THERAPEUTIC USE OF A MUTANT CAVEOLIN-1 PEPTIDE TO REDUCE
ATHEROSCLEROSIS INDUCED BY HYPERCHOLESTEROLEMIA AND DIABETES

by

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ABSTRACT

Endothelial dysfunction is a well-established response to cardiovascular risk factors, such as hypercholesterolemia and diabetes, and is the critical first step of atherogenesis. Nitric oxide (NO), the key regulator of endothelial function, is greatly diminished in atherosclerotic disease settings resulting in augmented oxidative stress and endothelial activation, a process that involves upregulation of endothelial adhesion molecules and increased leukocyte-endothelial interactions, all of which are major steps in the pathogenesis of atherosclerosis. Pharmacological inhibition of endothelial nitric oxide synthase (eNOS), the main vascular source of protective NO, induces endothelial dysfunction and promotes atherosclerosis. While there is little doubt that endothelial dysfunction is directly linked to atherosclerosis and cardiovascular disease in patients, whether the endogenous pool of eNOS and associated NO release can be considered a direct, therapeutically relevant primary target for atheroprotection is unknown. Caveolin-1 (Cav-1), the major coat protein of plasma membrane caveolae, binds to and inhibits endogenous eNOS. Previously, we have reported that a Cav-1-derived cell permeable peptide with an inactivated eNOS inhibitory domain, known as CavNOxin is able to increase basal NO release without interfering with the biological activities of Cav-1. Herein the current thesis, I hypothesize that ‘antagonizing’ the eNOS/Cav-1 interaction to specifically relieve eNOS from the inhibitory clamp of Cav-1, through the intracellular delivery of CavNOxin, is a potentially novel and unexplored anti-atherosclerotic therapeutic strategy. I show that CavNOxin is able to significantly attenuate hypercholesterolemia- and diabetes-induced atherosclerosis. In contrast, mice lacking eNOS showed resistance to CavNOxin treatment, indicating eNOS specificity. Mechanistically, I show that CavNOxin reduces oxidative stress, expression of pro-atherogenic mediators (in particular VCAM-1) and leukocyte-endothelial interactions. These data are the first to document the use of an eNOS-specific activator to directly reduce oxidative stress and increase atheroprotective endothelial function specifically through endogenous eNOS. In addition, this study provides target validation for the eNOS/Cav-1 interaction, which is highly endothelium-specific, as a strategy for the development of anti-atherosclerotic compounds.
PREFACE

Text from Section 1.5.2. and 1.14.1. from Chapter 1 was adapted from a review article entitled, “Targeting endothelial dysfunction in vascular complications associated with diabetes.” Sharma A, Bernatchez PN, de Haan JB. Int J Vasc Med. 2012, Epub 2011 Oct 13.

• As the first author of the review, I have written 100% of the manuscript. Dr Bernatchez and Dr de Haan edited the manuscript.

Text from Section 1.12. from Chapter 1 was based on a research article entitled, “A noninhibitory mutant of the caveolin-1 scaffolding domain enhances eNOS-derived NO synthesis and vasodilation in mice.” Bernatchez PN*, Sharma A*, Bauer PM, Marin E, Sessa WC. J Clin Invest. 2011; 121(9):3747-55.

• As the co-first author* of this article, I was responsible for producing 80% of the data and figures and writing the manuscript.

Chapter 4 and 5 are a submitted research paper entitled "Specific eNOS unclamping from Caveolin-1 decreases oxidative stress and atherosclerosis" Sharma A, Sellers S, Stefanovic N, Leung C, Tan M, Huet O, Granville D, Cooper M, de Haan JB and Bernatchez P.

• As the first author of this manuscript, I was responsible for producing 95% of the data and figures and writing the manuscript. Sellers S was responsible for generating the eNOS/ApoE double knockout mice. Dr Bernatchez and Dr de Haan edited the manuscript.

As an author of these articles, I was responsible for producing 50% of the data and figures.

All animal procedures and associated methods were approved by both the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics committee (Ethics number: E/1036/2010/B), Melbourne and the UBC animal care committee, Vancouver (Ethics number: A12-0136).
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<td>4-HNE</td>
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<td>a.u.</td>
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<td>Advanced glycation end products</td>
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<td>AP</td>
<td>Attenapedia</td>
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<td>eNOS</td>
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<td>ERK</td>
<td>Extracellular signal-related kinase</td>
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<td>Flavin mononucleotide</td>
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<tr>
<td>FAD</td>
<td>Flavin adenosine dinucleotide</td>
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<td>GEM</td>
<td>Genetic Engineered Models</td>
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<td>GST</td>
<td>Glutathione-S-Transferase</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>GPx1</td>
<td>Glutathione peroxidase 1</td>
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<td>HDL</td>
<td>High density lipoprotein</td>
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<td>HFD</td>
<td>High fat diet</td>
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<td>Intercellular cell adhesion molecule 1</td>
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<td>IEL</td>
<td>Internal elastic lamina</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH associated protein</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LNAME</td>
<td>N-nitro-L-arginine methyl ester</td>
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<td>MCP-1</td>
<td>Monocyte-chemotactic protein 1</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>Nox</td>
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<td>Nitrotyrosine</td>
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<td>NBF</td>
<td>Normal buffered formalin</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<td>NGS</td>
<td>Normal goat serum</td>
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<td>Neuronal nitric oxide synthase</td>
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<tr>
<td>NO</td>
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<td>NF-E2-related factor</td>
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<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
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<tr>
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<td>RAGE</td>
<td>Receptor of advanced glycation end products</td>
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<td>Reactive oxygen species</td>
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<td>Serine</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
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<tr>
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<td>Superoxide Dismutase</td>
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<td>soluble-guanylate cyclase</td>
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<td>Vascular cell adhesion molecule 1</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>Y</td>
<td>Tyrosine</td>
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ACKNOWLEDGEMENTS

A PhD is a challenging but rewarding journey. One of the joys of completing a PhD is to reflect over the journey and remember all the family and friends who have helped and supported me along this long but fulfilling road.

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Chapter 1. Introduction
1.1. Cardiovascular disease

Cardiovascular disease (CVD) is a general term to define diseases of the circulatory system: the heart, the blood vessels of the heart and the network of blood vessels (veins and arteries) throughout the body. CVD are the leading cause of mortality and morbidity in westernized societies with huge economic and social implications (source: World Health Organisation). Atherosclerosis, which is the buildup of fatty plaque in the arteries, is the most prevalent CVD and accounts for the majority of sudden death incidents. Clinical manifestations of atherosclerosis, such as coronary artery disease and peripheral artery disease, will typically occur in 2 out of 3 men and 1 out of 2 women over the age of 40[1]. Thus, understanding the pathogenesis of CVD and developing strategies to alleviate disease burden are highly sought after.

1.2. Atherosclerosis

Atherosclerosis is a disease found in large and medium size arteries, characterized by lipid accumulation, inflammatory cell migration and proliferation within the inner layer of the blood vessel wall. The early stage of the disease, in which fatty streaks begin to develop in the blood vessel wall, occurs over several years and is clinically silent. However, as the fatty plaque builds up, clinical symptoms can arise; the plaque can weaken, rupture and form blood clots, which can obstruct blood flow to various organs in the body, including the heart and brain resulting in heart attacks and strokes respectively [2, 3].

The development of atherosclerosis is a multi-step inflammatory process, which is initiated at the vascular endothelium, the innermost layer of the blood vessel [4](see Figure 1 for stages of atherosclerosis). Initially believed to be a mere anatomical boundary, the vascular endothelium is now considered a prime regulator of cardiovascular homeostasis, involved in maintaining vascular tone and an anti-atherogenic and anti-thrombotic surface[4]. A critical inducer of CVD is the impairment of the vascular endothelium, a condition known as endothelial dysfunction. The impaired endothelium undergoes a phenotypic conversion that favours adhesion of flowing leukocytes (mostly monocytes), enhanced vascular permeability and reduced antithrombotic properties, a process known as endothelial activation[5]. In settings of inflammation, such as atherosclerosis, pro-inflammatory cytokines (tumor necrosis factor-α
(TNF-α) and interleukin-1β (IL-1β)) and mechanical forces (sheer stress, stretch) mediate endothelial activation[5]. These stimuli remodel endothelial cell junctions enhancing vascular permeability and induce the expression of pro-inflammatory cell adhesion molecules, which promote leukocyte tethering, rolling, adhesion and subsequent translocation into the subendothelial space. In the subendothelial space, monocytes mature into macrophages and engulf lipids to form foam cells[6]. While normal endothelial cell uptake of cholesterol, in particular low-density lipoprotein (LDL) is tightly regulated, in disease settings, circulating levels of LDL are elevated and their extravasation into the endothelial wall increases[7]. Macrophages and other cells of the blood vessel secrete oxidative products and enzymes that initiate oxidation of LDL, yielding oxidized-LDL (ox-LDL)[7]. In turn, ox-LDL also contributes to the pathogenic process by causing LDL aggregation, upregulating adhesion molecule expression at the endothelium and potentiating the production of inflammatory cytokines[7]. Another early structural feature of atherosclerosis is “diffuse intimal thickening”, which is the circumferential thickening of the intima mainly composed of smooth muscle cells (SMC), elastin and proteoglycans. Lesion progression then ensues with migration and proliferation of SMC from the tunica media (middle layer of the blood vessel) to the vascular endothelium and the continued accumulation of foam cells[6]. In addition, there is heightened synthesis of extracellular matrix macromolecules by SMCs, which promotes cellular growth and results in the formation of a fibrous cap covering the crown of the lesion[6]. The initially deposited lipid and extracellular lipids derived from necrosis of cells can accumulate in the central core region of the plaque, leading to the second stage of atherosclerotic disease known as fibrous plaque. The last stage of the disease process, which results in a complicated lesion, arises when the fibrous plaque weakens and ruptures exposing the cholesterol and connective tissue underneath to clotting factors from the blood, thereby triggering the formation of a thrombus that extends out to the vessel lumen and ultimately impedes blood flow[6]. Since, the vascular endothelium plays a critical role in the protection against atherosclerosis and the improper regulation of endothelial function is implicated in the initiation of the disease process, it is important to discuss the physiological properties of this organ.
Figure 1: Stages of atherosclerosis

The normal blood vessel and the stages involved in atherogenesis are shown. (A) A normal blood vessel consists of three layers. The innermost layer, the intima, is lined by a single layer of endothelial cells that is in contact with the blood and underlying tissues. The middle layer, or media, contains SMCs embedded in a complex extracellular matrix. The outer layer, the adventitia, contains collagen, fibroblasts, nerve endings and microvessels. (B) The critical inducer for atherosclerosis is endothelial dysfunction followed endothelial activation. The steps involved are adhesion of leukocytes to the activated endothelial monolayer, migration of the adhered leukocytes into the subendothelial layer, maturation of leukocytes (mainly monocytes) into macrophages, and their uptake of lipid and transformation into foam cells. (C) Lesion progression involves the proliferation and migration of SMCs from the media to the intima, and the increased synthesis of extracellular matrix macromolecules such as collagen. Extracellular lipid derived from dead and dying foam cells can accumulate in the central region of a plaque, termed the lipid core. Advancing plaques also contain cholesterol crystals and immune T-cells. (D) The complicated lesion, known as atherothrombosis, occurs due to a physical disruption of the atherosclerotic plaque allowing blood coagulation components to come into contact with tissue factors in the plaque, causing thrombus formation that extends into the vessel lumen, resulting in a blockage of blood flow.
1.3. The vascular endothelium as a key source of nitric oxide

The vascular endothelium is not only a vital boundary between the blood and underlying tissues, but also an important regulator of vascular tone. A single layer of endothelial cells line the endothelium and are responsible for the maintenance of vascular tone and homeostasis by balancing the release of vasodilators and vasoconstrictors in response to various physiological stimuli, which include sheer stress, neurotransmitters (acetylcholine), peptides (bradykinin), circulating hormones and prostaglandins[8, 9]. In turn, these vasoactive substances modify the contractile state of vascular SMC, located in the tunica media. Of these vasoactive substances released by the endothelium, nitric oxide (NO) has emerged as one of the most critical mediators of endothelial function[10] and will be the focus of the current work.

NO was discovered in 1980 through the pioneering work of Furchgott and Zawadski. They demonstrated that acetylcholine-induced relaxation of blood vessels was strictly dependent on the presence of endothelial cells[11]. Their work proposed that acetylcholine, by activating its receptor on endothelial cells was able to stimulate the release of a substance (initially termed endothelium-derived relaxing factor), causing the downstream relaxation of vascular smooth muscle cells[11]. Louis Ignarro and colleagues subsequently identified this substance as NO, which is a soluble and highly versatile gaseous molecule with a relatively short half-life (6-30 seconds) and is readily inactivated by oxidation into nitrites and nitrates[10, 12, 13]. The field of NO has generated a tremendous amount of interest over the years and NO has proven to have many important cardiovascular functions, which will be addressed in the subsequent sections.

1.4. Vascular functions of NO

NO is well known as the most potent endogenous vasodilator through its ability to directly influence SMC relaxation[10, 11, 13]. In response to hemodynamic shear stress, neurotransmitters or agonists, the endothelium releases NO, through activation of the enzyme endothelial nitric oxide synthase (eNOS). NO then diffuses from the endothelium to the vascular SMC, where it interacts with and activates soluble-guanylate cyclase (s-GC), an enzyme that catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP)[14]. In turn, cGMP is the principal second messenger responsible for mediating the vasodilatory effects of NO, mainly by reducing intracellular calcium (Ca$^{2+}$) concentration.
resulting in SMC relaxation[14]. The ability of NO to effectively regulate vascular tone contributes to its role in regulating systemic vascular resistance, arterial relaxation, distensibility and blood pressure[15]. In addition, NO is an essential mediator in vascular endothelial growth factor (VEGF)-induced angiogenesis, which is the formation of new blood vessels from pre-existing ones[16].

Most importantly, NO contributes to preserving the non-thrombogenic and anti-atherosclerotic surface of the endothelium, allowing it to maintain the integrity of the vasculature and prevent the formation of plaques that could compromise downstream perfusion[13]. The anti-atherosclerotic properties of NO are attributed to its ability to 1) inhibit leukocyte recruitment, 2) protect against platelet aggregation, 3) reduce vascular SMC proliferation and 4) limiting oxidative stress (Figure 2)[17-20]. Indeed, endothelial dysfunction, which is the critical first step in the atherosclerotic disease process, is characterized by the reduced bioavailability of NO. In the next subsections, atherosclerotic processes and the anti-atherosclerotic role of NO will be addressed.

1.4.1. NO and leukocyte recruitment

Leukocyte recruitment to the vascular endothelium is a dynamic cellular and molecular process that involves leukocyte tethering, rolling, adhesion and ultimately migration to the subendothelial space[21]. In disease settings, the endothelium becomes activated and expresses cell adhesion molecules that increase leukocyte rolling and adhesion. Selectins, in particular E-selectin and P-selectin, are a family of cell adhesion molecules expressed on endothelial cells that facilitate early homing and rolling of leukocytes of the vessel wall. Pharmacological inhibition of NO by N-nitro-L-arginine methyl ester (L-NAME) enhances the endothelial expression of these selectins and causes a significant increase in leukocyte rolling, which is attenuated upon restoration of NO levels[22, 23]. In addition, NO influences the firm adhesion of circulating leukocytes to the endothelial wall, which is mediated mainly by vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule (ICAM-1)[24]. Inflammatory cytokines, including IL-1β and TNF-α, as well as oxidative stress molecules induce the expression of these adhesion molecules through activation of specific reduction-oxidation sensitive transcription factors, such as nuclear factor κB (NF-κB)[25]. Experimental evidence has demonstrated that NO attenuates gene expression of VCAM-1 and ICAM-1 in
isolated endothelial cells through modulation of NF-κB activation[24, 25]. Intravital microscopy studies have shown that upregulating NO levels, either pharmacologically or genetically, decreases leukocyte adhesion to the vascular wall[17, 26], lending credence to the pivotal role that NO plays in the prevention of focal adhesion and attachment of leukocytes.

1.4.2. NO and platelet activation

Platelet aggregation plays a key role in thrombus formation, the last stage of the atherosclerotic lesion, primarily by interacting with components of the subendothelial matrix including collagen, which then triggers platelet adhesion and further recruitment of platelets leading to formation of a thrombus[27]. A plethora of in vitro and in vivo studies have confirmed that endothelial-derived NO limits platelet activation, adhesion and aggregation[28]. The mechanism by which NO mediates its anti-platelet effects involve activation of s-GC to increase cGMP levels, enhancing intracellular Ca^{2+} stores and inhibiting the activation of phosphoinositide-3-kinase (PI3K)[28]. As expected, exogenous administration of NO donors and stimulation of endogenous NO has proven to be effective in protecting against platelet-rich thrombus formation[29].

1.4.3. NO and vascular SMC proliferation

In response to inflammation, vascular SMC undergo a phenotypic change from a quiescent "contractile" state to an active "synthetic" state, resulting in SMC proliferation and migration from the media to the intima, contributing to the formation of the intermediate atherosclerotic lesions[30]. Administration of pharmacological NO donors, overexpression of eNOS and its substrates can inhibit both vascular SMC proliferation and migration, reducing injury responses within the vasculature[19]. The mechanisms by which NO mediates its inhibitory effects on SMC migration and proliferation is rather complex, but is believed to revolve around the effect cGMP, its main second messenger, has on transcription factors, calcium entry and mobilization, phosphorylation of proteins and cell cycling events[31].
1.4.4. NO and oxidative stress

Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS), which include superoxide, hydrogen peroxide and peroxynitrite, by pro-oxidant enzymes and their detoxification by antioxidant enzymes, in favour of the former[32]. While low concentrations of ROS play a role in normal physiology, which include oxygen sensing, defence against infectious agents and signal transduction, moderate or high levels of ROS are detrimental and often associated with pathological conditions, stressing the need for tight control of oxidative signaling[33, 34]. Enhanced oxidative stress contributes significantly to the signaling pathways that trigger vascular inflammation in atherosclerosis, from the initiation of the fatty streak, through to lesion progression and ultimately plaque rupture[32]. Vascular sources of ROS that partake in atherosclerosis include nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (Nox), xanthine oxidase and mitochondrial ROS[32, 35]. In addition, improper regulation of eNOS can result in the production of superoxide instead of NO, a phenomenon termed eNOS uncoupling, which is considered a major contributor of oxidative stress in atherosclerotic disease settings[36] and will be discussed further in Section 1.6.3.

ROS, in particular superoxide, interacts with NO to form peroxynitrite, thus reducing NO bioavailability and accelerating endothelial dysfunction. In addition, ROS, especially hydroxyl radicals, can cause direct insults to cell membranes and nuclei[35]. One of the major roles that ROS play in the atherogenic process is the oxidation of lipids, leading to the formation of ox-LDL, a key atherosclerotic mediator of atherogenesis. Ox-LDL induces the production of monocyte-chemotactic protein 1 (MCP-1), which in turn promotes monocyte chemotaxis and differentiation to macrophages[37]. The accumulation of cholesterol ester in macrophages and the resultant foam cell formation is also facilitated by ox-LDL. In addition, ox-LDL upregulates the expression of adhesion molecules, stimulates pro-inflammatory cytokine production and extracellular matrix remodeling and promotes thrombosis, all of which contribute to atherosclerotic lesion development[35, 38]. Furthermore, macrophages and neutrophils recruited to the inflammatory site further augment the oxidative insult. Enhancing NO bioavailability, either by pharmacological NO donors or modulating eNOS protein and gene expression, has proven effective in limiting oxidative stress by scavenging superoxide in various disease settings[20, 39].
Figure 2: Anti-atherosclerotic functions of NO

The role of NO in normal endothelial function and endothelial dysfunction is shown. (Top) NO is an anti-atherogenic molecule that limits ROS, inhibits leukocyte-endothelial interactions, platelet aggregation and SMC proliferation and migration. (Bottom) Endothelial dysfunction, characterized by reduced NO bioavailability, results in increased ROS production (in part by uncoupled eNOS), leukocyte adhesion and migration to the subendothelial space, platelet aggregation and SMC proliferation and migration.
1.5. The link between atherosclerotic risk factors and NO

The major risk factors for atherosclerosis can be categorized as either modifiable or non-modifiable risk factors. Modifiable risk factors include hypercholesterolemia, hypertension, diabetes mellitus, obesity, smoking, psychosocial stress, poor diet and lack of physical activity, all of which can be controlled and the atherosclerotic disease progression reversed. On the other hand, non-modifiable atherosclerotic risk factors, such as age, genetics and gender, are harder to control[40]. Endothelial dysfunction is a well-established response to atherosclerotic risk factors and precedes the development of atherosclerosis. Furthermore, endothelial dysfunction is considered an independent clinical diagnostic tool for various CVD, including hypertension and atherosclerosis[41]. Hypercholesterolemia and diabetes are two modifiable risk factors that are highly prevalent in modern societies and their impact on endothelial function will be discussed further.

1.5.1. Hypercholesterolemia and atherosclerosis

Elevated levels of blood cholesterol characterize hypercholesterolemia. The excess lipoproteins carrying cholesterol, in particular LDL, in the blood can accumulate in the vascular wall and undergo oxidative modification and aggregation, which initiate and perpetuate the atherosclerotic disease process. Furthermore, a plethora of studies have demonstrated that hypercholesterolemia alters vascular reactivity; in particular, endothelium-dependent vasorelaxation is impaired in these settings[42]. This reduction in endothelium-dependent vasodilation is not due to an inability to react to NO, as vessels are able to respond normally to endothelium-independent NO donors, but rather a reduction in the bioavailability of NO within vasculature either via deficits in production or due to increased oxidant scavenging[43, 44]. Indeed, endothelial dysfunction in response to hypercholesterolemia occurs at an early stage prior to plaque formation[42]. Hypercholesterolemia can also lead to endothelial activation, leukocyte recruitment and adherence and platelet activation. A decrease in basal NO release and concomitant increase in endothelial adhesiveness was observed just 2 weeks after the introduction of a high cholesterol diet[45]. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, a class of drugs known as statins, have proven effective at lowering LDL cholesterol levels and reducing the risk of atherosclerosis[46]. In addition, statins have anti-oxidant
capabilities to reduce ROS, increase resistance of LDL to oxidation and upregulate eNOS gene expression[46, 47]. Recently, there have been experimental data suggesting that therapies that increase the bioavailability of NO in hypercholesterolemia may reverse endothelial dysfunction and result in regression of preexisting atherosclerotic lesions[48].

1.5.2. Diabetes and atherosclerosis

Diabetes is characterized by persistent elevation of blood glucose levels, often described as hyperglycemia. Diabetes occurs due to an inadequate production of insulin (type 1 diabetes) or resistance to endogenous insulin usually associated with the metabolic syndrome and obesity (type 2 diabetes)[49]. Diabetic patients have a much higher incidence of developing atherosclerosis than the general population due to abnormalities pertinent to a diabetic milieu[50]. Hyperglycemia plays a major role in inducing endothelial dysfunction such that impaired endothelium-dependent vasodilation is observed in cellular and experimental models of diabetes[51-53]. Moreover, several clinical studies have shown abnormal vessel reactivity in type 1 and type 2 diabetic patients[54, 55]. Thus, it is believed that decreased NO levels underlies the atherogenic disposition in diabetic subjects. In addition, various atherosclerotic proteins, such as VCAM-1, MCP-1 and connective tissue growth factor (CTGF) are upregulated in the diabetic condition[56]. A hyperglycemic environment is also known to activate NF-κB, leading to an induction in the expression of various inflammatory proteins[57]. Hyperglycemia triggers non-enzymatic glycation, characterized by the binding of glucose or by-products to the amino groups of proteins, leading to the formation of advanced glycation end products (AGEs) that alter the structure and function of proteins. The AGEs activate membrane receptors such as RAGE, which induces intracellular oxidative stress and a pro-inflammatory state and is considered to be a key mediator of diabetes-associated atherosclerosis[58]. The diabetic environment is also associated with a heightened state of oxidative stress. Indeed the increase in ROS production has been shown to augment monocyte infiltration, platelet activation, SMC migration, cell adhesion, release of CTGF and increased production of AGEs. Hyperglycemia and ROS such as superoxide anion can initiate the activation of protein kinase C (PKC), which in turn stimulates and regulates Nox enzymes to produce superoxide anions[59]. In addition, PKC inhibits the PI3K pathway, thereby limiting activation of Akt kinase and its downstream signaling molecule, eNOS, resulting in lowered NO bioavailability[59]. Lastly, diabetes results
in the significant increase of free fatty acids, namely triglycerides and VLDL, as a result of diminished lipid homeostasis by lower insulin levels, which contribute to the atherogenic process[60].

Figure 3: Link between cardiovascular risk factors and endothelial dysfunction

A schematic diagram is shown here demonstrating the link between cardiovascular risk factors and endothelial dysfunction resulting in the development of atherosclerotic lesions.
1.6. eNOS, the leading source of atheroprotective NO

NO is synthesized by a class of enzymes known as nitric oxide synthases (NOS), which exists in 3 known isoforms: neuronal NOS (nNOS or NOS I) is predominantly found in neuronal tissues, inducible NOS (iNOS or NOS II) is induced by numerous cell types during inflammation, and endothelial nitric oxide synthase (eNOS or NOS III) which is constitutively expressed in vascular endothelial cells[61]. All NOS isoforms catalyze the oxidation of the amino acid, L-arginine, to NO and L-citrulline, however they differ slightly in their structure and regulation[62]. Since eNOS is the main source of atheroprotective NO in the endothelium, the localization and structure of this NOS isoform will be described in greater detail in this section.

