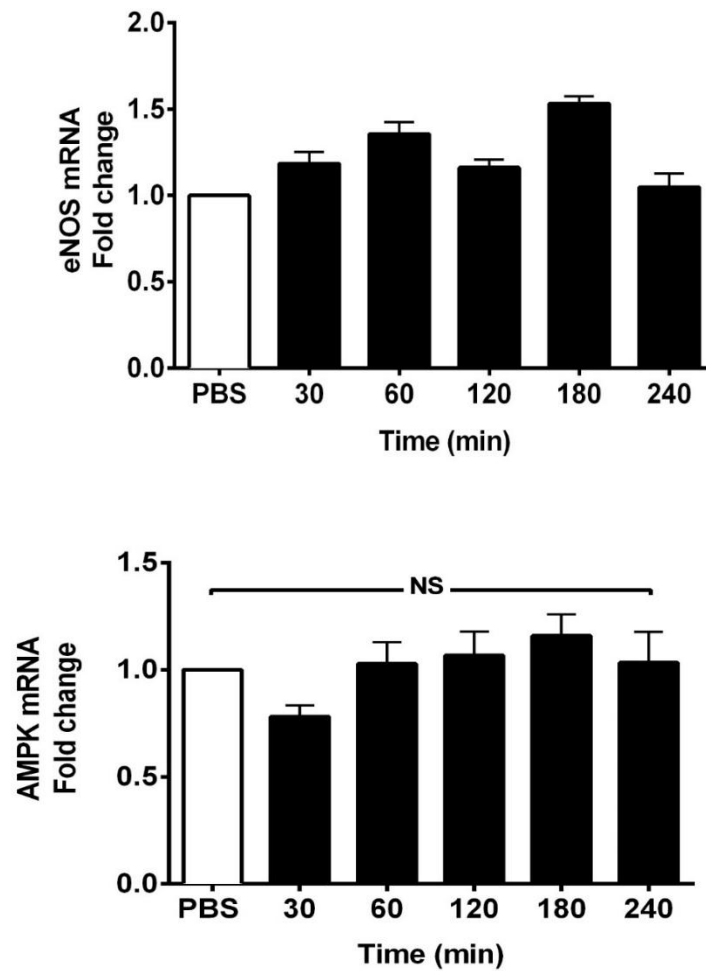


Figure 17: MPO Does Not Change mRNA Levels of Total eNOS, and AMPK. MMVEC cells were treated with 10 nmol/L MPO for 30, 60, 120, 180, and 240 min. mRNA levels of total eNOS, and AMPK were measured using TaqMan. Relative gene expression was calculated as ratio of target genes over housekeeping gene in the same sample. Data are mean \pm SEM of 4 independent experiments.

We also studied the effect of MPO treatment on AKT phosphorylation and found that MPO does decrease AKT phosphorylation at Ser⁴⁷³ but it does not appear to be calpain

dependent since we were not able to restore AKT phosphorylation when we inhibit calpain with 100 $\mu\text{mol/L}$ ZLLal (**Figure 18**).

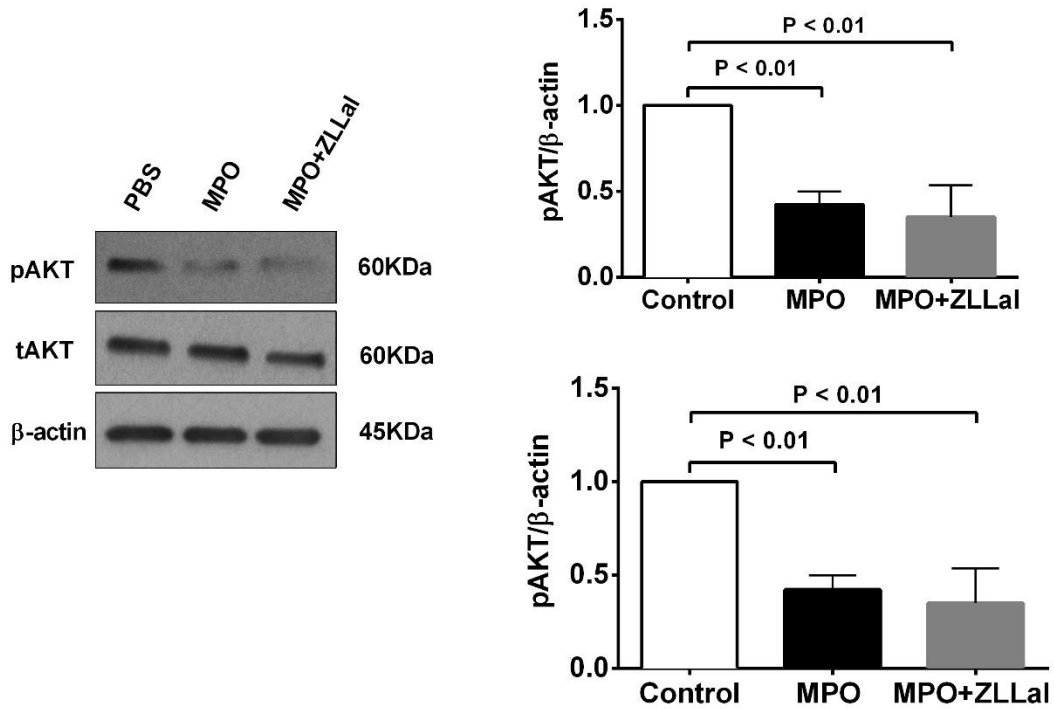


Figure 18: MPO Signaling Downregulates AKT Phosphorylation Independent of Calpain. Phosphorylation levels of Ser⁴⁷³ AKT, in MMVEC exposed to 10 nmol/L MPO, in the presence of absence of the calpain inhibitor ZLLal, were assessed by immunoblot analysis. MPO reduced AKT phosphorylation without affecting AKT total expression. Inhibition of calpain activity failed to restore AKT phosphorylation. Data are mean \pm SEM of 3 to 4 independent experiments.

The mechanism through which MPO/Calpain signaling affects Thr¹⁷²AMPK phosphorylation.

First, we studied the effect of MPO on the two major upstream kinases, serine/threonine Liver Kinase B1 (LKB1), which is the main kinase to regulate AMPK phosphorylation, and Phosphoinositide 3-kinase (PI3K), the main kinase to regulate AKT phosphorylation [129]. MPO did not significantly change LKB1 expression levels in MMVEC under our experimental conditions (**Figure 19, upper panel**). On the other hand, MPO treatment does decrease PI3K but it does not appear to be calpain dependent since we were not able to restore PI3K protein levels when we inhibit calpain with 100 $\mu\text{mol/L}$ ZLLal (**Figure 19 lower panel**). Indicating that the effects that MPO has on PI3K/AKT pathway is independent of calpain activation and probably involves other signaling mechanisms.

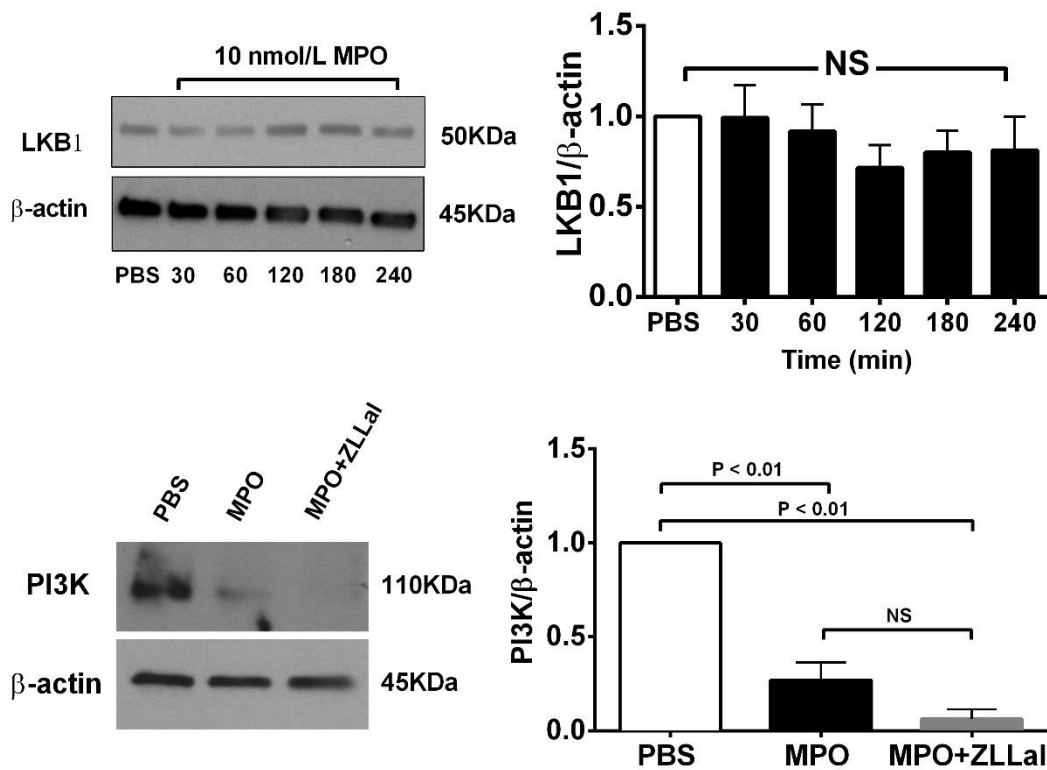


Figure 19: Role of LKB1 and PI3K in the Effect of MPO/Calpain Signaling on AMPK Phosphorylation. Serum starved MMVEC were incubated with vehicle (8 μ L PBS) or 10 nmol/L MPO for 30-180 min (LKB1 detection). Serum starved MMVEC were incubated with vehicle (8 μ L PBS) or 10 nmol/L MPO for 180 min (PI3K detection). With or without ZLLal (100 μ mol/L, 30 min) pretreatment. Expression levels of LKB1 and PI3K in MMVEC were assessed by immunoblot analysis. Data are mean \pm SEM of 3 independent experiments.

Second, we investigated the role of the protein phosphatase 2A (PP2A), a signaling partner of active calpains [130] that has been also shown to inhibit AMPK activity via dephosphorylation [75]. Interestingly MPO increased the expression level of the catalytic subunit of PP2A in MMVEC (**Figure 20**). In agreement with the data on AMPK/eNOS phosphorylation summarized in (**Figure 16**), the effect of MPO on PP2A were abolished by treatment of MMVEC with 100 μ mol/L ZLLal (**Figure 20**).

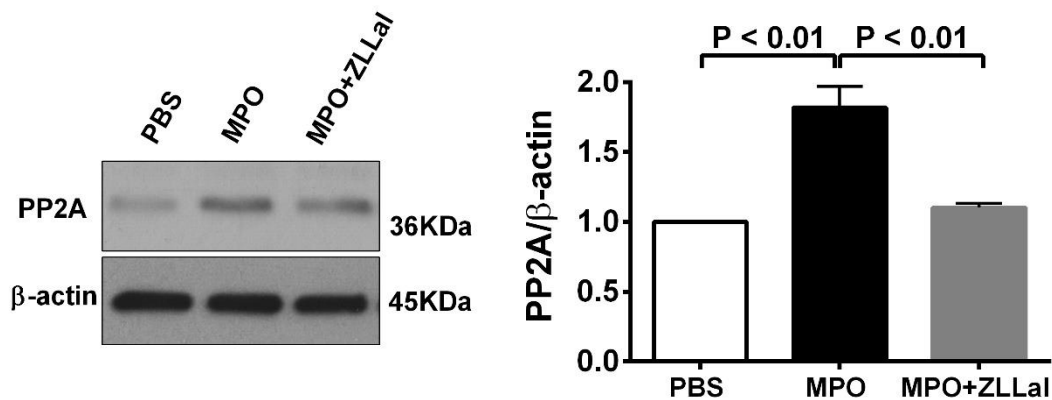


Figure 20: Role of PP2A in the Effect of MPO/Calpain Signaling on AMPK Phosphorylation. Serum starved MMVEC were incubated with vehicle (8 μ L PBS) or 10 nmol/L MPO for 180 min. With or without ZLLal (100 μ mol/L, 30 min) pretreatment. Expression levels of PP2A in MMVEC were assessed by immunoblot analysis. Data are mean \pm SEM of 3 independent experiments.

