THE EFFECTS OF CAVEOLIN-1 ON MITOCHONDRIAL DYNAMICS

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MASTER OF SCIENCE

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by
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ABSTRACT

Cardiovascular disease (CVD) is the leading global cause of death. Coronary Artery Disease (CAD) is a grouping of the most common cardiovascular diseases and is the current leading cause of death in developed countries. Treatments for CAD include pharmaceuticals as well as surgical interventions such as percutaneous coronary intervention (PCI) and coronary artery bypass grafting. However, these treatments do not completely remove the risk of adverse outcomes. Endothelial dysfunction is the underlying cause of CAD and is initiated by the chronic inflammation of the vasculature due to increased oxidative stress and production of reactive oxygen species (ROS). Previous studies have shown that the deletion of caveolin, a signaling molecules abundant within endothelial cells, can enhance inflammatory responses and lead to increased oxidative stress and ROS production. Mitochondrial ROS created from dysfunctional mitochondrial dynamics has also been shown to contribute to the inflammation of the endothelium. We hypothesize that due to the link between caveolin and endothelial dysfunction, and the link between mitochondria and endothelial dysfunction, caveolin has an important function in mitochondrial dynamics and that the loss of caveolin increases the mitochondrial fission via a Drp1-dependent pathway. Our data shows that adenoviral silencing of caveolin-1 in rat aortic endothelial cells increases Drp1 expression but does not significantly alter mitochondrial morphology. Overexpression of caveolin-1 via an adenoviral construct in these cells produces a decrease in Drp1 expression without altering mitochondrial morphology. This data provides insight into the pathophysiology of CAD and could provide us with new therapeutic targets in the future.

In dedication to my wonderful parents,

without whom I would not be the woman I am today.

ACKNOWLEDGMENTS

To my advisor, Dr. Rizzo: Words cannot express how thankful I am for the continual guidance and encouragement you have given me throughout my time in the lab. I will always consider you my “lab dad.”

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To my AMAZING lab: I don’t even know how to thank you!!! You have been supportive of me from the very day I started. Whether it was helping me with experiments or simply talking me through a rough day, you were always there. You are my lab family and I cannot wait to see what your wonderful and awesome things your futures hold.

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CHAPTER 1

INTRODUCTION

Heart disease is the leading global cause of death. In 2015, 17.9 million deaths were attributed to heart disease worldwide and in the United States, with cardiovascular disease (CVD) as an underlying cause accounting for 1 in 3 deaths (1). Within the broad category of cardiovascular disease fall multiple blood and vascular conditions including atherosclerosis, myocardial infarction, stroke, and heart failure, all of which if left untreated lead to death. In addition to taking a toll on global health, the cost of treating patients with these disease processes continues to rise each year. As of 2018, direct and indirect costs of CVD are approximately $329.7 billion. Although advancements in treatment and standard of care continue to improve the outlook of the CVD patient, complications of CVD frequently lead to hypertrophy and heart failure, both of which increase the risk of mortality (2).

CHAPTER 2

LITERATURE REVIEW

Coronary Artery Disease

Coronary artery disease (CAD), also known as ischemic heart disease (IHD), is a grouping of the most common cardiovascular disease processes and includes stable angina, unstable angina, myocardial infarction (MI), and sudden cardiac death. This disease is the current leading cause of death in developed countries and the percentage of patients with CAD in developing countries is on the rise. Risk factors which contribute to the development of CAD include hyperlipidemia, hypertension, diabetes, smoking, and rate of atherosclerotic lesion progression (3). Additionally, some genetic components have been identified. Multiple genetic linkage studies have implicated several loci involved in CAD (4-6). Most recently, the PROCARDIS genome-wide linkage analysis determined a like between CAD and MI in sibling pairs who have both been diagnosed with the disease, thus reaffirming evidence for a heritable component of CAD (7).

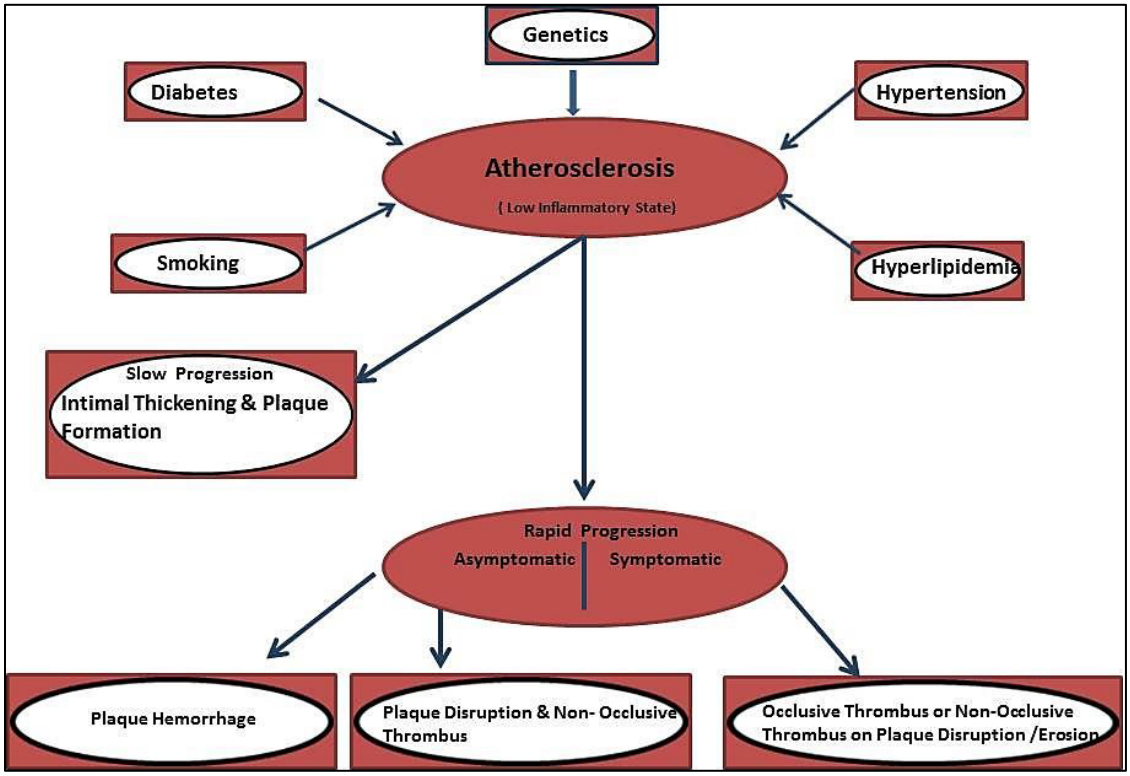

Figure 1. Risk factors contributing to the progresion of atheroslcerois and coronary artery disease (CAD) (3)

CAD begins with the development of coronary atherosclerosis, a low-grade and chronic state of inflammation of the intima of the vessels. Oxidized low density lipoprotein (oxLDL) activates NFκB signaling pathways, leading to the upregulation of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1). Expression of monocyte chemoattractant protein 1 (MCP-1) and Macrophage Colony Stimulating Factor (M-CSF) also increase, driving the recruitment of both monocytes and macrophages to the sites of oxLDL. These cells attempt to phagocytose the oxidized lipid, consequently becoming foam cells. Foam cell formation and activation and migration of smooth muscle cells (SMCs) from the vessel media into the intima produce fibrofatty lesions. Over time, these lesions become plaques of necrotic tissue covered by a fibrous cap. Rupture of the fibrous cap leads to the release of thrombogenic material

into the lumen of the vessel, in turn, causing thrombus formation, embolism, vessel occlusion, subsequent myocardial infarction, and possibly death (8).