1.6.1. Subcellular localization of eNOS

The subcellular localization of eNOS is unique amongst the NOS isoforms as it is a peripheral membrane protein that is dually acylated by co-translational N-myristoylation and post-translational cysteine palmitoylation, both of which are critical for the proper functioning of the enzyme[63]. N-myristoylation involves the attachment of myristic acid, a type of fatty acid, to glycine residue 2 at the N-terminal of eNOS. Through the use of non-myristoylated mutant eNOS, it was first demonstrated that this fatty acid modification was irreversible and required for Golgi membrane association and subsequently effective NO release[64, 65]. Later, it was revealed that N-myristoylation of eNOS was necessary for palmitoylation, another fatty acid modification at cysteine residues 15 and/or 26, which led to the common concept that eNOS is a dually acylated protein[66]. The importance of eNOS palmitoylation was elucidated through a series of in vitro fractionation and immunofluorescence studies, which determined that palmitoylation led to the enrichment of eNOS in specialized plasma membrane microdomains, known as caveolae, where eNOS co-localised with caveolin-1 (Cav-1), the major coat protein of caveolae[66]. Functionally, caveolae localization optimizes the ability of efficient NO production from eNOS, as demonstrated by palmitoylation-deficient eNOS mutants, which exhibited significantly attenuated NO release[67]. Unlike N-myristoylation, eNOS can cycle between palmitoylated and depalmitoylated states in response to extracellular signals, providing a mechanism for dynamic regulation of protein localization and activation[68].
1.6.2. eNOS structure and activation

Functionally active eNOS exists as a homodimer and each monomer consists of two domains, which are the N-terminal oxygenase domain and the C-terminal reductase domain linked by the calmodulin (CaM) regulatory domain. eNOS is homodimerized by cysteine residues 99 and 94 in the N-terminal oxygenase domain and stabilized by zinc thiolate clusters[69]. eNOS function is critically dependent on the availability of its substrates and cofactors. The N-terminal oxygenase domain binds to three co-factors; the substrate, L-arginine, an essential redox cofactor called tetrahydropterin (BH₄) and a heme prosthetic group, whilst the C-terminal reductase domain binds to the redox cofactors: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH)[61]. Another important role of the essential co-factor BH₄ is the maintenance of the dimeric state of eNOS. During NO synthesis, electrons donated by NADPH are transferred via FMN and FAD from the reductase domain of one monomer to the oxygenase domain of the other monomer, where it interacts with the heme prosthetic group and BH₄ to catalyze the reaction of oxygen and L-arginine to produce NO and L-citrulline[61, 69]. The classical pathway of eNOS activation is Ca²⁺-dependent. Agonists, such as acetylcholine and substance P, activate their respective G-protein coupled receptors at the cell membrane, stimulating a rise in cytosolic Ca²⁺ concentrations, which then binds to the CaM regulatory domain, forming a Ca²⁺-CaM complex that activates the eNOS enzyme. eNOS can also be activated in a Ca²⁺-independent manner involving phosphorylation of the enzyme, which will be discussed further in Section 1.7.1.

1.6.3. eNOS dysregulation and vascular diseases

In vascular diseases, such as atherosclerosis, the capacity of eNOS to function properly and produce NO is remarkably reduced. In such conditions, eNOS converts from producing NO to an enzyme that synthesizes superoxide anion. This phenomenon, commonly described as “eNOS uncoupling” occurs due to the depletion of L-arginine, BH₄ deficiency and/or BH₄ oxidation, the later being the predominant cause in vascular diseases[36]. Furthermore, in states of increased oxidative stress including hypercholesterolemia and diabetes, the likelihood of BH₄ oxidation to BH₂ by ROS species such as superoxide and peroxynitrite is dramatically increased, thereby enhancing eNOS uncoupling[70]. Indeed, cellular and experimental evidence
has clearly demonstrated the direct action of oxidative stress in inducing eNOS uncoupling which correlated with reduced BH4 levels[71-73]. Furthermore, the importance of proper eNOS regulation has been demonstrated elegantly in vivo with the use of eNOS knockout (eNOS KO) mice. Due to the profound importance of eNOS in the vasculature, it is not surprising that eNOS KO mice are hypertensive, develop left ventricular hypertrophy and have a significant reduction in growth-factor induced angiogenesis compared to their wild type (WT) counterparts[74-76]. At the endothelial level, eNOS KO mice lack endothelium-derived NO activity and exhibit increased platelet aggregation, leukocyte-endothelial adhesion, SMC proliferation and enhanced susceptibility to develop thrombosis[77]. Another relevant model to illustrate the significance of eNOS-derived NO is the Apolipoprotein E knockout (ApoE KO) mouse, which is genetically pre-disposed to develop atherosclerosis when placed on a high fat diet. Crossbreeding of the ApoE KO mice with eNOS KO mice increased severity of atherosclerotic lesions as compared to the single ApoE KO mice[78]. Interestingly, over-expression of eNOS specifically in the endothelium of the ApoE KO mice also resulted in exacerbated atherosclerosis associated with lower NO levels and enhanced superoxide production, despite elevated eNOS protein expression[79]. The elevated atherosclerosis observed in these mice was reversed upon BH4 supplementation, suggesting that eNOS had become uncoupled in these conditions[79]. Hence, these studies provide compelling data that substrate and co-factor bioavailability as well as proper regulation of the eNOS enzyme, rather than total eNOS protein expression, is required for the protective effects of the enzyme.

1.7. Regulation of eNOS

Due to its profound importance in vascular homeostasis, many studies have sought to improve the function of eNOS in efforts to boost endothelial NO production. However, it is first important to understand the key mechanisms that regulate eNOS activity. The main eNOS regulatory mechanisms can be subdivided into three categories: transcriptional regulation, post-translational modifications and protein-protein interactions.
1.7.1. Transcriptional regulation of eNOS

There are several physiological and pathophysiological stimuli that have been identified to modulate eNOS expression via mechanisms that alter steady-state eNOS mRNA levels, either by inducing eNOS gene transcription, stabilizing eNOS mRNA or both[80]. Increased transcription of the enzyme in turn, results in sustained activation of eNOS-dependent activities. For instance, sheer stress activates the extracellular signal-regulated kinases (ERK1/2) and NFκB, which bind to the promoter region of the eNOS gene, thereby upregulating eNOS expression[81]. Interestingly, increased expression of eNOS is even observed in oxidative stress settings. In cell culture systems, hydrogen peroxide and components of ox-LDL increase eNOS mRNA in a dose- and time-dependent manner[82, 83]. This increase in eNOS expression in settings of enhanced ROS production suggests that endothelial cells compensate for oxidative injury by increasing eNOS expression. The pleiotropic effects of cardiovascular drugs, such as statins and calcium channel blockers include the up-regulation of eNOS gene expression[84]. In addition, there are certain inflammatory cytokines that decrease eNOS mRNA expression. One important example of this is TNF-α, which reduces the eNOS mRNA half-life and destabilizes its gene expression[85]. Recently, a primary screening of chemical libraries for compounds that increase eNOS gene expression revealed two small molecular weight compounds, known as AVE9488 and AVE3085, that were able to increase endothelial NO production by simultaneous upregulation of eNOS gene expression and reversal of eNOS uncoupling[84]. Furthermore, these compounds demonstrated a potent anti-atherosclerotic and eNOS-specific effect in animal disease models, highlighting their potential therapeutic applications[84].

1.7.2. Post-translational modifications and phosphorylation of eNOS

In addition to myristoylation and palmitoylation sites described earlier, several consensus sequence sites for protein kinases have been identified on eNOS and are known to modify the activity of the enzyme through phosphorylation. The phosphorylation sites on human eNOS include tyrosine (Y) sites Y81 and Y567, serine (S) sites S114, S615, S633, and S1177, and threonine (T) sites T495, all of which regulate eNOS activity[86]. Phosphorylation of eNOS at the Y sites has opposite effects on eNOS activity, where Y81 is phosphorylated by pp60src and activates the enzyme while Y567 is phosphorylated by proline-rich tyrosine kinase 2 (PYK2)
and appears to attenuate eNOS activity, although the reason behind these opposing phosphorylation effects are less clear[86]. On the other hand, serine phosphorylation of eNOS is well established, with the phosphorylation of eNOS at S1177 being an important mechanism of physiologic regulation of NO production. The protein kinase Akt, which is activated and phosphorylated downstream of sheer stress and VEGF-induced signaling, is responsible for phosphorylating eNOS at S1177, thereby increasing eNOS activity[87]. Indeed, reduced phosphorylation of eNOS at S1177 is observed in patients with hypertension, diabetes and metabolic syndrome[88]. Mutant forms of eNOS have been utilized to study the eNOS phosphorylation at S1177 in animal models. Substitution of the serine residue to an alanine (S1177A) produces a form of eNOS that cannot be phosphorylated and does not exhibit Akt-dependent NO release[89], while substitution of the serine residue to an aspartate (S1177D) is constitutively activated by phosphorylation even in the absence of Akt[90]. Indeed, acetylcholine dependent vasodilation and VEGF-mediated angiogenesis was significantly reduced in the S1177A mouse and rescued in the S1177D mouse, highlighting the importance of eNOS phosphorylation at this site[89, 91]. Phosphorylation sites that reduce the catalytic activity of eNOS have also been characterized. In the absence of Ca\textsuperscript{2+}-CaM, AMP-activated kinase and PKC phosphorylate eNOS at T495 and inhibit eNOS activity[92].

1.7.3. Protein-protein interactions of eNOS

Protein-protein interactions represent an important post-translational mechanism of eNOS regulation with a variety of regulatory and structural proteins that positively or negatively regulate eNOS activity. Heat shock protein 90 (HSP90), Ca\textsuperscript{2+}-CaM and Cav-1 are recognized as the most important regulators of eNOS activity.

HSP90 belongs to a family of heat shock proteins that are ubiquitously expressed chaperone molecules responsible for the proper folding of synthesized proteins, coordinating trafficking and regulation of diverse signaling proteins. In resting endothelial cells, HSP90 was found to be associated with eNOS. In vitro immunoprecipitation studies have demonstrated that HSP90 is rapidly recruited to the eNOS complex by stimuli such as VEGF and sheer stress, resulting in enhanced eNOS activity[93]. In addition, HSP90 can activate eNOS directly, which suggests that HSP90 may act as an allosteric modulator of eNOS by inducing a conformational
change in the enzyme that results in increased activity by stabilizing the activated complex[94]. Lastly, HSP90 facilitates Akt-mediated phosphorylation and CaM binding to eNOS[95].

CaM is a Ca^{2+}-binding protein that activates a variety of intracellular Ca^{2+}-dependent enzymes, including eNOS. Mechanistically, the Ca^{2+}/CaM complex binds to the CaM regulatory domain of eNOS and displaces an adjacent autoinhibitory loop on eNOS, thus facilitating NADPH-dependent electron flux from the reductase domain through to the oxygenase domain[94]. The affinity for CaM binding to eNOS is highly dependent on intracellular Ca^{2+} levels. Moreover, CaM can activate CaM kinase II, which is involved in the phosphorylation of eNOS at S1177[96]. Another important feature of Ca^{2+}/CaM-induced increase in eNOS activity is the ability of the complex to aid in the dissociation of eNOS from Cav-1, a protein that negatively regulates eNOS activity.

As mentioned previously, for optimal NO release, localization of eNOS to caveolae microdomains at the plasma membrane is essential. Ironically, under basal conditions, eNOS enzyme activity is markedly reduced by its interactions with Cav-1, the main structural protein of caveolae[97]. This inhibition of eNOS by Cav-1 present in caveolae is mediated by a direct interaction between a specific amino acid sequence within Cav-1, called the scaffolding domain comprising of amino acids 82-101, and a motif in the oxygenase domain of eNOS that is rich in aromatic amino acid residues[97]. The eNOS/Cav-1 complex is in a dynamic state, cycling between states of dissociation and re-association depending on the affinity of eNOS for Ca^{2+}/CaM. Upon agonist (VEGF, acetylcholine)-induced increase in intracellular Ca^{2+}, eNOS is activated by recruiting large amounts of Ca^{2+}/CaM and HSP90, which facilitates the dissociation of eNOS from Cav-1, resulting in efficient stimulus-response coupling to effectively produce NO[96, 98]. Following prolonged agonist activation, eNOS is depalmitoylated and cytosolic translocation occurs, allowing the enzyme to be modulated by phosphorylation[99]. Repalmitoylation and re-interaction with Cav-1 facilitates rapid and efficient stabilization of the inactivated enzyme post-agonist stimulation[98].

The interaction between Cav-1 and eNOS in caveolae microdomains has attracted much attention over the recent few years. The subsequent sections will describe the biological functions of caveolae/caveolins and their significant role in regulating eNOS activity and vascular function.
1.8. Caveolae and caveolins

Caveolae are cholesterol and sphingolipid enriched flask-shaped invaginations at the plasma membrane discovered in the early 1950s by Yamada et al.[100]. These 50-100nm plasma membrane microdomains, although originally observed in the epithelium by electron microscopy, are expressed in a variety of different cell types such as myocytes and fibroblasts, and are particularly abundant in endothelial cells. The biochemical properties, structure and physiological roles of caveolae and their coat protein, caveolins, will be addressed in this section.

1.8.1. Biochemical properties and composition of caveolae

Lipid rafts are specialized regions within the plasma membrane that are high in cholesterol and glycosphingolipids. The lipid composition of these domains facilitates tight packing through interactions with their fatty acyl chains, giving rise to a highly liquid-ordered phase with less fluidity as compared to the surrounding plasma membrane, which is mainly composed of phospholipids[101]. Caveolae are a morphologically distinct subset of lipid rafts because of the ability of caveolar coat proteins, known as caveolins, to initiate caveolae biogenesis from raft-derived components, giving them their unique flask-like appearance[102]. The lipid composition of lipid rafts and caveolae give them distinct characteristics as compared to their phospholipid counterparts, which include higher buoyancy and resistance to solubilization by mild detergents such as Triton X-100[101]. Based on these characteristics, detergent and detergent-free methods involving sucrose gradients have been developed to isolate lipids rafts/caveolae and the proteins associated with them from all other cellular constituents. Caveolins are structural proteins and serve as specific markers for caveolae, thereby allowing the identification of caveolae microdomains from non-caveolae lipid rafts[103]. There are three distinct isoforms of caveolins; Cav-1, Caveolin-2 (Cav-2) and Caveolin-3 (Cav-3), which are expressed differentially in cells. Cav-1 is ubiquitously expressed in non-muscle caveolae-rich cells, such as endothelial cells, whereas Cav-3 expression is limited to vascular SMC, cardiomyocytes and skeletal muscle cells[104]. Cav-2 is considered an accessory protein that is co-expressed with and aids in the function of Cav-1-induced caveolae biogenesis. Genetic ablation of Cav-1 and Cav-3, but not Cav-2, results in the complete loss of caveolae organelles,
while re-expression of Cav-1 and Cav-3 drives organelle biogenesis, thereby stressing the importance of these proteins in the caveolae organelle assembly[105, 106]. Despite lacking caveolae, Cav-1 and Cav-3 knockout mice are viable but suffer a range of defects, which suggests additional compensatory mechanisms are present for these organelles[105, 106]. Since the predominant caveolin isoform in the endothelium is Cav-1, this discussion will focus on Cav-1’s structure and physiological roles.

1.8.2. Structural features of Cav-1

Cav-1 is a small 22kD protein comprised of 178 amino acids and exists as two isoforms, known as Cav-1α and Cav-1β, with the α isoform containing residues 1-178 and the β isoform containing residues 32-178[107]. Through the use of whole mount electron microscopy, both isoforms have demonstrated the ability to generate caveolae. However, the functional significance of the two isoforms is still relatively unknown but it is believed that the α isoform is more efficient at driving caveolae organelle biogenesis and is localized in deeply invaginated caveolae[107]. Structurally, Cav-1 contains a hydrophobic membrane-spanning region in between the cytoplasmic C- and N-terminus domains. The membrane-spanning region consists of the caveolin scaffolding domain (CSD; amino acids 82-101), which is important in mediating the biological activities of Cav-1, and the intramembrane domain (IMD; amino acids 101-134), which is responsible for the α-helical hairpin structure[102]. Cell-surface biotinylation studies have revealed no positive labeling of Cav-1, indicating that it is a purely intracellular protein. There are known phosphorylation sites in the N-terminus, which modulate the activity of Cav-1, and palmitoylation sites in the C-terminus, which is essential for membrane attachment[102, 108].

As mentioned earlier, Cav-1 is a structural protein that drives caveolae organelle biogenesis, which is largely attributed to two unique properties of Cav-1. Firstly, Cav-1 binds with a strong affinity to cholesterol and this Cav-1:cholesterol ratio is important for caveolae organelle formation. Indeed, cholesterol-depleting drugs, such as nystatin and cyclodextrin result in the complete loss of morphologically identifiable caveolae[109]. Secondly, Cav-1 has the tendency to homo-oligomerize with itself to form high molecular weight oligomers of approximately 400kDa, consisting of 14-16 Cav-1 monomers[109]. Sequence mapping of Cav-1
has determined that this homo-oligomerization, which occurs in the endoplasmic reticulum, is mediated by a region in Cav-1 protein that overlaps with the CSD, known as the oligomerization domain, comprising of amino acids 61-101[110]. Following this, the Cav-1 homo-oligomers undergo a second stage of oligomerization, possibly at the trans-Golgi network, where individual Cav-1 oligomers interact with each other via their C-terminal domains, forming an extensive meshwork on the underside of the plasma membrane. This large meshwork of oligomers acts synergistically with cholesterol to maintain the “flask-shape” invagination of caveolae[102].

### 1.8.3. Physiological roles of caveolae and Cav-1

Since their initial discovery, a plethora of studies have shed light on the important physiological roles of caveolae, which include cholesterol homeostasis, transcytosis, endocytosis and signal transduction. The important physiological roles of caveolae and Cav-1 are addressed in this section.

Cav-1 is a cholesterol binding protein and is sensitive to cellular fluctuations in cholesterol content. In addition, Cav-1 is able to directly regulate cholesterol homeostasis by modulating cholesterol efflux and influx. Cav-1 binds to newly synthesized cholesterol in the endoplasmic reticulum and transports cholesterol to caveolae, where it mediates cholesterol efflux to high-density lipoproteins (HDL) or the plasma membrane[111]. In addition, caveolae provide an alternative pathway for cholesterol influx from HDL into cells via scavenger receptor type B1, a HDL receptor that is localized in caveolae[112]. Thus, caveolae serve as a prime location for the exchange of plasma membrane cholesterol between HDL and the cell membrane, thereby maintaining cholesterol homeostasis. Interestingly, blood cholesterol levels are normal in Cav-1 KO mice, suggesting that there is another compensatory pathway for regulating cholesterol homeostasis in the absence of caveolae.

Another important physiological role of caveolae/Cav-1 is vesicular trafficking, which includes processes like transcytosis and endocytosis. Caveolae represent a “clathrin-independent” pathway for the internalization of extracellular ligands and plasma membrane components. Caveolae driven endocytosis is a dynamin-dependent process, which involves the re-structuring of the actin cytoskeleton, however, the exact molecular machinery that transforms
the invaginated caveolae to vesicles that bud off from the membrane is still relatively unknown\[113\].

Lastly, the role of caveolae as signaling hubs via the recruitment and interaction of signaling molecules, has generated tremendous amount of interest in the last few decades. Indeed, isolation and purification of caveolae from tissues and cultured cells reveal that they surprisingly contain high levels of several intracellular signaling proteins, including G-protein coupled receptors and eNOS\[114\]. These observations led to the concept that caveolae are docking points for cell surface receptors, which when activated by ligand binding are sequestered in caveolae and coupled to their downstream signaling molecules present in caveolae\[114, 115\]. In addition to being an important structural protein, Cav-1, is responsible for maintaining the efficient stimulus-to-response coupling in caveolae. The Cav-1 CSD has been shown to directly interact with distinct binding sites in caveolae-associated proteins and modulate the activity of these proteins\[115\]. Furthermore, Cav-1 oligomers serve to concentrate and compartmentalize signaling molecules within caveolae\[110\].

\[1.9. \textit{Caveolae and Cav-1 in the cardiovascular system} \]

Endothelial cells possess an abundant amount of caveolae, with caveolae organelles constituting approximately 30% of the endothelial surface in capillaries. Thus, it is not surprising that these organelles play a crucial role in the cardiovascular system. Cav-1 KO mice have proven to be an invaluable tool for investigating the importance of caveolae/Cav-1 in the development of cardiovascular pathologies. Pioneering studies by Drab et al demonstrated that Cav-1 KO mice exhibit aberrant endothelium-dependent vasodilation, contractility and myogenic tone, which was attributed to impaired NO and Ca\(^{2+}\) signaling in the cardiovascular system\[116\]. Even though cardiomyocytes do not express Cav-1, genetic ablation of Cav-1 caused dilated cardiomyopathy in the left ventricular chamber and chronic pulmonary hypertension\[117\]. These cardiopulmonary defects were a result of excessive NO production and increased endothelial cell permeability, suggesting that Cav-1 inhibition of eNOS may be critical in preventing these perturbations in systemic NO levels\[117\].

Endothelial caveolae have also been implicated in flow-mediated mechanotransduction, whereby these microdomains sense haemodynamic changes and transduce these changes to biochemical signals that regulate vascular function\[118\]. Indeed, signaling molecules that are
involved in flow-mediated activation of endothelial cells, such as eNOS and components of the MAP kinase pathway, are localized to caveolae and interact with Cav-1[119]. Moreover, Cav-1 KO mice have impaired regulation of vessel diameter, arterial remodeling and eNOS activation in response to flow in vivo, all of which are rescued by reconstitution of Cav-1 specifically in the endothelium[118]. In addition, Cav-1 modulates tumour angiogenesis, which is the formation of new blood vessels from pre-existing ones through the proliferation of endothelial and SMC and is a critical process involved in tumour growth. Endothelial cell migration, proliferation, cell sprouting from aortic rings, blood vessel infiltration and vessel density are significantly reduced in Cav-1 KO mice as compared to their wild-type counterparts[120, 121]. Furthermore, tumour growth and angiogenesis in vivo are significantly impaired in mice that lack Cav-1[120]. All of these experimental parameters are reversed upon pharmacological inhibition or genetic deletion of eNOS, indicating that dysregulated eNOS activity is a feature of Cav-1 KO mice and is responsible for impaired tumour angiogenesis[120]. Since Cav-1 KO mice have diminished responses to angiogenic factors and the ability to retard tumour growth, inhibition of Cav-1 is considered an anti-cancer therapeutic target. Lastly, caveolae and Cav-1 play an important role in modulating several aspects of the atherogenic process, which will be addressed in detail in the next section.

1.10. Cav-1 and atherosclerosis

Cav-1’s role in influencing atherosclerosis is complex as it can positively or negatively regulate the disease progression depending on the cell type examined[122]. Loss of Cav-1 in ApoE KO mice is associated with a 70% reduction in atherosclerosis, despite the fact these Cav-1/ApoE double KO (dKO) mice have a pro-atherogenic lipid profile[123]. Over-expression of endothelial specific Cav-1 in ApoE KO and re-expression of endothelial specific Cav-1 in Cav-1/ApoE dKO mice promotes atherosclerosis, stressing the essential role of endothelial Cav-1 in the progression of atherosclerosis[124, 125]. These studies have shed light on four important roles of endothelial Cav-1. Firstly, endothelial caveolae regulate transepithelial passage of LDL through the endothelial layer and into the intima, where they are retained and modified by oxidation to form ox-LDL, a major initiator in the atherosclerotic process[124]. Moreover, a receptor for native and modified LDL, known as CD36, is localized to caveolae and expression of this receptor was downregulated in Cav-1 KO mice[123]. Secondly, Cav-1 is an inhibitor of endothelial NO
production, which results in the decreased levels of anti-atherogenic NO production[124, 125]. Thirdly, Cav-1 influences the inflammatory component of atherosclerosis, in particular, by regulating proinflammatory mediators such as the TNF-α receptor and VCAM-1 expression[124, 125]. Gene expression studies in aortas from mice that overexpress endothelial Cav-1 demonstrate enhanced expression of VCAM-1[124, 125]. Lastly, Cav-1 reduces the proliferative and migrating properties of endothelial cells, which attenuates the capacity of endothelial cells to regenerate the intact lining of the blood vessel that could potentially limit lipid accumulation at lesion sites[122, 125]. Collectively, these studies demonstrate that Cav-1 in endothelial cells serves as a pro-atherogenic protein.

On the contrary, Cav-1 in vascular SMC is anti-atherogenic. Cav-1 is a negative regulator of SMC proliferation and migration, which is a key process in atherosclerotic lesion expansion. \textit{In vitro} and \textit{in vivo} studies have demonstrated that loss of Cav-1 results in hyper-proliferation of vascular SMC[126] and promotes neointimal formation in a model of arterial restenosis[127]. In addition, Cav-1 expression in vascular SMC is decreased in advanced human atheroma[126].

In macrophages, whether Cav-1 is anti-atherogenic or pro-atherogenic is still a matter of debate. Cav-1 is expressed at low levels in monocytes but is increased upon differentiation to macrophages when stimulated with the cytokine, macrophage- colony stimulating factor (M-CSF). A recent report revealed that Cav-1 plays a role in monocyte to macrophage differentiation as well as macrophage endocytosis of lipids, both of which result in the formation of macrophage-derived foam cells that form the necrotic core of atherosclerotic lesions[128]. On the other hand, another study demonstrated that Cav-1 is able to modulate cytokine production from macrophages by decreasing the production of pro-inflammatory cytokines (TNF-α) and increasing anti-inflammatory cytokine production (IL-10)[129]. These opposing actions make it difficult to deduce if Cav-1 expression in macrophages are pro-atherogenic or anti-atherogenic.