To obtain mechanistic evidence that the effect of MPO/calpain signaling on AMPK phosphorylation is indeed PP2A dependent, we pretreated MMVEC with the PP2A inhibitor okadaic acid. Results shown in (**Figure 21, and Figure 22**) demonstrate that direct inhibition of PP2A preserves phospho-AMPK/eNOS levels in MMVEC in the face

of MPO [75]. These data uncover PP2A as an important downstream mediator of endothelial dysfunction in response to MPO/calpain signaling.

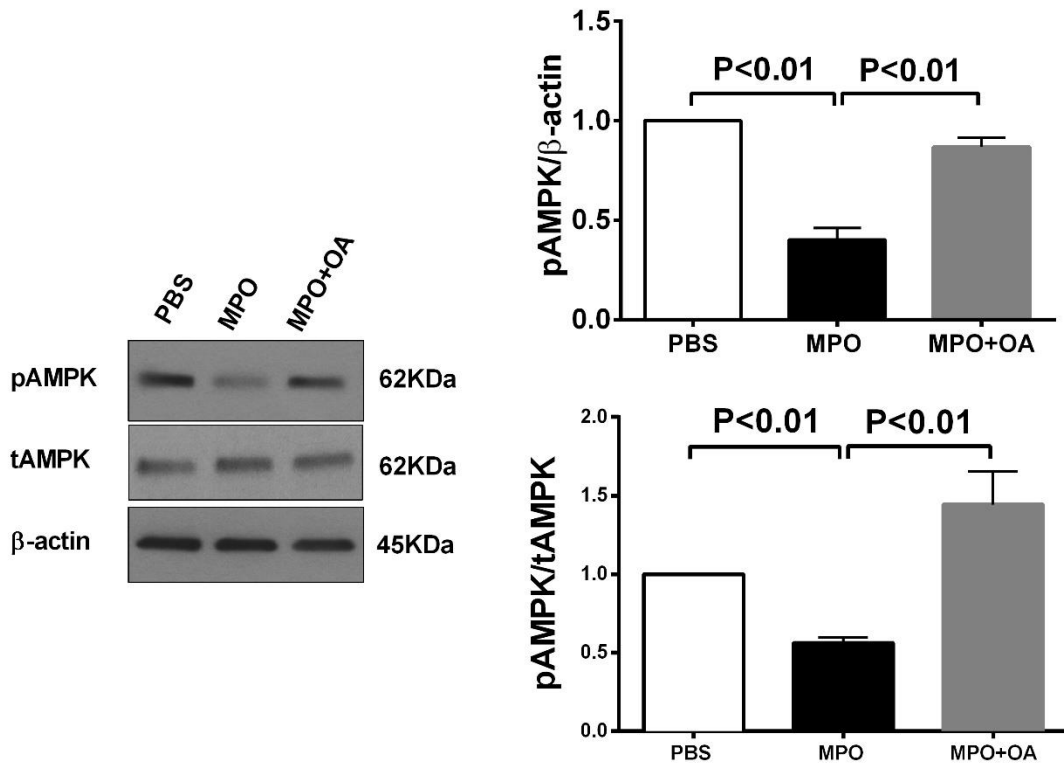


Figure 21: MPO/Calpain Signaling Decrease AMPK Phosphorylation in a PP2A Dependent Manner. Serum starved MMVEC were incubated with vehicle (8 μ L PBS) or 10 nmol/L MPO for 180 min. With or without okadaic acid (10 nmol/L, 30 min pretreatment). Levels of AMPK phosphorylation in MMVEC were assessed by immunoblot analysis. Data are mean \pm SEM of 3 independent experiments.

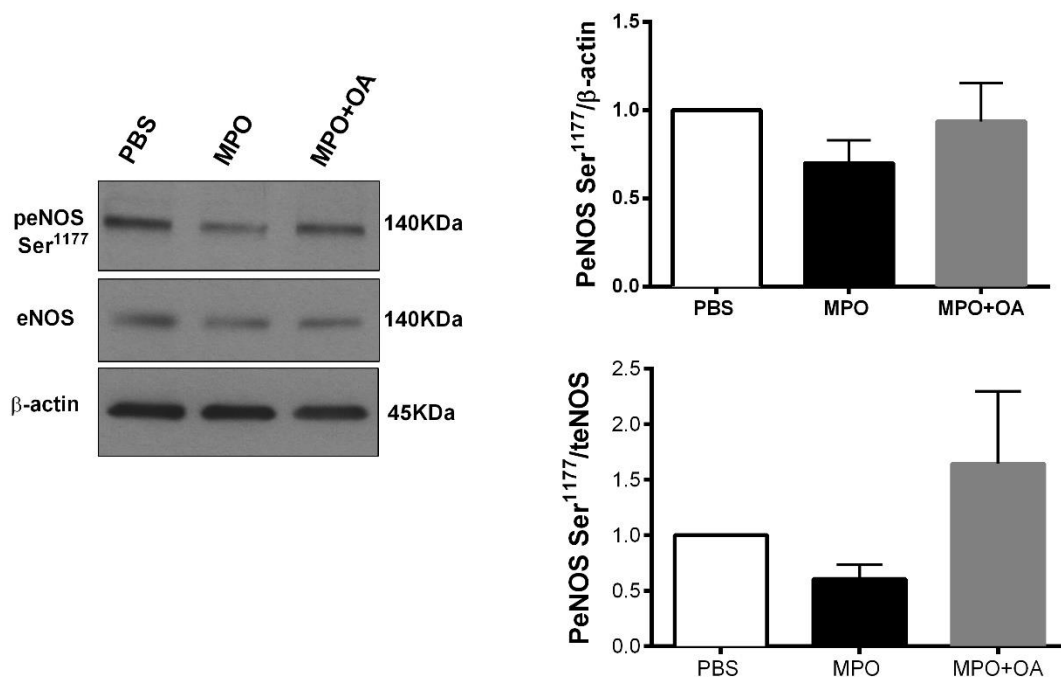


Figure 22: MPO/Calpain Signaling Decrease eNOS Phosphorylation in a PP2A Dependent Manner. Serum starved MMVEC were incubated with vehicle (8 μ L PBS) or 10 nmol/L MPO for 180 min. With or without okadaic acid (10 nmol/L, 30 min pretreatment). Levels of eNOS phosphorylation in MMVEC were assessed by immunoblot analysis. Data are mean \pm SEM of 3 independent experiments.

Mechanistic Role of Calpain in the MPO-induced Increased Endothelial Adhesiveness to Leukocytes.

To investigate the impact on MPO/calpain signaling on endothelial function we measured first the expression level of the endothelial cell adhesion molecule VCAM-1, an adhesion molecule upregulated by dysfunctional endothelia with impaired eNOS function [64]. Immunoblot analyses revealed that VCAM-1 expression levels are elevated in MMVEC cells treated with 10 nmol/L MPO for 180 minutes (**Figure 23**). Consistent with

the biochemical data reported above, VCAM-1 protein levels were attenuated by treatment with the calpain inhibitor ZLLal (**Figure 23**).

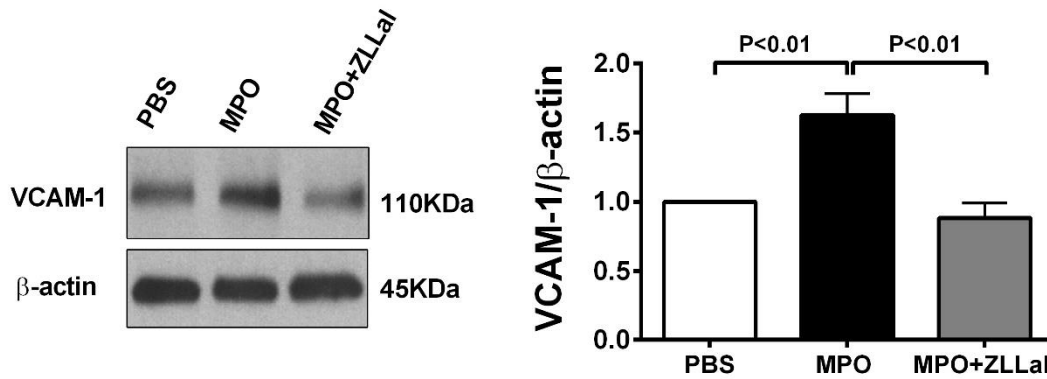


Figure 23: MPO Increases Endothelial Cell Adhesion Molecule (VCAM-1). Serum starved MMVEC were incubated with vehicle (8 μ M PBS) or 10 nmol/L MPO for 180 min with or without ZLLal (100 μ mol/L, 30 min) pretreatment. Expression levels of VCAM-1 in MMVEC were assessed by immunoblot analysis. Data are mean \pm SEM of 4 independent experiments.

Endothelia expressing VCAM-1 became adhesive to circulating leukocytes. Accordingly, we next studied the effect of MPO/Calpain signaling on the adhesion of leukocytes to the aortic endothelium. For these studies aortas and leukocytes were isolated from male wild-type C57BLK. Aortas were treated with MPO with or without ZLLal. Data in (**Figure 24**) show that in wild-type mice MPO increases the adhesion of leukocytes to the aortic endothelium. Thus, compared to control aortas, MPO-stimulated aortas showed a significantly high number of adhered leukocytes. Pretreating the aortas with ZLLal prevented leukocyte adhesion (**Figure 24**).

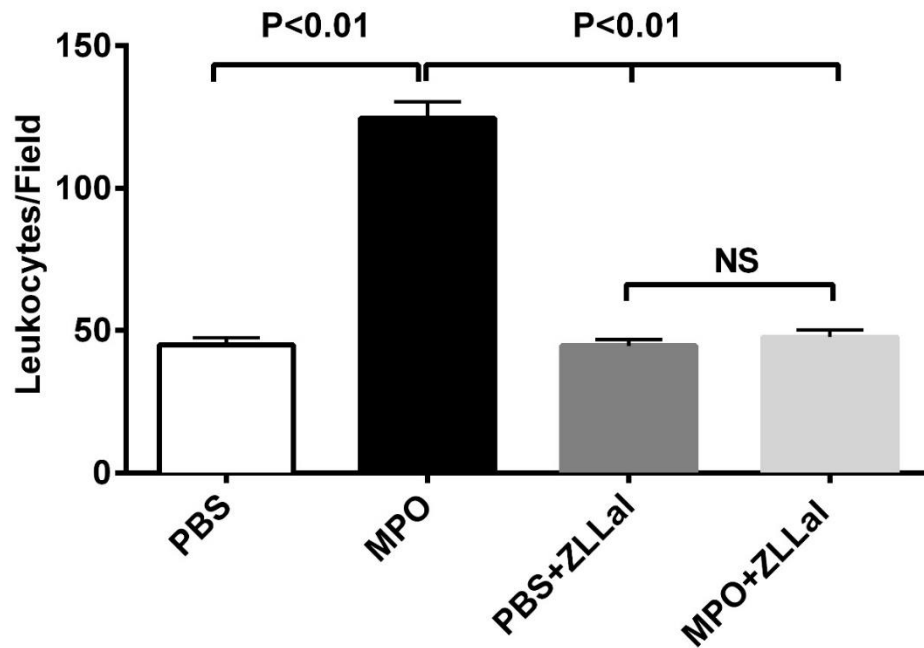


Figure 24: MPO Increases Endothelial Adhesiveness to Leukocytes. Circulating leukocytes and thoracic aortas were isolated from C57BLK donor mice. Leukocytes were fluorescently labeled as reported in the method section. Isolated 2 mm length aortic segments were first exposed to 10 nmol/L MPO for 180 min, with or without ZLLal (100 μ mol/L, 30 min). Aortic segments were then washed and co-incubated endothelial surface up with leukocytes for an additional 60 min. Inhibition of calpain activity by ZLLal also prevented adhesion of leukocytes to the wild-type aorta exposed to MPO. Data are mean \pm SEM of 6 aortic segments from 3 mice per group. A total of 54 aortic segments were studied.