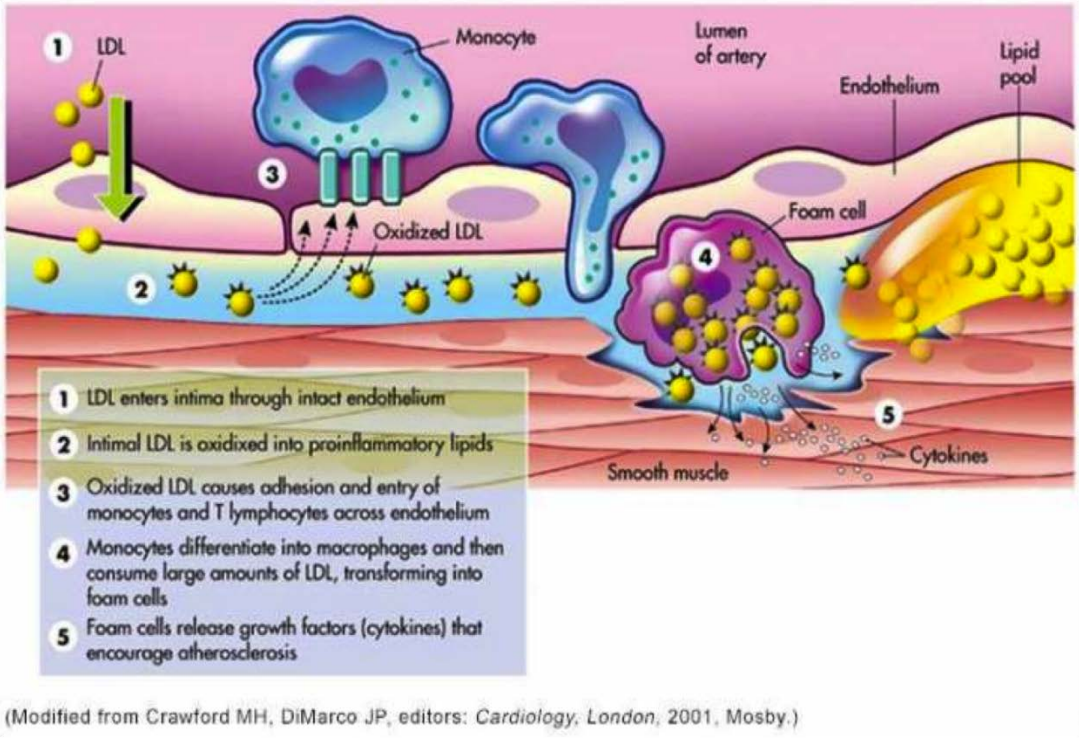

Figure 2. Development of atherosclerosis

Classical symptoms of acute coronary syndrome include chest pain, diaphoresis, back pain, shortness of breath, and dizziness. Diagnosis of coronary artery disease is based off the performed physical examination, echocardiogram (ECG), and the measurement of cardiac biomarkers (i.e. CK-MB, troponin, myoglobin) via blood samples (9). While the results of these tests provide clinicians with valuable information, the gold standard for diagnosing CAD is cardiac catheterization with angiogram, though, this test is not usually performed first due to the invasiveness of the procedure (3).

Treatment of CAD is dependent upon the severity of the disease. The main goal in treating CAD is to reduce the myocardial oxygen demand. Long term management of chronic CAD involves the use of β-blockers to reduce frequency and severity of angina and angiotensin-converting enzyme (ACE) inhibitors to decrease left ventricular afterload

as well as increase the microcirculation via vasodilation. Calcium channel blockers (CCBs) are also used to decrease myocardial oxygen demand by blocking L-type calcium channels (LTCCs) and preventing contraction of the cardiac myocytes. Nitrates are also used, albeit sparingly, to treat CAD through the induction of coronary dilation and inhibition of platelet aggregation (10). Additionally, surgical treatments can be used to treat the disease process and include percutaneous coronary intervention (PCI)—with or without stent placement—and coronary artery bypass graft (CABG). However, there are limitations to these treatments—pharmacological therapies often do not relieve patient symptoms; with PCI, there is a risk of recurrent symptoms within 6 months of therapy due to restenosis; complications with CABG can affect long term outcomes and the procedure does not prevent future MIs. Thus, further investigation into the treatment and prevention of CAD is necessary.

Endothelial Dysfunction

While the complete pathophysiology of coronary artery disease and other cardiovascular disease processes remains yet to be elucidated, dysfunction of the vascular endothelium has major role in its development. Under normal conditions, the vascular endothelium is an active organ which maintains vascular tone. The endothelium prevents the adhesion of platelets and leukocytes, inhibits smooth muscle cell migration, and acts as a barrier to low-density lipoprotein cholesterol (LDL-C) by degrading both very low-density lipoprotein cholesterol (VLDL-C) and chylotriglyceride. In response to inflammation, the vasculature no longer functions properly, and endothelial dysfunction occurs, leading to pathophysiology of cardiovascular disease.

Nitric oxide bioavailability reduces the vasodilation of the endothelium (11). Under normal conditions, NO is produced by the oxidation of L-arginine by nitric oxide

synthase (NOS). Within the endothelium, nitric oxide stimulates soluble guanylyl cyclase of smooth muscle cells to produce cyclic GMP (cGMP). Cyclic GMP activates protein kinase G (PKG), triggering the cytosolic calcium release from the sarcoplasmic reticulum. As the calcium leaves the cell, calcium-activated potassium channels open and intracellular calcium decreases. Because of this decrease, myosin light chain kinase (MLCK) can no longer phosphorylate myosin and smooth muscle cell relaxation occurs (12). Nitric oxide deficiency in cardiovascular disease is normally due to a reduction in NO precursors—such as decreased levels of L-arginine. Lower levels of L-arginine and L-citrulline, a precursor of arginine, have been found to contribute to endothelial dysfunction; supplementation of both arginine and citrulline successfully raises plasma levels of nitric oxide metabolites. This reduction in NO stimulates the upregulation of cell adhesion molecules and further propagates inflammatory signaling cascades.

In response to stressors (i.e. shear stress, acetylcholine, bradykinin, ATP), vasodilatory factors are released and activate signaling cascades that serve to regulate vascular tone and maintain normal homeostasis. Disruptions to the endothelium prevent the activation of these cascades and consequently the regulation of vascular tone. Interruptions to normal endothelial function can stem from multiple sources and lead to the upregulation of adhesion molecules such as VCAM-1 and intercellular adhesion molecule 1 (ICAM-1) (13). Increases in adhesion molecule expression lead to increased monocyte and macrophage recruitment to the vessel wall and, as a result, augmented expression of chemokines attracting inflammatory molecules such as T cells to the site of leukocyte adhesion. Additionally, reductions in nitric oxide accelerate NFκB signaling pathways by coordinating the expression of genes involved in endothelial activation. These genes are activated the most in areas of shear stress in response to inflammatory

cytokines such as IL-1β and TNF-α. These stimuli induce oxidative stress within the vasculature by increasing ROS production (14).

Elevated levels of reactive oxygen species (ROS) can occur from the xanithine oxidase, NADPH oxidase, cyclooxygenase, or eNOS uncoupling. The production of ROS by these means consequently activates inflammatory signaling pathways which, in turn, lead to the increase of related redox transcription factors and consequently the upregulation of adhesion molecules, as previously stated. Our lab has shown that caveolin-1 deficiency reduces oxidative stress and ER stress with a reduction in NADPH oxidase, a membrane-bound enzyme complex that leads to the generation of ROS.

Studies show that reduced nitric oxide availability and increased oxidative stress are related to perturbances in the plasma membrane of endothelial cells. The plasma membrane, both provides a separation of the intracellular and extracellular environment, and additionally, acts as a sensory organelle which can alter cellular physiology upon changes to these environments. The organization of the plasma membrane is vital to the function of the cell and disruptions in its subdomains have deleterious effects. Caveolae are 50-80 nm wide submicroscopic invaginations within the plasma membrane within the plasma membrane and are important signaling platforms for the cell (15). Caveolae contain the integral proteins termed, caveolins, which directly interact with nitric oxide synthase to form a caveolin-eNOS complex. Mice deficient in caveolin are known to have certain cardiovascular disease pathologies such pulmonary hypertension and dilated cardiomyopathies (16). Cassuto et. Al shows that caveolin-1 levels are decreased in diabetic patients with known endothelial dysfunction due to increased levels of the reactive nitrogen species (RNS) molecule, peroxynitrite ($ONOO^-$) (17). Implications of caveolae and caveolins in cardiovascular disease demonstrate the importance of research into this area.

Caveolae and Caveolins

While seemingly small alone, lipid rafts constitute a large percentage of the plasma membrane. Within this subdomain is a specialized microdomain: caveolae. While these organelles are almost completely absent in some cells such as those of the kidney, in others—such as endothelial cells and adipocytes—they represent up to 50% of the plasma membrane. Key signaling molecules of caveolae include caveolin and cavins; without the joint effort of the two proteins, caveolae formation does not occur.