1.11. Cav-1 regulation of eNOS-derived NO

In the above sections, a common theme that underlies cardiovascular pathologies associated with Cav-1 deficiency is the improper regulation of eNOS-derived NO. The functional relevance of Cav-1 as a negative regulator of signaling molecules was demonstrated for the first time \textit{in vitro} based on its direct protein-protein interaction with eNOS. Binding
assays performed with Glutathione-S-Transferase (GST)-linked Cav-1 deletion mutants, showed that the Cav-1 CSD is able to directly interact with eNOS and inhibit the activity of the enzyme[97]. In addition, co-immunoprecipitation studies revealed that eNOS co-immunoprecipitates with Cav-1 and this complex is dissociated upon the addition of Ca$^{2+}$-CaM in the presence of HSP90[130, 131]. The functional significance of this interaction in vivo was revealed with the use of a cell permeable peptide (Attenapedia; AP) linked to the Cav-1 CSD, a peptide known as AP-Cav or Cavtratin. This peptide was able to inhibit NO release from endothelial cells, attenuate acetylcholine-induced vasodilation, and behave as an anti-inflammatory and anti-tumour compound by reducing microvascular permeability and tumour growth in vivo, in an eNOS-dependent manner[132, 133].

Perhaps the most convincing functional relevance of the eNOS/Cav-1 interaction comes from investigations into the vascular and cardiopulmonary defects present in Cav-1 KO mice that are reversed by either pharmacological inhibition of eNOS, genetic deletion of eNOS or reexpressing endothelial-specific Cav-1[134-136]. Aorta isolated from Cav-1 KO mice exhibit an impaired vasoconstrictor response to phenylephrine, which was restored with the addition of the NOS inhibitor, nitro-L-arginine methyl ester (LNAME)[134]. Similarly, endothelium-dependent vasorelaxation induced by acetylcholine was potentiated by the lack of Cav-1 and reversed with LNAME[134]. Furthermore, in Cav-1 KO mice, persistent activation of eNOS due to the absence of the endogenous inhibitory clamp caused tyrosine nitration of protein kinase G (PKG), resulting in pulmonary hypertension, which is a phenotype rescued in eNOS/Cav-1 dKO mice[135]. Additionally, cardiac hypertrophy and increased eNOS activation in the pulmonary arteries of Cav-1 KO mice is reversed by re-expression of endothelial-specific Cav-1[136].

Recent studies have identified a novel regulatory role of Cav-1 in suppressing eNOS uncoupling. As mentioned previously, BH4 depletion or BH4 oxidation to BH2 is a molecular trigger for eNOS uncoupling, with the latter being more prominent, which led to the hypothesis that endothelial cells possess an internal regulatory mechanism for preventing eNOS uncoupling in conditions of BH4 depletion[137]. In such instances of reduced BH4 availability, Karuppiah et al found that the interaction between eNOS and Cav-1 was augmented, preventing eNOS from uncoupling and producing superoxide[137]. In support of this, vessels from chronic high fat diet fed Cav-1 KO mice displayed reduced acetylcholine-induced vasodilation, which was reversed with sepiapterin (BH4 precursor) and Tiron (superoxide scavenger) treatment,
suggesting that during settings of metabolic and oxidative stress, the eNOS/Cav-1 interaction is involved in preserving NO synthesis by preventing oxidation of BH4 (FASEB meeting abstract). Furthermore, Cav-1 has been implicated in mediating eNOS activation and uncoupling by Nox-derived ROS. Upon stimulation with Angiotensin II, Cav-1 recruits the Nox subunits that promote ROS production and activates eNOS through ROS-sensitive intracellular signaling but also promotes eNOS uncoupling resulting in increased ROS production and endothelial dysfunction[138]. Moderate downregulation of Cav-1, decreases the eNOS/Cav-1 interaction but preserves eNOS localization to caveolae, thereby reducing the amount of Nox-derived ROS that cause eNOS uncoupling[138].

1.12. Molecular dissection of the eNOS/Cav-1 interaction

A novel study by Bernatchez et al aimed to characterize the molecular mechanism by which the Cav-1 CSD inhibits eNOS by mapping this inhibitory domain using AP-Cav, a cell-permeable Cav-1 CSD peptide. The Cav-1 CSD in AP-Cav was truncated to three sub-sections and five modified peptides: AP-Cav-A (amino acids 82-88), AP-Cav-B (89-95), AP-Cav-C (amino acids 96-101), AP-Cav-AB (amino acids 82-95) and AP-Cav-BC (amino acids 89-101) were generated. These were tested for their functional capacity in eNOS-dependent biological assays[139]. The peptides that contained the hydrophobic Cav-B domain (amino acids 89-95: FTTFTVT) caused inhibition of 1) VEGF-induced NO release in vitro, 2) recombinant eNOS activity, 3) NO-dependent inflammation in the ear and 4) hydraulic conductivity in intact vessels[139]. Alanine scanning of the B domain revealed that threonine 90,91 (T90,91) and especially phenylalanine 92 (F92) are crucial for mediating eNOS inhibition in vitro and in vivo(see figure 4)[139].
Figure 4: Cav-1 regulation of eNOS-derived NO

A schematic diagram showing eNOS regulation by Cav-1. In basal conditions, eNOS is localised to caveolae, where it is under negative regulation by Cav-1 through a direct protein-protein interaction between eNOS and the Cav-1 CSD. Upon agonist activation or sheer stress, the inhibitory clamp of Cav-1 is relieved and effective NO production by eNOS can then occur.
My master’s project, carried out in the Bernatchez laboratory at the University of British Columbia, involved characterizing the mutant F92A Cav-1 protein with respect to the biological properties of WT Cav-1. F92A Cav-1 was able to not only restore NO release in endothelial cells, but also increased NO release in a dose-dependent fashion as compared to WT Cav-1[140]. Using sucrose gradient fractionation and velocity gradient centrifugation, I demonstrated that F92A Cav-1 is able to localize in caveolae microdomains and form high molecular weight oligomers similar to WT Cav-1[140]. Immunofluorescence and GST-pulldown analysis showed that F92A Cav-1 co-localizes and binds to eNOS in endothelial cells, suggesting an additional distinct binding site for eNOS in the Cav-1 CSD[140, 141]. Thus, these data led me to conclude that the increase in NO release observed with F92A Cav-1 in eNOS-expressing cells is due to its ability to bind eNOS and relieve the inhibitory clamp of endogenous Cav-1 toward eNOS. Furthermore, we generated a modified cell permeable AP Cav peptide, which contained alanine substitutions at T90,91 and F92. This peptide, known as CavNOxin, increased basal NO release in vitro, reduced blood pressure in vivo and attenuated potassium chloride and phenylephrine induced contractility in isolated vessels[140]. The vessel reactivity effects of CavNOxin were not observed in eNOS KO and Cav-1 KO mice, clearly indicating that CavNOxin requires both eNOS and Cav-1 to exert its actions. Moreover, CavNOxin reduced eNOS co-immunoprecipitation with Cav-1 in a dose-dependent manner[140], behaving like a “pharmacological antagonist” of the eNOS/Cav-1 interaction. In summary, my study demonstrated that it is possible to modify the eNOS/Cav-1 interaction to increase eNOS-derived NO without comprising the biological properties of Cav-1 (see Figure 5).
Figure 5: CavNOxin relieves the eNOS/Cav-1 inhibitory clamp

CavNOxin, a cell permeable Cav-1 peptide with an inactivated eNOS inhibitory domain, is able to compete with endogenous Cav-1 for eNOS, allowing an increase in basal NO production and could lead to atheroprotection.
1.13. Targeting NO and oxidative stress in CVD: a potential role for the eNOS/Cav-1 interaction

The proper regulation of endothelium-derived NO is essential in maintaining cardiovascular homeostasis, yet the development of pharmacological tools to bolster NO specifically to improve cardiovascular function is lacking. Organic nitrates, also known as nitroglycerin, which deliver NO have been used clinically since the early 1990s for the treatment of angina pectoris and heart failure. However, some limitations with this class of compounds include the rapid development of tolerance, lack of selectivity and low bioavailability[142-144]. On the other hand, inorganic nitrites, an intermediate precursor that is metabolized to NO in vivo, has demonstrated superior pharmacokinetic and pharmacodynamics properties[142]. Inorganic nitrites have shown promise in a variety of experimental and clinical models to ameliorate acute myocardial ischemia/reperfusion injury and recover left ventricular function in settings of heart failure[145]. However, more recent large-scale studies have failed to identify improved clinical outcome and overall benefit of these compounds. Improving eNOS activity by substrate (L-arginine) or co-factor (BH4 supplementation has proven to reverse endothelial dysfunction, limit atherosclerosis and reduce vascular oxidative stress in pre-clinical animal models[146-148]. However, each of these therapies has major clinical limitations. L-arginine supplementation lacks efficacy and can unexpectedly promote cardiovascular disease unless given in conjunction with BH4[149], whilst, chronic BH4 treatment is a less feasible option as it is quickly oxidized to BH2, which lacks eNOS co-factor activity[150]. Cardiovascular drugs currently used in the clinic, such as statins, have shown the potential to modulate eNOS independent of its lipid-lowering activities. Statins can upregulate eNOS expression by stabilizing eNOS mRNA and enhance eNOS activity by decreasing Cav-1 abundance, however statins lack the ability to directly improve endothelial function, therefore the modulation of eNOS by statins are considered a secondary or side-effect of the drug[151-153]. Thus, there is a need to develop targeted therapeutics that can increase NO availability to recover endothelial function in disease settings.

A promising and highly targeted approach to increase basal NO levels is by modulating the eNOS/Cav-1 interaction. Indeed, by identifying the eNOS inhibitory domain in the Cav-1 CSD, our group has been able to render this region inactive by alanine substitutions. By conjugating the mutated Cav-1 CSD to a cell permeable peptide (attenapedia; AP), the resultant
peptide termed CavNOxin, has become a valuable compound for studying antagonism of the eNOS/Cav-1 interaction. Cell permeable peptides are an excellent tool for delivering peptides in vitro and in vivo and in this case to aid with target validation of the eNOS/Cav-1 interaction. AP delivery, in particular, occurs via lipid-raft dependent endocytosis and has relatively low toxicity[154]. We have demonstrated that the CavNOxin peptide is delivered and expressed in endothelial cells within 6 hours. In addition, we were able to show that the CavNOxin peptide increased NO release and improved NO-dependent activities, without comprising the biological functions of caveolae and endogenous Cav-1. In my PhD thesis, I have aimed to extend the potential use of the CavNOxin peptide into pre-clinical models of cardiovascular diseases, in particular atherosclerosis.

1.14. Novel antioxidant therapies to limit CVD

Impairment of endogenous antioxidant defense systems is implicated in the heightened oxidative stress observed in CVD settings. Traditional antioxidants, such as vitamin E and vitamin C have proven to be protective in pre-clinical models of atherosclerosis[155], however, several clinical trials have reported conflicting results as to the overall benefit of these antioxidant vitamins in end-point CVD events[156-158]. The lack of clinical efficacy of these antioxidants could be attributed to non-specific targeting of ROS species and/or their cellular localization. A major focus of current therapeutics is the generation of small synthetic mimetics of endogenous antioxidant enzymes to bolster the activity of these enzymes that are often compromised in disease settings [158]. A summary of the antioxidant pathway and the targets of antioxidant mimetics is shown in Figure 6. In this section, I will discuss two novel antioxidant therapies that I investigated in Dr Judy de Haan’s laboratory as part of a secondary project.

1.14.1. GPx1 mimetic: m-hydroxy-ebselen

GPx1 is the most abundant isoform of the selenocysteine-containing GPx protein family, which are responsible for the detoxification of hydrogen peroxide. Amongst the ROS, hydrogen peroxide has a relatively long half-life, is highly membrane permeable and acts a putative second messenger for various inflammatory pathways[159]. GPx1 is considered a versatile antioxidant enzyme since it is able to neutralize hydrogen peroxide, eliminate lipid peroxides and reduce the potent peroxynitrite anion[160]. Recent pre-clinical and clinical data
provide strong evidence for the essential role GPx1 plays in protecting the cardiovascular system against oxidative stress. Indeed, impaired GPx1 activity is now considered an independent CVD event predictor in patients with coronary artery disease[49, 161].

In animal models, GPx1 KO mice exhibit an enhanced oxidative stress profile, impaired endothelial function and paradoxical vasoconstriction in response to endothelium-dependent agonists, which is indicative of lowered NO bioavailability[162]. In addition, GPx1 deficiency leads to impaired angiogenesis, increased infarct size and vascular permeability following ischemia/reperfusion injury[49]. Dr Judy de Haan laboratory and Torzewski and colleagues have shown that ApoE/GPx1 dKO mice have exacerbated atherosclerosis in response to diabetes and hypercholesterolemia respectively, as compared to their ApoE KO counterparts[163, 164]. This increase in atherosclerosis was associated with enhanced expression of pro-oxidative enzymes (Nox2), pro-inflammatory (VCAM-1) and pro-fibrotic (CTGF) mediators[164]. In cultured endothelial cells, it has been shown that GPx1 deficiency leads to oxidative stress, which contributes to increased VCAM-1 expression by augmenting NF-κB and JNK activation[165]. In addition, my unpublished data demonstrates that in response to the inflammatory cytokine, TNF-α, and hyperglycemia, the absence of GPx1 enhances leukocyte-endothelial interactions, which could explain the increased atherosclerosis observed in these settings (Appendix A: Figure 26). Overall these studies suggest that GPx1 contributes significantly to the pathogenesis of atherosclerosis and is a viable therapeutic target.

Ebselen [2-phenyl-1,2-benzisoselenazol-3[2H]-one] is a synthetic lipid soluble seleno-organic low molecular weight compound which mimics the activity of GPx1 in a kinetically similar fashion[49]. Ebselen exhibits neuroprotective effects in stroke patients and protective effects on the endothelium in hypertensive and diabetic rats[49]. Furthermore, Dr Judy de Haan’s group have demonstrated the anti-atherogenic effect of ebselen in diabetic ApoE KO and ApoE/GPx1 dKO mice, associated with a decrease in oxidative stress markers and reduced expression of proatherogenic cellularity and mediators[166]. Additionally, pretreatment with ebselen, diminished hydrogen peroxide induced increases in inflammatory cytokines, such as TNF-α and NFkB, and attenuated the TNF-α-induced expression of endothelial cell adhesion molecules (VCAM-1 and ICAM-1)[166]. Currently, research is focused on structural modifications of ebselen to increase its efficacy and potency. One such modification Dr de Haan’s group has worked on is the addition of a hydroxyl group on the phenyl ring of ebselen, a
compound known as m-hydroxy ebselen (ME). Biochemical and kinetic analysis have demonstrated that ME has higher catalytic activity and is able to reduce peroxynitrite at a much lower dose than ebselen, suggesting that ME behaves in a superior fashion to ebselen[49, 167]. Thus, with these biochemical and kinetic data in mind, I, together with colleagues in the de Haan laboratory, investigated the effects of ME in vitro and in vivo. In cultured endothelial cells, pre-treatment with ME was able to attenuate hydrogen peroxide induced increases in ERK and p38 by up to 80%, whereas ebselen had negligible effect on these pathways[167]. However, ME attenuated diabetes-associated atherosclerosis both within the total aorta (Appendix A: Figure 27) and the aortic sinus region (Appendix A: Figure 28) and oxidative stress (Appendix A: Figure 29) in diabetic ApoE/GPx1 KO mice to a similar extent as ebselen, indicating no additional benefit derived from the modified ebselen analogue in vivo[49, 143]. Importantly, we were able to conclude from our study that in vitro and cell based assays cannot be relied upon to predict the in vivo effectiveness of the Eb analogues.

### 1.14.2. Nrf2 agonist

Nf-E2-Related Factor 2 (Nrf2) is a redox sensitive transcription factor, which is activated in response to a range of oxidative stimuli. Once activated, Nrf2 up-regulates the expression of various endogenous anti-oxidant enzymes, including quinone reductase and heme-oxygenase 1 (HO-1)[158]. Nrf2 is ubiquitously expressed and normally resides in the cytoplasm bound to Kelch-like ECH-associated protein (Keap)-1[168]. Upon exposure to oxidative stimuli, the Keap protein is oxidized or covalently modified releasing Nrf2, which then enters the nucleus. By binding to antioxidant-response element consensus sequences, Nrf2 initiates transcription of antioxidant enzymes[158, 168]. Moreover Nrf2 has demonstrated anti-inflammatory properties driven through its ability to negatively regulate NF-kB, the transcription factor central to the inflammatory response[168].

Numerous small molecule activators of the Nrf2/Keap1 pathway have been identified including bardoxolone methyl (BM), which belongs to the antioxidant inflammation modulator drug class. BM has demonstrated structural and functional improvements in several rodent models of renal disease, along with an increase in protective antioxidant and anti-inflammatory genes[169]. In addition, the therapeutic potential of BM was extended to clinical trials in type 2 diabetic patients with chronic kidney disease[170, 171]. Although, BM showed promise in
Phase 1 and Phase 2 clinical trials, the larger Phase 3 BEACON trial was terminated prematurely due to unexpected heart failure in some stage 4 kidney disease patients[171]. Despite the setback experienced in the BEACON trial, which may have arisen due to improper dosage in renally impaired patients, the antioxidant and anti-inflammatory properties of Nrf2 activators make them desirable drug candidates to improve vascular health, since the onset of endothelial dysfunction is accompanied by pro-oxidative and pro-inflammatory conditions. In support of this, a study by Heiss et al have demonstrated that Nrf2 activators improve NO bioavailability, specifically by bolstering the antioxidant defense system and preventing eNOS uncoupling in cultured endothelial cells[172]. In addition newer synthetic Nrf2 activators improve endothelial dysfunction in murine models of kidney disease[173].

A recently published study by Dr de Haan’s group investigated a BM derivative compound, known as dh404. As part of this study, I have demonstrated that dh404 attenuates diabetes-associated atherosclerosis in an inverse dose-dependent manner with the lower doses being atheroprotective (Appendix B: Figure 30)[174]. Importantly, the data suggested that a window exists where the beneficial effects of protection may be lost due to off-target effects at higher doses. The atheroprotective effects of dh404 correlated with similar reductions in pro-inflammatory gene expression (TNF-α, MCP-1, ICAM-1 and VCAM-1) and oxidative stress[174], which suggests that the underlying endothelial dysfunction was also improved by the BM analogue leading to improvements in DAA (Appendix B: Figure 31 shows the reduction in VCAM-1 expression and protein levels caused by dh404; Figure 32 shows the effect of dh404 on urinary and plasma oxidative stress markers).
Figure 6: Novel synthetic mimetics to increase activity of endogenous antioxidant enzymes

The classic antioxidant pathway for the neutralization of ROS produced by vascular ROS sources (Nox, xanthine oxidase and uncoupled eNOS) in endothelial cells is shown here. Dotted arrows represent antioxidant mimetics that target endogenous antioxidant enzymes. Orange arrows represent the effect of the antioxidant mimetic. \( \text{O}_2^\cdot \): superoxide anion; \( \text{ONOO}^- \): peroxynitrite; \( \text{NO}_2^- \): nitrite; \( \text{H}_2\text{O}_2 \): hydrogen peroxide; \( \text{OH} \): hydroxyl radical; \( \text{LOOH} \): lipid hydroperoxide; \( \text{LOH} \): lipid alcohol.
Chapter 2. Research overview
2.1. Rationale

Cardiovascular diseases, such as atherosclerosis, remain a major cause of mortality and morbidity in Westernized societies and are on the increase in developing nations. Atherosclerotic risk factors, including hypercholesterolemia and diabetes, are well known to attenuate the protective nature of the vascular endothelium, a condition often described as endothelial dysfunction. In fact, endothelial dysfunction is the critical first step in the pathogenesis of atherosclerosis, which is often preceded by endothelial activation, a process that upregulates the endothelial expression of adhesion molecules resulting in the recruitment of inflammatory cells and atherosclerotic lesion expansion. Endothelial dysfunction is now widely considered an independent clinical predictor for cardiovascular disease. A salient feature of endothelial dysfunction is reduction in the bioavailability of NO, a potent mediator of endothelial homeostasis. NO is considered an anti-atherogenic molecule through its ability to regulate vascular tone as well as reduce adhesion molecule expression, platelet aggregation and SMC proliferation and migration. A plethora of pre-clinical and clinical studies have demonstrated that hypercholesterolemic and diabetic subjects have attenuated vascular reactivity and increased adhesion of inflammatory cells, prior to the development of advanced atherosclerotic lesion. In addition, atherosclerosis is linked to a heightened state of oxidative stress, which can scavenge NO and further reduce NO bioavailability. Current therapeutics for the treatment of atherosclerosis includes lipid-lowering or anti-hypertensive drugs with secondary effects on endothelial function. Since, endothelial dysfunction is the prime initiator of atherosclerosis, the development of targeted therapies that directly improve endothelial function, in particular NO bioavailability, are highly warranted.

NO is synthesized in endothelial cells by the constitutively expressed synthase, eNOS. Effective production of atheroprotective NO requires eNOS trafficking to endothelial caveolae, which are lipid-enriched flask shaped organelles at the plasma membrane responsible for compartmentalizing signal transduction molecules. Interestingly, within caveolae, basal eNOS activity is inhibited by Cav-1, the major coat protein of caveolae, via a direct protein-protein interaction between the Cav-1 scaffolding domain (amino acids 82-101) and eNOS. Earlier work from our group has elucidated through functional mapping of the Cav-1 scaffolding domain that the amino acids responsible for inhibiting eNOS-derived NO release are T90, T91 and F92. A cell permeable peptide, AP, when linked to the Cav-1
scaffolding domain, reduced NO release in cultured endothelial cells, vessel reactivity and NO-dependent inflammation. This led to the generation of a modified cell permeable Cav-1 peptide, consisting of an inactivated eNOS inhibitory domain through alanine substitutions. This peptide, termed CavNOxin, is able to increase NO release in eNOS-expressing cells and improve endothelium-dependent vascular tone, without comprising the biological properties of endogenous Cav-1. More importantly, these observations raised the concept of regulated eNOS/Cav-1 antagonism, whereby CavNOxin prevents eNOS from binding to endogenous inhibitory Cav-1 resulting in increased eNOS-derived NO release. However, the therapeutic potential of the CavNOxin peptide in atherosclerotic disease settings, which are highly governed by reduced NO bioavailability is unknown.

2.2. Hypothesis

I hypothesize that CavNOxin, a cell permeable Cav-1 peptide that is able to increase NO release can attenuate endothelial processes associated with atherosclerosis in vivo.

2.3. Aims

Aim 1: To determine if CavNOxin can reduce atherosclerosis in a hypercholesterolemic setting in an eNOS-dependent manner (to be covered in Chapter 4)

Specifically, I aim to investigate the effect of CavNOxin on:

1) Oxidative stress markers
2) Inflammatory mediators
3) Leukocyte-endothelial interactions
4) Atherosclerosis in eNOS-deficient mice

Aim 2: To determine if CavNOxin can reduce diabetes-associated atherosclerosis (to be covered in Chapter 5)

Specifically, I aim to determine the effect of CavNOxin on:

1) Blood glucose levels
2) Oxidative stress parameters
3) Inflammatory markers
4) Leukocyte-endothelial interactions
Chapter 3. Materials and methods
In this chapter, the materials and methods for both the hypercholesterolemic (aim 1) and diabetic study (aim 2) will be addressed in sections 3.1. to 3.15. In addition, for the diabetic study, supplementary experiments were done, which will be addressed in sections 3.16. to 3.18.

3.1. Experimental mice

For the hypercholesterolemic study, ApoE KO mice were purchased from the Animal resource Centre (Western Australia) and Jackson Laboratories (Bar Harbor, ME) and housed at the Precinct Animal Centre (PAC) at the Baker IDI Heart and Diabetes Institute and the Genetic Engineered Models (GEM) facility at the UBC James Hogg Research Centre respectively. To generate eNOS/ApoE dKO mice, eNOS KO (stock #002684) mice were purchased from Jackson Laboratories and cesarean derived from crossing to a C57BL/6 background to remove pathogens endemic to the JAX population. The resulting pathogen free eNOS heterozygous mice were used to generate eNOS KO mice, which were maintained on a C57BL/6 background. eNOS KO (first generation) mice were initially crossed to ApoE KO mice (third generation or earlier). The resulting mice heterozygous for both eNOS and ApoE were then crossed again to an ApoE KO mouse. Finally, resulting littermates that were ApoE KO/eNOS heterozygous were bred together to give age matched littermates which were either ApoE KO, eNOS KO or eNOS/ApoE dKO. All experiments were approved by the animal ethics committee at both institutions.

3.2. Animal experimental procedures

At 8 weeks of age, male ApoE KO mice and male eNOS/ApoE dKO mice were placed on a high-fat Western-type (HFD) diet containing 22% fat and 0.15% cholesterol (Speciality feeds, SF00-219 and Harlan Teklad TD88137) for 12 weeks till 20 weeks of age. A control group of male ApoE KO mice were placed on a standard chow diet. Published data has demonstrated that this time period is sufficient to induce atherosclerosis[175]. Furthermore, the ApoE KO mouse fed a HFD is a useful pre-clinical model to mimic diet-induced atherosclerosis, as it develops lesions similar to those seen in humans. During the 12-week time period, mice were either injected intraperitoneally (IP) with CavNOxin at 2.5mg/kg (prepared by dissolving in 1%
DMSO in sterile water) or vehicle peptide (AP; at equimolar dose) every 3 days (for peptide sequences see Table 1). This dose was based on the previously published anti-tumour effect of Cavtratin in vivo[133].