Additional mechanistic studies with μ -calpain deficient mice (Capn1^{-/-}) mice were undertaken to dissect the individual contribution of endothelial-expressed and/or

leukocyte-expressed μ -calpain. As shown in **(Figure 25)**, MPO fails to increase the adhesion of leukocytes isolated from wild-type mice to the aortic endothelium of μ -calpain deficient mice **(Figure 25)**. In contrast, increased leukocyte adhesion by MPO was observed in wild-type mouse aortas incubated with $Capn1^{-/-}$ mouse leukocytes. Thus, endothelial expressed μ -calpain is a requirement for MPO to increase leukocyte-endothelium interactions. These data agree with data on VCAM-1 expression and they demonstrate that in the setting of increased MPO signaling, the vascular endothelium develops a calpain-dependent, pro-adhesive phenotype to leukocytes. Male wild-type C57BLK and μ -calpain deficient mice ($Capn1^{-/-}$) do not differ in body weight or blood pressure **(Table 1)**, nor they differ in basal levels of eNOS and AMPK **(Figure 26)**.

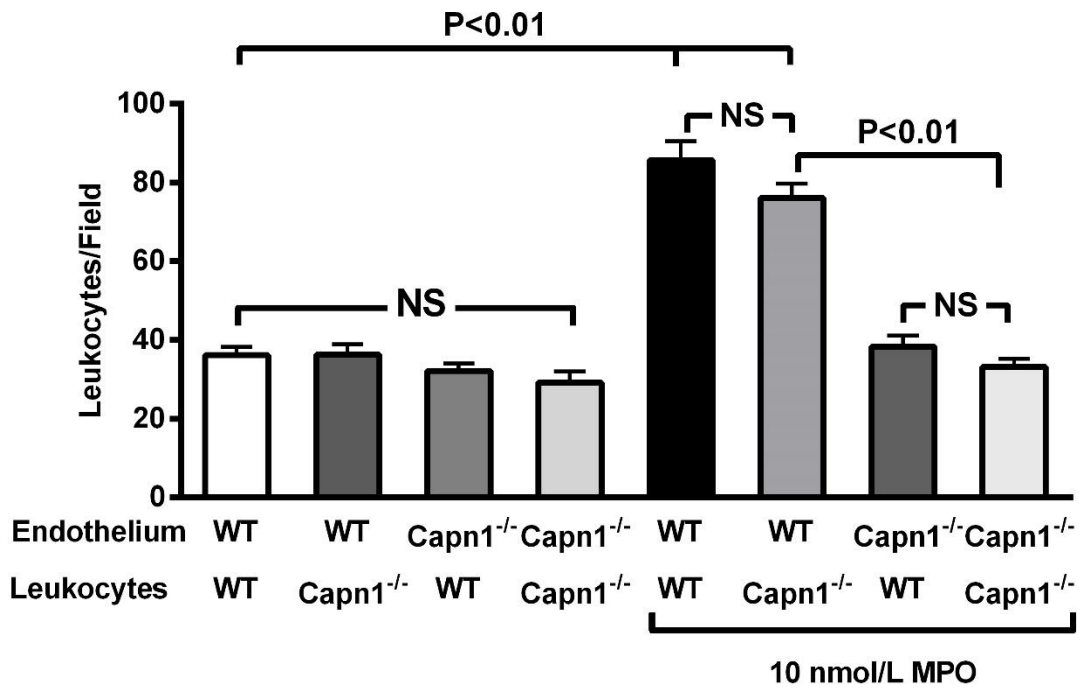


Figure 25: MPO Increases Endothelial Adhesiveness to Leukocytes in an Endothelial μ -Calpain Dependent Manner. Circulating leukocytes and thoracic aortas were isolated from C57BLK and μ -calpain^{-/-} donor mice. Leukocytes were fluorescently labeled as reported in the method section. Isolated 2 mm length aortic segments were first exposed to 10 nmol/L MPO for 180 min. Aortic segments were then washed and co-incubated endothelial surface up with leukocytes for an additional 60 min. Data are mean \pm SEM of 6 aortic segments from 3 mice per group. A total of 54 aortic segments were studied.

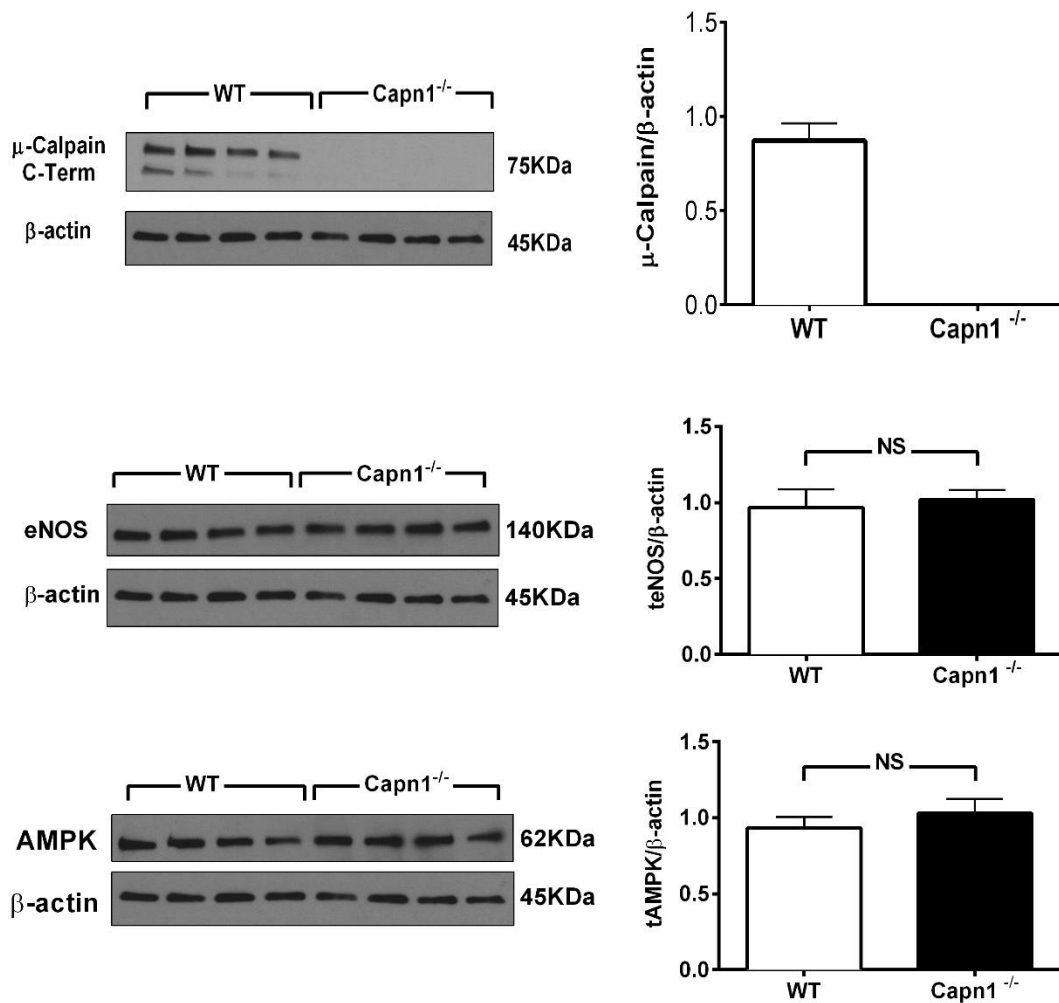


Figure 26: Expression Levels of μ -Calpain, eNOS and AMPK in the Aortas of Wild-Type and *Capn1*^{-/-} Mice. Representative immunoblot demonstrate total expression levels of μ -calpain, eNOS AMPK and β -actin. No difference in total eNOS and AMPK was observed in the aortas of wild-type and *Capn1* deficient mice. Lack of μ -calpain expression in *Capn1*^{-/-} mice was also confirmed by immunoblot analysis. Data are expressed as total protein/ β -actin ratio and they are mean \pm SEM from 4 mice per group.

Variable	WT	CAPN1 ^{-/-}	P value
	Mean ± SEM	Mean ± SEM	
HR	581.7±72.41	586.8±23.65	0.9743
SBP	127.5±9.114	118.7±6.783	0.4579
DBP	100.4±7.615	88.61±6.647	0.2703
MABP	113±8.618	103.7±6.482	0.4121
BW(g)	22.77±0.9251	21.35±0.7784	0.2684

Table 1: Telemetry reading for male wild-type C57BLK and μ -calpain deficient mice (Capn1^{-/-}). Blood pressure and heart rate were evaluated in conscious mice 4 days after telemetry implantation (DSI equipped with ADInstrument 6 software) via carotid catheter (PA-C10). Data are mean±SEM of 3 mice per group.

MPO Decreases Adiponectin receptor Levels in a μ -Calpain dependent manner.

As mentioned previously in chapter one, reduced levels of AdipoR1 and AdipoR2 has long been associated with inflammation and endothelial dysfunction [84]. Interestingly, adiponectin is negatively correlated with myeloperoxidase (MPO) [89]. Since adiponectin receptors are upstream of both AMPK and eNOS, we tested the hypothesis that MPO decreases expression levels of adiponectin receptors. Data shown in **(Figure 27)**, demonstrate that 10 nmol/L MPO significantly decreases AdipoR1, and AdipoR2 protein levels in MMVEC. These effects appear to be exclusive to the protein since, AdipoR2 mRNA levels were not significantly changed by MPO **(Figure 28)**.