Caveolin: Structure and Function

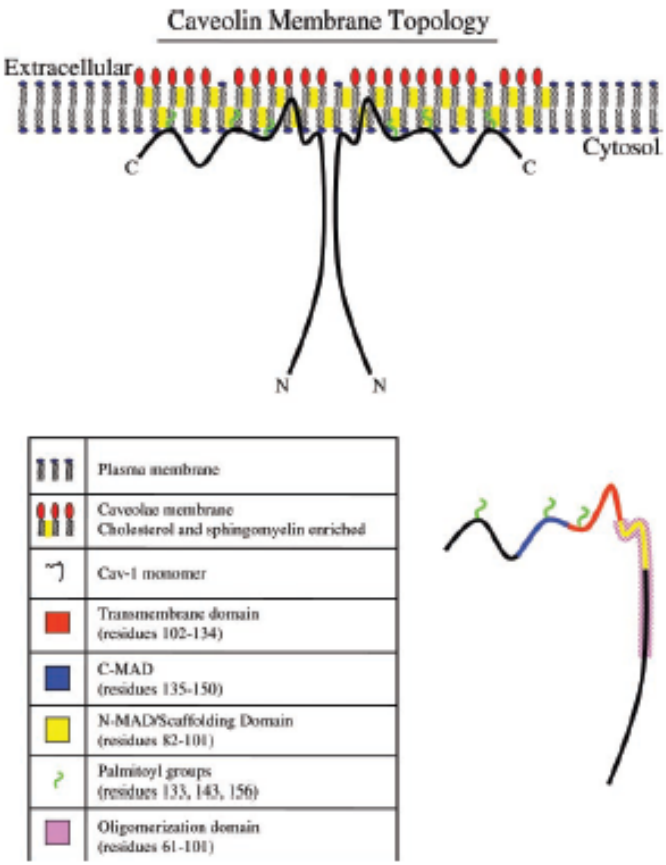

Figure 3. Caveolin proteins consist of four main signaling domains. Transmembrane domain (TMD), caveolin-scaffolding domain (CSD)/N-terminal domain, oligomerization domain, and C-terminal domain

Caveolin (Cav) are ubiquitously expressed structural proteins that are crucial for the formation of caveolae. This protein is critical to the curved arrangement of the caveolae and interacts with a myriad of other signaling molecules which encounter the plasma membrane. There are three mammalian variations of caveolin: Cav-1, Cav-2, and Cav-3. Caveolins 1 and 2 are generally expressed together in cells other than skeletal muscle while Cav-3 is exclusively expressed in skeletal, cardiac and some smooth muscle cells. While the expression of Cav-2 appears to be dependent on Cav-1 expression, Cav-2 deletion is still known to produce deleterious effects such as inducing a pulmonary hypertensive phenotype (18).

Caveolin proteins consist of four main signaling domains: a C-terminal domain, a transmembrane domain (TMD), a caveolin scaffolding domain (CSD), and an N-terminal domain. The C-terminus of the protein includes three palmitoylated cysteines which are important in assisting oligomerization and protein stability. The transmembrane domain (TMD) is a hydrophobic hairpin loop within the cytoplasmic face of the plasma membrane. The CSD is required for oligomerization of caveolin with various proteins; this domain can inhibit the catalytic activity of multiple signaling molecules (i.e. eNOS, heme oxygenase) by binding to the caveolin binding motif (CBM). The oligomerization domain overlaps with the CSD; this domain assists the caveolin protein with homo- and hetero-oligomerization. Caveolins 1 and 2 form homo- or hetero-oligomers while Cav-3 is only able to form hetero-oligomers with Cav-1.

Caveolin and Endocytic/Membrane Trafficking

The budding of caveolae from the plasma membrane is driven by GTP hydrolysis and conducted by the small GTPase, dynamin. In the endothelia, caveolae bud from the luminal surface of the membrane to fuse with abluminal surface of the membrane. This assists trans-endothelial transport from the blood stream to the tissues. In other cell types, the caveolae fuse with early or late endosomes and become 'caveosomes.' At this point, the components of the caveolae can be recycled during endocytosis. This process mediates caveolin trafficking back to the surface of the plasma membrane to continue the propagation of signal transduction.

The trafficking of non-caveolar is much different from that of caveolar trafficking in that it requires the monoubiquitination of caveolin and might possibly regulate its degradation. This pathway includes a myriad of different proteins including valosin-containing protein (VCP), which binds to monoubiquitinated caveolin in a complex along

with UBX domain-containing protein 1 (UBXD1). The binding of these proteins allows the incorporation of caveolin into microvesicles. This pathway mediates the lysosomal degradation of caveolins, more specifically Cav-1 and Cav-3, and assists in managing the recycling of caveolae components, as well as the removal of unneeded caveolin (19).

Caveolin proteins are most well known for their actions as scaffolding proteins and their facilitations of a multitude of protein signaling pathways. Caveolins are synthesized in the endoplasmic reticulum (ER), where they then undergo stabilization via the binding of cholesterol. After exiting the ER, caveolin is transported to the Golgi complex by coat protein II vesicles, where they form oligomers of higher molecular weights and can then be incorporated into lipid rafts and consequently, exit the plasma membrane of the cell.

Caveolae and Caveolins: Interactions with Other Organelles

Caveolin deficiency has been implemented in multiple disease processes including cancer, cardiovascular disease, diabetes, lipodystrophy, and pulmonary fibrosis. Our lab continues to investigate the effects of caveolin-1 deletion on cardiovascular disease processes. Not all mechanisms underlying cardiovascular pathologies have been elucidated; however, the interactions that both caveolae and caveolin have with other organelles in the cell can assist in explaining some of the phenomena seen and described.

Nucleus

Caveolin localized to the nucleus is thought to play a role in gene regulation and signaling. In the cell, Vascular Endothelial Growth Factor (VEGF) stimulates cell growth and migration via the activation of endothelial Nitric Oxide Synthase (eNOS). In the nucleus, eNOS colocalizes with Cav-1 within the caveolae after myristoylation and

palmitoylation. eNOS is regulated by its direct interaction with Cav-1 and the binding of calmodulin to eNOS will displace the enzyme from caveolae and activate the molecule. Upon activation, both eNOS and caveolin translocate to the nucleus; thus, the actions of eNOS and caveolin are thought to be one of many mechanisms to control nitric oxide production and gene activation.

Additionally, caveolins are thought to be involved in the regulation of the cell cycle. Caveolin-1 expression downregulates the expression of Cyclin D1 through the upregulation of p53 and p21. Caveolin-1 can also prevent Cyclin D1 expression by downregulating the signaling pathways which lead to its expression (i.e. MAP kinase, PI3 Kinase, and Wnt pathways) (20).

Endoplasmic Reticulum (ER) and Golgi Network

As the ER is the organelle responsible for the synthesis of cholesterol, it is no surprise that caveolin is involved. Newly synthesized cholesterol is transported from the ER to caveolae and distributed to other areas of the plasma membrane as necessary; this movement occurs within minutes of cholesterol synthesis. This dispersal of cholesterol is dependent upon caveolin. Cholesterol oxidation leads to caveolin translocation to the ER and eventually to the Golgi apparatus, where it collects in the presence of cholesterol. After cholesterol oxidase is removed from the Golgi, caveolin once again returns to the cell surface and to the plasma membrane.

Our lab has found that Cav-1 is a critical mediator of vascular remodeling and that silencing has a protective effect against increases in vascular remodeling as well as collagen synthesis and hypertrophy (21). We have shown that caveolin-1 depletion decreases oxidative stress and ER stress in abdominal aortic aneurysm (AAA) (22). Both oxidative stress and ER stress are involved in AAA establishment, continuing formation,

and rupture; additional studies show that pharmacological ER stress inhibition is effective in preventing AAA formation (23).

Mitochondria

The spatial relationship between caveolae and mitochondria has been highlighted for years through electron microscope imagery. Mitochondria and caveolae share some of the same structural proteins; this explains the proximity seen in many images between the two structures. Functional studies examining the relationship between caveolae and mitochondria show an increase in oxidative stress and ROS production when caveolin-1 is depleted. When both Cav-1 and adiponectin—a key signaling molecule which regulates multiple metabolic processes—are deleted, the increase in oxidative stress is even more evident (24).

While caveolin deficient mice appear relatively normal, it is known that compared to wild-type (WT) mice, these animals are lean and much more resistant to developing diet-induced obesity. Additionally, caveolin knockout (KO) mice are prone to developing lipodystrophy, cancer, and cardiovascular disease (CVD) among other disorders. It is hypothesized that, due to the lack of caveolin, cholesterol transport and homeostasis in the mitochondria, an already cholesterol-poor organelle, is disrupted. This, in turn, interrupts proper metabolic function. For example, mitochondrial-associated membranes (MAMs) are extensions of the ER which assist in signaling between the ER and the mitochondrion. Recent studies suggest caveolin indirectly regulates mitochondrial cholesterol by controlling its influx and efflux from the ER (25). Without caveolin, excess cholesterol accumulates within the mitochondrial outer membrane which reduces fluidity, signal transduction, and overall metabolism, subsequently increasing the output of ROS from the cell. Conversely, Wang et al.

demonstrate that increased translocation of caveolae to the mitochondria have been shown to increase mitochondrial structural stability and function, which serves to promote healthy levels of oxidative stress (26). Due to the conflicting data in the literature, it is imperative that more research is done to pinpoint the actions of caveolin-1 in the endothelium.

Mitochondrial Fusion and Fission in Cardiovascular Disease

Mitochondrial fusion occurs in environments of increased ATP demand and leads to elongated and interconnected mitochondria. Fusion provides a more uniform mitochondrion by integrating the genetic components of two mitochondria. Fusion in mammals is controlled by outer membrane proteins mitofusins 1 and 2 (MFN1/2), along with inner mitochondrial membrane protein optic atrophy 1 (OPA1). MFN1 and MFN2 assist with the fusion of the outer mitochondrial membrane by heterodimerization or homodimerization of MFN2. MFN1 and MFN2 are essential for fusion and cells lacking these proteins have significantly decreased mitochondrial fusion as well as mitochondrial membrane potential (27). Inner membrane fusion is mediated by OPA1 through mRNA splicing and proteolytic cleavage (28). Without OPA1, mitochondrial fusion is superficial and only the outer mitochondrial membrane fuses.