For the diabetic study, 8-week old male ApoE KO mice were rendered diabetic by IP injections of streptozotocin (STZ; Sigma-Aldrich, St. Louis, Mo) on 2 consecutive days, at a dose of 100 mg/kg/day. STZ is a diabetogenic agent that destroys pancreatic β-cells, resulting in insulin deficiency and hyperglycemia. Sham-injected (citrate buffer) ApoE KO mice served as non-diabetic controls. After establishment of diabetes (approximately 2 weeks after STZ injections), diabetic mice were randomized and injected IP every 3 days with either sterile water, vehicle peptide (AP; dissolved in 1% DMSO in sterile water) or CavNOxin at 2.5mg/kg and 5.0mg/kg for a period of 14 weeks. Non-diabetic mice received the identical treatment regimens for 14 weeks. This time-point has been previously demonstrated by our group to be sufficient in establishing atherosclerotic lesions in diabetic ApoE KO mice[167]. Non-fasted blood glucose readings were measured every week from the tail vein to ensure that the mice were diabetic throughout the study. Only mice with a blood glucose reading of more than 18mmol/L were included in the study (see section 3.17.). Animals had unrestricted access to water and standard mouse chow. All experimental procedures on animals were performed in accordance with experimental protocols approved by the animal ethics committee at the respective institutes.

Table 1: Peptide sequences
Amino acid sequences of peptides are shown. Alanine substitution of the eNOS inhibitory domain in the Cav-1 CSD is indicated in red.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP (Vehicle)</td>
<td>RQIKIWFQNRRMKWKK</td>
</tr>
<tr>
<td>CavNOxin</td>
<td>RQIKIWFQNRRMKWKK-DGIWKASFAATVTKYWFYR</td>
</tr>
</tbody>
</table>

3.3. Organ collection

At termination, animals were fasted for 12 hours but were allowed free access to water. Mice were anaesthetised by an IP injection of Avertin (2,2,2- tribromoethanol) dissolved in saline. Deep anaesthesia was monitored as lack of pedal reflexes after applying pressure to the toes of the right limb as well as a lack of corneal reflexes. Next, the thoracic cavity was opened
and blood drawn (0.8-1ml) by direct puncture of the right ventricle and placed in heparinised tubes. The blood was then immediately spun down at 4,000rpm at room temperature for 10 minutes to separate the plasma for analytical purposes. Once the plasma was separated, it was stored at -80°C. The remaining red blood cells were mixed with an equal volume of saline, in order to maintain osmotic pressure and kept at 4°C for the measurement of glycated haemoglobin (diabetic study). Following which, the kidneys, liver and lungs were collected. Each organ was weighed and then processed by fixing a portion in 10% neutral buffered formalin (NBF) and snap freezing the remainder in liquid nitrogen. Subsequently, the organs were fixed in 10% NBF overnight and paraffin embedded for histological analysis. The heart and aorta were dissected out and placed in cold saline. The aorta was cut close to the heart, cleaned of peripheral fat under a microscope and fixed in 10% NBF for Sudan IV staining to quantitate atherosclerotic lesions. The top of the heart was collected after dissection with a sharp scalpel blade through the heart at a plane parallel to the tips of the external atria. The heart top, which encompasses the aortic sinus, was fixed in Paraformaldehyde/Lysine/Periodate (PLP) prior to being frozen in optimal cutting temperature (OCT) compound.

In another cohort of mice (12 weeks of HFD and 10 weeks of diabetes), aortas were cleaned of peripheral fat and snap frozen in Trizol. A portion of the aortic arch was frozen in OCT compound for DHE analysis and VCAM-1 immunohistochemical analysis (section 3.9.2 and 3.10).

### 3.4. Plasma biochemistry

Plasma cholesterol (CHOL), triglycerides, LDL, HDL, and LDL/HDL (LH) ratio were measured by standard commercial enzymatic assays using a Beckman Coulter LX20PRO Analyser, with reagents and calibrators supplied by Beckman Coulter Diagnostics. CHOL reagent is used to measure cholesterol concentration by a timed-endpoint method. In the reaction, cholesterol esterase (CE) hydrolyses cholesterol esters to free cholesterol and fatty acids. Free cholesterol is oxidized to cholestene-3-one and hydrogen peroxide by cholesterol oxidase (CO). Peroxidase catalyses the reaction of hydrogen peroxide with 4-aminoantipyrine (4-AAP) and phenol to produce a coloured quinoneimine product. The system monitors the change in absorbance at 520nm and this change is directly proportional to the concentration of CHOL.
Triglycerides GPO reagent is used to measure the triglycerides concentration by a timed endpoint method. Triglycerides in the sample are hydrolyzed to glycerol and free fatty acids by the action of lipase. A sequence of three coupled enzymatic steps using glycerol kinase, glycerophosphate oxidase and horseradish peroxidase causes the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) with 4-AAP to form a red quinoneimine dye. The system monitors the change in absorbance at 520nm and this change is directly proportional to the concentration of triglycerides.

To measure HDL cholesterol, a unique detergent was used, which solubilizes only the HDL lipoprotein particles and releases HDL cholesterol to react with cholesterol esterase and cholesterol oxidase in the presence of chromogens, to produce a color product. The same detergent also inhibits the reaction of the cholesterol enzymes with LDL, VLDL and chylomicrons lipoproteins by adsorbing to their surfaces. A polyanion contained in the reagent enhances the selectivity for HDL cholesterol assay by complexing LDL, VLDL and chylomicrons lipoproteins. The system monitors the change in absorbance at 560nm and this change is directly proportional to the concentration of cholesterol in the sample and is used by the system to calculate and express the HDL-cholesterol concentration. The LDL and LH ratio are then calculated based on the following equations:

\[
LDL = \text{CHOL} - \text{HDL} - \left(\frac{\text{triglycerides}}{2.25}\right)
\]

\[
\text{LH ratio} = \frac{LDL}{\text{HDL}}
\]

3.5. Plasma NO

Plasma NO was determined using the total NO detection kit as per the manufacturer’s instructions (Enzo Life Sciences). The kit involves the enzymatic conversion of nitrate to nitrite, by the enzyme nitrate reductase, followed by colourimetric detection of nitrite. This allows the total determination of both NO products in the sample. Firstly, plasma was diluted 1:5 in the reaction buffer and ultrafiltration was performed through a 10,000 molecular-weight cut off filter (Amicon Ultra-0.5 centrifugal devices; Merck Millipore). The samples were added to the filter, spun at 14000g for 15 minutes at room temperature and the resulting filtrate was then used for the assay. Nitrate standards (3.125, 6.25, 12.5, 25, 50 and 100µM/L) were prepared using the 1mM/L nitrate standard solution provided. Next, 50µl of standards and samples were added in duplicate to assigned wells of the 96-well microtitre plate. In addition, 25µl of NADH and 25µl
of nitrate reductase enzyme was added to the wells and the plate was incubated for 30 minutes at 37°C. Following which, 50µl each of Greiss Reagent I and II were added into the wells and allowed to incubate for a further 10 minutes at room temperature. The colorimetric detection of nitrite as a colored azo dye product of the Greiss reaction was measured by reading the optical density (OD) of each well at 540nM after normalising against the blank wells. The average net OD bound for each standard and sample was calculated by subtracting the average zero standard OD from the average OD for each standard and sample. The average net OD for each standard concentration was plotted and the total NO concentration for each sample was extrapolated from the graph using the Microsoft Excel software.

3.6. Plasma dROMs assay

The dROMs test allows for the determination of the blood concentration of reactive oxygen metabolites (ROMs), particularly hydroperoxides, which are markers and amplifiers of free radical-induced oxidative damage[176]. Plasma dROMs were measured as per Chew et al[177]. Briefly, 10µl of the heparanized plasma collected was dissolved in an acidic buffer supplied by the manufacturer in the dROMs kit. The hydroperoxides present in the sample then react with transition metal ions liberated from the proteins by the acidic medium and are converted into alkoxy and peroxy radicals. These newly formed radicals oxidize N,N-diethyl-para-phenylenediamine to its corresponding radical cation. The concentration of this species was determined spectrophotometrically at 505nm using the Free Radical Analyser System 4 (FRAS 4) set at a k-factor of 18. dROMS are measured in Carratelli Units where one CARR Unit is equivalent to 0.8 mg/L hydrogen peroxide[176].

3.7. Atherosclerotic lesion analysis

The extent of atherosclerosis was examined by two approaches: 1) Total aortic plaque was determined in the aorta by the en face technique after Sudan IV staining and 2) Aortic sinus plaque area was determined by histological analysis using Oil Red O staining. 1) For the en face technique, formalin-fixed aortas were stained with Sudan IV (Sigma), a fat-soluble dye used for the staining of lipids. Briefly, aortas were rinsed in 70% ethanol and stained with Sudan IV for 20 minutes in the dark. Following which, aortas were rinsed for 20 minutes with 80% ethanol and then washed under running tap water for an hour. Next, the aortas were
opened longitudinally and cut into 3 sections; namely the arch, thoracic and abdominal aorta, under a dissecting microscope. The aortas were pinned out flat and photographs of the stained aortas were digitized by use of a dissecting microscope (Olympus SZX9, Olympus Optical) equipped with a high-resolution camera (Fujix HC-2000, Fujifilm). Digitized images were then evaluated manually with Image Pro Plus and lesion area measurements were performed by calculating the proportion of aortic intimal surface area occupied by the red stain in each of the 3 aortic segments and expressed as a percentage of the total aortic area. After *en face* analysis, aortic tissues were embedded in paraffin, and cross-sectional serial sections 4µm thick were sectioned for immunohistochemical analysis using a microtome.

2) Lesions within the aortic sinus were assessed by Oil Red O, a fat-soluble dye used to stain lipids, in frozen heart sections. Briefly, 8-µm-thick cryostat sections were cut and sections that were 40 µm apart and covering 320 µm of the sinus were selected for staining with Oil red O to detect lesions. The area of lesion, which was defined as the red stain below the three leaflets of the aortic sinus, was quantitated with Image Pro Plus. For each mouse, lesion size (µm²) was determined from the average of 5 cross sections.

### 3.8. Quantitative reverse-transcription polymerase chain reaction

#### 3.8.1. RNA extraction

Aortic tissues (frozen in Trizol) were homogenized using a bullet blender (10 minutes, 4°C at speed 9). After homogenization, the addition of chloroform:isoamylalcohol (49:1 ratio) causes phase separation, where the RNA separates into the aqueous phase. RNA was then isolated from the aqueous phase by mixing with an equal volume of 100% isopropyl alcohol and precipitation overnight at -20°C. Following this, the RNA pellet was obtained after centrifugation at 13200rpm for 15 minutes at 4°C. Lastly, the RNA pellet was washed with 75% ethanol and redissolved in 10µl of nuclease-free water. RNA concentration was determined using the nanodrop spectrophotometer at 260nm and 280nm.
3.8.2. cDNA synthesis

Firstly, contaminating DNA was removed by treatment with DNA-free™ DNase and DNase inactivation agent according to the manufacturer's specifications (Ambion Inc., Austin, TX). Next, DNA-free RNA (2µg) was reverse-transcribed into cDNA using the Superscript First Strand Synthesis System (Life Technologies BRL, Grand Island, NY). 50ng/µl of random primers were first added to RNA samples and incubated at 70°C for 5 minutes, followed by immediate placement on ice. Meanwhile, a master mix was made from 4µL of 5X first strand buffer, 2µL of 10mM dNTPs, 2µL of 0.1M dithiothreitol (DTT), 0.1µL of RNase inhibitor (Promega; 20U/ µL) and 1µL of M-MuLV Reverse Transcriptase (200U/ µL). The master mix was then added to the RNA mixture and incubated at room temperature (25°C) for 10 minutes and subsequently incubated at 37°C for 60 minutes and at 70°C for 10 minutes to complete the reverse transcription. The mixture was then pulse centrifuged to capture any condensation built up within the PCR tube and cDNA was then stored at -20°C until further use.

3.8.3. qRT-PCR analysis

Quantitative Real Time-PCR (qRT-PCR) is a quantitative method for the determination of mRNA gene expression. qRT-PCR measures the degradation of a fluorescent labelled oligonucleotide (referred to as a probe) in real-time, concomitant with PCR amplification[178]. The probe has a reporter dye at the 5’ end and a quencher dye at the 3’ end, which is designed to anneal between the 5’ and 3’ oligonucleotides sites. When the probe first anneals it is intact and thus no fluorescence is emitted. However, during PCR the probe is cleaved by the 5’ nucleolytic activity of Taq DNA polymerase (Invitrogen) resulting in the separation of the reporter and quencher dyes and subsequently resulting in an energy transfer from the quencher dye to the reporter dye. This process is repeated in every cycle, resulting in the emission of energy as fluorescence. Thus, the accumulation of PCR products is directly monitored by measuring the increase in fluorescence. A passive reference dye, MGB, is included in the reaction mixture and acts as an internal control (normalising the reporter dye signal) and correcting for any fluctuations in fluorescence caused by changes in volume or concentration. A threshold line is set in the linear phase of the exponential curve representing PCR amplification. This threshold line is set above background fluorescence and fluorescence emitted from no template controls.
(NTC). When the fluorescence of a particular sample rises significantly above background and crosses the threshold a cycle number (Ct) is then recorded. Thus, if a given sample contains more cDNA for the gene of interest it will result in an earlier emission of fluorescence rising above the threshold and thus recording a lower Ct. qRT-PCR reactions contained 500nmol/L of forward and reverse primer and 50nmol/L of FAM/MGB probe and VIC/MGB 18S ribosomal probe, in 1X Taqman universal PCR master mix (PE Biosystems, Foster City, C.A., USA). Probes and primer sequences of genes of interest were designed and purchased from Applied Biosystems (ABI, Foster City, CA, USA) and are shown in Table 2. PCR amplifications (ABI Prism 7500; Perkin-Elmer Inc, Foster City, CA) were performed with the following time course: Holding stage at 95ºC followed by a cycling stage consisting of 50 cycles of 95ºC for 3 seconds and 60ºC for 30 seconds. The method used to obtain relative gene expression data is the comparative Ct method previously described by K. Livak[178] (Applied Biosystems; Sequence Detector User Bulletin 2). The reporter dye on the probe is typically labelled with a FAM dye and the reporter dye on endogenous control (18S) probe is labelled with a VIC dye, thereby allowing the amplification and measurement of both the gene of interest and the endogenous control gene in the same reaction. Each dye emits fluorescence at different wavelengths when excited by the laser. The Ct are extrapolated from the results graph for both the FAM probe and the VIC probe and the difference in Ct (ΔCt) is calculated by subtracting the VIC Ct from the FAM Ct. The average ΔCt value is then calculated for the calibrator group (HFD + Vehicle group for hypercholesterolemic study and Non-diabetic group for diabetic study) and this is subtracted from the ΔCt value of every sample, including the calibrator group. The resulting value is the ΔΔCt and this value is used in the equation, $2^{-\Delta_{\text{Ct}}}$ which yields the fold induction value. Results are expressed relative to the calibrator group in each study, which were arbitrarily assigned a value of 1. All means, standard error of means and statistics are calculated using this value.
<table>
<thead>
<tr>
<th>Gene</th>
<th>forward primer 5’-3’</th>
<th>reverse primer 5’-3’</th>
<th>probe 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>AAACATTCTGTATGGCTCTGAGACT</td>
<td>CTCTAGGGACACCCACATCATACTCAT</td>
<td>6-FAM CCAGAGCTACGCACACGC</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>CTGCTCAAGTGATGCGATAACCA</td>
<td>ATCGTCCCTTTTTTGATGACATGAAG</td>
<td>6-FAM CCAAAATCTCTGGAGCGAG</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>GGAAGGTGCCGGGGAAGTT</td>
<td>TCCAGCCGAGGACCACATACAG</td>
<td>6-FAM CCCTGGAACGTCACAGG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GTCTGTGGCTGACCCACAAAGAG</td>
<td>TGGTTCGATCCAGGGTTTTA</td>
<td>6-FAM FAM AATGGGCTCAGGATAC</td>
</tr>
<tr>
<td>NFKB</td>
<td>TCTCACATCCGATTGTGATAACC</td>
<td>CGAGGCAGCTCCCAGAGTT</td>
<td>6-FAM FAM AGCTCAAGATCTGCGC</td>
</tr>
<tr>
<td>Nox1</td>
<td>GACCAATTGGGGGACATGAGTT</td>
<td>CCCCCACCGCAGACTTG</td>
<td>6-FAM FAM CTAGAATAGCTACTGGCC</td>
</tr>
<tr>
<td>Nox2</td>
<td>AGTGGCGTGGTTGCTGCACAAG</td>
<td>CCAAGCTACCATTTAGTTCCAGAAAGGG</td>
<td>6-FAM FAM CAACTGGACGGAACCT</td>
</tr>
<tr>
<td>Nox4</td>
<td>AAAAAATATCACCACAACCTGATTGCCAGA</td>
<td>TGGTCCACAGCAGAAAACCTTGTGGAAGTGGTTTAGCTA</td>
<td>6-FAM FAM CTTGGTCTTCTCACCAGG</td>
</tr>
<tr>
<td>MnSOD</td>
<td>GGGACATATTAATCACACATCCATTCTTCTG</td>
<td>CCCAAAGTCACGGCTTGATAGC</td>
<td>6-FAM FAM CCTGAGCCCTAAGGGG</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>GGACGGTGTTGGCCTGACATG</td>
<td>CGGCAAATGATGGAAGTCAGCG</td>
<td>6-FAM MAM TGGTCTCTACTCTCGAG</td>
</tr>
<tr>
<td>Rac-1</td>
<td>CGAAAGAGATCGCTGTACA</td>
<td>AGAGAACCGCTCGGAAGATC</td>
<td>6-FAM MAM ACACGGAGACTCAA</td>
</tr>
<tr>
<td>GPx-1</td>
<td>CCCCACCTCGCTCATAAAGGC</td>
<td>GCCACACCGGAGACCAGGGGGCGGGGGGGGGGGGGG</td>
<td>6-FAM MAM CGACCCCAAGTACATC</td>
</tr>
<tr>
<td>18S</td>
<td>TGGTACCAGATGACGATCG</td>
<td>TGGTGGCCTGGGAAGATCC</td>
<td>6-FAM MAM TGCTGGCACCAGGACCA</td>
</tr>
</tbody>
</table>

Table 2: qRT-PCR primers and probes
Sequences of primers and probes used in the qRT-PCR analysis
3.9. Immunohistochemical analysis

3.9.1. Nitrotyrosine immunohistochemistry on paraffin-embedded aortic sections

Paraffin-embedded aortic sections were cut at 4µm and stained for nitrotyrosine (NT), a marker of peroxynitrite-induced protein damage. Tissue sections were de-waxed by placing the slides in xylene (2 rounds of 5 minutes each) and hydrated through a graded series of alcohol. Next, tissue sections were incubated with tris-buffered saline (TBS; 10 mM Tris-Cl, 150 mM NaCl, pH 7.6.) and endogenous peroxidases were inactivated with 0.3% H₂O₂ in TBS. Non-specific proteins were then blocked by incubating tissue sections with 5% skim milk in TBS for 30 minutes. Following which, tissue sections were incubated with the NT primary antibody (Millipore; 1:75 dilution) overnight at 4°C. The next day, biotinylated anti-rabbit immunoglobulin 1:100 (Dako) was then added for 10 minutes, followed by horseradish peroxidase–conjugated streptavidin, diluted 1:500 (Dako) for an additional 30 minutes. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co). Tissue sections were counterstained with hematoxylin, dehydrated in graded series of alcohol solutions, cleared with xylene and mounted with DePex mounting medium. Bright field images were captured by Olympus BX-50 (Olympus Optical) at 20X magnification and positive immunostaining was quantitated using Image Pro Plus. Threshold between positive and negative staining was defined by using positive and negative control slides and applied by a colour cube manual algorithm in Image Pro Plus with manual selection of positive stained colours. An average of 3-5 sections were assessed per mouse, and 7 to 9 mice were analyzed per group using the same colour thresholds. Manual tracing of the media and intimal layers using Image Pro Plus was used to determine the measurable area in each image, where the intima was defined as the area between the lumen and the internal elastic lamina (IEL), and the media was defined as the area between IEL to the external elastic lamina (EEL). Values for positive staining divided by the measurable area were used to calculate the percentage of positively stained tissue. Results are expressed relative to the HFD + vehicle or non-diabetic groups for the hypercholesterolemic and diabetic studies respectively, which were arbitrarily assigned a value of 1.
3.9.2. VCAM-1 immunohistochemistry on frozen aortic and aortic sinus sections

Frozen aortic sections and aortic sinus sections were cryosectioned at 4µm and 8 µm respectively and stained for VCAM-1, an important endothelial cell adhesion molecule. Tissue sections were fixed with cold acetone for 15 minutes at -20°C, washed in TBS and endogenous peroxidases were inactivated with 0.3% H₂O₂ in TBS. Non-specific proteins were then blocked by incubating the tissue sections with 10% normal rabbit serum in TBS for 30 minutes at room temperature, followed by the biotin-avidin blocking kit (Vector Laboratories). Following which, tissue sections were incubated with the VCAM-1 primary antibody (BD Pharminogen; 1:75 dilution) overnight at 4°C. The next day biotinylated anti-rat immunoglobulin (1:200) (Vector Laboratories) was added for 10 minutes, followed by horseradish peroxidase–conjugated streptavidin, diluted 1:500 (Dako) for an additional 30 minutes. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co). Tissue sections were counterstained with hematoxylin, dehydrated in graded series of alcohol solutions, cleared with xylene and mounted in DePex mounting medium. Images were captured and analysed as described in section 3.9.1.

3.9.3. 4-hydroxy-2-nonenal immunohistochemistry on paraffin-embedded aortic sections

Paraffin-embedded aortic sections were sectioned at 4µm and stained for 4-hydroxy-2-nonenal (4-HNE), an important marker of tissue lipid peroxidation. Tissue sections were dewaxed by placing the slides in xylene (2 rounds of 5 minutes each) and hydrated through a graded series of alcohol. Next, tissue sections were incubated with tris-buffered saline (TBS; 10 mM Tris-Cl, 150 mM NaCl, pH 7.6.) and endogenous peroxidases were inactivated with 0.3% H₂O₂ in TBS. Following which, a mouse on mouse (M.O.M) kit (Vector labs) was used to localise mouse antibodies on the mouse tissues. First, the tissues were incubated for 1 hour at room temperature in M.O.M Ig blocking reagent followed by 5 minutes with M.O.M diluents. Next, the sections were incubated with 4-HNE primary antibody (Genox; 1:100 dilution) diluted in M.O.M diluents for 1 hour at room temperature. After primary antibody incubation, biotinylated anti-mouse IgG reagent was applied to the sections for 10 minutes. The sections were then incubated with horseradish peroxidase–conjugated streptavidin, diluted 1:500 (Dako) for an additional 15 minutes. Peroxidase activity was identified by reaction with 3,3′-
diaminobenzidine tetrahydrochloride (Sigma Chemical Co). Tissue sections were counterstained with haematoxylin, dehydrated in graded series of alcohol solutions, cleared with xylene and mounted in DePex mounting medium. Images were captured and analysed as described in section 3.9.1.

**3.10. DHE staining for superoxide analysis in situ**

Dihydroethidium (DHE; 10µM) was used to detect superoxide formation in frozen aortic sections. Serial sections of frozen aorta were cryosectioned at 10µm and mounted onto slides (Menzel-Glaser SuperFrost Plus). For each aorta, DHE staining was performed in the presence or absence of 1mM tempol (Fluka), a superoxide dismutase (SOD) mimetic, enabling the measurement of superoxide-specific DHE. Aortic sections were air-dried and were then incubated with physiological Krebs Buffer (that was bubbled with a carbogen (95% O₂, 5% CO₂) with or without 1mM tempol for 30 minutes at 37 °C. Subsequently, aortic sections were incubated with 10µM DHE in the presence or absence of 1mM tempol for an additional 30 minutes at 37 °C in the dark. Each aortic section also had a negative control, which consisted of Krebs buffer only. Lastly, aortic sections were washed in Krebs buffer and mounted using an aqueous mounting medium. A Zeiss 510 Meta confocal microscope equipped with a krypton/argon laser was used to image the fluorescence of 2-hydroxyethidium. Laser settings were identical for each image acquired, with excitation and emission spectra set at 488 and 543 nm, respectively. The intensity of the fluorescence was quantified using Image J (version 1.42q) with the following equation:

\[
\text{Superoxide-specific DHE staining} = \frac{\text{Percentage intensity of DHE stain}}{-\text{Percentage intensity of DHE stain with tempol}}
\]

**3.11. Cell culture**

Human aortic endothelial cells (HAEC) were purchased from Lonza (Cell Application, San Diego, CA). HAEC were grown in endothelial cell basal medium (EBM-2; Lonza) supplemented with EGM-2 MV SingleQuots (Lonza) and 10% fetal bovine serum (FBS) as recommended by the manufacturer. Cells between passages 4 to 9 were used for experiments. For static cell adhesion assays, THP-1 cells, which are a human monocytic cell line derived from an acute monocytic leukemia patient, were obtained from Dr Raelene Pickering (Baker IDI
Heart and Diabetes Institute). THP-1 cells are non-adherent cells and were grown in RPMI media (Invitrogen) supplemented with L-Glutamine and 10% heat-inactivated FBS. Both cell lines were placed in a humidified incubator at 37°C with 7% CO₂ to ensure optimal growth conditions.