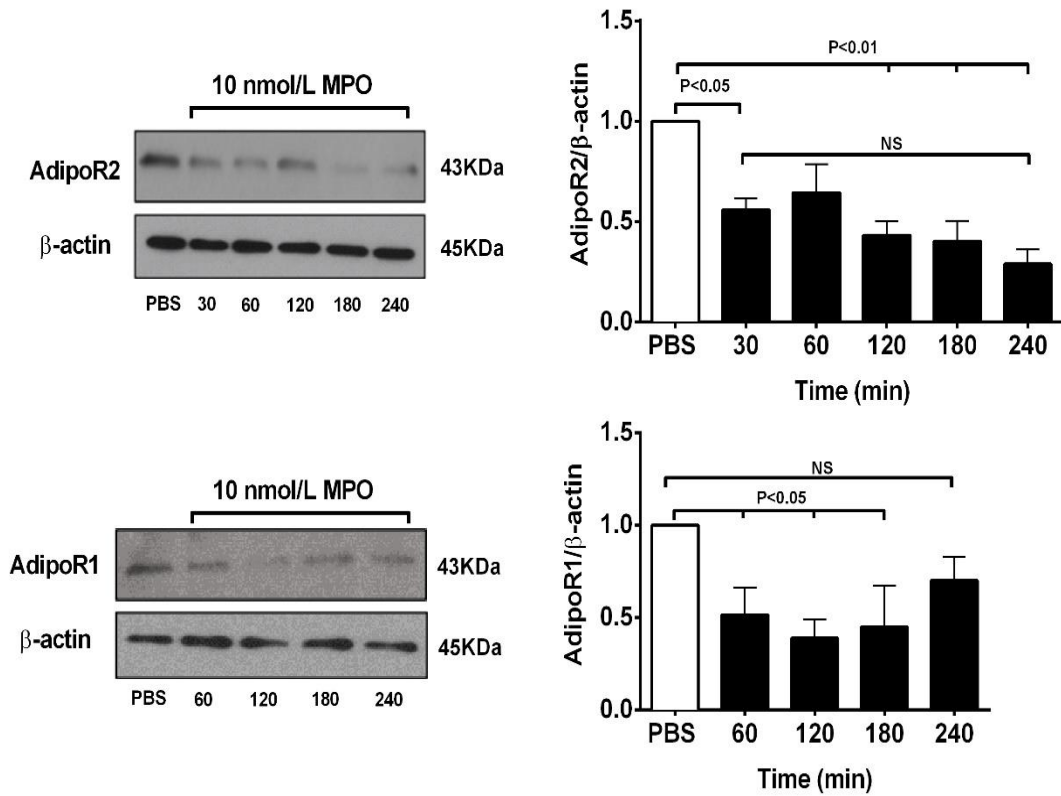


Figure 27: MPO Causes Degradation of Adiponectin Receptors. Serum starved MMVEC were incubated with vehicle (8 μ l PBS) or 10 nmol/L MPO for 30, 60, 120, 180, and 240 min. The expression of both AdipoR1, and AdipoR2 were measured by immunoblot analysis and quantified by densitometry. Beta actin detection was used as a loading control. Data are mean \pm SEM of 4 independent experiments.

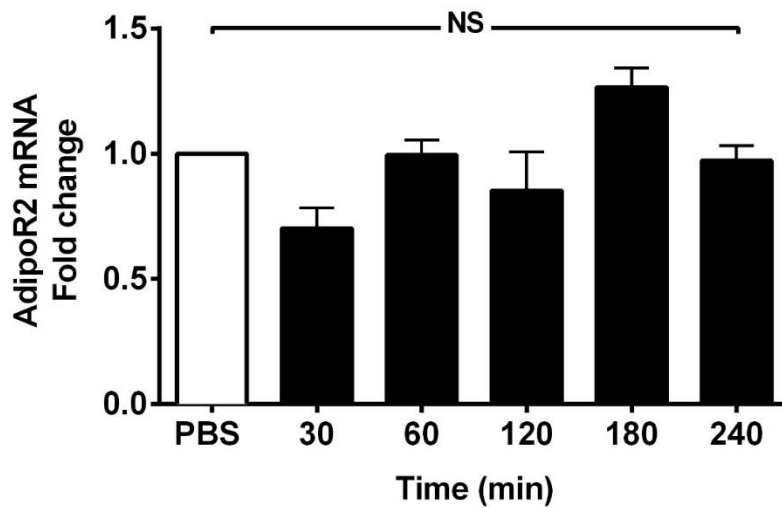


Figure 28: MPO Does Not Change mRNA Levels of AdipoR2. MMVEC cells were treated with 10 nmol/L MPO for 30, 60, 120, 180, and 240 min. mRNA levels of AdipoR2 were measured using TaqMan. Relative gene expression was calculated as ratio of target genes over housekeeping gene in the same sample. Data are mean \pm SEM of 4 independent experiments.

We also studied the effect of calpain inhibition on AdipoR2 in MMVEC exposed to MPO. Treatment of MMVEC with 100 μ mol/L ZLLal restored AdipoR2 protein levels in the face of MPO (**Figure 29**).

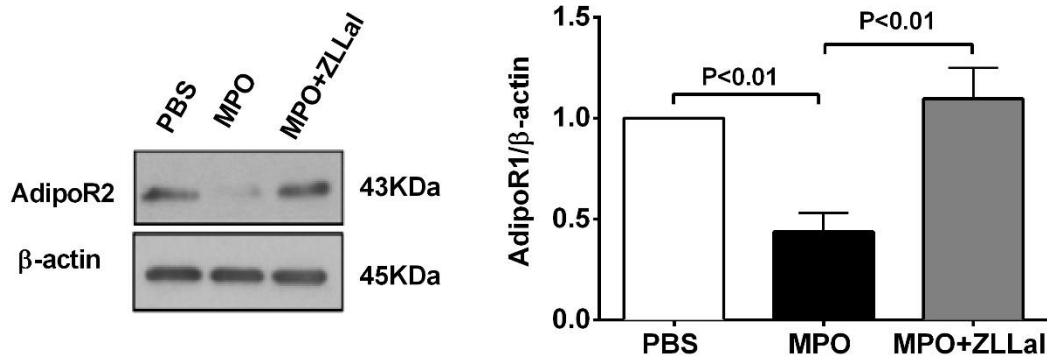


Figure 29: MPO Decreases endothelial AdipoR2 Levels in a Calpain Dependent Manner. Serum starved MMVEC were incubated with vehicle (8 μ M PBS) or 10 nmol/L MPO for 180 min with or without ZLLal (100 μ mol/L, 30 min) pretreatment Expression levels of AdipoR2 in MMVEC were assessed by immunoblot analysis. Data are mean \pm SEM of 4 independent experiments.

Data shown in **(Figure 29)** indicate that the effects of MPO on AdipoR2 is dependent on calpain activation.

To further understand the effects of MPO on the adiponectin receptor we studied the effects of MPO on AdipoR2 in the presence and absence of adiponectin. MMVEC cells were treated with 10 μ g/ml of adiponectin with or without MPO **(Figure 30)**.

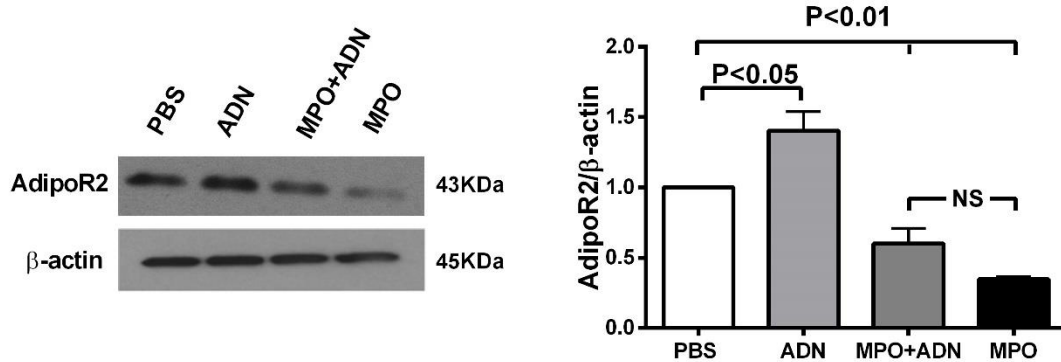


Figure 30: MPO Decreases endothelial AdipoR2 Levels in the Presence of Adiponectin. Serum starved MMVEC were incubated with vehicle (8 μ M PBS), Adiponectin (ADN) or 10 nmol/L MPO for 180 min with or without ADN (10 μ g/ml). Expression levels of AdipoR2 in MMVEC were assessed by immunoblot analysis. Data are mean \pm SEM of 3 independent experiments.

Data in **Figure 30** show that treatment of endothelial cells with adiponectin resulted in a significant increase in AdipoR2 expression levels. This increase was lost when cells were treated with MPO and adiponectin together.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Myeloperoxidase (MPO) has long been associated with increased risk of cardiovascular disease and endothelial dysfunction [22]. Human studies have found a direct correlation between serum MPO levels and endothelial dysfunction; this correlation has been found to be stronger than that of C-reactive protein (CRP), a protein that is widely used in the clinic as an indicator of cardiovascular health [25]. MPO is known to react with nitric oxide, thereby enhancing its catabolism [36]. One of the first studies investigating the cause of MPO induced endothelial dysfunction was completed by Eiserich et al in 2002. This study hypothesized that the direct interaction of MPO with nitric oxide is the primary mechanism underlying MPO-dependent alteration in endothelial function. This theory is based on seminal findings in animal models of endotoxemia demonstrating that MPO released by active leukocytes localizes in and around endothelial cells of the aorta, where it drives catalytic consumption of nitric oxide in endothelial cells without altering its function in the underlying smooth muscle cells [32]. Interestingly, this phenomenon was not observed in MPO^{-/-} mice with endotoxemia [32]. Although this theory served as the working model for the past decade, it does not provide an explanation for more recent studies that have demonstrated a direct inhibitory action of MPO and its derived metabolites on eNOS activity [131, 132]. In order to understand the mechanisms underlying the actions of MPO on eNOS, we investigated the effects of MPO on positive regulators (e.g. Adiponectin signaling), and negative regulators (e.g. calpains) of eNOS activity.

Calpains are a family of calcium dependent proteases [91]. Clinical studies have found a direct association between increased calpain activity and the risk of cardiovascular disease. Polymorphisms in calpain-10 has been associated with type 2 diabetes, ischemic stroke, coronary artery disease, and sleep apnea in humans [133, 134]. In this study, we focused on μ -and m-calpains because they are the only two calpains present in endothelial cells with known crystal structures [91]. This body of work is, to our knowledge, the first to uncover the role of the cytosolic protease μ -calpain in the endothelial dysfunction associated with increased levels of myeloperoxidase. We show that 10nmol/L of MPO, which is within the range of found in the plasma of humans with cardiovascular disease, activates the μ -calpain isoform in endothelial cells. Results from **Figures 6 and 7** provide evidence that MPO increases both the autolysis of the NH₂-terminus domain and the proteolytic activity of the μ -calpain isoform but not that of the m-calpain isoform. However, in our study we found some discrepancies in the time points between autolysis and the proteolytic activity of μ -calpain; this could be explained by recent findings indicating that in some cases NH₂-terminus autolysis could occur before or in the absence of proteolytic activity [94]. This could may also be why calpain became active at a 120 min while the NH₂-terminus domain was autolyzed as early as 30 min. Interestingly, we also show that activation of μ -calpain by MPO is amenable to pharmacological inhibition, which makes the endothelial calpain system an attractive, novel therapeutic target in cardiovascular disease.

Calpastatin is the indigenous inhibitor of both μ - and m-calpains, where one calpastatin molecule has the ability to inhibit four calpain molecules [91]. **Figure 10** shows

decreased calpastatin levels with MPO treatment, however upon the use of a specific calpain inhibitor we were able to restore calpastatin levels to that of control, indicating that the decrease in calpastatin levels with MPO treatment is a result of degradation by calpain **Figure 11**. This is consistent with published literature presenting evidence of calpastatin degradation by both μ - and m-calpain *in vitro* and *in vivo* [135]. It is believed that this process is initiated in order to increase the number of calpastatin inhibitory units however, with prolonged calpain activation and no increase in calpastatin levels, this process leads to full degradation of calpastatin [135].

A number of post translational modifications have been found to affect the activity of calpain. Such modifications include: chlorination, nitrosylation, and phosphorylation/dephosphorylation. Our results demonstrate that MPO treatment did not alter total cell chlorination, eliminating it as the possible mechanism of calpain activation. A large body of evidence has found increased catalytic consumption of nitric oxide by MPO [32], as well as decrease nitric oxide bioavailability by active calpains. Others have also reported inhibition of calpain activity by nitric oxide [136, 137]. One recent study reported a role for nitrosylation in the regulation of μ -calpain activity, where increase calpain nitrosylation results in a decrease in its activity [126]. Altogether, our data demonstrate that reduced calpain nitrosylation occurs in endothelial cells exposed to MPO at a time point where calpain is most active. Thus, a feedback regulation is likely to exist between the eNOS/NO biosynthetic machinery and the calpain system in the inflamed vascular endothelium. Since eNOS/NO biosynthetic machinery mainly exists in endothelial cells, this is particularly important as it introduces calpain denitrosylation by

MPO as a specific mechanism of endothelial μ -calpain activation. It is important to note that both phosphorylation and dephosphorylation positively regulate calpain activity [138]. However, we did not test if phosphorylation/dephosphorylation of μ -calpain plays a role in regulating calpain activity in this body of work and future studies need to be done to further clarify the effects of phosphorylation/dephosphorylation in our experimental model.