Mitochondrial fission is controlled by the large GTPase dynamin related protein 1 (Drp1), mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial dynamic proteins of 49 and 51 kDa (MiD49/51). The process of fission degrades non-reusable mitochondrial material and distributes mitochondria during mitosis. This process is also necessary during apoptosis when the release of cytochrome c causes cell death.

Impairment of or excessive mitochondrial fusion and fission along with pathological changes results in abnormalities of the vasculature. When cells are exposed to such pathological conditions, abnormal mitochondrial ROS formation can occur. This response to impairment of normal function can lead to cellular damage. Mitochondrial ROS formation has also been shown to be linked to mitochondrial membrane potential and oxidative mitochondrial metabolism. Many of the risk factors of cardiovascular disease also affect mitochondrial metabolism, two of which include hyperglycemia and hypoxia. Continued unbalanced ROS production leads to oxidative damage in many parts of the cell; this induces further ROS production, exacerbating stresses on the already dysfunctional mitochondria. Additionally, superoxide produced from excessive ROS reacts with nitric oxide, reducing its bioavailability. This increases NOX uncoupling and ROS production. As previously stated, ROS uncoupling leads to further propagation of inflammatory signals through the production of harmful cytokines such as IL-1β, TNF-α, and IL-6

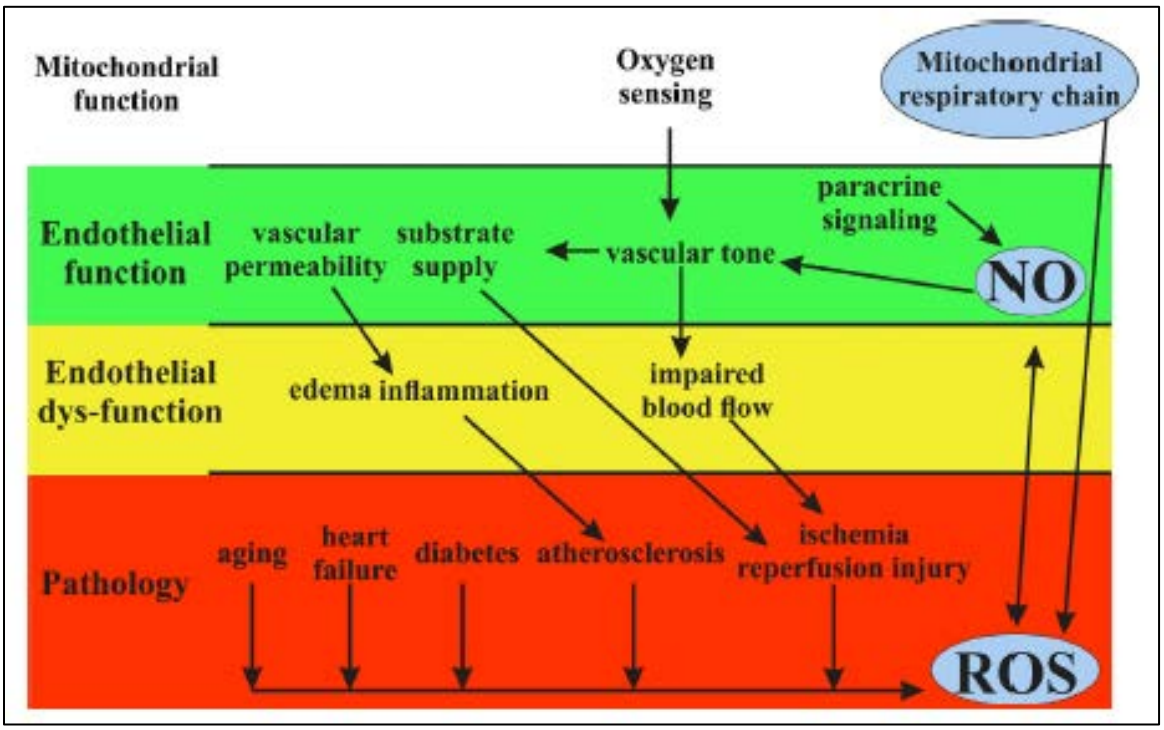

Figure 4. The influence of mitochondrial function on the endothelium (29)

Mitochondrial dynamics has many implications within the pathologies of cardiac hypertrophy and heart failure. When the heart is unable to provide adequate cardiac output to meet the demands of the body, compensatory mechanisms are triggered; one of the main mechanisms an increase in size of cardiac myocytes, termed cardiac hypertrophy. Cardiac hypertrophy can be physiological or pathological, the first of which is harmful to the cardiovascular system. Pathological hypertrophy is a risk factor for heart failure, arrhythmia, and death. Studies show that OPA1 expression is decreased in both human and rat ischemic heart failure while MFN1, MFN2, Fis1, and Drp1 remained unchanged. Explanted hearts with dilated non-ischemic cardiomyopathy showed no OPA1 decrease but did show reduced expression of MFN1, MFN2, and DRP1 (30). Pennanen et al. have reported that norepinephrine can trigger mitochondrial fission in a Drp1-dependent manner and that adenoviral silencing of Drp1 prevents mitochondrial fission and hypertrophic growth (31).

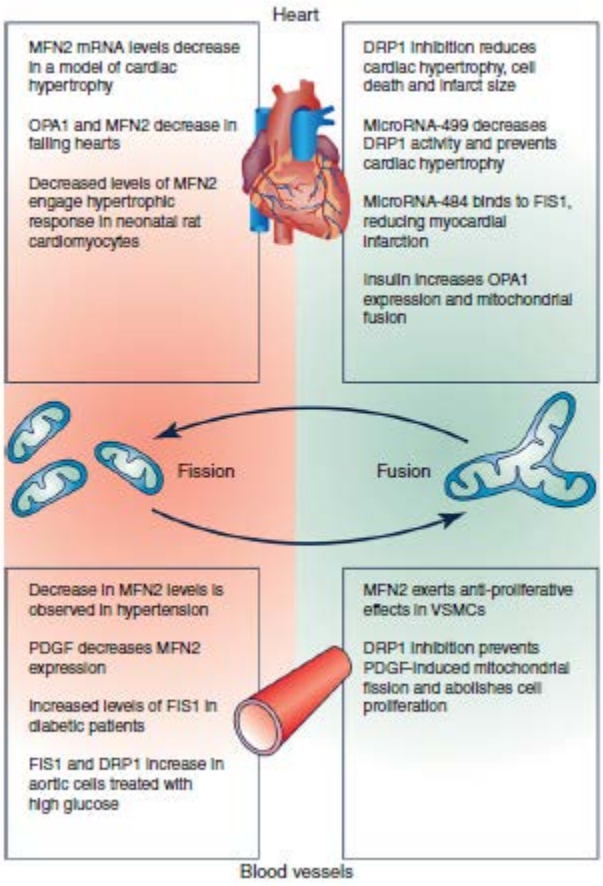

Figure 5. A summary of mitochondrial fusion and fission events associated with cardiovascular disease pathologies (32)

CHAPTER 3

HYPOTHESIS

The literature shows a link between caveolin and endothelial dysfunction as well as mitochondria and endothelial dysfunction. Additionally, mitochondria and caveolae are in close spatial proximity. It has previously been shown that this caveolae-mitochondria interaction regulates cellular adaptation to stress via the modulation of mitochondrial structure and function (33). Thus, we hypothesize that caveolin-1 has an important function in mitochondrial dynamics, namely fission and fusion. Loss of caveolin-1 increases Drp1 and consequently, mitochondrial fragmentation.

CHAPTER 4

RESULTS

Determination of Cav-1 silencing efficiency

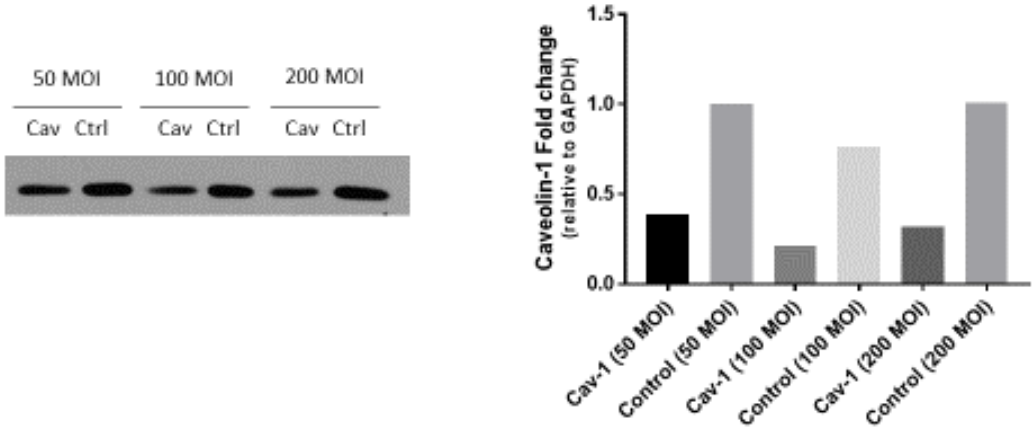

Figure 6. MOIs of 50, 100, and 200 were used to determine Caveolin-1 AV silencing efficiency. Data was analyzed via WB and densitometry was completed using ImageJ software.