3.12. Cytochrome c reduction assay for superoxide analysis in vitro

In order to detect and quantify CavNOxin’s effect on superoxide production in endothelial cells, the Cytochrome c reduction assay adapted from Dikalov et al (with modifications) was performed[179]. Ferricytochrome c receives an electron from superoxide and is reduced to ferrocytochrome c, which results in increased absorbance specifically at 550nm whereas absorbance at 540nm and 560nm remain the same, allowing for the quantification of superoxide production. However, ferricytochrome c can be reduced by other electron donating molecules and ferrocytochrome c can be reoxidized by oxidants such as H₂O₂. For this reason, the reaction mixture has to include catalase (to catalyze the decomposition of H₂O₂) and has to be carried out in the presence and absence of SOD, an inhibitor of superoxide, in order to accurately quantify the superoxide-induced signal. Another important point to note is that the assay is light sensitive, therefore preparation of solutions and incubation of cells were carried out in the dark. Endothelial cells were seeded in two 24-well plates (BD Biosciences) and treated with vehicle or CavNOxin peptide (1-5µM; 6 hours). Subsequently, cells were washed twice with PBS and incubated with a PBS (Ca²⁺/Mg²⁺)-based buffer (150µl per well) containing 50µM acetylated ferricytochrome c (Sigma Aldrich) and catalase (125 units/ml; Sigma Aldrich) for 30 minutes in a 37°C incubator. The post-incubation supernatant was then removed from each well, transferred into labelled Eppendorf tubes (one tube/well) and placed on ice. Immediately, the cells were incubated for an additional 30 minutes at 37°C with a PBS (Ca²⁺/Mg²⁺)-based buffer (150µl per well) containing 50µM acetylated ferricytochrome c, catalase (125 units/ml) including SOD (200 units/ml; Sigma Aldrich). In the meantime, the supernatant collected from each well was transferred into a 96-well plate (BD Biosciences) and the absorbance of each well was measured at 540nm, 550nm and 560nm using a spectrophotometer (Magellan, TECAN). The optical density (OD) values obtained represent the reduction of ferricytochrome c by any molecule present in the cellular system. The post-incubation supernatant containing SOD was then collected from cells and the measurement
process was repeated. The second set of OD values would represent reduction of ferricytochrome c by any molecule other than O$_2^-$. Therefore, to calculate the amount of superoxide produced from each well, Equations 1 and 2 were used:

Equation 1.

$$\Delta \text{OD } 550\text{nm} = \text{OD } 550\text{nm} - \left[ \left( \text{OD } 540\text{nm} + \text{OD } 560\text{nm} \right)/2 \right]$$

Equation 2.

$$\text{superoxide} = \frac{\left( \Delta \text{OD } 550\text{nm} \text{ without SOD} - \Delta \text{OD } 550\text{nm} \text{ with SOD} \right)}{21.1 \text{mmol/L} \cdot \text{cm}^{-1}}$$

where $\Delta$ represents change in absorbance at 550nm and 21.1 mmol/L $\cdot$ cm$^{-1}$ is the extinction coefficient for superoxide. In this regard the value for superoxide is in mmol/L.

3.13. VCAM-1 western blot analysis

HAECs were seeded in a 6-well culture plate and grown to 90% confluency. HAECs were serum starved and treated with vehicle or CavNOxin peptide (5uM; 6 hours) in the presence or absence of TNF-a (0.5ng/ml; 4 hours). HAECs were then washed with ice-cold PBS and lysed with 200ul of lysis buffer (Tris based buffer containing protease inhibitors). Cellular aggregates were dissociated by sonication (three 10-s bursts) and the solubilised lysate was separated by centrifugation (132,000 rpm, 10 minutes, 4°C). Protein concentration of the lysate was determined using the BCA protein assay kit as per manufacturer’s instructions (Biorad). Following this, proteins were separated by SDS/Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was performed. Briefly, the gel was transferred onto a polyvinylidene difluoride membranes and were blocked overnight at 4°C in TBS containing 5% skim milk. VCAM-1 protein expression was detected with anti-VCAM-1 antibody (BD Pharminogen, 1:1000 dilution), and equal loading was verified by blotting with anti-β-tubulin (Millipore Inc, 1:2500 dilution). Following which, the membranes were washed with TBS-Tween 20 and incubated with Alexa Fluor 780 anti-rabbit (Life technologies, 1:5000) and Alexa Fluor 780 anti-mouse (Life technologies, 1:5000) for 1 hour at room temperature. The membranes were then scanned using the Odyssey infra-red scanner and densitometry scans of western blots were performed by using the Odyssey software.

Monocyte-endothelial interactions were determined by performing an *in vitro* static cell adhesion assay. HAECs were seeded on fibrinogen-coated 6-well culture plates and grown to 90% confluency. HAECs were then treated with vehicle or CavNOxin peptide (5µM; 6 hours) in the presence or absence of TNF-a (0.5ng/ml; 4 hours). In the meantime, THP-1 cells were labelled with the CellVue Burgundy labeling kit (Affymetrix), which incorporates a fluorescent dye into lipid regions of the cell membrane. THP-1 cells were grown to a density of 1 x 10⁶ cells/ml. Thereafter, THP-1 cells were counted using trypan blue for cell viability on a haemocytometer and re-suspended in 1ml RPMI media without serum to yield 2 x 10⁷ cells. The cells were then gently centrifuged at room temperature for 5 minutes (400g, acceleration 5, deceleration 5). Next, media was removed and the cells were resuspended in 2ml of Diluent C (labelling solution) containing 1nM of dye and incubated for 2-5 minutes at room temperature. The cell labeling reaction was stopped through the addition of 2mls of FBS. The labelled THP-1 cells were gently centrifuged as before and washed three times with RPMI media to reduce background signal. The last wash was kept and used as the blank. The cells were then resuspended in RPMI media and counted using trypan blue for cell viability on a haemocytometer. The labelled THP-1 cells (300,000 cells/ml) were then added to the treated HAECs for 20 minutes at 37°C. Thereafter, HAECs were washed twice with PBS, fixed with 10% NBF (30 minutes, room temperature) and plates were scanned at a fluorescence intensity of 700nm using the Odyssey infra-red scanner and the fluorescence of adhered cells per well was quantified.

3.15. In vivo dynamic flow adhesion assay

In order to confirm our *in vitro* adhesion results in an *in vivo* setting, we performed an *ex vivo* perfusion chamber and intravital microscopy assay. This enables real-time analysis of leukocyte-endothelial cell adhesion in intact vessels using perfused whole blood. Firstly, aortas from 10-12 week old ApoE KO mice were carefully dissected out and cleaned of peripheral fat in pre-warmed Krebs Buffer. For the diabetic study, ApoE KO mice were injected with STZ or citrate buffer as per Section 3.2. and aortas were isolated for the experiment after 5-6 weeks of diabetes. Cleaning of the aortas was carefully performed to avoid any tears which could cause
leakage during the perfusion stage. The isolated aortas were then incubated with vehicle or CavNOxin peptide (10µM; 12 hours) in phenol-red free Dulbecco’s Modified Eagle Medium (DMEM; high glucose, no sodium pyruvate). At the 8-hour time point, TNF-α (5ng/ml) was added to some vessels for 4 hours. Addition of TNF-a activates the endothelium, promoting leukocyte-endothelial cell interactions. Vessels were then carefully mounted on each end of a cannula in a vessel chamber containing pre-warmed Krebs buffer. Whole human blood (5ml/vessel) was collected in 40Units/ml of heparin from multiple healthy volunteers. The blood was labelled with VybrantDil (1:1000), which is a lipophilic dye to label leukocytes, for 10 minutes at 37°C in the dark. VybrantDil labelled blood was then allowed to flow through the vessel at a rate of 100µl/min. Labelled leukocyte adhesion to the vascular wall was visualized using a fluorescent microscope coupled to a digital camera recorder. Readings were taken at two fields along the perfused vessel for 10 seconds at the 5, 10 and 15 minute time-points. The average reading from the two fields at each time-point was plotted on a curve using the GraphPad Prism 6 software.

3.16. 8-isoprostane assay

Urinary 8-isoprostane was determined using the 8-isoprostane EIA kit as per manufacturer’s instructions (Cayman Chemicals). This assay is based on the competition between 8-isoprostane and 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. Because the concentration of the 8-isoprostane tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-isoprostane tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-isoprostane in the well. Initially, urinary samples (diluted 1:50) were added to the plate, which was pre-coated with mouse anti-rabbit IgG and blocked with a protein buffer. Following which, the AChE tracer and antiserum were added and allowed to incubate with the sample for 18 hours at 4°C. The plate was washed 5 times with the wash buffer to remove any unbound reagents and Ellman’s Reagent (which contains the substrate to AChE) was added to the well and allowed to develop on the shaker for 90-120mins at room temperature. The product of the enzymatic reaction had a distinct yellow colour and absorbed strongly at 412nm. The intensity of the colour, determined spectrophotometrically was proportional to the amount of 8-isoprostane tracer bound to the well,
which was inversely proportional to the amount of free 8-isoprostanote present in the well during the incubation. The average OD for non-specific binding and maximum binding wells were determined. The difference between the maximum binding and non-specific bindings gives us the corrected maximum binding. The average net OD bound for each standard and sample was calculated by subtracting the average blank OD from the average OD for each standard and sample. The average value for each sample and standard was then divided by the corrected maximum binding to obtain the ratio of the absorbance of the particular sample or standard to that of the maximum binding. The standard values were then plotted and the 8-isoprostanote concentration (ng/ml) for each sample was extrapolated from the graph using the GraphPad Prism software. The 8-isoprostanote concentration was then normalized to the urine volume over a 24 hour period per sample.

3.17. F4/80 immunohistochemistry

Paraffin-embedded aortic sections and frozen aortic sinus sections were sectioned at 4µM and cryosectioned 8 µM respectively, and stained for F4/80, an important macrophage marker. In the frozen aortic sinuses, staining was performed as per section 3.9.2. with anti-rat F4-80 primary antibody (Serotac; dilution 1:100) and biotinylated anti-rat immunoglobulin (1:200) (Vector Laboratories). Paraffin-embedded aortic sections were de-waxed by placing the slides in xylene (2 rounds of 5 minutes each) and hydrated through a graded series of alcohol. Next, tissue sections were washed under running water and antigen retrieval was performed. Briefly, tissue sections were placed in pre-heated citrate buffer antigen retrieval solution (1M sodium citrate, 1M citric acid, pH 6.2) and boiled for 10 minutes in a microwave and subsequently allowed to cool for 20-30 minutes in the buffer. Next, the tissue sections were rinsed with water and TBS; (10 mM Tris-Cl, 150 mM NaCl, pH 7.6.) and endogenous peroxidases were inactivated with 0.3% H2O2 in TBS. Non-specific proteins were then blocked by incubating tissue sections with 5% skim milk in TBS for 30 minutes. Following which, tissue sections were incubated with the F4/80 anti-rat primary antibody (Serotac; 1:75 dilution) overnight at 4°C. The next day, biotinylated anti-rabbit immunoglobulin 1:100 (Dako) was then added for 10 minutes, followed by horseradish peroxidase-conjugated streptavidin, diluted 1:500 (Dako) for an additional 30 minutes. Peroxidase activity was identified by reaction with 3,3′-dianinobenzidine tetrahydrochloride (Sigma Chemical Co). Tissue sections were counterstained with hematoxylin,
dehydrated in graded series of alcohol solutions, cleared with xylene and mounted with DePex mounting medium. Images were captured and analysed as described in section 3.9.1.

3.18. Blood glucose and glycated haemoglobin

Blood glucose measurements were recorded weekly to ensure that diabetes was maintained throughout the study in diabetic cohorts. A needle was used to prick the mouse tail and a tiny amount of blood (0.5µl) was squeezed out and measured using the Sensocard blood glucose monitor (Point of Care Diagnostics, Hungary). A reading above 18mmol/L was considered diabetic. Glycated haemoglobin was measured using High Performance Liquid Chromatography (HPLC). Firstly, 3µl of the red blood cell/saline mixture was added to 200µl MilliQ water and centrifuged at 10,000g for 10 minutes. Following which, 50µl of the supernatant was added to a HPLC vial and the analysis was performed using Agilent Chemstation software. Glycated haemoglobin was calculated using the following formula:-

\[
\text{Glycated Haemoglobin} = \left( \frac{\text{Area Peak B}}{\text{Area Peaks A + B}} \right) \times 100
\]

Where:
- Peak B = peak of glycated Hb
- Peak A = peak of non-glycated Hb

3.19. Statistical analysis

All results are expressed as mean ± SEM and considered statistically significant if P < 0.05 by one-way ANOVA followed by Newman-Kuels post-hoc test. In the hypercholesterolemic study, comparison of vehicle treated and CavNOxin treated HFD ApoE KO mice were performed using an unpaired t test. For the dynamic flow adhesion assay, a two-way ANOVA followed by a Bonferroni post-hoc test was performed. For the immunohistochemical and DHE staining, data is normalized to HFD + vehicle group for the hypercholesterolemic study and non-diabetic control group for the diabetic study.
Chapter 4. The effect of CavNOxin on hypercholesterolemia-induced atherosclerosis
4.1. Introduction

A characteristic feature in the early pathogenesis of hypercholesterolemia-induced atherosclerosis is the diminished bioavailability of anti-atherogenic NO, a phenomenon known as endothelial dysfunction. Indeed, endothelial dysfunction is prominent in the initial stages of hypercholesterolemia prior to lesion development or clinical manifestations of atherosclerosis[180]. In hypercholesterolemic subjects, endothelial function, as assessed by vasodilatory responses to endothelium-dependent stimuli such as acetylcholine, were attenuated, while responses to the endothelial-independent NO donor, sodium nitroprusside were preserved[180]. Recently, the causal link between cholesterol status and initial vascular abnormalities that trigger endothelial dysfunction have been elucidated. Ox-LDL is considered a key mediator that triggers hypercholesterolemia-induced endothelial dysfunction. Ox-LDL is chemotactic for monocytes/T-lymphocytes and activates the endothelium to produce adhesion molecules such as VCAM-1 and MCP-1[181-183]. More importantly, ox-LDL causes the displacement of eNOS, the enzyme responsible for endothelial NO production, from its subcellular localization in caveolae[184-186].

For optimal NO production, eNOS is localized to caveolae, which are cholesterol-enriched plasma membrane invaginations. Ironically, in caveolae, eNOS is maintained under basal inhibitory control by Cav-1, the major coat protein of endothelial caveolae. Upon agonist activation or sheer stress, the eNOS/Cav-1 inhibitory clamp is relieved by the Ca\(^{2+}\)-CaM complex, resulting in efficient production of eNOS-derived NO. The integrity of caveolae organelles is highly dependent on membrane cholesterol content. Thus, it is not surprising that perturbations in cholesterol homeostasis, such as those observed in settings of hypercholesterolemia, have a negative impact on eNOS localization and signaling. Indeed, incubation of cultured endothelial cells with hypercholesterolemic serum or ox-LDL caused the redistribution of eNOS/Cav-1 out of caveolae into cytosolic compartments[184, 186]. In addition, increased Cav-1 abundance, stabilization of the eNOS/Cav-1 inhibitory complex and impaired basal NO release was observed in hypercholesterolemic settings[184, 186]. Collectively, these studies strengthen the notion that eNOS-derived NO is compromised in settings of hypercholesterolemia. As hypercholesterolemia progresses, oxidative stress ensues and NO bioavailability is further reduced through inactivation of NO by superoxide anion[186]. The impaired NO release results in reduced vascular reactivity, increased leukocyte adherence to
the vascular wall, SMC migration and proliferation and platelet aggregation, all of which are key steps in the pathogenesis of atherosclerosis. Thus, strategies to directly improve NO bioavailability are warranted and the eNOS/Cav-1 interaction has the potential to be a highly relevant therapeutic target.

The Cav-1 scaffolding domain (CSD) is responsible for binding to eNOS and inhibiting eNOS-derived NO. Through careful analysis of the Cav-1 CSD, it was revealed that the amino acids responsible for the inhibition of eNOS-derived NO were T90, T91 and F92. This led to the generation of a modified cell permeable mutant Cav-1 peptide with an inactivated eNOS inhibitory domain, known as CavNOxin. Intracellular delivery of CavNOxin was able to increase basal NO release in cultured endothelial cells, reduce phenylephrine-induced constriction in isolated aortic rings and lower blood pressure in vivo, whilst still retaining the ability to bind eNOS. In this chapter, I hypothesize that CavNOxin, through its ability to improve NO release, can attenuate hypercholesterolemia-induced atherosclerosis. In particular, atherosclerotic processes that are NO-dependent, such as oxidative stress and leukocyte-endothelial interactions, are investigated.

4.2. Results

4.2.1. CavNOxin does not alter metabolic parameters of ApoE KO mice fed a HFD

Body weight and metabolic parameters of ApoE KO mice treated with either vehicle or CavNOxin (2.5mg/kg) at the conclusion of the HFD study are shown in Table 3. ApoE KO mice fed a HFD for 12 weeks had similar body weight as their counterparts that were fed a normal chow diet. As expected, HFD ApoE KO mice displayed significantly elevated triglycerides, total cholesterol, HDL and LDL levels compared to normal chow fed ApoE KO mice (Table 3; P<0.001). In the HFD treated mice, treatment with the CavNOxin peptide did not significantly alter these lipid parameters when compared to the vehicle treated group (a control AP cell permeable peptide in DMSO/water), which suggests that CavNOxin does not affect cholesterol homeostasis and the lipoprotein pathways that are involved in atherogenesis in HFD ApoE KO mice (Table 3).
Table 3: CavNOxin does not alter lipid parameters in HFD ApoE KO mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow + Vehicle</th>
<th>Chow + CavNOxin</th>
<th>HFD + Vehicle</th>
<th>HFD + CavNOxin</th>
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<tr>
<td>Body Weight (g)</td>
<td>28.0±1.6</td>
<td>30.8±0.8</td>
<td>28.8±1.5</td>
<td>29.4±1.7</td>
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<tr>
<td>TGs (mmol/L)</td>
<td>1.6±0.2</td>
<td>1.0±0.2</td>
<td>2.8±0.2*</td>
<td>2.6±0.3*</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>7.9±0.5</td>
<td>9.3±0.6</td>
<td>24.2±1.1***</td>
<td>24.5±1.4***</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.5±0.1</td>
<td>2.1±0.1</td>
<td>4.9±0.2***</td>
<td>4.6±0.2***</td>
</tr>
<tr>
<td>Non-HDL cholesterol (mmol/L)</td>
<td>5.7±0.3</td>
<td>6.8±0.4</td>
<td>18.0±0.8***</td>
<td>18.7±1.1***</td>
</tr>
<tr>
<td>LDL/HDL ratio (mmol/L)</td>
<td>4.0±0.1</td>
<td>3.2±0.1</td>
<td>3.7±0.1*</td>
<td>4.0±0.1##</td>
</tr>
</tbody>
</table>

Table 3: CavNOxin does not alter lipid parameters in HFD ApoE KO mice

Body weight, triglycerides (TG), cholesterol, HDL, LDL and LDL/HDL ratio were determined after 12 weeks of HFD in the presence or absence of CavNOxin treatment. *P<0.05, ***P<0.001 vs chow + vehicle group; ##P<0.01 vs HFD + vehicle group. n=10/group. Results are expressed as mean ± SEM.

4.2.2. CavNOxin ameliorates HFD-induced atherosclerosis

The effect of CavNOxin treatment on hypercholesterolemia-induced atherosclerosis was determined by evaluating the extent of plaque area in the aorta and aortic sinus region of ApoE KO mice by Sudan IV and Oil red O staining respectively. Sudan IV and Oil red O are lipid-soluble dyes that stain lipids, giving them a characteristic red colour that is easy to distinguish and quantitate.

After 12 weeks of HFD, the percentage of plaque in the total aorta, arch, thoracic and abdominal sub-regions was increased 6.4-, 7.4-, 8.5- and 4.0-fold respectively compared to chow fed vehicle-treated ApoE KO mice (Figure 7A and 7B, %Total plaque area: HFD + vehicle: 6.80 ± 0.48% vs chow+vehicle: 1.05± 0.14%). Treatment with CavNOxin had no effect in chow fed mice (Figure 7A; quantification Figure 7B) when compared to vehicle treated chow fed mice. However, in the HFD ApoE KO mice, CavNOxin treatment led to a 48% attenuation of total aortic plaque area in whole aortas as compared to vehicle-treated HFD ApoE KO mice.
(Figure 7A and 7B; %Total plaque area: 6.80 ± 0.48% vs 3.98 ± 0.56%, P=0.0039). The arch, thoracic and abdominal sections showed 50%, 48% and 47% reduction in atherosclerosis, respectively (Figure 7A and 7B). In the aortic sinus, HFD induced a 2.8-fold increase in lesion area as compared to normal chow fed ApoE KO mice (Figure 7C and 7D; HFD + vehicle: 386612 ± 24044\(\mu\)m\(^2\) vs chow + vehicle: 135658 ± 22787\(\mu\)m\(^2\) lesion area). CavNOxin treatment significantly reduced atherosclerotic plaque in the aortic sinus compared to vehicle-treated HFD ApoE KO mice by 22% (Figure 7C and 7D; HFD + vehicle: 386612 ± 24044\(\mu\)m\(^2\) vs HFD + CavNOxin: 299896 ± 21607\(\mu\)m\(^2\) lesion area), with no observed differences in normal chow fed ApoE KO mice.

Thus, these data demonstrate that CavNOxin treatment attenuates hypercholesterolemia-induced atherosclerosis. Since, no observed differences were seen between vehicle and CavNOxin treated ApoE KO mice fed a normal chow diet, the remainder of the study focused mostly on the differences between vehicle- and CavNOxin-treated HFD ApoE KO mice.
A

Chow

CavNOxin Vehicle

Arch Thoracic Abdominal

HFD

Vehicle

CavNOxin

Arch Thoracic Abdominal

B

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Arch</th>
<th>Thoracic</th>
<th>Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow + Vehicle</td>
<td>[Data]</td>
<td>[Data]</td>
<td>[Data]</td>
<td>[Data]</td>
</tr>
<tr>
<td>Chow + CavNOxin</td>
<td>[Data]</td>
<td>[Data]</td>
<td>[Data]</td>
<td>[Data]</td>
</tr>
<tr>
<td>HFD + Vehicle</td>
<td>[Data]</td>
<td>[Data]</td>
<td>[Data]</td>
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</tr>
<tr>
<td>HFD + CavNOxin</td>
<td>[Data]</td>
<td>[Data]</td>
<td>[Data]</td>
<td>[Data]</td>
</tr>
</tbody>
</table>

% Plaque Area

0 5 10 15 20

*** ** #
Figure 7: CavNOxin reduces atherosclerosis in ApoE KO mice fed a HFD

(A) Sudan IV stained aortas and (B) quantification of % plaque area in total, arch, thoracic and abdominal aorta from chow + vehicle, chow + CavNOxin, HFD + Vehicle and HFD + CavNOxin treated mice. (C) Oil Red O stained sinuses and (D) quantification of lesion area from chow + vehicle, chow + CavNOxin, HFD + Vehicle and HFD + CavNOxin treated mice. ***P<0.001, **P<0.01 and *P<0.05 vs chow + vehicle; ###P<0.001, ##P<0.01 and #P<0.05 vs HFD + vehicle. n=5-13/group. Results expressed as mean ± SEM. Scale bar = 50µm.
4.2.3. CavNOxin attenuates oxidative stress markers

NO bioavailability is a delicate balance between NO and ROS production in the vasculature, since ROS, in particular superoxide, is able to quench NO to produce peroxynitrite. Thus, it is not surprising that increased oxidative stress represents a common characteristic feature of vascular diseases. Previously, I have shown that the F92A Cav-1 mutant improves basal NO release and reduces superoxide levels in cultured endothelial cells[140]. Therefore, in this study I wanted to investigate the effect of CavNOxin on a range of known oxidative stress markers and overall NO bioavailability in plasma samples and aortic tissues from HFD ApoE KO mice treated with vehicle or CavNOxin peptide.

Nitrites and nitrates are the oxidation products of NO. In the Greiss reaction assay, nitrates are converted enzymatically to nitrites and total nitrite concentration is indicative of NO bioavailability. Measurement of plasma nitrites revealed that HFD ApoE KO mice had similar plasma nitrite levels as their chow fed counterparts at the conclusion of the 12 week study (Figure 8A). CavNOxin treatment led to a significant increase in plasma nitrite levels in HFD ApoE KO mice as compared to vehicle treated HFD ApoE KO mice (Figure 8A; P<0.05), indicating an increase in NO bioavailability. Plasma free radicals/hydroperoxides were quantified using the dROMs test. HFD ApoE KO mice demonstrated elevated levels of plasma dROMs as compared to chow fed mice (Figure 8B). However, CavNOxin had no effect on dROMs levels in HFD mice (Catarelli units; Figure 8B). Next, basal superoxide levels in cultured endothelial cells treated with vehicle or CavNOxin peptide (1µM and 5µM) was determined using the Cytochrome C Reduction assay. As shown in Figure 8C, CavNOxin treatment significantly reduced basal superoxide levels as compared to vehicle-treated endothelial cells at both doses (330.10 ± 38.92 vs 124.78 ± 15.37 nmol superoxide/million cells at 5µM of peptide), further confirming our previously published observations that the mutant F92A Cav-1 plasmid suppresses basal superoxide production.