This work uncovers a novel endothelial signaling mechanism, involving the calpain system, through which MPO decreases eNOS Ser¹¹⁷⁷ phosphorylation, thus causing endothelial dysfunction beyond catalytic consumption of nitric oxide. This gives rise to the possibility that following initial degradation of nitric oxide, more stable signaling alterations of eNOS take place in the vascular endothelium exposed to MPO. The activation of calpain seen in our study could then help explain recent data reporting decreased eNOS function following exposure of endothelial cells to MPO byproducts [131]. Our results also provide a molecular explanation to the evidence of increased nitric oxide bioavailability following calpain inhibition in cultured endothelial cells [139] and animal models of vascular inflammation [140]. It is important to note the existence of other phosphorylation sites for activation or inhibition of eNOS activity mentioned in the first chapter. This suggests that further experiments need to be done to study the effect of the MPO/calpain system on the other eNOS phosphorylation sites.

As mentioned in Chapter 1, it has been stated that there is another form of NOS called inducible nitric oxide synthase (iNOS). In the setting of inflammation, large amounts of NO can be produced by iNOS, and the literature demonstrates that MPO can increase

the catalytic activity of iNOS *in vitro* [141]. However, others have demonstrated that during inflammation MPO downregulates iNOS expression *in vivo* [142]. It has been reported in the literature that induction of iNOS requires a longer period of time than the 3 hour protocol adopted in this study [143], and therefore iNOS is not likely to play a significant role in this work.

Interestingly, both eNOS and calpain require elevated cytosolic calcium levels for activation, which, in theory, should consistently hinder physiological eNOS function since active calpain can downregulate eNOS [144]. This paradox could be explained by three possible lines of evidence. First, in the presence of physiological calcium signaling, eNOS binds HSP90, forming a complex that renders eNOS resistance to calpain degradation [145]. However, the presence of MPO, which is known for its ability to cause Ca²⁺ overload in endothelial cells [146], and therefore, leads to abnormal activation of calpain, is likely to impair this physiological protective mechanism. Second, our laboratory and others have demonstrated that sustained calpain activation disrupts HSP90/eNOS association, which further reduces eNOS activity [147, 148]. Third, studies have found that loss of eNOS phosphorylation at Ser¹¹⁷⁷ reduces eNOS sensitivity to calcium [149]. When taken together, these data demonstrate that MPO further downregulates eNOS function by reducing eNOS Ser¹¹⁷⁷ phosphorylation, thus favoring the action of calpain.

Several kinases regulate eNOS activity by phosphorylating its activation and/or inhibition sites. Relevant here, AMPK activates eNOS by phosphorylating the Ser¹¹⁷⁷ activation site [127], thereby increases NO production [150]. We report evidence that MPO reduces eNOS Ser¹¹⁷⁷ phosphorylation via a calpain-dependent inhibition of

AMPK phosphorylation at Thr¹⁷². Other kinases that were tested in this study were AKT, and PI3K. We found that MPO significantly decreases PI3K expression levels, as well as significantly decrease AKT phosphorylation at Ser⁴⁷³. These effects of MPO do not appear to be calpain dependent, as inhibition of calpain failed to restore both AKT phosphorylation and PI3K levels. In agreement with our results, inhibition of μ -calpain has been associated with decrease PI3K/AKT *in vitro* [151, 152], and *in vivo* [153]. However, the effects of MPO on PI3K/AKT is not fully understood. AKT activity is regulated by phosphorylation and dephosphorylation at different sites (Ser⁴⁷³, and Thr³⁰⁸), which is regulated by a number of kinases and phosphatases including: mammalian target of rapamycin complex 2 (mTORC2), phosphoinositide dependent kinase 1 (PDKP1), Phosphatase and tensin homolog (PTEN), and PH domain and Leucine rich repeat Protein Phosphatases family [154]. Further studies are required in order to fully understand how MPO effects the PI3K/AKT system.

Dephosphorylation of AMPK is been shown to be regulated by protein phosphatase 2A (PP2A) [155]. PP2A is a serine/threonine phosphatase. It is composed of a scaffolding subunit, catalytic subunit, and a regulatory subunit [155]. Regulation of PP2A activity is not fully understood, though one study suggests that partial degradation of the regulatory subunit (PR72, and PR130) by calpain renders PP2A active and significantly increases its sensitivity to activation by polycation [156]. We found that MPO significantly increase PP2A expression levels in a calpain dependent manner. Our data also show that the decreased AMPK/eNOS phosphorylation by μ -calpain is PP2A dependent. Furthermore, we have identified in PP2A a novel molecular mechanism through which the MPO/calpain

pathway downregulates AMPK/eNOS signaling as shown in **Figures 21, and 22**. Additional studies are needed to understand the mechanisms by which MPO/calpain system activates PP2A, as well as the impact that the MPO/calpain signaling has on the other kinases, molecular chaperones, and cofactors that are known to regulate AMPK, eNOS, AKT functions in different pathophysiological conditions of the cardiovascular system.

In addition to its vasodilatory properties, nitric oxide has been known to maintain an anti-adhesive phenotype in the vascular endothelium, thus preventing abnormal leukocytes infiltration and adhesion to the vascular wall [64, 157]. Interestingly, endothelial nitric oxide has been shown to prevent upregulation of VCAM-1 in response to inflammatory stimuli [64]. We found that MPO increases expression levels of VCAM-1 in endothelial cells, in addition to increasing leukocyte adhesion to the mouse aorta via a calpain-dependent mechanism as shown in **Figure 24**. Consistent with our results, others have demonstrated an association between MPO and increased leukocyte adhesion to the vascular endothelium [158]. Data also demonstrate that μ -calpain increases expression of cell adhesion molecules in endothelial cells [159]. Conversely, inhibiting calpain reduces the adverse outcomes of ischemia reperfusion injury by reducing ICAM-1 expression and preventing neutrophil infiltration [160]. Studies from our lab have also reported that calpain inhibition significantly decreases adhesion of leukocytes in the microcirculation of diabetic rats [112]. Therefore, inhibition of calpain activity attenuates adhesion of leukocytes to the vascular endothelium of both large conduit vessels and the microcirculation which is in agreement with our *in vitro* and *ex vivo* results.

As discussed in Chapter 1, leukocyte-endothelium interactions are mediated by adhesion molecules expressed on the cell surface of both cell types. Importantly, μ -calpain is constitutively expressed both in circulating leukocytes [161], and endothelial cells [108]. Thus, we asked whether μ -calpain expressed by leukocytes played a role in these studies. In order to answer this question, we implemented our *ex vivo* model using mice that are deficient in μ -calpain. Mice deficient in μ -calpain are characterized with erythrocytes deformability, and abnormal platelet aggregation [162]. We found evidence that aortas from mice deficient in μ -calpain are protected against leukocyte adhesion induced by MPO. This indicates an obligatory role for endothelial expressed calpains in the initiation of vascular inflammation in response to MPO. Interestingly, leukocytes deficient in μ -calpain were not able to attenuate their adhesion to the vascular endothelium of wild type mice. These results are supported by previous data from our laboratory and others demonstrating that the interaction between leukocytes and the vascular endothelium can be largely abrogated by direct blockade of adhesion molecules expressed on the vascular endothelium only [116, 163]. It should be noted that endothelial expressed calpains have been shown to influence the adhesive and migratory behavior of circulating leukocytes [164]. A study by Dreolini, et al in 2007 revealed that activation of LFA-1 (CD11/CD18) can occur independent from calpain [165]. Others have found that m-calpain is responsible for controlling the turnover of integrins in leukocytes [166]. It is important to note that calpain activity is required for the shape change in leukocytes from spherical to flattened, which is required for their extravasation into the endothelium [167]. A recent study has shown that leukocytes deficient in μ -calpain significantly decreased the levels of

angiotensin II induced atherosclerosis in LDL receptor knockout mice [168]. Therefore, more studies need to be conducted to understand if μ -calpain deficiency in leukocytes would affect the extravasation step into the endothelium, as well as the effects of μ -calpain on the leukocytes integrin levels and function. Also, further studies are needed to fully dissect how different inflammatory stimuli and/or duration of inflammation affect the calpain system of leukocytes and endothelial cells.

A number of studies have shown that adiponectin and its receptors are involved in AMPK/eNOS signaling [65, 80, 83]. In our study, we provide evidence of reduced adiponectin receptor protein levels after treatment with MPO. We also show that the effects of MPO on AdipoR2 is calpain dependent. However, whether the effects that the MPO/calpain signaling had on AdipoR2 is related to the effects on AMPK/eNOS signaling remains unknown. These data provide some explanation to a phenomenon seen in some cases of obesity known as “adiponectin resistance”, a condition in which adiponectin’s effects are diminished [85, 86]. MPO levels are elevated in obesity [169], which could mean a decrease in the adiponectin receptor levels and therefore, decrease adiponectin signaling. Similarly, in this study, we found that treating the cells with adiponectin in the presence of MPO was not able to restore AdipoR2 expression levels as shown in **Figure 30**. However, additional studies are needed to fully understand the effects of different inflammatory stimuli. It would also be interesting to see if overexpression of adiponectin receptors would have any therapeutic effects.

Conclusion

Preclinical and clinical research has indisputably confirmed a role for MPO in cardiovascular disease. The endothelium and eNOS are now well-established cellular and molecular targets of the cardiovascular damaging actions of MPO. However, using MPO as a therapeutic target has been associated with a large number of complications and side effects, especially with the need to preserve the immunodefensive functions of MPO. Thus, identifying the downstream inflammatory pathways of MPO in endothelial cells will provide more selective pharmacological targets that would hamper the pathological activation of MPO, while preserving its innate host defense functions. Our work is the first to identify endothelial-expressed μ -calpain as a new molecular target of MPO in the vascular wall thus introducing. We also are the first to report that increased calpain activity downregulates AMPK/eNOS signaling but not PI3K/AKT signaling in endothelial cells via activation of the PP2A phosphatase. This helps explain the inhibitory effect of MPO on eNOS activity, while at the same time questions the current working model that direct catalytic consumption or nitric oxide is the main mechanism through which MPO causes endothelial dysfunction. Furthermore, we report that increased calpain activity downregulates important endothelial cell functions, thus causing adhesion of circulating white blood cells to the vascular wall. In summary, our study could provide new molecular targets in the treatment of a number of acute and chronic vascular disorders associated with metabolic disorders and chronic activation of leukocytes. **(Figure 31).**