To study the effects of Cav-1 deletion on mitochondrial morphology, we infected RAECs with either a control-miR adenoviral construct or a Cav-1 silencing adenoviral construct. Knockdown efficiency was determined via western blot. After infection, RAECs were stimulated with TNF-α at 6h and 3h. Compared to unstimulated control AV infected cells, Cav-1 silenced cells appeared more fragmented and less interconnected at 0h.

Cav-1 deficiency alters mitochondrial morphology and increases mitochondrial fragmentation

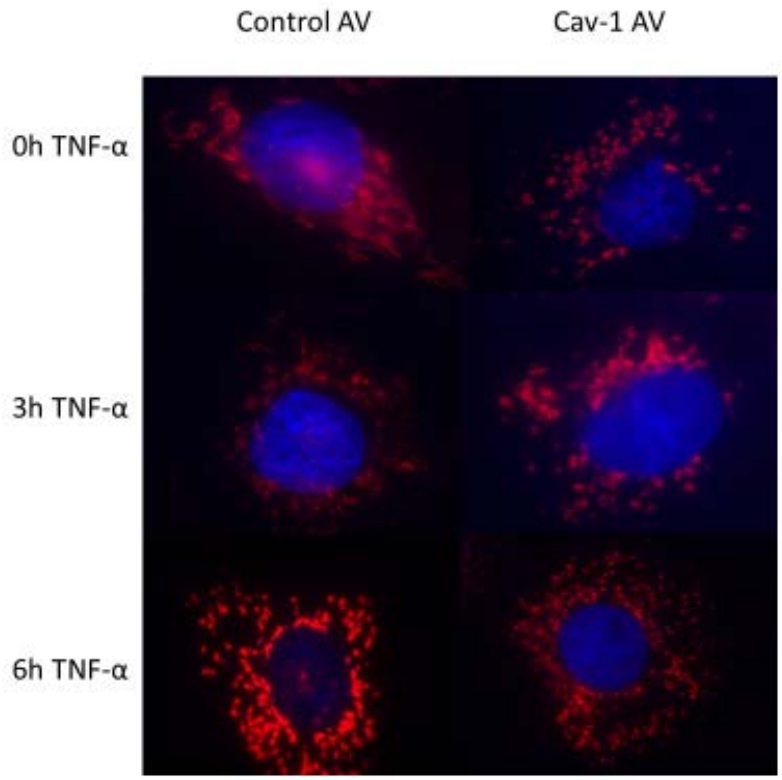

Figure 7. Representative images of control AV and Cav-1 silenced RAECs infected with mito-DS Red and stained with dapi.

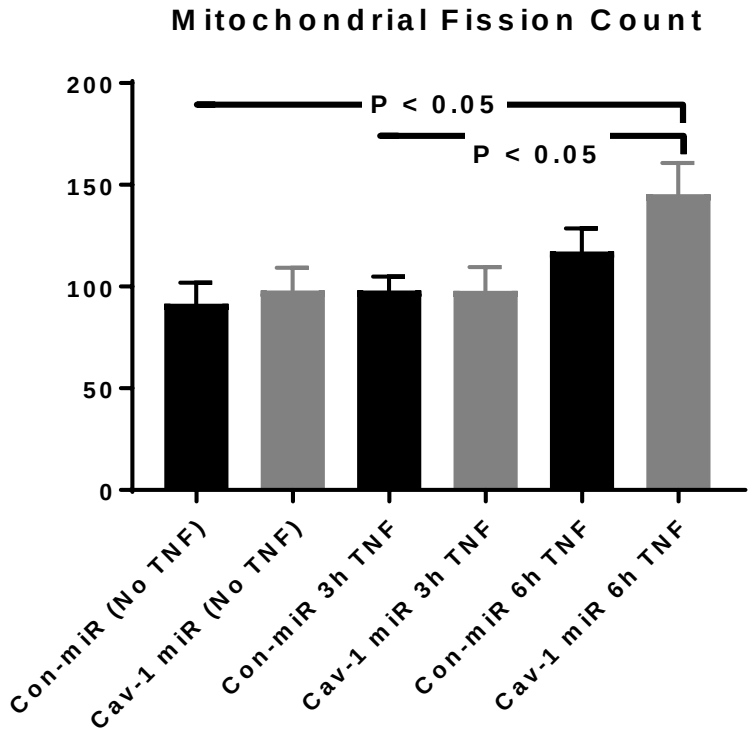

Figure 8. Representative analysis of mitochondrial fission count in control AV infected cells compared to Cav-1 silenced cells. Mitochondrial images were processed using ImageJ iterative deconvolution software and averages were taken along with S.E.M measurements.

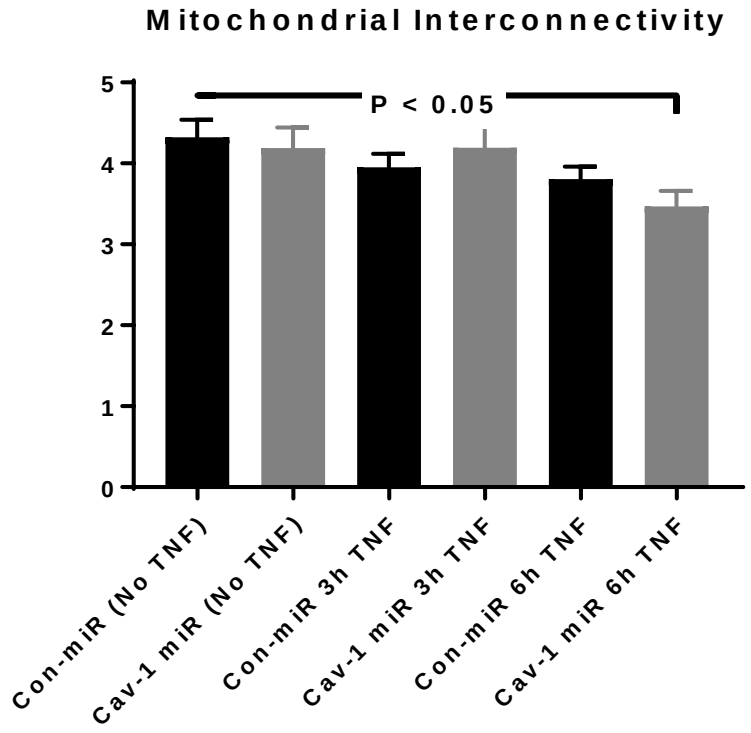

Figure 9. Representative analysis of mitochondrial interconnectivity in control AV infected cells compared to Cav-1 silenced cells. Mitochondrial images were processed using ImageJ iterative deconvolution software and averages were taken along with S.E.M measurements.

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Cav-1 deficiency increases levels of total Drp1

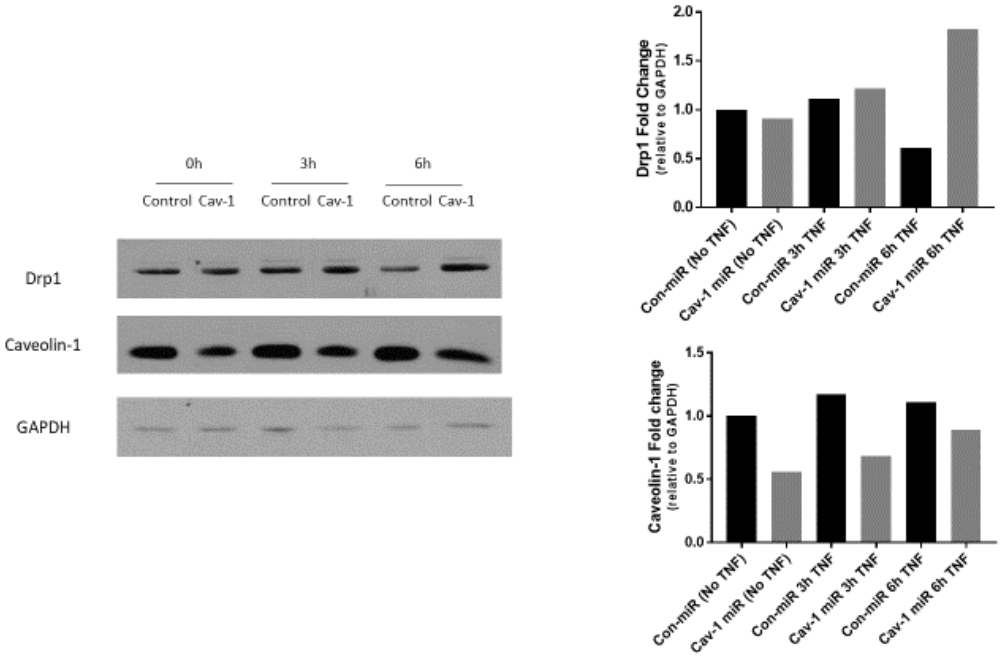

Figure 10. Representative western blot images of Drp1 and Cav-1 levels in control AV infected RAECs compared to Cav-1 silenced RAECs. Densitometry of western blot is shown (right) relative to GAPDH.

