MOLECULAR AND PHARMACOLOGICAL CHARACTERIZATION OF MUTANT (F92A) CAV-1: A DIRECTION TOWARDS INCREASING NITRIC OXIDE BIOAVAILABILITY

by

Arpeeta Sharma

B.Sc., King’s College London, UK, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty Of Graduate Studies

(Pharmacology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2010

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Nitric Oxide (NO) produced by the endothelium is a critical mediator of vascular function and plays an important role in the protection against various cardiovascular diseases. In fact, a central feature of most cardiovascular diseases is reduced bioavailability of NO resulting from impaired endothelial function. Consequently, therapies that improve NO synthesis and availability in disease settings are relevant. Endothelial nitric oxide synthase (eNOS) is a membrane enzyme expressed exclusively in vascular endothelial cells and is responsible for NO production. Improper regulation of the enzyme results in production of eNOS-derived superoxide anion (O$_2^-$) instead of NO. O$_2^-$ is an oxidative stress mediator and scavenges NO, thereby contributing to lowered NO bioavailability.

Extensive research has demonstrated a number of factors involved in positively regulating eNOS activity. However, one of the few proteins that bind to eNOS under basal conditions and inhibit NO release is Caveolin-1 (Cav-1), the major coat protein of plasma membrane lipid-enriched invaginations known as caveolae. Recently, it was demonstrated that a single amino acid substitution of the Cav-1 protein, mutant known as F92A Cav-1, is unable to inhibit eNOS. Furthermore, preliminary data indicates that high expression of F92A Cav-1 can increase basal NO release. Due to the significance of NO in vascular function, the current work explores the possible mechanisms by which F92A Cav-1 potentiates eNOS activity and NO release. We report that F92A Cav-1 preserves the unique properties of Cav-1, including targeting to caveolae and forming high molecular weight oligomers, which are essential for caveolae organelle biogenesis. Moreover, F92A Cav-1 still retains the ability to bind to eNOS without altering its subcellular localization, indicating that F92A Cav-1 can prevent eNOS binding to endogenous Cav-1, which could rationalize the increased NO release observed. Lastly, we provide evidence.
that over-expression of F92A Cav-1 reduces the release of basal $O_2^-$ in endothelial cells as compared to WT Cav-1, revealing another potential positive effect of the mutant Cav-1. Hence, this report compares the biological properties of WT and F92A Cav-1 and the data collected is aimed at describing a therapeutically relevant pharmacological target to increase NO bioavailability in cardiovascular disease settings.
PREFACE

The appendices of this thesis contains a manuscript in preparation entitled “Increased eNOS-derived nitric oxide release and vasodilation through delivery of mutant non-inhibitory Caveolin-1 scaffolding domain”, which has been just been submitted to JCI on August 5th 2010. In this manuscript, I am a co-author with Dr Bernatchez and both of us have contributed equally to the design of experiment, data acquisition and analysis and the preparation of the manuscript. The article presented has been approved by the respective co-authors. I would like to specially thank Dr Bernatchez for his facilitation in the article.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>..........................................................</td>
<td>ii</td>
</tr>
<tr>
<td>PREFACE</td>
<td>..........................................................</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>..........................................................</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>..........................................................</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>..........................................................</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>..........................................................</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>..........................................................</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>..........................................................</td>
<td>xi</td>
</tr>
<tr>
<td>Chapter 1. Introduction</td>
<td>..........................................................</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Scope of the thesis</td>
<td>..........................................................</td>
<td>2</td>
</tr>
<tr>
<td>1.2. The endothelium and regulation of vascular tone</td>
<td>..........................................................</td>
<td>2</td>
</tr>
<tr>
<td>1.3. The discovery of NO</td>
<td>..........................................................</td>
<td>3</td>
</tr>
<tr>
<td>1.4. Physiological roles of NO</td>
<td>..........................................................</td>
<td>3</td>
</tr>
<tr>
<td>1.5. NO synthesis</td>
<td>..........................................................</td>
<td>4</td>
</tr>
<tr>
<td>1.6. eNOS structure</td>
<td>..........................................................</td>
<td>5</td>
</tr>
<tr>
<td>1.7. eNOS “uncoupling”</td>
<td>..........................................................</td>
<td>6</td>
</tr>
<tr>
<td>1.8. The link between eNOS-derived NO and vascular disease</td>
<td>..........................................................</td>
<td>7</td>
</tr>
<tr>
<td>1.9. eNOS regulation</td>
<td>..........................................................</td>
<td>8</td>
</tr>
<tr>
<td>1.9.1. Transcriptional regulation of eNOS</td>
<td>..........................................................</td>
<td>8</td>
</tr>
<tr>
<td>1.9.2. Phosphorylation</td>
<td>..........................................................</td>
<td>9</td>
</tr>
<tr>
<td>1.9.3. Subcellular localization of eNOS</td>
<td>..........................................................</td>
<td>9</td>
</tr>
<tr>
<td>1.9.4. Protein-protein interactions</td>
<td>..........................................................</td>
<td>10</td>
</tr>
<tr>
<td>1.10. Caveolae and lipid rafts as key regulators of eNOS function</td>
<td>..........................................................</td>
<td>13</td>
</tr>
<tr>
<td>1.11. Caveolins are structural proteins of caveolae</td>
<td>..........................................................</td>
<td>13</td>
</tr>
<tr>
<td>1.12. Structure of Cav-1</td>
<td>..........................................................</td>
<td>14</td>
</tr>
<tr>
<td>1.13. Physiological roles of caveolae and caveolins</td>
<td>..........................................................</td>
<td>16</td>
</tr>
<tr>
<td>1.14. Caveolins and caveolins in the endothelium</td>
<td>..........................................................</td>
<td>16</td>
</tr>
<tr>
<td>1.15. Cav-1 regulation of eNOS-derived NO</td>
<td>..........................................................</td>
<td>17</td>
</tr>
<tr>
<td>1.16. Direct interaction of Cav-1 and eNOS</td>
<td>..........................................................</td>
<td>17</td>
</tr>
<tr>
<td>Chapter 2. Research Overview</td>
<td>..........................................................</td>
<td>20</td>
</tr>
<tr>
<td>2.1. Rationale</td>
<td>..........................................................</td>
<td>21</td>
</tr>
<tr>
<td>2.2. Hypothesis</td>
<td>..........................................................</td>
<td>22</td>
</tr>
<tr>
<td>2.3. Specific aims</td>
<td>..........................................................</td>
<td>22</td>
</tr>
<tr>
<td>Chapter 3. Methods And Materials</td>
<td>..........................................................</td>
<td>23</td>
</tr>
<tr>
<td>3.1. Cell culture</td>
<td>..........................................................</td>
<td>24</td>
</tr>
<tr>
<td>3.2. Cell transfection and infection</td>
<td>..........................................................</td>
<td>24</td>
</tr>
<tr>
<td>3.2.1. Cell transfection</td>
<td>..........................................................</td>
<td>24</td>
</tr>
<tr>
<td>3.2.2. Cell infection</td>
<td>..........................................................</td>
<td>25</td>
</tr>
<tr>
<td>3.2.3. Amplification of AdWT Cav-1 and AdF92A Cav-1</td>
<td>..........................................................</td>
<td>25</td>
</tr>
<tr>
<td>3.2.4. Cytopathic effect assay (CPE assay) to determine adenovirus virulence</td>
<td>..........................................................</td>
<td>26</td>
</tr>
<tr>
<td>3.3. Western blot analysis</td>
<td>..........................................................</td>
<td>27</td>
</tr>
<tr>
<td>3.4. Isolation of caveolae/lipid rafts by sucrose gradient fractionation</td>
<td>..........................................................</td>
<td>27</td>
</tr>
<tr>
<td>3.5. Determination of protein molecular weight by velocity gradient centrifugation</td>
<td>..........................................................</td>
<td>28</td>
</tr>
</tbody>
</table>
Chapter 6. Conclusions And Future Directions