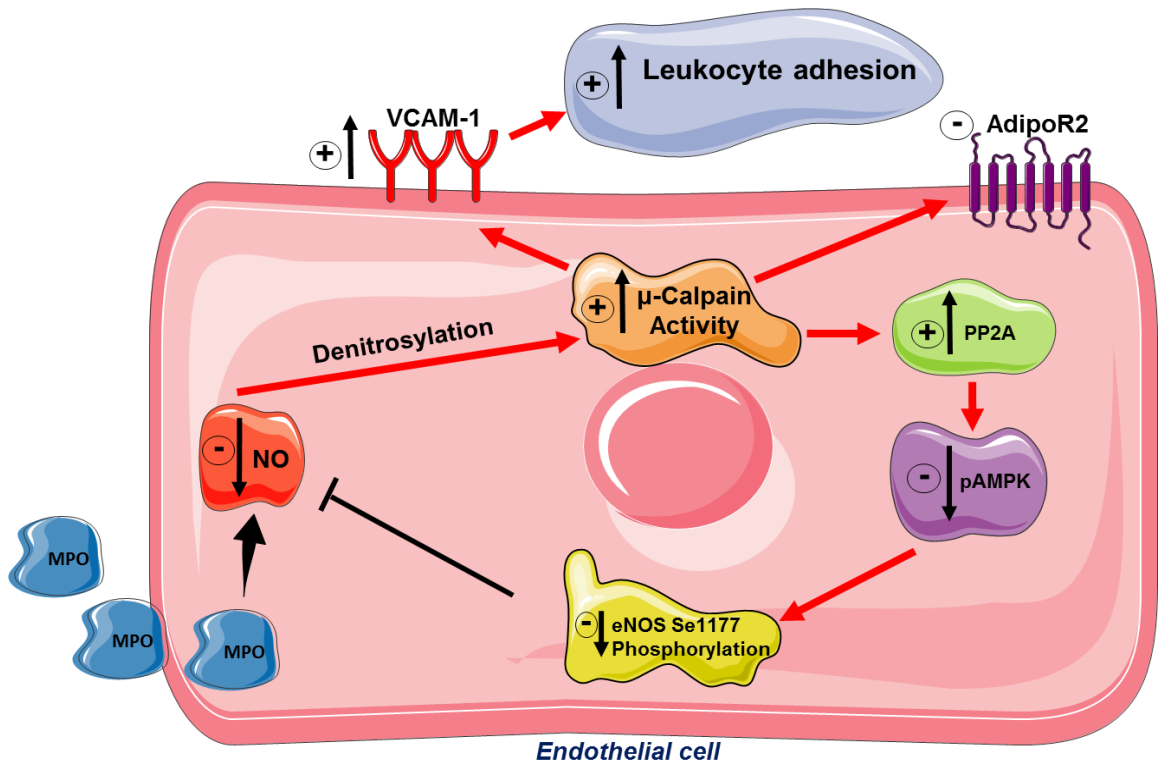


Figure 31: MPO/Calpain Signaling in Endothelial Dysfunction: our data show that MPO decreases calpain nitrosylation possibly due to decrease in NO bioavailability leading to calpain activation, and increases endothelia adhesiveness to leukocytes. Activation of calpain downregulates AMPK/eNOS signaling via PP2A. Impairment of AMPK/eNOS phosphorylation leads to further decrease in NO production which results in increased expression levels of leukocyte adhesion molecules (VCAM-1) that leads to further recruitment of leukocytes.

Future Directions

- Our study demonstrated that the possible mechanism of μ-calpain activation by MPO is through denitrosylation. However, more studies are needed to understand the specific μ-calpain sites that are denitrosylated by MPO.

- As mentioned previously both eNOS, and AKT activity are regulated by phosphorylation at different sites; therefore, we need to investigate the effects of the MPO/calpain system on the other eNOS, and AKT phosphorylation sites.
- More studies are needed to fully understand the involvement of adiponectin receptors in the MPO/Calpain system, this could be done by using siRNA AdipoR2.
- The data provided in this work are *in vitro* data. *in vivo* studies to understand the effects of elevated plasma MPO on calpain activity and downstream targets are required.
- Due to the discrepancies in the literature about the mechanisms of calpain's involvement in endothelial dysfunction under different stimuli, further studies are needed to fully dissect how different inflammatory stimuli and/or duration of inflammation affect the calpain system of leukocytes and endothelial cells.

REFERENCES CITED

1. Benjamin, E.J., et al., *Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association*. Circulation, 2017. **135**(10): p. e146-e603.
2. Lee, C.D., et al., *White blood cell count and incidence of coronary heart disease and ischemic stroke and mortality from cardiovascular disease in African-American and White men and women: atherosclerosis risk in communities study*. Am J Epidemiol, 2001. **154**(8): p. 758-64.
3. Sattler, A.R. and J.M. Olefsky, *Inflammatory mechanisms linking obesity and metabolic disease*. J Clin Invest, 2017. **127**(1): p. 1-4.
4. Sweetnam, P.M., et al., *Total and differential leukocyte counts as predictors of ischemic heart disease: the Caerphilly and Speedwell studies*. Am J Epidemiol, 1997. **145**(5): p. 416-21.
5. Munir, T.A., M.N. Afzal, and R. Habib ur, *Baseline leukocyte count and acute coronary syndrome: predictor of adverse cardiac events, long and short-term mortality and association with traditional risk factors, cardiac biomarkers and C-reactive protein*. J Ayub Med Coll Abbottabad, 2009. **21**(3): p. 46-50.
6. Dutta, P., et al., *Myocardial infarction accelerates atherosclerosis*. Nature, 2012. **487**(7407): p. 325-9.
7. Soehnlein, O. and F.K. Swirski, *Hypercholesterolemia links hematopoiesis with atherosclerosis*. Trends Endocrinol Metab, 2013. **24**(3): p. 129-36.
8. Ganda, A., et al., *Mild renal dysfunction and metabolites tied to low HDL cholesterol are associated with monocytosis and atherosclerosis*. Circulation, 2013. **127**(9): p. 988-96.
9. Haumer, M., et al., *Association of neutrophils and future cardiovascular events in patients with peripheral artery disease*. J Vasc Surg, 2005. **41**(4): p. 610-7.
10. Nauseef, W.M., *Myeloperoxidase in human neutrophil host defence*. Cell Microbiol, 2014. **16**(8): p. 1146-55.

11. Nauseef, W.M. and H.L. Malech, *Analysis of the peptide subunits of human neutrophil myeloperoxidase*. Blood, 1986. **67**(5): p. 1504-7.
12. Cowland, J.B. and N. Borregaard, *The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules*. J Leukoc Biol, 1999. **66**(6): p. 989-95.
13. Koeffler, H.P., J. Ranyard, and M. Pertcheck, *Myeloperoxidase: its structure and expression during myeloid differentiation*. Blood, 1985. **65**(2): p. 484-91.
14. Nauseef, W.M., *Posttranslational processing of a human myeloid lysosomal protein, myeloperoxidase*. Blood, 1987. **70**(4): p. 1143-50.
15. Olsson, I., et al., *The biosynthesis of neutrophil and eosinophil granule proteins*. Folia Histochem Cytobiol, 1986. **24**(2): p. 89-97.
16. Yamada, M., M. Mori, and T. Sugimura, *Myeloperoxidase-catalyzed binding of 3-amino-1-methyl-5H-pyrido[4,3-b]indole, a tryptophan pyrolysis product, to protein*. Chem Biol Interact, 1980. **33**(1): p. 19-33.
17. Babior, B.M., *Phagocytes and oxidative stress*. Am J Med, 2000. **109**(1): p. 33-44.
18. Pullar, J.M., M.C. Vissers, and C.C. Winterbourn, *Living with a killer: the effects of hypochlorous acid on mammalian cells*. IUBMB Life, 2000. **50**(4-5): p. 259-66.
19. Naskalski, J.W., J. Marcinkiewicz, and R. Drozd, *Myeloperoxidase-mediated protein oxidation: its possible biological functions*. Clin Chem Lab Med, 2002. **40**(5): p. 463-8.
20. Eiserich, J.P., et al., *Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils*. Nature, 1998. **391**(6665): p. 393-7.
21. Jiang, Q. and J.K. Hurst, *Relative chlorinating, nitrating, and oxidizing capabilities of neutrophils determined with phagocytosable probes*. J Biol Chem, 1997. **272**(52): p. 32767-72.

22. Baldus, S., et al., *Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes*. *Circulation*, 2003. **108**(12): p. 1440-5.
23. Brennan, M.L., et al., *Prognostic value of myeloperoxidase in patients with chest pain*. *N Engl J Med*, 2003. **349**(17): p. 1595-604.
24. Podrez, E.A., H.M. Abu-Soud, and S.L. Hazen, *Myeloperoxidase-generated oxidants and atherosclerosis*. *Free Radic Biol Med*, 2000. **28**(12): p. 1717-25.
25. Vita, J.A., et al., *Serum myeloperoxidase levels independently predict endothelial dysfunction in humans*. *Circulation*, 2004. **110**(9): p. 1134-9.
26. Zhang, R., et al., *Association between myeloperoxidase levels and risk of coronary artery disease*. *JAMA*, 2001. **286**(17): p. 2136-42.
27. Yang, J.J., et al., *Internalization of proteinase 3 is concomitant with endothelial cell apoptosis and internalization of myeloperoxidase with generation of intracellular oxidants*. *Am J Pathol*, 2001. **158**(2): p. 581-92.
28. Baldus, S., et al., *Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration*. *J Clin Invest*, 2001. **108**(12): p. 1759-70.
29. Astern, J.M., et al., *Myeloperoxidase interacts with endothelial cell-surface cytokeratin 1 and modulates bradykinin production by the plasma Kallikrein-Kinin system*. *Am J Pathol*, 2007. **171**(1): p. 349-60.
30. Tiruppathi, C., et al., *Albumin mediates the transcytosis of myeloperoxidase by means of caveolae in endothelial cells*. *Proc Natl Acad Sci U S A*, 2004. **101**(20): p. 7699-704.
31. Huang, P.L., *Endothelial nitric oxide synthase and endothelial dysfunction*. *Curr Hypertens Rep*, 2003. **5**(6): p. 473-80.
32. Eiserich, J.P., et al., *Myeloperoxidase, a leukocyte-derived vascular NO oxidase*. *Science*, 2002. **296**(5577): p. 2391-4.

33. Zhang, C., et al., *L-arginine chlorination products inhibit endothelial nitric oxide production*. J Biol Chem, 2001. **276**(29): p. 27159-65.
34. Rocha-Penha, L., et al., *Myeloperoxidase in Hypertensive Disorders of Pregnancy and Its Relation With Nitric Oxide*. Hypertension, 2017. **69**(6): p. 1173-1180.
35. Teng, N., et al., *The roles of myeloperoxidase in coronary artery disease and its potential implication in plaque rupture*. Redox Rep, 2017. **22**(2): p. 51-73.
36. Baldus, S., et al., *Myeloperoxidase enhances nitric oxide catabolism during myocardial ischemia and reperfusion*. Free Radic Biol Med, 2004. **37**(6): p. 902-11.
37. Asselbergs, F.W., et al., *Myeloperoxidase polymorphism related to cardiovascular events in coronary artery disease*. Am J Med, 2004. **116**(6): p. 429-30.
38. Bonetti, P.O., L.O. Lerman, and A. Lerman, *Endothelial dysfunction: a marker of atherosclerotic risk*. Arterioscler Thromb Vasc Biol, 2003. **23**(2): p. 168-75.
39. Vallance, P., *Importance of asymmetrical dimethylarginine in cardiovascular risk*. Lancet, 2001. **358**(9299): p. 2096-7.
40. Feletou, M. and P.M. Vanhoutte, *Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture)*. Am J Physiol Heart Circ Physiol, 2006. **291**(3): p. H985-1002.
41. Feletou, M., in *The Endothelium: Part 2: EDHF-Mediated Responses "The Classical Pathway"*. 2011: San Rafael (CA).
42. Moncada, S. and E.A. Higgs, *Nitric oxide and the vascular endothelium*. Handb Exp Pharmacol, 2006(176 Pt 1): p. 213-54.
43. Davignon, J. and P. Ganz, *Role of endothelial dysfunction in atherosclerosis*. Circulation, 2004. **109**(23 Suppl 1): p. III27-32.