Next, we wanted to determine if the observed mitochondrial fragmentation was due to the disruption of normal mitochondrial fusion and fission. Increased levels of Drp1 expression were analyzed via western blot in caveolin silenced RAECs compared to control infected RAECs. Statistical significance was analyzed using ANOVA.

Overexpression of Cav-1 does not alter mitochondrial morphology

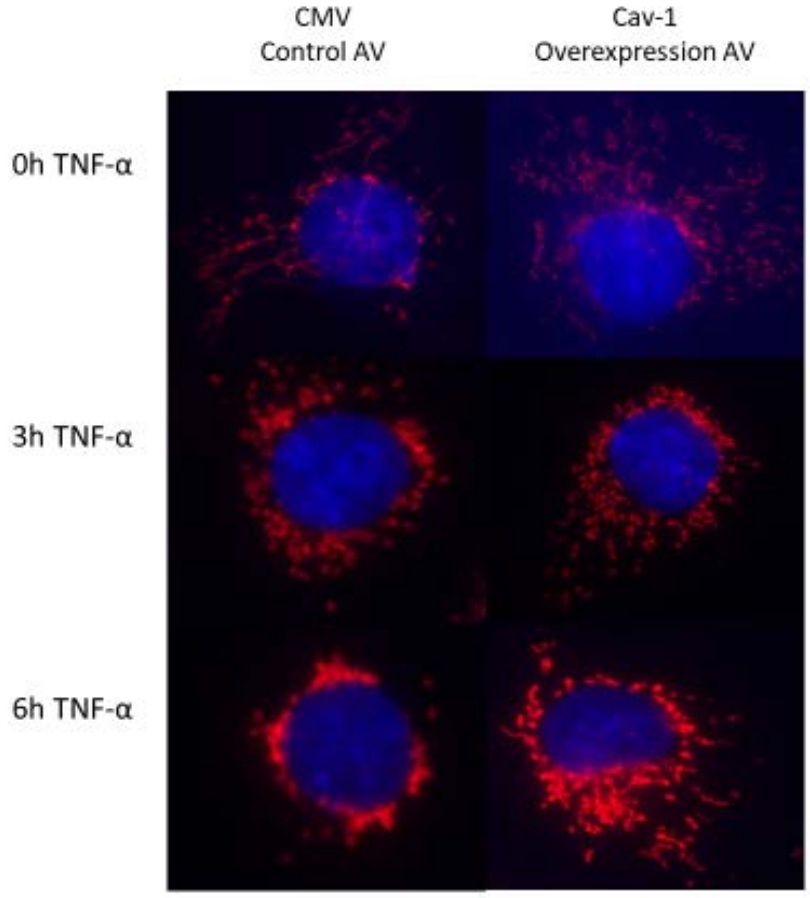

Figure 11. Representative images of control CMV and Cav-1 overexpressed RAECs infected with mito-DS Red and stained with Dapi.

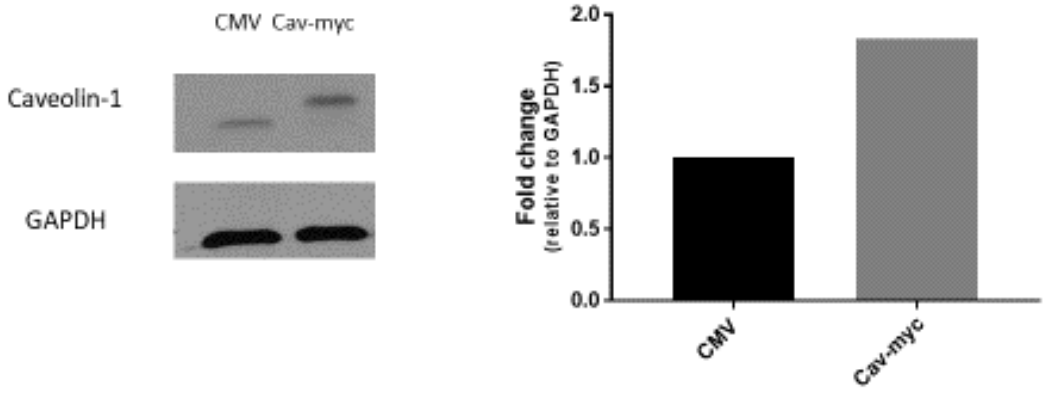

Figure 12. Caveolin-1 overexpression efficiency determination by western blot analysis and densitometry analysis using ImageJ. Cells were treated infected at 50 MOI of either Control CMV or Cav-myc caveolin-1 overexpressing vector for 48h after 24h serum starvation.

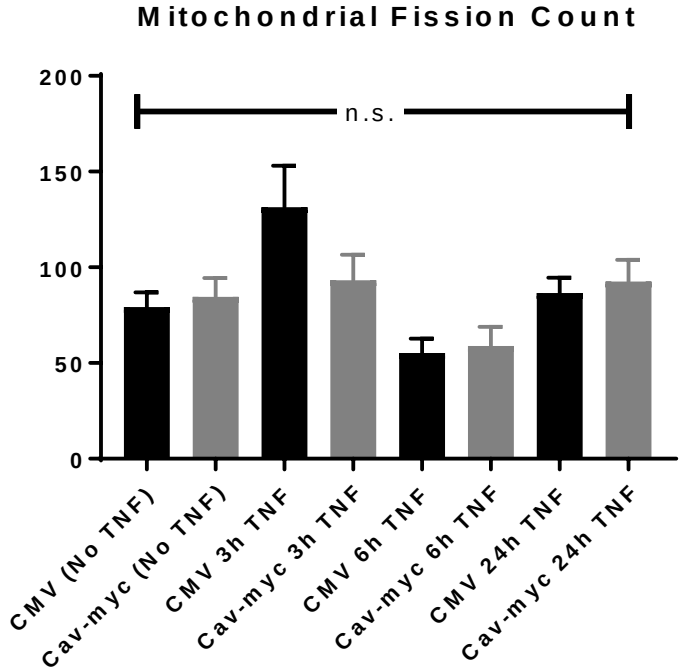

Figure 13. Representative analysis of mitochondrial fission count in control CMV infected cells compared to Cav-1 overexpressed cells. Mitochondrial images were processed using ImageJ iterative deconvolution software and averages were taken along with S.E.M measurements

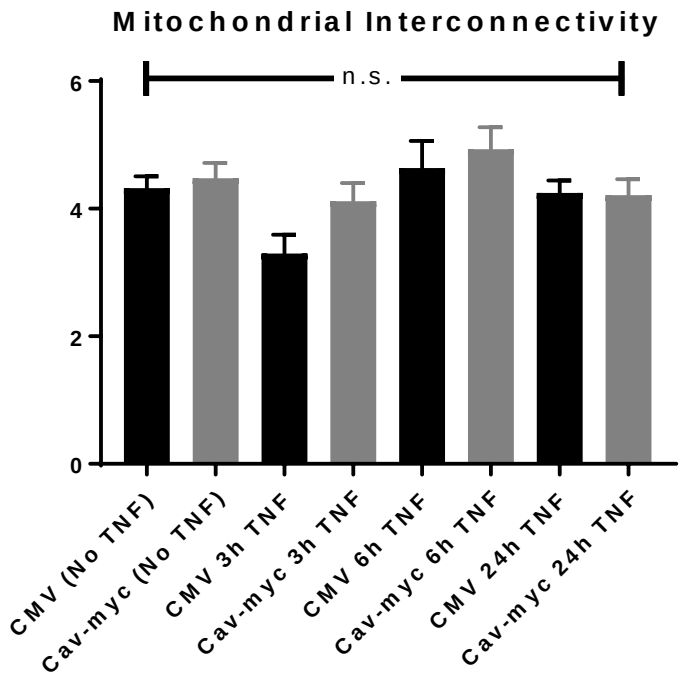

Figure 14. Representative analysis of mitochondrial interconnectivity in control CMV infected cells compared to Cav-1 overexpressed cells. Mitochondrial images were processed using ImageJ iterative deconvolution software and averages were taken along with S.E.M measurements

To determine an opposing viewpoint, we infected RAECs with an adenoviral construct overexpressing Cav-1; efficiency was determined via western blot. After infection, cells were again stimulated with TNF-α at 3h and 6h. Mitochondrial morphology was then evaluated. Statistical significance was determined using ANOVA.