In vivo, I assessed DHE, NT and 4-HNE, which are well-known markers of superoxide, peroxynitrite-mediated damage and a lipid peroxidation by-product, respectively, by fluorescence (DHE) and immunohistochemical (NT and 4-HNE) staining. DHE is a cell permeable compound that is converted to oxyethidium when oxidized in cells. Oxyethidium intercalates with nucleic acid to emit a bright red colour that is detectable quantitatively on a
confocal microscope. In order to determine superoxide-specific DHE oxidation, a sample including tempol (a superoxide dismutase mimetic) was included. Superoxide-specific DHE staining was significantly reduced by 66% in CavNOxin treated HFD ApoE KO mice compared to vehicle-treated mice (Figure 8D; P<0.01). NT is a product of tyrosine nitration of proteins mediated by ROS species, in particular peroxynitrite. NT staining was predominantly observed in the endothelial and medial layers of the aorta (brownish staining) in HFD ApoE KO mice. Treatment with CavNOxin significantly decreased NT levels by 52% in HFD ApoE KO mice compared to vehicle-treated mice (Figure 8E; P<0.01). 4-HNE is a by-product of oxidation of lipids in cells. In HFD ApoE KO mice, 4-HNE staining was dispersed in the aortic cross section with no significant difference in 4-HNE staining in CavNOxin treated HFD ApoE KO mice (Figure 8F).
**Figure 8: CavNOxin attenuates oxidative stress \textit{in vitro} and \textit{in vivo}**

(A) Total plasma nitrite concentration for HFD ApoE KO mice treated with vehicle or CavNOxin. *P<0.05 vs chow + vehicle. (B) dROMs analysis is shown in HFD ApoE KO mice treated with vehicle or CavNOxin. ***P<0.001 vs chow + vehicle. (C) Basal superoxide levels measured in cultured endothelial cells treated with vehicle or CavNOxin peptides (1 and 5µM) by the Cytochrome C Reduction assay. **P<0.01. (D) Representative images and quantification of superoxide detection by fluorescence imaging of DHE and DHE plus tempol (inset) in aortas of HFD ApoE KO mice treated with vehicle or CavNOxin. (E) Representative images and quantification of aortas stained with anti-NT antibody from HFD ApoE KO mice treated with vehicle or CavNOxin. (F) Representative images and quantification of HNE stained aortas from HFD ApoE KO mice treated with vehicle or CavNOxin. **P<0.01 vs vehicle (for D and E). n=10/group. Results expressed as mean ± SEM. Scale bar = 50µm.
4.2.4. Atherosclerotic gene analysis by qRT-PCR

NO is known to regulate the gene expression of various proteins involved in the atherosclerotic process[13]. Hence, to determine if CavNOxin can influence the expression of pro-atherogenic markers that contribute to atherosclerosis, we compared their gene expression in aortas from HFD ApoE KO mice treated for 12-weeks with either vehicle or CavNOxin peptide. In particular, gene expression of proteins involved in inflammation and cellular adhesion (VCAM-1, ICAM-1, MCP-1 and the p65 subunit of NF-κB), ROS production (eNOS, Nox1, Nox2 and Nox4) as well as antioxidant genes (MnSOD and CuZnSOD) were analyzed.

There was a trend for a decrease in the gene expression of ICAM-1, MCP-1, Nox2, and the p65 subunit of NFκB with CavNOxin treatment as compared to vehicle treated aortas, although these did not reach significance (Figure 9). The gene expressions of eNOS, MnSOD and CuZnSOD were relatively unaltered in CavNOxin treated aortas (Figure 9). Importantly, there was a significant reduction in VCAM-1 gene expression (Figure 9A; vehicle: 1.06 ± 0.17 arbitrary units (a.u.) vs CavNOxin 0.59 ± 0.05a.u.; P<0.01), a pro-adhesion surface molecule that causes adhesion of leukocytes/monocytes to the vascular wall, in the aortas of CavNOxin treated HFD ApoE mice.
Figure 9: Atherosclerotic gene expression analysis

Atherosclerotic genes assessed by qRT-PCR in HFD ApoE KO mice treated for 12 weeks with either vehicle or CavNOxin peptide (2.5 mg/kg). **P<0.01 vs vehicle. n=10/group. Results expressed as mean ± SEM
4.2.5. CavNOxin decreases the pro-atherogenic marker VCAM-1.

As demonstrated in Figure 9 above, VCAM-1 gene expression was reduced after CavNOxin treatment in HFD ApoE KO mice. Therefore, next I investigated the effect of CavNOxin on protein expression in cultured endothelial cells and in vivo in HFD-fed mouse aortas. As expected, aortic VCAM-1 protein expression as assessed by immunohistochemistry was found to be predominantly in the endothelium (brown staining) of vehicle treated HFD ApoE KO mice (Figure 10A). CavNOxin treatment significantly reduced VCAM-1 staining in the aorta by 40% (Figure 10A; P<0.05). Within the aortic sinus region, VCAM-1 staining was focused around the plaque area (Figure 10B). There was a trend towards a reduction in VCAM-1 levels in the CavNOxin-treated aortic sinus as compared to the vehicle-treated sinus, however this just fell out of significance (Figure 10B; P=0.07).

In unstimulated HAECs, basal levels of VCAM-1 protein were found to be low (Figure 10C). Stimulation with TNF-α induced VCAM-1 protein expression by 13-fold, whilst CavNOxin treatment attenuated this TNF-α-induced VCAM-1 protein expression by 66% (Figure 10C).
Figure 10: CavNOxin decreases VCAM-1 expression levels *in vivo* and *in vitro*

Representative images (top panel) and quantification (bottom panel) of VCAM-1 stained (A) aortas and (B) aortic sinus of HFD ApoE KO mice treated with vehicle and CavNOxin peptides. *P<0.05. (C) Western blot analysis (top panel) and densitometry (bottom panel) of VCAM-1 to β-actin (internal control) ratio in HAECs treated with either vehicle or CavNOxin peptide (5µM for 6 hours) in the presence of TNF-α (0.5ng/ml for 4 hours). * P<0.05 vs TNF-α and **P<0.01 vs TNF-α + Vehicle. n=6-10/group. Results expressed as mean ± SEM. Scale bar = 50µm.
4.2.6. CavNOxin reduces leukocyte-endothelial interactions

To determine whether the attenuated VCAM-1 levels described above translate into decreased leukocyte-endothelial cell interactions, I investigated the effect of CavNOxin in an \textit{in vitro} static adhesion assay and \textit{ex vivo} dynamic flow adhesion assay. Fluorescently labelled THP-1 monocytc cells (red dots in Figure 11A) adhered to unstimulated endothelial cells. A 6-hour pre-treatment with CavNOxin (5µM) led to a 40% decrease in the adhesion of fluorescently labelled THP-1 cells (Figure 11A). Upon stimulation with TNF-α, there was a significant increase in THP-1 cell adhesion to endothelial cells (Figure 11B; control: 72.4 ± 7.8 vs TNF-α: 762.7 ± 13.1 fluorescence intensity/mm²; P<0.001). Treatment with CavNOxin significantly reduced TNF-α-induced THP-1 cell adhesion by 17% (P<0.05), while treatment with the vehicle peptide had no effect.

With these positive \textit{in vitro} findings, the effect of CavNOxin on real-time leukocyte adherence to aortas exposed to normal blood flow was determined. In unstimulated aortas, there was no difference in leukocyte recruitment and adhesion between vehicle and CavNOxin treatment. In response to TNF-α stimulation in the presence of vehicle peptide, leukocyte adhesion to the aortic wall increased 2.7-fold after 15 minutes of blood flow (Figure 11C). However, the increase in leukocyte adhesion to the vascular wall was diminished in aortas co-incubated with CavNOxin (Figure 11C; vehicle + TNF-α: 16 ± 3 cells vs CavNOxin + TNF-α: 8 ± 2 cells after 15 min of flow; P<0.01 vs vehicle + TNF-α).
Figure 11: CavNOxin reduces leukocyte-endothelial interactions in vitro and ex vivo

(A) and (B) Representative images of one well of a 6-well plate scanned using an infra-red scanner to visualize adhered THP-1 monocytic cells (top panel; red dots) and quantification of intensity of adhered THP-1 cells (bottom panel) in HAECs treated with vehicle or CavNOxin peptide (5µM) in the (A) absence or (B) presence of TNF-α (0.5ng/ml). *P<0.05; ***P<0.001 vs control (ctrl) and #P<0.05 vs TNF-α and TNF-α + vehicle. n=6/group. (C) Representative images showing leukocytes (arrows pointing to white dots) binding to the aortic surface (left panel) and quantitation of average leukocyte binding per field every 5 minutes over a 15-minute period (right panel). ## P<0.05 and ### P<0.01 vs vehicle; ** P<0.01 and ***P<0.001 vs CavNOxin + TNF-α. n=6-8/group. Results expressed as mean ± SEM.

(A) and (B) Representations of one well of a 6-well plate scanned using an infra-red scanner to visualize adhered THP-1 monocytic cells (top panel; red dots) and quantification of intensity of adhered THP-1 cells (bottom panel) in HAECs treated with vehicle or CavNOxin peptide (5µM) in the (A) absence or (B) presence of TNF-α (0.5ng/ml). *P<0.05; ***P<0.001 vs control (ctrl) and #P<0.05 vs TNF-α and TNF-α + vehicle. n=6/group. (C) Representations showing leukocytes (arrows pointing to white dots) binding to the aortic surface (left panel) and quantitation of average leukocyte binding per field every 5 minutes over a 15-minute period (right panel). ## P<0.05 and ### P<0.01 vs vehicle; ** P<0.01 and ***P<0.001 vs CavNOxin + TNF-α. n=6-8/group. Results expressed as mean ± SEM.
4.2.7. ApoE KO mice lacking the eNOS gene are resistant to CavNOxin treatment

In order to confirm that CavNOxin mediates its effect via an eNOS-dependent mechanism, mice lacking the eNOS gene on an ApoE KO background were generated. When fed a HFD, male eNOS/ApoE dKO mice showed a 2.6-fold increase in atherosclerotic burden compared to male single HFD ApoE KO mice (Total plaque: ApoE KO: 6.80 ± 0.48% vs ApoE/eNOS dKO: 18.00 ± 2.28%) confirming previously published studies on protective role of eNOS in atherosclerosis. Interestingly, treatment with CavNOxin peptide (Figure 12A) did not significantly affect the extent of atherosclerotic lesions in the HFD fed eNOS/ApoE dKO when compared to vehicle treated counterparts. In addition, plasma nitrite levels in eNOS/ApoE dKO mice were significantly lower compared to ApoE KO mice (Figure 12B). This lack of effect of the CavNOxin peptide on atherosclerotic lesions in the eNOS/ApoE dKO mice clearly indicates that the CavNOxin peptide requires and interacts with eNOS to exert its anti-atherogenic effect.
Figure 12: CavNOxin lacks the anti-atherosclerotic effect in eNOS/ApoE dKO mice

(A) Sudan IV stained aortas (top panel) and quantification of %plaque area in the total, arch, thoracic and abdominal aorta from HFD eNOS/ApoE dKO mice treated with vehicle or CavNOxin peptides. (B) Total plasma nitrite concentration for ApoE KO and eNOS/ApoE dKO mice prior to the HFD. *P<0.05 vs ApoE KO, n=5-6/group. Results are expressed as mean ± SEM.
4.3. Discussion

Our study exhibits for the first time in vivo in an experimental model of atherosclerosis that targeted mutation of the Cav-1 scaffolding domain, which specifically relieves the inhibitory block of Cav-1 on eNOS-derived NO, leads to the marked attenuation of hypercholesterolemia-induced atherosclerosis. It is generally well accepted that endothelial dysfunction and subsequent reduced NO bioavailability contribute significantly to early atherogenesis, predominantly by modulating oxidative stress and leukocyte-endothelial interactions. Through the use of CavNOxin, a cell permeable Cav-1 peptide with an inactivated eNOS inhibitory domain, I was able to demonstrate that the reduction in atherosclerotic plaque observed correlated with an increase in plasma NO, a decrease in oxidative stress and a reduction in leukocyte adherence to the vascular wall.

A key finding of the current study was the identification of eNOS as the molecular target of CavNOxin’s anti-atherosclerotic effect. The fact that CavNOxin had no effect on the extent of atherosclerosis in mice that lacked the eNOS gene, confirms that CavNOxin specifically targets the eNOS/Cav-1 pathway. This was an important finding because the contribution of other NOS isoforms to the development of atherosclerosis cannot be discounted. While eNOS is the predominant NOS isoform in the healthy vascular wall, iNOS expression is increased in vascular injury states and nNOS is expressed in medial SMCs and in the adventitia; the expression of both of these latter isoforms of NOS are upregulated in advanced atherosclerotic plaques[187, 188]. Furthermore, genetic deficiency of eNOS and nNOS worsens atherosclerosis, however, iNOS deficiency is protective against atherosclerosis[187, 189]. To my knowledge, these results are the first to pharmacologically target the endogenous eNOS/Cav-1 interaction to improve eNOS activity in an in vivo cardiovascular disease model.

Atherosclerosis represents a heightened state of oxidative stress, attributed to the increased production of superoxide and reactive nitrogen species by enzymes present in vascular cells, including Noxs, xanthine oxidases and uncoupled eNOS. Hypercholesterolemic vessels display a 3-fold increase in superoxide anion production as compared to normocholesterolemic vessels, and this increase in superoxide production is able to uncouple eNOS, resulting in further ROS production[190]. Oxidative stress in atherosclerosis mainly manifests as lipid peroxidation or protein nitration on the vascular wall, which can alter the structure and activity of
lipids/proteins, thereby accelerating the disease process[191]. In atherosclerotic vessels, the reactive nitrogen species that result in nitration are likely to be derived from NOS, in particular eNOS and iNOS[192]. As compared to normal C57/Bl6 mice, atherosclerosis-prone ApoE KO mice have higher vascular superoxide levels[193]. Genetic deletion or pharmacological inhibition of eNOS results in the reduction of vascular superoxide formation in the HFD ApoE KO mouse but not in WT C57/Bl6 mice, suggesting that eNOS is uncoupled only in atherogenic ApoE KO mice and is a contributor of endothelial superoxide production[193]. eNOS inhibition, deletion and overexpression worsens atherosclerosis, suggesting that eNOS-derived NO plays a protective role in atherosclerosis, but the proper regulation and balance between eNOS-derived NO and superoxide is critical in the prevention of the disease[78, 79].

Since, CavNOxin has the ability to modulate eNOS function, I investigated the effect of CavNOxin on superoxide production as well as downstream lipid peroxidation and protein nitration. It is important to note, that due to the short half-life of NO and superoxide, quantitation of vascular bioavailable NO and superoxide can be a challenging task. Therefore, it is widely accepted that the assessment of stable secondary oxidative damage, such as lipid peroxidation (4-HNE, plasma dROMs) and protein nitration (NT) levels, is an alternative yet accurate way to assess oxidative stress. In this study, CavNOxin attenuated basal superoxide production in cultured endothelial cells and in aortas of HFD ApoE KO mice. In addition, CavNOxin reduced peroxynitrite-induced damage, as determined by NT staining in hypercholesterolemic settings in vivo. However, CavNOxin had limited effect on hypercholesterolemia-induced aortic 4-HNE levels and plasma dROMs levels, both of which indicate lipid peroxidation. This could be due to the fact that iNOS instead of eNOS contributes to lipid peroxidation, since iNOS/ApoE dKO mice exhibit protection against atherosclerosis with lowered plasma lipid peroxidation levels[189]. Importantly, this reduction in superoxide and peroxynitrite levels observed in our study was accompanied by an increase in NO bioavailability, indicated by elevated total plasma nitrite levels after CavNOxin treatment in HFD ApoE KO mice. However, the upregulation of NO bioavailability induced by CavNOxin did not alter the aortic gene expression of eNOS. These findings are consistent with Fernandez-Hernando et al, who demonstrated in an atherosclerotic setting that genetic deletion of Cav-1 or reconstitution of endothelial Cav-1 into Cav-1 knockout mice had no impact on eNOS gene and protein expression, whilst NO levels were increased in the Cav-1 knockouts and decreased in the
mice that overexpressed endothelial specific Cav-1[124, 125]. This suggests that higher eNOS gene or protein expression does not always translate to higher levels of eNOS derived NO, but rather NO levels are dependent on a functional ‘coupled’ eNOS rather than an atherogenic ‘uncoupled’ eNOS. Although the exact source of oxidant generation was not addressed in this study, the potential anti-oxidant effect of CavNOxin is still encouraging and could prove beneficial; considering multiple studies have reported no clinical benefit of traditional antioxidants in limiting atherosclerosis[156, 157].

Leukocytes play a vital role in atherogenesis, from initiation of lesion formation, through their recruitment to endothelial cells, transformation into foam cells, lesion expansion and promoting development of the fibrous cap. The recruitment and transmigration of leukocytes is mediated through the activation of endothelial cells, which express leukocyte adhesion molecules such as VCAM-1, ICAM-1 and E-selectin. Leukocyte rolling and adherence to the vascular wall has been shown to be increased just two weeks after the implementation of a high cholesterol diet[194]. This was correlated with impaired basal NO release and progressive endothelial surface expression of adhesion molecules, in particular VCAM-1[194]. Increased eNOS activity has been associated with the downregulation of VCAM-1 and the subsequent reduction in the adhesion of leukocytes/monocytes to the vascular endothelium, while eNOS gene ablation results in increased leukocyte-endothelial interactions on the vessel wall[193]. Hence, it is not surprising that Cav-1/ApoE dKO mice exhibit reduction in atherosclerotic plaque size, which is associated with significantly less leukocyte adhesion to the vessel wall and reduced expression of endothelial VCAM-1 and MCP-1[124, 125, 195]. Furthermore, the reduction in plaque size observed in Cav-1/ApoE dKO mice correlated with fewer macrophages, T-cells and neutrophils, strengthening the notion that Cav-1 modulates the inflammatory response in atherosclerosis[195]. Thus, with this knowledge in mind, I set out to determine if CavNOxin is able to regulate leukocyte-endothelial interactions at the vessel wall. In the current study, I showed that CavNOxin was able to reduce TNF-α-induced VCAM-1 protein expression in endothelial cells. Additionally, CavNOxin downregulated VCAM-1 gene expression and aortic VCAM-1 protein levels in hypercholesterolemic settings. Moreover, these effects on VCAM-1 gene and protein expression were translated to functional assays both in vitro and ex vivo in which leukocyte-endothelial interactions were examined. In vitro, I show that CavNOxin treatment is able to attenuate monocyte adhesion in unstimulated endothelial cells as well as in
response to pro-inflammatory cytokines, in this instance TNF-α. In a functional dynamic flow adhesion assay ex vivo, it was interesting to observe that CavNOxin had little effect on basal leukocyte-endothelial interactions, whilst in response to TNF-α, there was a marked reduction in leukocyte adhesion to the vessel wall. From a therapeutic perspective, it is advantageous that CavNOxin appears to be more effective in inflammatory settings. The differences observed in the in vitro and ex vivo assays could be due to the differential conditions and protein expressions of endothelial cells cultured in vitro as opposed to the endothelial lining in vivo[193].

It has been postulated that the extent of atherosclerosis is variable across the vascular tree, primarily due to regional differences in haemodynamic factors. The high-susceptibility vascular sites are usually exposed to low shear stress, oscillatory flow, turbulent flow or exhibit high vessel curvature[196]. In addition, endothelial cells, eNOS-derived NO and endothelial caveolae play an integral role in sensing mechanical forces and translating these forces to downstream signals that modulate pro-atherosclerotic gene expression in different vascular regions[196]. Thus, it is recommended when studying atherosclerosis that analysis of more than one site is required to fully elucidate the therapeutic potential of a drug or compound being investigated. Indeed, several studies have reported a site-specific effect of modulators of atherosclerosis. For example, probucol, a lipid lowering antioxidant compound, decreased lesion formation in most regions of the aorta but increased lesion area in the aortic sinus[197]. Similarly, ebselen, an antioxidant mimetic, reduced atherosclerosis in the aorta with no effect in the aortic sinus[166]. The lack of effect of certain anti-atherosclerotic compounds at the aortic sinus could be due to haemodynamic factors that regulate the region or the bioavailability of these compounds at these specific sites. In this chapter, we studied the extent of atherosclerosis in the total aorta, in particular the aortic arch, thoracic and abdominal aorta, as well as the aortic sinus. CavNOxin, through its ability to regulate the eNOS/Cav-1 interaction and increase eNOS-derived NO, is able to attenuate atherosclerotic lesion area in both the total aorta and the aortic sinus region, thereby supporting the therapeutic potential of increasing NO bioavailability through regulation of the eNOS/Cav-1 interaction.

In summary, we have demonstrated the anti-atherosclerotic effect of CavNOxin, a novel cell permeable peptide that improves eNOS activity, attenuates oxidative stress and pro-inflammatory genes in hypercholesterolemia-induced atherosclerosis. By specifically modulating the eNOS/Cav-1 interaction, our study provides target validation for the
development of peptide therapeutics to ameliorate atherosclerosis in a clinical setting of hypercholesterolemia.
Chapter 5. The effect of CavNOxin on diabetes-associated atherosclerosis
5.1. Introduction

Diabetes, whether of type 1 or type 2 origin, is considered an independent risk factor for CVD with nearly 80% of diabetes-related mortality being linked to a CVD event. The predominant CVD associated with diabetes includes macrovascular complications, such as atherosclerosis, and microvascular complications, such as retinopathy and nephropathy. In particular, diabetic patients have a 4-fold higher incidence of developing atherosclerosis as compared to healthy individuals[198, 199]. Diabetes-associated atherosclerosis is generally attributed to the adverse effects that the hyperglycemic environment and its accompanying oxidative stress has on vascular biology, in particular endothelial function. Indeed, endothelial dysfunction is a consistent finding in clinical and pre-clinical models of diabetes. In fact, diabetic patients exhibit paradoxical vasoconstriction instead of vasodilation when exposed to increasing amounts of acetylcholine in the coronary circulation[200], which indicates the extent of endothelial injury. When the vascular endothelium is exposed to hyperglycemic stress, an array of deleterious intracellular events occur, including activation of PKC and the AGE/RAGE pathway, which promote endothelial dysfunction and reduce NO bioavailability. In addition, these pathways induce an increase in ROS production through the activation of Nox enzymes, mitochondrial oxidative phosphorylation and uncoupling of eNOS, which further inactivates NO. Moreover, in diabetic vessels, superoxide anion produced can induce vasoconstriction not only through the inactivation of NO and resulting peroxynitrite formation, but also through the generation of hydrogen peroxide and hydroxyl radical, which stimulate the activity of endothelium derived vasoconstrictors[201, 202]. Lastly, another important factor that contributes to endothelial dysfunction and oxidative stress in diabetes is the resultant dyslipidemia and elevation of free fatty acids[203].

Since vascular endothelial dysfunction is closely linked to NO bioavailability, it is not surprising that hyperglycemia modulates various aspects of eNOS function. In experimental diabetic conditions, eNOS-derived NO production is significantly inhibited while endothelial superoxide production is markedly increased. These observations are paradoxical as eNOS gene and protein expression are maintained or even increased most likely as a compensatory mechanism, suggesting that eNOS uncoupling plays a major role in diabetic vascular diseases[204-206]. Indeed, eNOS monomer to dimer ratio is increased in cultured endothelial
cells exposed to high glucose conditions[206]. This is further confirmed by the fact that BH4 supplementation, an essential eNOS co-factor that stabilizes the eNOS dimeric conformation and prevents eNOS uncoupling, is able to recover NO activity[206]. Interestingly, in the early stages of glucose exposure, the eNOS/HSP-90 interaction is enhanced leading to recruitment of Akt and eNOS phosphorylation at the serine 1177 site, an important post-translational modification site of enzyme activity. Following longer-term glucose exposure, the eNOS/HSP90 interaction is dissociated leading to attenuation in eNOS activity[207, 208].

In the introduction to this thesis (Chapter 1), the importance of endothelial caveolae and the protein-protein interaction between eNOS and Cav-1 in regulating eNOS activity was extensively reviewed and discussed. In the past, several studies have suggested that diabetes-mediated alterations in Cav-1 protein levels that reduce eNOS-derived NO bioavailability play a role in regulating endothelial permeability in the renal and lung vasculature[209, 210]. However, it was only recently that the direct impact of diabetes on endothelial caveolae and the eNOS/Cav-1 interaction has been addressed. In diabetic settings in vitro and in vivo, it was found that peroxynitrite levels were increased, which resulted in disassembly of caveolae organelles, decreased expression of endothelial Cav-1 and uncoupling of eNOS[211]. Functionally, this resulted in significantly reduced flow-mediated dilation of coronary arterioles and increased risk of coronary artery disease[211]. The effect of disruption of caveolae assembly and eNOS uncoupling was further corroborated in isolated micro-vessels from Cav-1 KO mice in which endothelial caveolae are absent[211]. These data further strengthen the concept that proper eNOS coupling and regulation are critical in protecting the vasculature against diabetes-associated vascular diseases.

In the current chapter, I postulate that CavNOxin, a cell permeable peptide that is capable of improving NO release while preserving the function of caveolae, can ameliorate diabetes-associated atherosclerosis. The effect of CavNOxin on atherogenic pathways pertinent to NO, such as oxidative stress, modulation of inflammatory genes and leukocyte-endothelial interactions as well as its effect on diabetes-associated atherosclerosis were investigated.
5.2. Results

5.2.1. CavNOxin does not influence glucose and lipid parameters in a diabetic setting

Blood glucose levels were monitored in the mice on a weekly basis to ensure the level of diabetes was maintained throughout the study. Diabetic animals had elevated blood glucose levels during the course of the study, irrespective of treatment, as compared to their non-diabetic counterparts (Figure 13). At the conclusion of the study, body weight and plasma analysis of glucose and lipid parameters were performed. As published previously, diabetic ApoE KO mice weighed significantly less than non-diabetic mice (Table 4; P<0.05). Treatment with vehicle peptide did not have an impact on body weight in diabetic ApoE KO mice. On the contrary, CavNOxin peptide treatment at both doses (2.5mg/kg and 5mg/kg), had higher body weights than untreated and vehicle treated diabetic ApoE KO mice, which were similar to the body weights of non-diabetic mice (Table 4). As expected, diabetic ApoE KO mice had elevated glucose and glycated haemoglobin levels compared to non-diabetic counterparts (Table 4; P<0.001). In addition, diabetic ApoE KO mice displayed significantly elevated total cholesterol and LDL levels with modest increases in triglycerides and HDL levels compared to non-diabetic ApoE KO mice (Table 4; P<0.001). Treatment with either vehicle or CavNOxin did not alter glucose and lipid parameters in diabetic mice (Table 4), which suggests that any anti-atherogenic effect of CavNOxin is glucose and lipid independent.
Blood glucose measurements were taken every week to monitor diabetic mice. An average of blood glucose measurements per animal group at the 5, 10 and 15 week timepoint is shown here. n=6-12/group. Results are expressed as mean ± SEM.