44. Cade, W.T., *Diabetes-related microvascular and macrovascular diseases in the physical therapy setting*. Phys Ther, 2008. **88**(11): p. 1322-35.
45. Laughlin, M.H., S.C. Newcomer, and S.B. Bender, *Importance of hemodynamic forces as signals for exercise-induced changes in endothelial cell phenotype*. J Appl Physiol (1985), 2008. **104**(3): p. 588-600.
46. Taddei, S., et al., *Mechanisms of endothelial dysfunction: clinical significance and preventive non-pharmacological therapeutic strategies*. Curr Pharm Des, 2003. **9**(29): p. 2385-402.
47. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation*. Nat Rev Immunol, 2013. **13**(3): p. 159-75.
48. Krieglstein, C.F. and D.N. Granger, *Adhesion molecules and their role in vascular disease*. Am J Hypertens, 2001. **14**(6 Pt 2): p. 44S-54S.
49. Nussbaum, C., et al., *Myeloperoxidase: a leukocyte-derived protagonist of inflammation and cardiovascular disease*. Antioxid Redox Signal, 2013. **18**(6): p. 692-713.
50. Geng, J.G., *Interaction of vascular endothelial cells with leukocytes, platelets and cancer cells in inflammation, thrombosis and cancer growth and metastasis*. Acta Pharmacol Sin, 2003. **24**(12): p. 1297-300.
51. Vestweber, D. and J.E. Blanks, *Mechanisms that regulate the function of the selectins and their ligands*. Physiol Rev, 1999. **79**(1): p. 181-213.
52. Pober, J.S. and W.C. Sessa, *Evolving functions of endothelial cells in inflammation*. Nat Rev Immunol, 2007. **7**(10): p. 803-15.
53. Deanfield, J.E., J.P. Halcox, and T.J. Rabelink, *Endothelial function and dysfunction: testing and clinical relevance*. Circulation, 2007. **115**(10): p. 1285-95.
54. Matsushita, K., et al., *Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor*. Cell, 2003. **115**(2): p. 139-50.

55. Davenpeck, K.L., T.W. Gauthier, and A.M. Lefer, *Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation*. Gastroenterology, 1994. **107**(4): p. 1050-8.
56. Gauthier, T.W., K.L. Davenpeck, and A.M. Lefer, *Nitric oxide attenuates leukocyte-endothelial interaction via P-selectin in splanchnic ischemia-reperfusion*. Am J Physiol, 1994. **267**(4 Pt 1): p. G562-8.
57. Ahluwalia, A., et al., *Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment*. Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1386-91.
58. Stuehr, D.J., *Mammalian nitric oxide synthases*. Biochim Biophys Acta, 1999. **1411**(2-3): p. 217-30.
59. Sessa, W.C., *eNOS at a glance*. J Cell Sci, 2004. **117**(Pt 12): p. 2427-9.
60. Mount, P.F., B.E. Kemp, and D.A. Power, *Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation*. J Mol Cell Cardiol, 2007. **42**(2): p. 271-9.
61. Bauer, P.M., et al., *Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase*. J Biol Chem, 2003. **278**(17): p. 14841-9.
62. Fulton, D., et al., *Regulation of endothelium-derived nitric oxide production by the protein kinase Akt*. Nature, 1999. **399**(6736): p. 597-601.
63. Michell, B.J., et al., *Identification of regulatory sites of phosphorylation of the bovine endothelial nitric-oxide synthase at serine 617 and serine 635*. J Biol Chem, 2002. **277**(44): p. 42344-51.
64. De Caterina, R., et al., *Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines*. J Clin Invest, 1995. **96**(1): p. 60-8.

65. Chen, H., et al., *Adiponectin stimulates production of nitric oxide in vascular endothelial cells*. J Biol Chem, 2003. **278**(45): p. 45021-6.
66. Dimmeler, S., et al., *Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation*. Nature, 1999. **399**(6736): p. 601-5.
67. Harris, M.B., et al., *Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors*. Am J Physiol Heart Circ Physiol, 2004. **287**(2): p. H560-6.
68. Kolluru, G.K., J.H. Siamwala, and S. Chatterjee, *eNOS phosphorylation in health and disease*. Biochimie, 2010. **92**(9): p. 1186-98.
69. Mihaylova, M.M. and R.J. Shaw, *Metabolic reprogramming by class I and II histone deacetylases*. Trends Endocrinol Metab, 2013. **24**(1): p. 48-57.
70. Sanders, M.J., et al., *Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade*. Biochem J, 2007. **403**(1): p. 139-48.
71. Cacicedo, J.M., et al., *AMPK inhibits fatty acid-induced increases in NF-kappaB transactivation in cultured human umbilical vein endothelial cells*. Biochem Biophys Res Commun, 2004. **324**(4): p. 1204-9.
72. Okayasu, T., et al., *PPARalpha activators upregulate eNOS activity and inhibit cytokine-induced NF-kappaB activation through AMP-activated protein kinase activation*. Life Sci, 2008. **82**(15-16): p. 884-91.
73. Suzuki, K., et al., *Cilostazol activates AMP-activated protein kinase and restores endothelial function in diabetes*. Am J Hypertens, 2008. **21**(4): p. 451-7.
74. Hattori, Y., et al., *High molecular weight adiponectin activates AMPK and suppresses cytokine-induced NF-kappaB activation in vascular endothelial cells*. FEBS Lett, 2008. **582**(12): p. 1719-24.
75. Wu, Y., et al., *Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase*. J Biol Chem, 2007. **282**(13): p. 9777-88.

76. Mugabo, Y., Y. Mukaneza, and G. Renier, *Palmitate induces C-reactive protein expression in human aortic endothelial cells. Relevance to fatty acid-induced endothelial dysfunction*. *Metabolism*, 2011. **60**(5): p. 640-8.
77. Zhang, Y., et al., *AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress*. *Arterioscler Thromb Vasc Biol*, 2006. **26**(6): p. 1281-7.
78. Michell, B.J., et al., *Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase*. *J Biol Chem*, 2001. **276**(21): p. 17625-8.
79. Boo, Y.C., et al., *Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A*. *J Biol Chem*, 2002. **277**(5): p. 3388-96.
80. Yamauchi, T., et al., *Adiponectin receptors: a review of their structure, function and how they work*. *Best Pract Res Clin Endocrinol Metab*, 2014. **28**(1): p. 15-23.
81. Yamauchi, T., et al., *Cloning of adiponectin receptors that mediate antidiabetic metabolic effects*. *Nature*, 2003. **423**(6941): p. 762-9.
82. Yamauchi, T., et al., *Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions*. *Nat Med*, 2007. **13**(3): p. 332-9.
83. Yamauchi, T., et al., *Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase*. *Nat Med*, 2002. **8**(11): p. 1288-95.
84. Tsuchida, A., et al., *Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity*. *J Biol Chem*, 2004. **279**(29): p. 30817-22.
85. Coletta, D.K., et al., *Genome-wide linkage scan for genes influencing plasma triglyceride levels in the Veterans Administration Genetic Epidemiology Study*. *Diabetes*, 2009. **58**(1): p. 279-84.

86. Wu, Y., et al., *Genome-wide association study for adiponectin levels in Filipino women identifies CDH13 and a novel uncommon haplotype at KNG1-ADIPOQ*. Hum Mol Genet, 2010. **19**(24): p. 4955-64.
87. Hug, C., et al., *T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin*. Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10308-13.
88. Zhang, P., et al., *Overexpression of adiponectin receptors potentiates the antiinflammatory action of subeffective dose of globular adiponectin in vascular endothelial cells*. Arterioscler Thromb Vasc Biol, 2009. **29**(1): p. 67-74.
89. Bobbert, P., et al., *High molecular weight adiponectin correlates positively with myeloperoxidase in patients with type 2 diabetes mellitus*. Diabetes Res Clin Pract, 2008. **82**(2): p. 179-84.
90. Sorimachi, H. and K. Suzuki, *The structure of calpain*. J Biochem, 2001. **129**(5): p. 653-64.
91. Goll, D.E., et al., *The calpain system*. Physiol Rev, 2003. **83**(3): p. 731-801.
92. Cong, J., et al., *The role of autolysis in activity of the Ca²⁺-dependent proteinases (mu-calpain and m-calpain)*. J Biol Chem, 1989. **264**(17): p. 10096-103.
93. Suzuki, K., *Nomenclature of calcium dependent proteinase*. Biomed Biochim Acta, 1991. **50**(4-6): p. 483-4.
94. Goll, D.E., et al., *Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin?* Bioessays, 1992. **14**(8): p. 549-56.
95. Saido, T.C., K. Mizuno, and K. Suzuki, *Proteolysis of protein kinase C by calpain: effect of acidic phospholipids*. Biomed Biochim Acta, 1991. **50**(4-6): p. 485-9.
96. Saido, T.C., et al., *Positive regulation of mu-calpain action by polyphosphoinositides*. J Biol Chem, 1992. **267**(34): p. 24585-90.

97. Pontremoli, S., et al., *Isovalerylcarnitine is a specific activator of calpain of human neutrophils*. *Biochem Biophys Res Commun*, 1987. **148**(3): p. 1189-95.
98. Ma, H., et al., *Characterization and expression of calpain 10. A novel ubiquitous calpain with nuclear localization*. *J Biol Chem*, 2001. **276**(30): p. 28525-31.
99. Michetti, M., et al., *Reversible inactivation of calpain isoforms by nitric oxide*. *Biochem Biophys Res Commun*, 1995. **207**(3): p. 1009-14.
100. Murachi, T., *Intracellular regulatory system involving calpain and calpastatin*. *Biochem Int*, 1989. **18**(2): p. 263-94.
101. Otsuka, Y. and D.E. Goll, *Purification of the Ca²⁺-dependent proteinase inhibitor from bovine cardiac muscle and its interaction with the millimolar Ca²⁺-dependent proteinase*. *J Biol Chem*, 1987. **262**(12): p. 5839-51.
102. Geesink, G.H., D. Nonneman, and M. Koohmaraie, *An improved purification protocol for heart and skeletal muscle calpastatin reveals two isoforms resulting from alternative splicing*. *Arch Biochem Biophys*, 1998. **356**(1): p. 19-24.
103. Emori, Y., et al., *All four repeating domains of the endogenous inhibitor for calcium-dependent protease independently retain inhibitory activity. Expression of the cDNA fragments in Escherichia coli*. *J Biol Chem*, 1988. **263**(5): p. 2364-70.
104. Maki, M., et al., *Analysis of structure-function relationship of pig calpastatin by expression of mutated cDNAs in Escherichia coli*. *J Biol Chem*, 1988. **263**(21): p. 10254-61.
105. Kapprell, H.P. and D.E. Goll, *Effect of Ca²⁺ on binding of the calpains to calpastatin*. *J Biol Chem*, 1989. **264**(30): p. 17888-96.
106. Nishimura, T. and D.E. Goll, *Binding of calpain fragments to calpastatin*. *J Biol Chem*, 1991. **266**(18): p. 11842-50.