Overexpression of Cav-1 decreases levels of Drp1

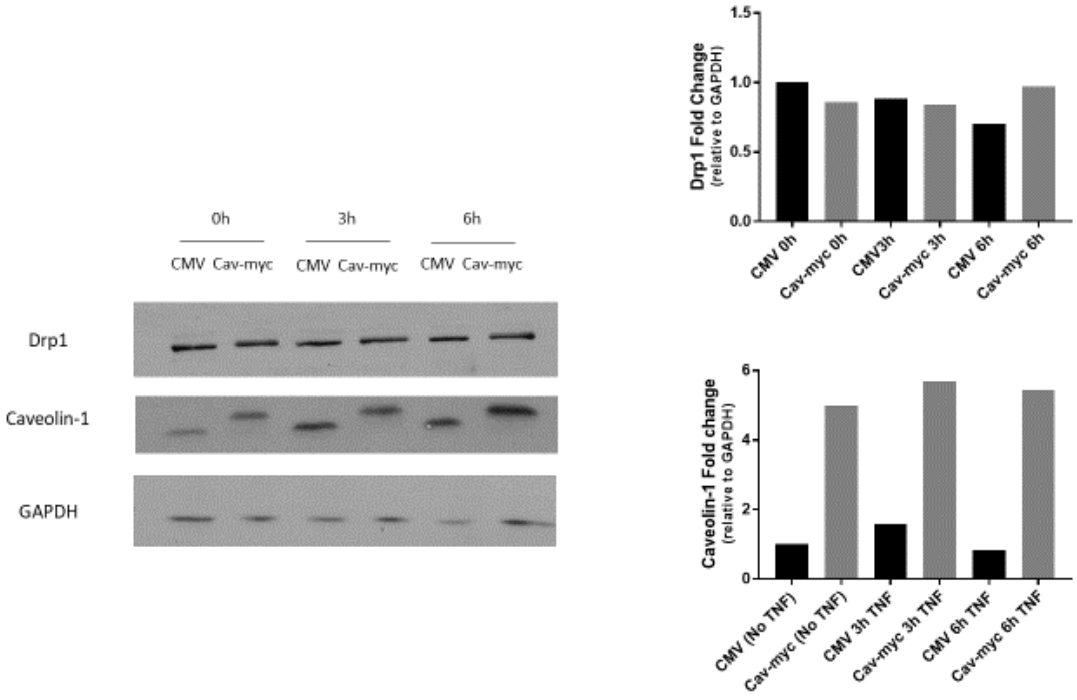

Figure 15: Representative western blot images of Drp1 and Cav-1 levels in CMV control AV infected RAECs compared to Cav-1 overexpressing RAECs. Densitometry of western blot is shown relative to GAPDH.

After noting a difference in mitochondrial morphology between caveolin-1 silenced cells and caveolin-1 overexpressing cells we wanted to discern if these differences were once again due to disrupted mitochondrial dynamics. Statistical significance was determined using ANOVA.

CHAPTER 5

DISCUSSION

Our data shows an increase in Drp1 expression when endothelial cells are infected with a caveolin-1 silencing miRNA. Analysis of samples by western blot does not show much of a decrease in caveolin-1 expression after treatment with the silencing miRNA. This can be due to the abundance of Cav-1 in endothelial cells or the long half-life of the protein (>24h). Drp1 is a GTPase that regulates mitochondrial fission. Though an increase in expression is seen after TNF stimulation in caveolin-1 silenced endothelial cells, mitochondrial fragmentation and interconnectivity analysis show no significance between control infected cells and caveolin-1 adenovirus infected cells. This leads us to believe that there is an accumulation of Drp1 before morphological changes occur; once enough Drp1 has been produced, mitochondrial fragmentation begins to occur. Longer timepoints are needed to examine this hypothesis.

Our data also shows that Drp1 expression is decreased with the overexpression of caveolin-1. Analysis of samples by western blot shows a clear increase in caveolin-1, indicating the efficiency of the virus. This data matches our previous data which shows an increase in Drp1 expression and strengthens our hypothesis. Because caveolin-1 is abundant in many signaling pathways, overexpression of the protein can either dampen signaling or augment signaling. In order to elucidate which process is happening, further studies need to be performed. Also, because Drp1 is an early step in the mitochondrial fission process, once again, our time points may not be long enough to give adequate

information, especially since cardiovascular disease processes include chronic inflammation.

During endothelial dysfunction, inflammatory stimuli are chronically produced. In vitro, TNF-α stimulation is given at 3 hours and 6 hours. While this provides us with important information about the inflammatory process, it is not completely physiologically relevant. Longer time courses are needed to determine more true effects of Cav-1 silencing or overexpression on the endothelium. Chronic inflammation leads to the production of other stimuli such as IL-1β or IFN-γ. Varghese et al. showed an increase in the release of IL-1β in human coronary plaques in comparison to normal arteries (34). In the atherosclerotic plaque, IFN-γ is released by T-lymphocytes which decreases the production of collagen by smooth muscle cells and prevents smooth muscle cell proliferation. IFN-γ also acts on macrophages and augments their production of the collagen-degrading enzymes, matrix metalloproteinases (MMPs). Analysis of the effects of both cytokines on the vascular endothelium at various stages of inflammation with and without caveolin-1 silencing or overexpression can provide important information on treating and preventing endothelial dysfunction.

CHAPTER 6

CONCLUSION

Our data has shown an increase in levels of the mechanoenzyme Drp1 in caveolin-1 silenced endothelial cells as well as a decrease in the protein in endothelial cells overexpressing caveolin-1. Despite mitochondrial morphology changes not being statistically significant, this data provides us with additional information regarding the relationship between caveolin-1 signaling and mitochondrial dysfunction. More comprehensive studies need to be performed to obtain a more complete picture at the steps taken.

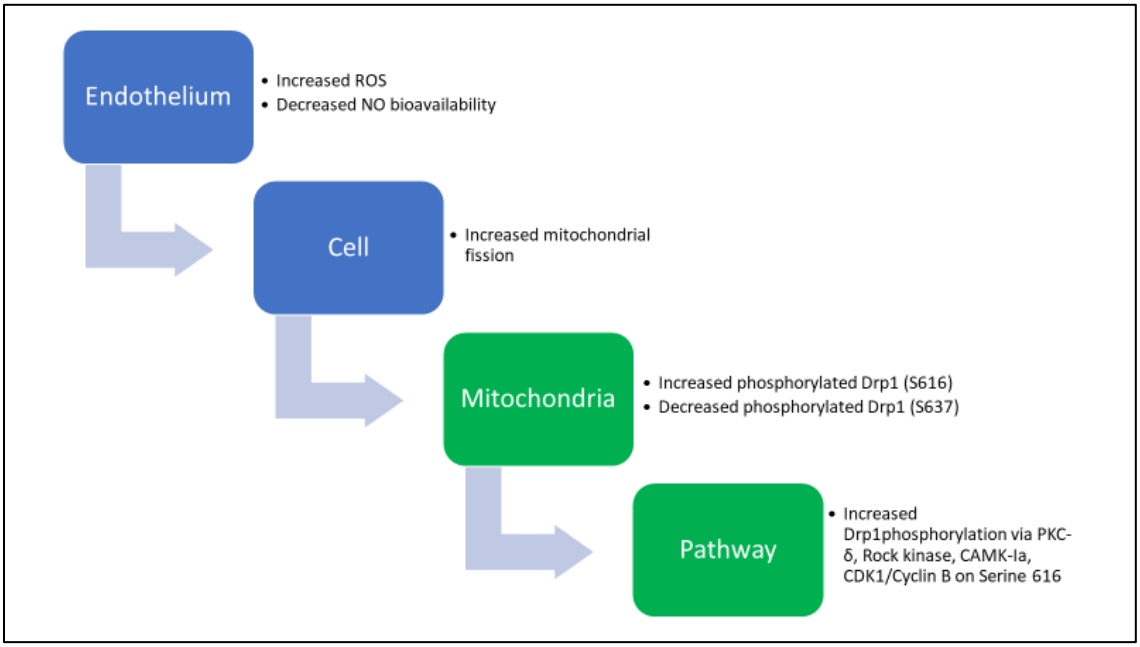

Figure 16. Proposed mechanism describing the effects of caveolin silencing on mitochondrial dynamics in endothelial dysfunction. The data shows us that increased inflammation of the endothelium contributes to mitochondrial fission (blue); this could be due to increased phosphorylation of Drp1 at Ser616, a site which when phosphorylated is known to activation fission, by a multitude of kinases (green).