Table 4: CavNOxin does not alter glucose and lipid parameters in diabetic ApoE KO mice

Metabolic parameters of mice at the 10-week time point of the study. Body weight, blood glucose, glycated haemoglobin (Hb), triglycerides, cholesterol, HDL, LDL and LDL/HDL ratio were determined after 10 weeks of diabetes in the presence or absence of CavNOxin treatment. *P<0.05, **P<0.01 and ***P<0.001 vs Non-diabetic. n=6-12/group. Results are expressed as mean ± SEM.
5.2.2. CavNOxin ameliorates diabetes-associated atherosclerosis in a dose-dependent manner

Diabetic ApoE KO mice revealed greater atherosclerotic burden than HFD-induced atherogenesis (Total plaque HFD ApoE KO: 6.80 ± 0.48% vs diabetic ApoE KO: 10.37 ± 2.31%). Diabetic ApoE KO mice had a 9.4-fold increase in total plaque area as compared to non-diabetic ApoE KO mice on chow (Figure 14A and 14B), with increased plaque burden observed in the arch, thoracic and abdominal areas (9.2-, 9.2-, 7.5-fold increase, respectively; Figure 14A-14B). Treatment with vehicle peptide in diabetic mice had no significant impact on plaque compared with untreated diabetic mice (Figure 14A-14B, grey vs black bar). However, treatment with CavNOxin at 5mg/kg attenuated total aortic plaque growth by 70%; this reduction by CavNOxin was observed in the arch and thoracic regions of the aorta (Figure 14A and 14B; arch 80%, thoracic 84%), suggesting a far greater efficacy of CavNOxin in diabetic atherosclerosis than in HFD-induced atherosclerosis albeit that abdominal aorta plaque reduction was not significant. Moreover, the therapeutic effect of CavNOxin was dose-dependent, since a lower dose (2.5 mg/kg) reduced total atherosclerosis burden by a lesser 56%, with reduced plaque area in the arch and thoracic region at 47% and 60% respectively (Figure 14A and 14B).

In addition, diabetes induced a significant increase in lesion area in the aortic sinus, which was attenuated by 68% and 81% with CavNOxin (2.5mg/kg) and CavNOxin (5.0mg/kg) treatment respectively (Figure 14C and 14D; diabetic: 526704 ± 324261 vs diabetic + CavNOxin (5mg/kg): 282586 ± 23635µm² lesion area). Together, these data demonstrate that CavNOxin decreases diabetes-induced atherosclerosis.
Figure 14: CavNOxin reduces atherosclerosis in diabetic ApoE KO aorta

(A) Sudan IV-stained aortas from non-diabetic (ND), diabetic, diabetic + vehicle, diabetic + CavNOxin (2.5mg/kg) and diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks after sham or STZ-induced diabetes. (B) Percentage plaque area of total, arch and thoracic aorta is shown in respectively. (C) Oil-red O stained aortic sinuses from (i) ND, (ii) diabetic, (iii) diabetic + vehicle, (iv) diabetic + CavNOxin (2.5mg/kg) and (v) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks after sham or STZ-induced diabetes. (D) Analysis of lesion area in the sinus. *P<0.05 and **P<0.001 vs ND; # P<0.05 and ## P<0.01 vs diabetic and diabetic + vehicle (for B); ###P<0.05 vs diabetic and #### P<0.001 vs diabetic + Vehicle (for D). n=6-12/group. Results are expressed as mean ± SEM. Scale bar = 50µm.
5.2.3. *CavNOxin reduces diabetes-associated oxidative stress*

Diabetes induced oxidative stress has a deleterious impact on vascular biology and greatly facilitates atherosclerotic disease progression. Improving NO release can mitigate oxidative stress burden in disease settings. Therefore, to assess if CavNOxin, through its ability to upregulate eNOS function, can improve oxidative stress in diabetes-associated atherosclerosis, I investigated a range of known ROS markers in the plasma and aortic tissues of diabetic ApoE KO mice in the presence or absence of CavNOxin treatment.

Plasma free radicals/hydroperoxides were quantified using the dROMs test. Diabetic ApoE KO mice demonstrated a significant 2.4 fold increase in plasma dROMs as compared to their non-diabetic counterparts (Figure 15A). Treatment with the vehicle peptide had no effect on diabetes-induced increase in plasma dROMs levels. However, CavNOxin treatment at both doses significantly reduced plasma dROMs levels by up to 48% in diabetic ApoE KO mice (Figure 15A; P<0.05 vs diabetic controls).

Next, urinary 8-isoprostanes, a known biomarker for oxidative stress, was assessed. Diabetes induced a significant 3.7-fold increase in urinary 8-isoprostanes (Figure 15B; p<0.001 vs control). Treatment with the vehicle peptide had no effect on the diabetes-induced increase in urinary 8-isoprostanes, while, CavNOxin treatment significantly reduced levels of this prominent oxidative stress biomarker in a dose-dependent manner (Figure 15B; 76% and 97% reduction with CavNOxin (2.5mg/kg) and CavNOxin (5mg/kg) respectively; P<0.05 vs diabetic + vehicle).

NO bioavailability was measured based on plasma nitrites/nitrates levels, which are the oxidative products of NO. Untreated and vehicle-treated diabetic ApoE KO mice displayed a modest non-significant decrease in plasma nitrite levels as compared to their non-diabetic counterparts (Figure 15C), indicating reduced NO bioavailability in the diabetic setting. In CavNOxin treated diabetic ApoE KO mice, plasma nitrite levels returned to that of their non-diabetic counterparts albeit the data was non-significant, suggesting improvement on NO bioavailability (Figure 15C).

Following assessment of systemic oxidative stress, I investigated the level of oxidative stress in aortic tissue by DHE, NT and 4-HNE staining to detect the levels of superoxide and the extent of protein and lipid oxidation respectively. Diabetes resulted in an increase in superoxide-
specific DHE staining, which was normalized back to the levels observed in non-diabetic ApoE KO mice after both 2.5 and 5.0mg/kg CavNOxin treatment, however these data did not reach significance (Figure 16). There was a significant 3-fold increase in NT staining in the aortas of diabetic ApoE KO mice as compared to their non-diabetic controls (Figure 17; P<0.001). Vehicle peptide treatment did not alter aortic NT levels, however, CavNOxin treatment at the low and high dose reduced NT levels by 41% and 84% respectively, indicating a dose-dependent effect (Figure 17). Similarly, diabetes induced a significant upregulation of 4-HNE levels in ApoE KO mice, which was attenuated by 64% and 105% with low and high dose of CavNOxin respectively (Figure 18; P<0.05). Collectively, these results demonstrate that the diabetic environment is associated with a significant increase in vascular oxidative stress and importantly this data highlights the protective antioxidant effect of CavNOxin.
Figure 15: CavNOxin attenuated systemic oxidative stress

(A) Plasma dROMS, (B) urinary 8-isoprostanes and (C) total plasma nitrite concentration measured from non-diabetic (ND), diabetic, diabetic+ vehicle, diabetic + CavNOxin (2.5mg/kg) and diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks (A and B) and 10 weeks (C) after sham or STZ-induced diabetes. **P<0.01 and ***P<0.001 vs ND; # P<0.05 vs diabetic (for A) and diabetic + vehicle (for A and B); ### P<0.001 vs diabetic (for B). n=5-11/group. Results are expressed as mean ± SEM.
Figure 16: CavNOxin influences superoxide-specific DHE staining

Representative images of DHE and DHE plus tempol (inset) aortas to detect superoxide from (A) non-diabetic (ND), (B) diabetic, (C) diabetic + vehicle, (D) diabetic + CavNOxin (2.5mg/kg) and (E) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 10-weeks after sham or STZ-induced diabetes. (F) Quantification of DHE stained area in the aorta. n= 4-8/group. Results are expressed as mean ± SEM.
Figure 17: CavNOxin reduces diabetes-induced nitrotyrosine levels

Representative images of NT stained aortas (A to F) from (A) non-diabetic (ND), (B) diabetic, (C) diabetic + vehicle, (D) diabetic + CavNOxin (2.5mg/kg) and (E) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks after sham or STZ-induced diabetes. (F) Negative aortic staining with no primary antibody. (G) Quantification of NT staining in the aortic wall. ***P<0.001 vs ND; # P<0.05 vs diabetic and diabetic + vehicle; ## P<0.01 vs diabetic + vehicle; ### P<0.001 vs diabetic. n=4-10/group. Results are expressed as mean ± SEM. Scale bar = 50µm.
Figure 18: CavNOxin decreases diabetes-induced 4-HNE levels

Representative images of 4-HNE stained aortas from (A) non-diabetic (ND), (B) diabetic, (C) diabetic + vehicle, (D) diabetic + CavNOxin (2.5mg/kg) and (E) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks after sham or STZ-induced diabetes. (F) negative aortic staining with no primary antibody added. (G) Quantification of 4-HNE staining in the aortic wall. **P<0.01 vs ND; # P<0.05 vs diabetic and diabetic + vehicle. n=4-11/group. Results are expressed as mean ± SEM. Scale bar = 50µm.
5.2.4. CavNOxin’s effect on diabetes-induced gene expression

To determine if CavNOxin influences the expression of pro-atherosclerotic markers that contribute to cellular adhesion and oxidative stress in a diabetic environment, I compared the gene expression of specific pro-inflammatory (Figure 19A; VCAM-1, ICAM-1, MCP-1 and the p65 subunit of NF-κB), pro-oxidative (Figure 19B; Nox1, Nox2, Nox4, Rac-1, eNOS) and anti-oxidant (Figure 19C; MnSOD, CuZnSOD, GPx-1) genes in the aorta of non-diabetic and diabetic ApoE KO mice in the presence or absence of CavNOxin.

Diabetes resulted in a substantial increase in VCAM-1, ICAM-1 and MCP-1 gene expression by 2.6-, 2.7 and 8.8-fold respectively as compared to their non-diabetic counterparts (Figure 19A; P<0.05 (VCAM-1, ICAM-1) and P<0.01 (MCP-1) vs non-diabetic controls). These findings are in line with published literature on the effect of hyperglycemia on pro-inflammatory genes[212]. CavNOxin treatment attenuated VCAM-1, ICAM-1 and MCP-1 gene expression by up to 95%, 99% and 85% respectively in a dose-dependent manner (Figure 19A; P<0.05 vs diabetic controls). In our study, we observed no differences in the expression of one of the subunits of NF-κB, namely p65, with diabetes and CavNOxin treatment. Diabetic ApoE KO mice demonstrated increased gene expression of the Nox enzymes, in particular Nox1 and Nox2 (Figure 19B; P<0.05 vs non-diabetic control), with minimal impact on Nox4 expression. CavNOxin treatment did not modulate Nox1 gene expression but decreased Nox2 expression by 87%, however this just fell out of significance (Figure 19B). Diabetes caused a trend toward increase in Rac-1 and eNOS gene expression, which was decreased upon CavNOxin treatment, however the data was not significant (Figure 19B). Diabetes resulted in a trend towards increased MnSOD gene expression, which was decreased with CavNOxin treatment at the higher dose, but the data was not significant (Figure 19C). Diabetes did not have a significant impact on the other antioxidant gene expressions.
Figure 19: Gene expression analysis

(A) Pro-inflammatory, (B) pro-oxidative and (C) anti-oxidant gene expression assessed by qRT-PCR in diabetic ApoE KO mice treated with vehicle or CavNOxin. **P<0.01 and *P<0.05 vs non-diabetic, #P<0.05 vs diabetic and $P<0.05 vs diabetic + vehicle. n=7-10/group. Results expressed as mean ± SEM.
5.2.5. **CavNOxin decreases VCAM-1 protein expression and F4/80 levels**

Based on the gene expression changes observed in the previous section and in the published literature, it is clear that diabetes influences the expression of pro-inflammatory adhesion molecules, in particular VCAM-1 and MCP-1, which contribute to accelerated atherogenesis[212, 213]. To confirm that these changes at the gene level translate into changes at the protein level, I investigated if CavNOxin could modulate VCAM-1 protein expression in a diabetic setting by immunohistochemistry in the aorta and the aortic sinus.

Diabetes caused an increase in aortic VCAM-1 protein levels, which was predominantly confined to the endothelial region (Figure 20A, B and G; non-diabetic: 1.04 ± 0.15 a.u. vs diabetic: 2.46 ± 0.61 a.u; P<0.05 vs non-diabetic control). Vehicle treatment did not have an impact on diabetes-induced VCAM-1 protein levels in the aorta (Figure 20B, C and G). Interestingly, the lower dose of CavNOxin significantly attenuated aortic VCAM-1 protein expression by 83%, but the higher dose did not influence this parameter (Figure 20; P<0.05 vs diabetic and diabetic + vehicle). In the aortic sinus, VCAM-1 protein levels were significantly increased by diabetes with maximal staining in the plaque region (Figure 21A, B and G; non-diabetic: 1.00 ± 0.17 a.u. vs diabetic: 2.30 ± 0.20 a.u.; P<0.01 vs non-diabetic). CavNOxin treatment significantly reduced VCAM-1 levels in the aortic sinus at both the 2.5mg/kg and 5mg/kg dose by 78% and 110% respectively (Figure 21; P<0.05 vs diabetic and diabetic + vehicle).

MCP-1 exhibits chemotactic activity for monocytes. Since we observed an upregulation of MCP-1 aortic gene expression, we investigated if the expression of F4/80, a monocyte/macrophage marker was modulated by diabetes and CavNOxin treatment. In the diabetic aorta, F4/80 protein levels was significantly elevated as compared to non-diabetic aortas, with staining observed in both the endothelial and medial layers (Figure 22A,B and G; non-diabetic: 1.10 ± 0.30 a.u. vs diabetic: 2.51 ± 0.38 a.u.; P<0.01). While vehicle treatment did not alter F4/80 protein levels, CavNOxin treatment decreased diabetes-induced F4/80 protein levels by up to 134% in a dose-dependent manner (Figure 22B, D, E and G; P<0.01). Similarly, a significant increase in F4/80 staining was observed in the plaque region of the aortic sinus in diabetic mice (Figure 23A, B and G; non-diabetic: 1.00 ± 0.21 vs diabetic: 2.94 ± 0.41 a.u.), which was significantly reduced by 67% and 120% with CavNOxin treatment at the low and high dose respectively (Figure 23B, D, E and G; P<0.01).
Collectively, this data show that CavNOxin is able to normalize diabetes-induced expression of the pro-inflammatory markers, VCAM-1 and F4/80, to levels that were seen in their non-diabetic counterparts.
Figure 20: CavNOxin reduces aortic VCAM-1 protein levels in diabetic ApoE KO mice

Representative images of VCAM-1 stained aortas (A to F) from (A) non-diabetic (ND), (B) diabetic, (C) diabetic + vehicle, (D) diabetic + CavNOxin (2.5mg/kg) and (E) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 10-weeks after sham or STZ-induced diabetes. (F) is a negative control section with no primary antibody added. (G) Quantification of VCAM-1 staining in the aortic wall. *P<0.05 and **P<0.01 vs ND; # P<0.05 vs diabetic and diabetic + vehicle. n=4-9/group. Results are expressed as mean ± SEM. Scale bar = 50µm.
Figure 21: CavNOxin attenuates VCAM-1 protein levels in the aortic sinus of diabetic ApoE KO mice

Representative images of VCAM-1 stained aortic sinuses (A to F) from (A) non-diabetic (ND), (B) diabetic, (C) diabetic + vehicle, (D) diabetic + CavNOxin (2.5mg/kg) and (E) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks after sham or STZ-induced diabetes. (F) is a negative control section with no primary antibody added. (G) Quantification of VCAM-1 staining in the aortic sinus. **P<0.01 vs ND. # P<0.05 vs diabetic and diabetic + vehicle. n= 4-8/group Results are expressed as mean ± SEM. Scale bar = 50µm.
Figure 2: CavNOxin decreases aortic F4/80 levels in diabetic ApoE KO mice

Representative images of F4/80 stained aortas (A to F) from (A) non-diabetic (ND), (B) diabetic, (C) diabetic + vehicle, (D) diabetic + CavNOxin (2.5mg/kg) and (E) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks after sham or STZ-induced diabetes. (F) are negative tissue sections with no primary antibody added. (G) Quantification of F4-80 staining in the aortic wall. **P<0.01 vs ND, ## P<0.05 vs diabetic, $P<0.01$ vs diabetic + vehicle. n= 4-8/group. Results are expressed as mean ± SEM. Scale bar = 50µm.
Figure 23: CavNOxin attenuates F4/80 levels in the aortic sinus of diabetic ApoE KO mice

Representative images of F4/80 stained aortic sinuses (A to F) from (A) non-diabetic (ND), (B) diabetic, (C) diabetic + vehicle, (D) diabetic + CavNOxin (2.5mg/kg) and (E) diabetic + CavNOxin (5.0mg/kg) treated ApoE KO mice, 14-weeks after sham or STZ-induced diabetes. (F) are negative tissue sections with no primary antibody added. (G) Quantification of F4-80 staining in the aortic wall. **P<0.01 vs ND, #P<0.05 and ## P<0.01 vs diabetic, $P<0.05 and $$ P<0.01 vs diabetic + vehicle. n= 4-7/group. Results are expressed as mean ± SEM. Scale bar = 50µm.
5.2.6. CavNOxin attenuates leukocyte-endothelial interactions in diabetic settings

The functional relevance of diabetes-induced increases in pro-inflammatory adhesion molecule expression and their subsequent attenuation with CavNOxin treatment was determined by assessing leukocyte-endothelial interactions in an in vitro static adhesion assay and in an ex vivo dynamic flow adhesion assay. In cultured endothelial cells, long-term exposure (3 days) resulted in a significant 1.6-fold increase in adhesion of fluorescently labelled THP-1 cells to the endothelial cells (Figure 24A and B; P<0.05 vs low glucose). Vehicle treatment did not alter THP-1 cell adhesion to endothelial cells in low or high glucose conditions. However, CavNOxin treatment (5µM, 6 hours) significantly reduced THP-1 cell adhesion to high-glucose exposed endothelial cells by 84% (Figure 24A and B; P<0.05 vs high glucose and high glucose + vehicle). It is important to note that CavNOxin did not alter THP-1 cell adhesion in low glucose conditions, unlike the experiment highlighted in chapter 4 (Figure 11A). A possible explanation for this discrepancy between experiments may have arisen as a consequence of different cell culture conditions. In these experiments, cells were cultured for a 3 day period with the media being changed every day to mimic treatment of cells receiving high glucose, unlike the previous experiment where the endothelial cells were grown to 90% confluency before treatment, which results in data that cannot be compared.

Next, an ex vivo dynamic flow adhesion assay was performed to explore real-time leukocyte adherence to aortas isolated from non-diabetic and diabetic mice. In non-diabetic aortas, there was no difference in leukocyte recruitment and adhesion between vehicle and CavNOxin treatment (Figure 24C and D). In diabetic aortas, leukocyte adhesion to the aortic wall increased 2.5-fold after 15 minutes of blood flow as compared to non-diabetic aortas. Interestingly, the levels of adhesion were similar to levels observed in aortas stimulated with the pro-inflammatory cytokine TNF-α (Chapter 4, Figure 11C). This increase in leukocyte adhesion to the vascular wall in diabetic aortas was diminished in aortas that were treated with CavNOxin for 6 hours (Figure 24C and D; diabetic + vehicle: 15 ± 2 cells vs diabetic + CavNOxin: 5 ± 2 cells after 15 min of flow; P<0.001 vs diabetes).

Overall, these data highlight the anti-adhesive effect of CavNOxin on hyperglycemia-induced leukocyte-endothelial interactions, which may contribute to its protective effects against atherosclerosis.
Figure 24: CavNOxin reduces hyperglycemia induced leukocyte-endothelial interactions in vitro and ex vivo

(A) HAECs grown in either low glucose or high glucose for 72 hours were treated with either vehicle or CavNOxin peptide (5uM for 6 hours) and incubated with fluorescently labelled THP-1 monocytic cells. (B) Graph represents quantification of intensity of adhered THP-1 cells. *P<0.05 as indicated. (C) Representative images showing leukocytes (arrows pointing to white dots) binding to the aortic surface. (D) Quantitation of average leukocyte binding per field every 5 minutes over a 15 minute period. Data expressed as mean ± SEM. *P<0.05 (for B). **P<0.01 vs Non-diabetic + vehicle, # P<0.05 and ###P<0.01 vs Diabetic + CavNOxin (for D). n=4-5/group. Results are expressed as mean ± SEM.
5.3. Discussion

The current study has demonstrated that administration of a cell permeable peptide, CavNOxin, to diabetic ApoE KO mice is able to ameliorate atherosclerotic lesion formation in most regions of the aorta and the aortic sinus, indicating that CavNOxin is an effective anti-atherosclerotic agent against the diabetic macrovascular disease. This anti-atherosclerotic effect was accompanied with protective effects against oxidative stress, reflected by decreased plasma peroxides and urinary isoprostanes as well as reduced aortic NT and lipid peroxide levels. Moreover, CavNOxin was able to modulate diabetes-induced pro-atherosclerotic gene and protein expression, in particular VCAM-1 and MCP-1, which correlated with attenuated leukocyte-endothelial interactions.

Presently, the most common mouse models used to study diabetes-associated atherosclerosis are the STZ-induced ApoE KO mouse, which represents a type 1 (non-insulin dependent) diabetes model and the leptin deficient (ob/ob) ApoE KO mouse, which represents a type 2 (insulin-dependent) diabetes model; both of these exhibit increased atherosclerotic lesions associated with hyperglycemia[214]. In the current study, the type 1 STZ-induced ApoE KO mouse model was chosen to study the effects of diabetes-associated atherosclerosis since this model is accepted as most closely replicating the human condition with well developed, cholesterol-rich pro-inflammatory plaque[215, 216]. In addition, while hyperglycemia is strongly associated with early fatty streak formation in atherosclerosis-prone mice, progression to advanced atherosclerotic plaques requires dyslipidemia, which was observed in the diabetic ApoE KO mice in this study[217]. Another important point to consider is that the insulin receptor is localized in caveolae and Cav-1 is implicated in insulin signaling as Cav-1 deficient mice develop insulin resistance when placed on a HFD[218, 219]. Thus, the STZ-diabetic model being an insulin-deficient model, eliminates confounding effects of Cav-1 driven insulin signaling on atherogenesis[219], thereby allowing us to focus on the eNOS/Cav-1 interaction per se and its role in NO signaling, endothelial dysfunction and diabetes-associated atherosclerosis.

Previous work by Dr de Haan’s group has established that the 14-week experimental time-point is sufficient in establishing atherosclerotic lesions in diabetic ApoE KO mice. Indeed, the atherosclerotic lesion burden is much greater in diabetic ApoE KO mice compared to ApoE
KO mice on a HFD. With this in mind, in the current study, I chose two different doses of CavNOxin to study its effects on diabetes-associated atherosclerosis. Most importantly, CavNOxin reduced diabetes-associated atherosclerosis in a dose-dependent manner, lending credence to its therapeutic potential as an anti-atherosclerotic agent.

NO bioavailability is critically dependent on the delicate balance between eNOS-derived NO synthesis and superoxide inactivation of NO, with the balance tipping in favour of the later in pathological conditions. Therefore, limiting ROS production and oxidative stress is an important therapeutic avenue in maintaining NO bioavailability. In comparison to the hypercholesterolemic ApoE KO mice (Chapter 4), we observed a greater extent of systemic oxidative stress and ROS-related tissue damage in diabetes, which correlated with the increased atherosclerotic burden in diabetic mice. This observation is supported by a biomarker study, which analysed oxidative stress in blood samples from diabetic and hypercholesterolemic patients and found significantly higher levels of the specific biomarker in diabetic patients indicative of greater oxidative stress in this population[220]. Moreover, several additional pathways are activated in the diabetic environment that contributes to the heightened state of oxidative stress. These include the polyol pathway flux, increased activity of PKC and the AGE/RAGE pathway, which stimulate ROS production, pro-inflammatory pathways and cause long-lasting epigenetic changes which drive persistent activation of proinflammatory genes even after blood glucose levels are normalized, resulting in a phenomenon known as ‘hyperglycemic memory’[221]. Recent studies have uncovered that predominant sources of ROS in diabetic macrovascular diseases are, uncoupled eNOS and Nox enzymes, particularly Nox1[222, 223]. Indeed, eNOS uncoupling is so profound in the diabetic endothelium that eNOS-derived superoxide causes augmentation of vascular remodeling and dysfunction, strengthening the notion that proper regulation of eNOS is required to protect against atherosclerosis[224]. In the present chapter, we demonstrated a novel “anti-oxidant effect” of CavNOxin, as it was able to significantly decrease systemic oxidative stress as well as vascular tissue damage induced by peroxynitrite and lipid peroxidation. Although the source of oxidant generation was not fully elucidated in the study, it would most likely be due to CavNOxin’s effect on eNOS signaling since we have previously demonstrated the eNOS specificity of CavNOxin’s actions. On the other hand, CavNOxin treatment resulted in a slight improvement in NO bioavailability, albeit non-significant, in the diabetic setting. This non-significant increase in nitrite levels is not
surprising since NO bioavailability is a relatively difficult parameter to measure \textit{in vivo} due to its short half-life and the higher amounts of ROS present in the diabetic milieu can lead to radical scavenging of NO, resulting in less obvious changes in NO bioavailability.