107. Kawasaki, H., Y. Emori, and K. Suzuki, *Calpastatin has two distinct sites for interaction with calpain--effect of calpastatin fragments on the binding of calpain to membranes*. Arch Biochem Biophys, 1993. **305**(2): p. 467-72.
108. Fujitani, K., et al., *Identification of mu-, m-calpains and calpastatin and capture of mu-calpain activation in endothelial cells*. J Cell Biochem, 1997. **66**(2): p. 197-209.
109. Averna, M., et al., *Functional role of HSP90 complexes with endothelial nitric-oxide synthase (eNOS) and calpain on nitric oxide generation in endothelial cells*. J Biol Chem, 2008. **283**(43): p. 29069-76.
110. Cuvelier, S.L., et al., *Eosinophil adhesion under flow conditions activates mechanosensitive signaling pathways in human endothelial cells*. J Exp Med, 2005. **202**(6): p. 865-76.
111. McDonald, M.C., et al., *Calpain inhibitor I reduces the activation of nuclear factor-kappaB and organ injury/dysfunction in hemorrhagic shock*. FASEB J, 2001. **15**(1): p. 171-186.
112. Stalker, T.J., Y. Gong, and R. Scalia, *The calcium-dependent protease calpain causes endothelial dysfunction in type 2 diabetes*. Diabetes, 2005. **54**(4): p. 1132-40.
113. Peltier, J., et al., *Calpain activation and secretion promote glomerular injury in experimental glomerulonephritis: evidence from calpastatin-transgenic mice*. J Am Soc Nephrol, 2006. **17**(12): p. 3415-23.
114. Rask-Madsen, C., et al., *Loss of insulin signaling in vascular endothelial cells accelerates atherosclerosis in apolipoprotein E null mice*. Cell Metab, 2010. **11**(5): p. 379-89.
115. Lorant, D.E., et al., *Activation of polymorphonuclear leukocytes reduces their adhesion to P-selectin and causes redistribution of ligands for P-selectin on their surfaces*. J Clin Invest, 1995. **96**(1): p. 171-82.

116. Scalia, R., et al., *A novel role for calpain in the endothelial dysfunction induced by activation of angiotensin II type 1 receptor signaling*. *Circ Res*, 2011. **108**(9): p. 1102-11.
117. Tsubuki, S., et al., *Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine*. *J Biochem*, 1996. **119**(3): p. 572-6.
118. Gabel, S., et al., *Protein phosphatases 1 and 2A maintain endothelial cells in a resting state, limiting the motility that is needed for the morphogenic process of angiogenesis*. *Otolaryngol Head Neck Surg*, 1999. **121**(4): p. 463-8.
119. Stabile, L.P., et al., *Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen*. *Cancer Res*, 2002. **62**(7): p. 2141-50.
120. Robaszekiewicz, A., G. Bartosz, and M. Soszynski, *Detection of 3-chlorinated tyrosine residues in human cells by flow cytometry*. *J Immunol Methods*, 2011. **369**(1-2): p. 141-5.
121. Forrester, M.T., et al., *Detection of protein S-nitrosylation with the biotin-switch technique*. *Free Radic Biol Med*, 2009. **46**(2): p. 119-26.
122. Jaffrey, S.R. and S.H. Snyder, *The biotin switch method for the detection of S-nitrosylated proteins*. *Sci STKE*, 2001. **2001**(86): p. pl1.
123. Wang, Y., S.E. Thatcher, and L.A. Cassis, *Blood Pressure Monitoring Using Radio Telemetry Method in Mice*. *Methods Mol Biol*, 2017. **1614**: p. 75-85.
124. Fong, S.W., et al., *Systemic and coronary levels of CRP, MPO, sCD40L and PlGF in patients with coronary artery disease*. *BMC Res Notes*, 2015. **8**: p. 679.
125. Yang, Y.T., M. Whiteman, and S.P. Gieseg, *HOCl causes necrotic cell death in human monocyte derived macrophages through calcium dependent calpain activation*. *Biochim Biophys Acta*, 2012. **1823**(2): p. 420-9.

126. Liu, R., et al., *Effect of protein S-nitrosylation on autolysis and catalytic ability of μ -calpain*. Food Chem, 2016. **213**: p. 470-7.
127. Chen, Z.P., et al., *AMP-activated protein kinase phosphorylation of endothelial NO synthase*. FEBS Lett, 1999. **443**(3): p. 285-9.
128. Otani, K., et al., *Inhibition of calpain results in impaired contraction-stimulated GLUT4 translocation in skeletal muscle*. Am J Physiol Endocrinol Metab, 2006. **291**(3): p. E544-8.
129. Lizcano, J.M., et al., *LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1*. Embo j, 2004. **23**(4): p. 833-43.
130. Jin, N., et al., *C-terminal truncation of GSK-3 β enhances its dephosphorylation by PP2A*. FEBS Lett, 2017. **591**(7): p. 1053-1063.
131. Liu, Z., et al., *Critical role of vascular peroxidase 1 in regulating endothelial nitric oxide synthase*. Redox Biol, 2017. **12**: p. 226-232.
132. Rabelink, T.J. and A.J. van Zonneveld, *Coupling eNOS uncoupling to the innate immune response*. Arterioscler Thromb Vasc Biol, 2006. **26**(12): p. 2585-7.
133. Zhang, W., et al., *Correlation between Calpain-10 single-nucleotide polymorphisms and obstructive sleep apnea/hypopnoea syndrome with ischemic stroke in a Chinese population: A population-based study*. Medicine (Baltimore), 2017. **96**(16): p. e6570.
134. Buraczynska, M., et al., *Calpain-10 gene polymorphisms in type 2 diabetes and its micro- and macrovascular complications*. J Diabetes Complications, 2013. **27**(1): p. 54-8.
135. De Tullio, R., et al., *Differential degradation of calpastatin by μ - and m-calpain in Ca(2+)-enriched human neuroblastoma LAN-5 cells*. FEBS Lett, 2000. **475**(1): p. 17-21.

136. Forsythe, P. and A.D. Befus, *Inhibition of calpain is a component of nitric oxide-induced down-regulation of human mast cell adhesion*. J Immunol, 2003. **170**(1): p. 287-93.
137. Koh, T.J. and J.G. Tidball, *Nitric oxide inhibits calpain-mediated proteolysis of talin in skeletal muscle cells*. Am J Physiol Cell Physiol, 2000. **279**(3): p. C806-12.
138. Du, M., et al., *Phosphorylation regulated by protein kinase A and alkaline phosphatase play positive roles in mu-calpain activity*. Food Chem, 2018. **252**: p. 33-39.
139. Cheng, Z., et al., *Hyperhomocysteinemia and hyperglycemia induce and potentiate endothelial dysfunction via mu-calpain activation*. Diabetes, 2015. **64**(3): p. 947-59.
140. Cuzzocrea, S., et al., *Calpain inhibitor I reduces the development of acute and chronic inflammation*. Am J Pathol, 2000. **157**(6): p. 2065-79.
141. Galijasevic, S., et al., *Myeloperoxidase up-regulates the catalytic activity of inducible nitric oxide synthase by preventing nitric oxide feedback inhibition*. Proc Natl Acad Sci U S A, 2003. **100**(25): p. 14766-71.
142. Kumar, A.P., et al., *Inducible nitric oxide synthase expression is inhibited by myeloperoxidase*. Nitric Oxide, 2005. **13**(1): p. 42-53.
143. Cunha, F.Q., et al., *Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF-alpha and IL-1-beta*. Immunology, 1994. **81**(2): p. 211-5.
144. Averna, M., et al., *Proteolytic degradation of nitric oxide synthase isoforms by calpain is modulated by the expression levels of HSP90*. Febs j, 2007. **274**(23): p. 6116-27.
145. Averna, M., et al., *In vivo degradation of nitric oxide synthase (NOS) and heat shock protein 90 (HSP90) by calpain is modulated by the formation of a NOS-HSP90 heterocomplex*. Febs j, 2008. **275**(10): p. 2501-11.

146. Cook, N.L., et al., *Myeloperoxidase-derived oxidants inhibit sarco/endoplasmic reticulum Ca²⁺-ATPase activity and perturb Ca²⁺ homeostasis in human coronary artery endothelial cells*. *Free Radic Biol Med*, 2012. **52**(5): p. 951-61.
147. Su, Y. and E.R. Block, *Role of calpain in hypoxic inhibition of nitric oxide synthase activity in pulmonary endothelial cells*. *Am J Physiol Lung Cell Mol Physiol*, 2000. **278**(6): p. L1204-12.
148. Stalker, T.J., C.B. Skvarka, and R. Scalia, *A novel role for calpains in the endothelial dysfunction of hyperglycemia*. *Faseb j*, 2003. **17**(11): p. 1511-3.
149. McCabe, T.J., et al., *Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation*. *J Biol Chem*, 2000. **275**(9): p. 6123-8.
150. Morrow, V.A., et al., *Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells*. *J Biol Chem*, 2003. **278**(34): p. 31629-39.
151. Tan, W.J., et al., *Calpain 1 regulates TGF-beta1-induced epithelial-mesenchymal transition in human lung epithelial cells via PI3K/Akt signaling pathway*. *Am J Transl Res*, 2017. **9**(3): p. 1402-1409.
152. Noma, H., et al., *Calpain inhibition induces activation of the distinct signalling pathways and cell migration in human monocytes*. *Immunology*, 2009. **128**(1 Suppl): p. e487-96.
153. Rao, S.S., et al., *Calpain-activated mTORC2/Akt pathway mediates airway smooth muscle remodelling in asthma*. *Clin Exp Allergy*, 2017. **47**(2): p. 176-189.
154. Sarbassov, D.D., et al., *Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex*. *Science*, 2005. **307**(5712): p. 1098-101.
155. O'Connor, C.M., et al., *Therapeutic targeting of PP2A*. *Int J Biochem Cell Biol*, 2018. **96**: p. 182-193.

156. Janssens, V., et al., *Specific regulation of protein phosphatase 2A PR72/B'' subunits by calpain*. *Biochem Biophys Res Commun*, 2009. **386**(4): p. 676-81.
157. Kurose, I., et al., *Microvascular responses to inhibition of nitric oxide production. Role of active oxidants*. *Circ Res*, 1995. **76**(1): p. 30-9.
158. Victor, V.M., et al., *Insulin Resistance in PCOS Patients Enhances Oxidative Stress and Leukocyte Adhesion: Role of Myeloperoxidase*. *PLoS One*, 2016. **11**(3): p. e0151960.
159. Prangsaengtong, O., et al., *Calpain 1 and -2 play opposite roles in cord formation of lymphatic endothelial cells via eNOS regulation*. *Hum Cell*, 2012. **25**(2): p. 36-44.
160. Marzocco, S., et al., *Calpain inhibitor I reduces intestinal ischemia-reperfusion injury in the rat*. *Shock*, 2004. **21**(1): p. 38-44.
161. Dewitt, S. and M.B. Hallett, *Cytosolic free Ca(2+) changes and calpain activation are required for beta integrin-accelerated phagocytosis by human neutrophils*. *J Cell Biol*, 2002. **159**(1): p. 181-9.
162. Wieschhaus, A., et al., *Calpain-1 knockout reveals broad effects on erythrocyte deformability and physiology*. *Biochem J*, 2012. **448**(1): p. 141-52.
163. Alvarez, A., et al., *Direct evidence of leukocyte adhesion in arterioles by angiotensin II*. *Blood*, 2004. **104**(2): p. 402-8.
164. Hussain, A.M., Q.X. Zhang, and A.G. Murray, *Endothelial cell calpain activity facilitates lymphocyte diapedesis*. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, 2005. **5**(11): p. 2640-8.
165. Dreolini, L. and F. Takei, *Activation of LFA-1 by ionomycin is independent of calpain-mediated talin cleavage*. *Biochem Biophys Res Commun*, 2007. **356**(1): p. 207-12.

166. Svensson, L., et al., *Calpain 2 controls turnover of LFA-1 adhesions on migrating T lymphocytes*. PLoS One, 2010. **5**(11): p. e15090.
167. Dewitt, S., R.J. Francis, and M.B. Hallett, *Ca(2)(+) and calpain control membrane expansion during the rapid cell spreading of neutrophils*. J Cell Sci, 2013. **126**(Pt 20): p. 4627-35.
168. Howatt, D.A., et al., *Leukocyte Calpain Deficiency Reduces Angiotensin II-Induced Inflammation and Atherosclerosis But Not Abdominal Aortic Aneurysms in Mice*. Arterioscler Thromb Vasc Biol, 2016. **36**(5): p. 835-45.
169. Rensen, S.S., et al., *Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis*. Am J Pathol, 2009. **175**(4): p. 1473-82.