Tetramethylrhodamine (TMRE) experiments in endothelial cells will provide our lab with data related to changes in mitochondrial membrane potential. Mitochondria provide the majority of the cell's ATP through oxidative phosphorylation which involves the transfer of positively charged proteins across the inner membrane of the mitochondrion, creating mitochondrial membrane potential. Energy from the resulting proton gradient is used to produce ATP by combining ADP with free phosphate (35). TMRE accumulates in the mitochondria due to the relative negative charge. Decreased sequestration of TMRE by the mitochondrion of caveolin-1 silenced or overexpressing cells means there is decreased mitochondrial membrane potential.

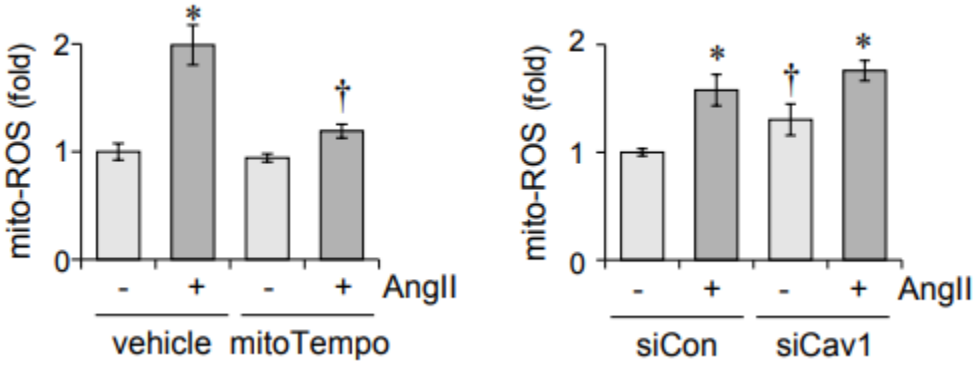

Figure 17: Cav-1 silencing does not block angiotensin-II induced mitochondrial ROS production in VSMCs (21)

Measuring mitochondrial superoxide production will provide us with beneficial information. We have previously shown that Cav-1 deletion does not decrease mito-ROS production in VSMCs but have not yet determined if the same is true in ECs (21). Staining cells with MitoSox fluorescent dye will provide us with this data. This reagent selectively permeates live cells and is oxidized by superoxide only. Upon binding to nucleic acid, the dye fluoresces, allowing for visualization using fluorescent microscopy.

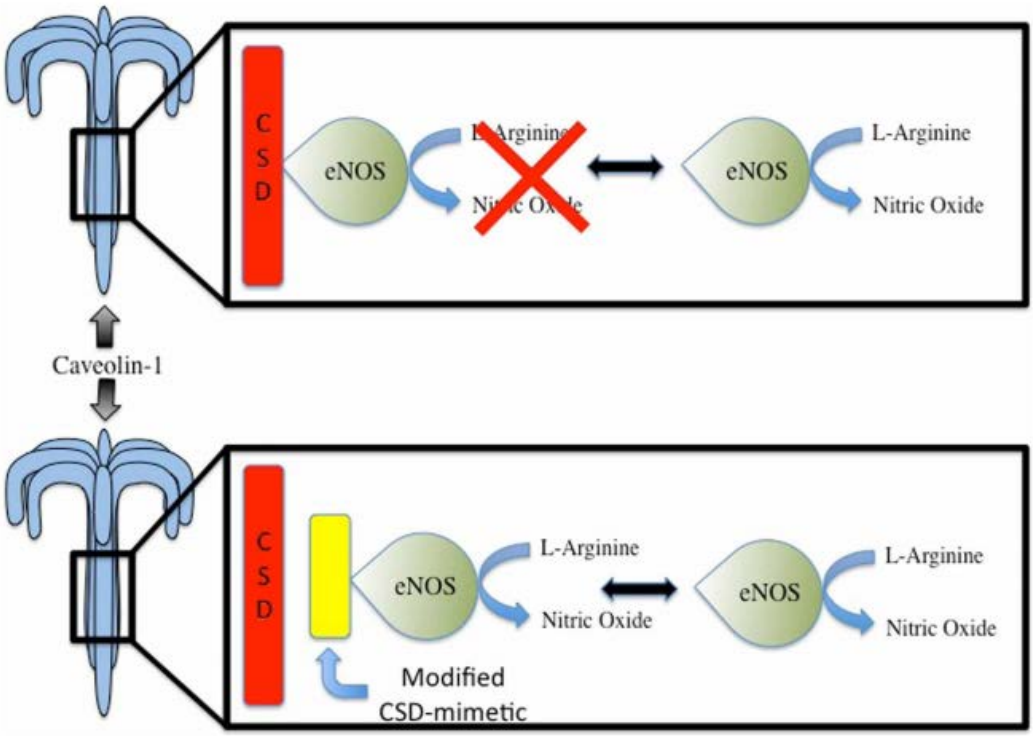

Figure 18: Pathway of the modified Cav-1 CSD derived peptide. Under normal conditions, the binding of eNOS to the CSD of caveolin-1 prevents the formation of NO from its precursors. The binding of eNOS to the modified CSD-mimetic promotes the formation of nitric oxide in addition to its release into the endothelium (36, 37).

While there is much information left to be discovered about caveolin-1 and its role in mitochondrial dynamics and cardiovascular disease, various laboratories have started to target the protein as a therapeutic. A modified caveolin-1 CSD derived peptide has been used as an antagonist to promote the release of nitric oxide; currently, this is the most promising therapeutic regarding cav-1 since studies show this therapy decreases (36). Additionally, amlodipine, an angio-selective CCB, has been found to inhibit the binding of eNOS to caveolin-1, promoting the formation of nitric oxide (38, 39).

CHAPTER 7

METHODS

Growth and maintenance of Endothelial Cells

Rat aortic endothelial cells (RAECs) purchased (Jackson Labs, Boston, MA) were cultured to 90-100% confluency in low glucose (1 g/L) 10% FBS DMEM (Corning Life Sciences, Tewksbury, MA) with 1% PenStrep (Corning Life Sciences, Tewksbury, MA). All cells were incubated at 37°C with 5% CO_2. After proper preparation, RAECs were stimulated with 10ng/mL of TNF-α. Cells for western blot analysis were collected in either 1X SDS lysis buffer or M-PER reagent (Thermo Scientific, Waltham, MA). Cells for mitotracker analysis were fixed on coverslips and analyzed using ImageJ software.

Genetic Manipulation

For caveolin-1 silencing or overexpression, RAECs were transduced with adenovirus in serum-free DMEM supplemented with 0.5 µg/mL poly-L-lysine for 2 hours followed by the addition of fresh serum-free DMEM. After 24 hours, cells were washed, and new serum-free media was added. Prior to stimulation, fresh serum-free media was added, and cells rested for 1 hour. Adenoviral silencing constructs include miRNA targeting Cav-1 (200 MOI) while adenoviral overexpression constructs include an overexpression vector targeting Cav-1 with a myc tag (50 MOI). Cells were also infected with mito-DS Red (SignaGen Laboratories, Gaithersburg, MD) at an MOI of 30 for mitochondrial viewing.

Western Blot Analysis

Cell lysates were separated by SDS-PAGE using bis-acrylamide gels of various percentages and then transferred to a nitrocellulose membrane. Following overnight transfer, the membranes were blocked for 1 h using dry milk powder (Research Products International, Mt. Prospect, IL) in TBST (1g in 20 mL), washed, and incubated with primary antibody overnight at 4°C. Membranes were washed and incubated in secondary antibody for 2 h, washed, and developed using an SRX-101A film processor (Konica Minolta, Tokyo, Japan). The following primary antibodies were used; all were diluted in TBST: Caveolin-1 (BD 610060 at 1:10,000; BD Biosciences, San Jose, CA), Drp1 (BD 611112 at 1:10,000; BD Biosciences, San Jose, CA), GAPDH (MAB374 at 1:20,000; EMD Millipore, Burlington, MA).

Mitochondrial Fragmentation and Morphology Analysis

Quantitative analysis of mitochondrial fission was conducted using ImageJ. RAECs or 3T3-L1s were cultured on coverslips and transduced as indicated in the results. After stimulation, cells were fixed in 3.7% paraformaldehyde (Electron microscopy sciences) for 15 minutes at 37° C. Cells were then washed 3 times in PBS for 3-5 minutes and mounted on glass slides using ProLong Gold with Dapi (Thermo Fisher Scientific). Pictures of mitochondria were taken using a 60X oil objective lens with a 1.5X adjustment attached to an Olympus IX81 inverted fluorescent microscope and a Photometrics Cool SNAP HQ camera. Images were acquired using MetaMorph software and imported into ImageJ. ImageJ processing included iterative deconvolution followed by background subtraction. From there, the threshold of the deconvoluted image was adjusted to match the original image and the particle count was then able to be measured.

Fission was measure using Mitochondrial Fission Count—the number of mitochondria per cell divided by the total mitochondrial area of the given cell.

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