A central mechanism driving diabetic-associated vascular diseases is inflammation, which triggers endothelial dysfunction and enhances the pro-adhesive and pro-thrombotic properties of the endothelium, thereby promoting the recruitment of inflammatory cells to the vascular wall. In this study, I have confirmed previously published data, which demonstrated that aortic lesions from diabetic ApoE KO mice display elevated gene expression of pro-inflammatory molecules VCAM-1, ICAM-1, MCP-1 and the p65 subunit of NF-κB compared to their non-diabetic controls\cite{212, 223}. NF-κB is a redox-sensitive transcription factor responsible for the induction of a host of pro-inflammatory genes, including VCAM-1, ICAM-1 and MCP-1. Oxidative stress and cytokines pertinent to the inflammatory state, such as TNF-α and IL-1, are common stimuli that activate NF-κB and its downstream pro-inflammatory molecules\cite{225}. NO has been directly implicated in the inhibition of VCAM-1, ICAM-1 and MCP-1 expression via a NF-κB-dependent mechanism\cite{25, 226, 227}. On the contrary, in my study, I show that CavNOxin treatment decreased VCAM-1, ICAM-1 and MCP-1 gene expression but had no effect on the gene expression of the p65 subunit of NF-κB in the aorta of diabetic ApoE KO mice. While the gene expression of the NF-κB subunits is not necessarily indicative of its activity, my results may indicate that CavNOxin reduces the expression of its downstream pro-inflammatory molecules by an NF-κB independent pathway that involves the lowering of oxidative stress.

There is now a large body of evidence that suggests that hyperglycemia is a potent inducer of leukocyte/endothelial interactions. Monocytes isolated from diabetic patients demonstrate increased adhesiveness to the human endothelium, while diabetic mouse models exhibit leukocyte accumulation on the vascular wall\cite{228-231}. Similarly, we have shown increased monocyte adhesion in cultured endothelial cells exposed to high glucose conditions. In addition, aortas isolated from diabetic mice had augmented leukocyte adhesion to the vascular wall as compared to their non-diabetic controls. It is important to note that hyperglycemic injury to the vascular endothelium was evident after a short period (5 weeks) of diabetes and prior to the development of atherosclerotic lesions, which strengthens the need to combat endothelial dysfunction at an early stage before clinical manifestations of the disease. Remarkably,
CavNOxin was able to attenuate leukocyte adhesion to the vascular wall back to levels observed in non-diabetic control mice, confirming that CavNOxin is a potent inhibitor of leukocyte-endothelial interactions.

Diabetes is considered an independent risk factor for cardiovascular disease with endothelial dysfunction playing a major role in initiating vascular injury. In this chapter of my thesis, I have provided evidence that CavNOxin, is a valuable tool in limiting oxidative stress, leukocyte-endothelial interactions and atherosclerosis in a diabetic setting, demonstrating that CavNOxin is a credible anti-atherosclerotic agent.
Chapter 6. Perspectives
6.1. Endothelial dysfunction as a prognostic tool for CVD

Our understanding of clinical predictors and the diagnostic tools for the treatment and prevention of CVD have significantly improved over the past decade. The endothelium, once considered just a simple barrier separating blood from underlying tissues has now emerged as a key player in the maintenance of vascular homeostasis. CVD patients invariably display endothelial dysfunction, which is characterized by a reduction in NO bioavailability and the presence of heightened oxidative stress[232]. Evidence suggests that endothelial dysfunction is therapeutically reversible especially if detected early; therefore, diagnostic tools to assess endothelial function and the design of drugs to specifically target and improve endothelial dysfunction is at the forefront of vascular research. Assessment of endothelial function is now considered an independent prognostic predictor for the risk of future CVD events[41, 233].

In the past, invasive methods that involved infusion of acetylcholine into the coronary artery to evaluate flow-mediated dilation were utilized to assess endothelial function. Although, this assessment of endothelial function was highly informative, the invasiveness of the method led to the discovery of non-invasive approaches to determine endothelial function[233, 234]. Currently, flow-mediated dilation in response to NO-stimulating agonists (acetylcholine) in the brachial artery is the gold standard for assessing endothelium function[233, 234]. Moreover, the indication of NO bioavailability for vascular tone has also led to the discoveries of the other important functions of NO, including cell adhesion and proliferation. Indeed, flow mediated dilation data correlates with progression of preclinical atherosclerosis, in particular coronary artery disease[233]. Thus, improvements in endothelial dysfunction are now recognized as important first steps towards lessening CVD burden. A major approach to do so is through the repletion of NO and the reduction of oxidative stress, however, current strategies have their merits and pitfalls, which will be discussed in the next section.

6.2. NO therapeutics: strengths and weaknesses

Due to the vital importance of NO bioavailability on endothelial function, therapeutic strategies to upregulate NO levels have been highly sought after in an attempt to alleviate CVD burden. NO donors, such as organic nitrates and sodium nitroprusside, are pharmacologically active compounds that can release NO in biological systems, however, their efficacy has been
impacted by their rapid NO release, poor distribution to target tissues, toxicity, and development of tolerance, limiting their effectiveness as a long-term therapeutic target[235]. Protecting eNOS function through increasing substrate, L-arginine, and co-factor, BH4, availability has proven successful in improving endothelial function and atherosclerosis in pre-clinical models, however, their specificity towards eNOS have not been validated using eNOS KO mice. Furthermore, clinical investigation has revealed major limitations with L-arginine and BH4 supplementation. L-arginine was shown to improve cardiovascular end-points for hypercholesterolemic patients with coronary artery disease in one study[236] while another study was terminated prematurely due to high incidences of sudden death[237]. Furthermore, L-arginine proved ineffective in directly improving endothelial function in diabetic patients[238].

Clinical studies of BH4 therapy in vascular disease have been restricted due to the fact that BH4 is quickly oxidized, is temperature and light sensitive and exhibits poor uptake by endothelial cells, limiting its use as a chronic drug treatment[239]. Many cardiovascular drugs currently used in the clinic for co-morbidities associated with atherosclerosis can improve endothelial function; statins can promote increased NO bioavailability by improving eNOS phosphorylation, expression and activity in a fashion that is completely independent of its lipid lowering actions, whereas amlodipine, a myocyte Ca\(^{2+}\)-channel blocker does so by reducing superoxide production and increases NO release by relieving the eNOS/Cav-1 inhibitory clamp[240, 241]. However, the direct effect on endothelial function is difficult to assess as these biological functions have been described as beneficial but ancillary or side effects of these compounds. Since oxidative stress is a major determinant of NO bioavailability, great interest was generated to explore the cardioprotective benefits of antioxidant therapy. However, despite the strong evidence that implicates oxidative stress as an inducer of atherogenesis and endothelial dysfunction, clinical trials with traditional antioxidants, including vitamin C and E, have yielded disappointing outcomes with no cardiovascular benefit[156-158]. Current therapeutics are aimed at generating synthetic mimetics of endogenous antioxidant enzymes to bolster the activity of these enzymes, which are often compromised in disease settings. My additional studies with the GPx1 mimic, ME, and the Nrf2 transcription factor agonist, the Bardoxolone methyl derivative, dh404, discussed in Section 1.14. demonstrates the therapeutic potential of increasing antioxidant activity as alternative treatments for diabetes-associated atherosclerosis. (Supplementary data in Appendix A and B)
With this clinical data in mind, it is clear that novel approaches to directly target endogenous eNOS-derived NO are warranted. However, simply augmenting eNOS expression in settings of CVD risk factors, such as hypercholesterolemia and diabetes, could lead to an increase in superoxide production instead of NO, resulting in more detrimental rather than beneficial effects[242]. Thus, an approach to improve NO release whilst maintaining eNOS coupling is needed. My current and previous studies[140] have shown that through careful modulation of the eNOS/Cav-1 interaction, CavNOxin is able to restore NO release and NO-dependent biological activities in CVD settings.

6.3. The novelty of CavNOxin

Cav-1 is a physiological intracellular inhibitor of eNOS activity, keeping the enzyme in a tonically inactive state through a direct protein-protein interaction between eNOS and the Cav-1 CSD. In my previous studies, thorough analysis of the eNOS/Cav-1 interaction revealed that three amino acids (T90, T91 and F92) were responsible for inhibiting eNOS-derived NO release[139]. This led to the generation of a modified cell permeable Cav-1 CSD peptide with an inactivated eNOS inhibitory domain, a peptide termed CavNOxin. Previously, I demonstrated that CavNOxin not only lacks the ability to inhibit eNOS, but is able to increase eNOS-derived NO release in cultured endothelial cells in a dose-dependent manner[140]. Co-immunoprecipitation studies have confirmed that CavNOxin competes with endogenous Cav-1 for eNOS binding, thereby exerting its positive actions of NO release through regulated eNOS/Cav-1 antagonism. Furthermore, CavNOxin improves vascular tone, an effect that is blunted in both eNOS and Cav-1 KO, indicating specificity of the peptide for the eNOS/Cav-1 interaction. However, modulating the eNOS/Cav-1 interaction to increase NO release in vivo in settings of endothelial dysfunction and CVD has never been determined. Our studies reveal a novel anti-atherogenic role for CavNOxin in mouse models of atherosclerosis induced by hypercholesterolemia and diabetes. CavNOxin exerted its protective effect by inhibiting atherosclerotic processes, in particular oxidative stress and leukocyte/endothelial interactions, both of which are positively regulated by NO. More importantly, the anti-atherosclerotic effect of CavNOxin was not observed in eNOS KO mice, further confirming that eNOS is the specific molecular target for CavNOxin. To our knowledge, these studies are the first to demonstrate an
eNOS-specific pharmacological activator that targets the endogenous eNOS/Cav-1 interaction to improve endothelial function, oxidative stress and atherosclerosis.

Genetic approaches to either globally knockout Cav-1 or overexpress endothelial specific Cav-1 in mice have highlighted the importance of endothelial-specific Cav-1 in the maintenance of cardiovascular homeostasis. Genetic ablation of Cav-1 confers protection against atherosclerosis, while endothelium-specific rescue of Cav-1 restores normal atherosclerosis, providing direct evidence of endothelial Cav-1’s role in the pathogenesis of atherosclerosis[123, 124]. Interestingly, endothelial Cav-1 re-introduction also corrects most of the cardiovascular and pulmonary defects observed in Cav-1 KO animals which are widely associated with eNOS hyperactivation, attesting to the fact that eNOS is likely the most significant target of endothelial Cav-1[135]. However, genetic ablation of Cav-1 is directly linked to loss of whole caveolae organelles and dislocation of signaling molecules associated with caveolae/Cav-1[106], therefore making it extremely difficult to delineate between caveolae and Cav-1 dependent signaling. Hence, approaches to dissociate Cav-1 dependent signaling, whilst preserving caveolae organelle formation are highly sought after. With this in mind, the novel peptide, CavNOxin, is able to manipulate the endogenous protein-protein interaction between Cav-1 and eNOS, in order to promote eNOS-derived NO activity, whilst still preserving the other biological functions of Cav-1[140]. Moreover, it was hoped that the current PhD study would pave the way for a breakthrough in caveolae research, in which “knock-in” transgenic models can be developed expressing the modified Cav-1 CSD with intact caveolae but inactivated eNOS signaling sites, enabling the differentiation of Cav-1 dependent cell signaling pathways from caveolae functions.

Collectively, these data provide evidence that CavNOxin is an attractive candidate for the development of targeted therapeutics to increase NO in endothelial dysfunction and CVD settings with minimal interference to caveolae/Cav-1 biology.

6.4. Peptide-based approach for drug development and target validation

A plethora of research has shown that cell permeable peptides are a highly innovative approach for drug discovery. With more than 100 peptide drug candidates in clinical development since 2001 and recent advances in peptide drug delivery, this is a rapidly advancing field[243, 244]. Cell permeable peptides are generally short, water-soluble and partly
hydrophobic in nature, with a net positive charge at physiological pH[243]. Their main characteristic feature is their ability to penetrate cell membranes and transport cargo into cells, tissues and organs at low concentration with high efficacy and minimal toxicity, making these peptides an ideal tool for drug delivery[154, 243].

In my studies, I utilized the AP peptide delivery system, which is a 16-amino acid long polypeptide corresponding to the third helix of the DNA binding domain of a Drosophila transcription factor[245]. AP exhibits rapid cellular uptake, typically between 1-3 hours, in a receptor-independent manner with low cellular toxicity even at high concentrations[154, 243, 245]. Cavtratin, an AP peptide fused to the Cav-1 CSD, was reported to attenuate endothelium-dependent relaxations and eNOS-derived NO release, without any effect on iNOS-induced NO release[132]. Furthermore, cellular uptake was monitored by surface biotinylation of Cavtratin and revealed high endothelial cell expression in 6 hours[132]. Acute systemic administration of Cavtratin markedly reduced interstitial edema, inflammation and vascular leakage associated with tumour progression in mice[132, 133]. eNOS KO mice showed reduced effectiveness of Cavtratin in tumour microvascular leakage and progression, which strongly supports the notion that eNOS is necessary for the actions of Cavtratin[133]. Based on these positive findings of peptide delivery *in vivo*, the Bernatchez group generated a modified Cavtratin peptide, CavNOxin, with the premise of improving NO release by inactivating the eNOS inhibitory domain of Cavtratin[140]. Similar to Cavtratin, rhodamine labeling of CavNOxin confirmed time-dependent cellular uptake with maximal enrichment in endothelial cells observed at 6 hours[140]. Additionally, CavNOxin improved biological activities of eNOS, such as increased endothelial cell NO release, improved endothelial-dependent relaxation and attenuated blood pressure[140].

In my studies, I examined the anti-atherogenic potential of chronic administration of CavNOxin. With respect to adverse effects of chronic peptide exposure, I observed no differences in the health and well-being of animals exposed to either CavNOxin (2.5mg/kg or 5.0mg/kg) or vehicle (AP) peptide in both the hypercholesterolemic and diabetic milieu. CavNOxin did however exhibit anti-atherogenic, anti-oxidative and anti-adhesive properties most likely through its actions on eNOS-derived NO. This was confirmed by reduced effectiveness of the CavNOxin peptide in eNOS KO mice. Nevertheless, it is noted that the high degree of specificity of Cavtratin and CavNOxin for eNOS, does not completely rule out the possibilities of other targets for these peptides, which is an important consideration that needs to
be taken into account for future use of these peptides.

There is an urgent need for targeted therapeutics to reverse endothelial dysfunction and alleviate CVD, for example atherosclerosis, given its prevalence in western societies. As such, my study paves the way for future studies to optimize CavNOxin or for CavNOxin to serve as a template for the development of small molecules to directly target endothelial function and reduce CVD burden.
Chapter 7. Conclusion
A large body of evidence has indicated that endothelial dysfunction is an early marker and critical inducer of atherosclerosis. Endothelial dysfunction leads to enhanced oxidative stress and endothelial activation, which encompasses the upregulation of endothelial adhesion molecules and subsequent accumulation of leukocytes to the vascular wall, a key process in atherogenesis. NO is a potent gaseous molecule released in endothelial cells by the constitutively expressed enzyme, eNOS. NO is the key determinant of endothelial function and is considered an anti-atherogenic autocoid as it is protective against oxidative stress, leukocyte-endothelial interactions, SMC proliferation and migration and platelet aggregation. Atherosclerotic risk factors, such as hypercholesterolemia and diabetes, directly cause endothelial dysfunction with reduced eNOS function and vascular NO levels. Whether improvement of endothelial function by specifically targeting the endogenous pool of eNOS to improve eNOS-derived NO release results in atheroprotection is unknown.

In endothelial cells, eNOS is localized to plasma membrane caveolae and bound to Cav-1, the structural coat protein of caveolae. Cav-1 serves as a negative regulator of eNOS activity in basal conditions through a direct protein-protein interaction between eNOS and the Cav-1 CSD. Through careful analysis of the eNOS/Cav-1 interaction, a cell permeable peptide linked to the Cav-1 CSD with an inactivated eNOS inhibitory domain was previously generated. This peptide, known as CavNOxin, is able to increase eNOS-derived NO release and improve vascular tone by competing with endogenous Cav-1 for the eNOS binding site. In the current thesis, I investigated the therapeutic potential of CavNOxin in pre-clinical models of hypercholesterolemia- and diabetes-induced atherosclerosis. Importantly, I was able to show that CavNOxin treatment results in marked attenuation of atherosclerosis in both settings. In the hypercholesterolemic setting, CavNOxin exerted its athero-protective effects by reducing oxidative stress, in particular superoxide production and protein tyrosine nitration-mediated oxidative tissue damage and improved NO bioavailability. Additionally, CavNOxin modulated VCAM-1 expression and inhibited leukocyte-endothelial interactions. Mice lacking eNOS show resistance to CavNOxin treatment in the setting of hypercholesterolemia, indicating eNOS-specific actions of the peptide. In the diabetic setting, CavNOxin exhibited anti-atherosclerotic effects by reducing systemic oxidative stress and oxidative tissue damage by protein tyrosine nitration and lipid peroxidation. CavNOxin also influenced the expression of pro-inflammatory mediators, particularly VCAM-1 and MCP-1, and caused a block in hyperglycemia-induced
leukocyte adhesion to the vascular wall. Collectively, these studies demonstrate that CavNOxin is an effective anti-atherosclerotic agent by improving eNOS-derived NO release, reducing oxidative stress and inflammation.

To my knowledge, these data are the first to document the use of an eNOS-specific pharmacological agent to directly increase atheroprotective endothelial function. My studies in atherosclerotic disease models \textit{in vivo} now provide the needed proof-of-principle that strategies targeting the endogenous eNOS/Cav-1 interaction is a rational and effective approach to augment NO levels and reduce atherosclerotic disease burden. More importantly, the unique ability of CavNOxin to regulate eNOS activity while preserving caveolae/Cav-1 biology makes it a desirable therapeutic agent. Future studies aimed at the optimization of CavNOxin or small molecules based on CavNOxin could be efficient in protecting endothelial function in a myriad of CVD.
Figure 25: Schematic diagram of the eNOS/Cav-1 interaction with CavNOxin

Top: eNOS exists in a dynamic balance between a “less active” state (bound to and inhibited by endogenous Cav-1) and a more active state (free from the Cav-1 inhibitory clamp).

Bottom: CavNOxin can bind eNOS and prevent its inhibition by endogenous Cav-1, resulting in the creation of a third dynamic state whereby eNOS is bound to a noninhibitory Cav-1 scaffolding domain thereby shifting the eNOS equilibrium toward a “more active” state by preventing the inhibitory clamp of endogenous Cav-1. Our data show that CavNOxin is able to attenuate atherosclerosis by reducing oxidative stress and leukocyte-endothelial interactions. Adapted from Bernatchez P, Sharma A et al, Journal of Clinical Investigation (2011)[140].
REFERENCES


APPENDICES
APPENDIX A: GPx KO and m-hydroxy-ebselen study

Figure 26: Lack of GPx1 increases leukocyte-endothelial interactions in response to hyperglycemia and TNF-α.

(A) Aortic endothelial cells were isolated from wild type (WT) and GPx1 KO mice and grown in low glucose (5mM) or high glucose (20mM) media. Endothelial cells were then incubated with fluorescently labelled THP-1 monocyte cells. Increased adhesion of THP-1 cells to GPx1 KO endothelial cells were observed in high glucose conditions. (B) Aortas were isolated from WT and GPx1 KO mice and incubated in the presence or absence of TNF-α (5ng/ml). Next, whole blood was perfused through the aortas and leukocyte adhesion was monitored. The graph shows quantitation of average leukocyte binding per field every 5 minutes over a 15-minute period. *P<0.05. n=4-5/group. Results expressed as mean ± SEM.
**Figure 27: M-hydroxy-ebselen attenuates diabetes-associated atherosclerosis**

(A) Chemical structures of ebselen (Eb) and M-hydroxy-ebselen (ME). (B) 14 weeks of diabetes resulted in a significant ~8-fold increase in plaque (stained red) within the aorta of ApoE/GPx1 dKO mice. Treatment with both ME and Eb at 10 mg/kg twice daily significantly attenuated atherosclerotic plaque deposition within the diabetic aorta to a similar extent. ***$P<0.001$ vs ND control; ###$P<0.001$ vs diabetic aorta; NS = not significant. ND = non-diabetic; ME = m-hydroxy ebselen; Diab = diabetic; Eb =ebselen. Results expressed as mean ± SEM. Figure adapted from Tan M, Sharma A et al. Plos One (2013)[167].
Diabetes resulted in a significant 4-fold increase in plaque within the aorta sinus region. Treatment with both ME and ebselen (Eb) at 10 mg/kg twice daily significantly attenuated atherosclerotic plaque within the sinus. ***$P<0.001$ vs ND control; ###$P<0.01$ vs diabetic sinus. ND = non diabetic; ME = m-hydroxy ebselen; Diab = diabetic; Eb = ebselen. Results expressed as mean ± SEM. Figure adapted from Tan M, Sharma A et al. Plos One (2013)[167].

**Figure 28: ME attenuates lesion area in the aortic sinus of diabetic ApoE/GPx1 dKO mice.**
Figure 29: Oxidative stress is reduced in the aortic sinus region after treatment with ME

Aortic sinus regions of ApoE/GPx1 dKO mice were immunostained with the nitrotyrosine (NT) antibody to detect peroxynitrite-induced damage. Diabetes resulted in a significant 2-fold increase in NT staining within the aorta sinus region of dKO mice. Treatment with ME and ebselen (Eb) significantly attenuated the level of NT staining observed within the aortic sinus region. *P<0.05 vs ND control; #P<0.05 vs diabetic sinus. ND = non-diabetic; Diab = diabetic. Results expressed as mean ± SEM. Figure adapted from Tan M, Sharma A et al. Plos One (2013)[167].
APPENDIX B: DH404 diabetes-associated atherosclerosis study

Figure 30: Dh404 attenuates diabetes-associated atherosclerosis in the aorta and aortic sinus of diabetic ApoE KO mice

(A) Representative images of Sudan IV stained aortas. Treatment with dh404 at 3 and 10 mg/kg/day for 18 weeks resulted in an attenuation of aortic plaque formation of diabetic mice in the (B) total, (C) arch, (D) thoracic and (E) abdominal aorta. (F) Representative images of Oil Red O stained aortic sinus to detect plaque formation after 18 weeks of dh404 treatment. (G) Graph shows quantification of lesion area. At 3 and 10 mg/kg/day, plaque formation was significantly attenuated in diabetic mice when compared with their vehicle treated counterparts. **P < 0.01, ***P < 0.001 vs. ND+SO; #P < 0.05, ##P < 0.01 vs. D+SO; ^P < 0.05, ^^P < 0.01 vs. as indicated. Abd: abdominal; D, diabetic mice; dh-3, -10, and -20, dh404 at 3, 10, or 20 mg/kg/day; ND: non-diabetic mice; SO: sesame oil (vehicle); Thor: thoracic. Results expressed as mean ± SEM. Figure is published in Tan M, Sharma A et al. Diabetes (2014)[174].
Figure 31: VCAM-1 gene and protein expression are reduced by dh404 treatment

VCAM-1 protein expression was examined in the aortic plaque using immunohistochemistry. (A) Representative images and (B) quantification is shown. (C) VCAM-1 gene expression in the aorta was assessed by qRT-PCR after 5 weeks of treatment. *P < 0.05, **P < 0.01 vs. ND+SO; #P < 0.05, ##P < 0.01 vs. D+SO. A.U.: arbitrary units; D: diabetic mice dh-3, -10, and -20, dh404 at 3, 10, or 20 mg/kg/day; ND: non-diabetic mice; SO: sesame oil (vehicle). Results expressed as mean ± SEM. Figure is published in Tan M, Sharma A et al. Diabetes (2014)[174].
Figure 32: Diabetes-associated oxidative stress is attenuated with dh404 treatment

Oxidative stress was assessed by urinary levels of (A) 8-isoprostane and (B) 8-OHdG and (C) plasma levels of dROMs. P < 0.05, **P < 0.01, ***P < 0.001 vs. ND+SO; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. D+SO. D: diabetic mice; dh-3, -10, and -20, dh404 at 3, 10, or 20 mg/kg/day; ND: non-diabetic mice; SO: sesame oil (vehicle). Results expressed as mean ± SEM. Figure is published in Tan M, Sharma A et al. Diabetes (2014)[174].
APPENDIX C: PhD highlights: list of publications, conferences and awards

Publications (Year 2010-2014)


7) Bernatchez P*, Sharma A*, Bauer PM, Marin E and Sessa WC. A non-inhibitory mutant of caveolin-1 scaffolding domain enhances eNOS-derived NO synthesis and vasodilation in mice. JCI (2011); 121: p3747-3755.


10) **Sharma A**, Andy Trane, Carol Yu, Jean-Francois Jasmin and Pascal Bernatchez. *Amlodipine increases endothelial nitric oxide release by modulating binding of native eNOS complex to caveolin-1.* European Journal of Pharmacology (2011); 659: p206-212.

* indicates co-authorship

**Conferences (Year 2010-2014)**


**Awards (Year 2010-2014)**

Young Investigator Oral Presentation (1st Place) – Australian Atherosclerosis Society, Melbourne, Australia (2013).


Harold Mitchell Travel Fellowship (2013)
Best Presentation for PhD student – Department of Anesthesiology, Pharmacology and Therapeutics, UBC (2013)


Early Career Poster Awards (1st place) - International Symposium of Atherosclerosis Satellite Meeting, Melbourne (2012)

Baker IDI Bright Sparks top-up scholarship (April 2011- April 2014)

Canada Governor General Gold Award for Best Master’s Thesis (2011)

The Cornelis van Breeman Outstanding Young Investigator Award (2011)

Alexander Graham Bell- Canada Graduate Scholarship (Doctoral) (September 2010 – June 2012)