5.10. Clinical implications of the study

5.9. Other cellular mechanisms that could rationalize F92A Cav

5.8. Superoxide production: a factor in determining NO bioavailability

5.7. Differences in intracellular Cav

5.6. Cav

5.5. Similar eNOS binding to WT and F92A Cav

5.4. F92A Cav quantification.

5.3. Protein

5.1. Caveolae disruption: increased or decreased eNOS activation?

Chapter 5. Discussion

4.5. Over expression of F92A Cav

4.4. F92A Cav

4.2. WT and F92A Cav

4.1. WT and F92A Cav

Chapter 4. Results

3.9. Statistical analysis

3.8. Superoxide anion release quantification

3.7. Co-localization of Cav-1 proteins with eNOS by immunocytofluorescence

3.6. Glutathione-S-Transferase (GST) pull-down experiments

3.6.1. F92A Cav-1 cDNA synthesis by PCR

3.6.2. Cloning of F92A Cav-1 PCR product and transformation into competent cells

3.6.3. Overnight culturing and isolation of DNA plasmids from bacterial plasmids

3.6.4. Digestion and linearization of DNA using restriction enzymes

3.6.5. Ligation and transformation of F92A Cav-1 with GST vector

3.6.6. Sequencing, re-transformation and re-culturing of GST plasmids

3.6.7. Purification of GST and GST-fused proteins

3.6.8. Binding of purified GST fused proteins to glutathione beads

3.6.9. Binding experiments with BAEC lysates and recombinant eNOS

3.6.10. Binding exper...
LIST OF TABLES

Table 1: Forward and reverse primer sequences for F92A Cav PCR reaction.................................30
Table 2: Temperature and duration cycles for PCR. *indicates repeated for 35 cycles..................30
LIST OF FIGURES

Figure 1. Mutant F92A Cav-1 does not block eNOS-derived NO release ........................................... 19
Figure 2. Detection of $O_2^-$ using the Cytochrome C Reduction Assay ........................................... 40
Figure 3. WT and F92A Cav-1 target similarly to caveolae/Lipid Rafts .................................................. 44
Figure 4. Velocity Gradient Centrifugation determines native protein size ................................... 47
Figure 5. WT and F92A Cav-1 auto-assemble to form HMW oligomers ............................................. 48
Figure 6. GST, GST Cav and GST F92A Cav purification to glutathione beads ................................... 52
Figure 7. eNOS binds equally to GST Cav and GST F92A Cav .............................................................. 53
Figure 8. F92A Cav-1 preserves eNOS subcellular localization ......................................................... 56
Figure 9. Validation of the Cytochrome C Reduction Assay ................................................................. 60
Figure 10. F92A Cav-1 decreases $O_2^-$ release in endothelial cells as compared to WT Cav-1 .... 61
Figure A1. Summary of steps involved in GST F92A Cav generation ............................................. 89
Figure A2. eNOS dimer/monomer interaction in BAECs infected with AdWT Cav-1 and F92A Cav-1 ...................................................................................................................................... 90
Figure A3. F92A Cav-1 increases basal NO release .............................................................................. 128
Figure A4. CavNoxin increases eNOS activity and NO dependent activities ................................ 130
Figure A5. CavNoxin’s effect are eNOS and Cav-1 specific ............................................................ 132
Figure A6. F92A Cav-1 does not alter biochemical properties of Cav-1 ............................................ 133
Figure A7. F92A Cav-1 still binds eNOS and retains its subcellular localisation ........................... 134
Figure A8. Supplementary data 1 ..................................................................................................... 135
Figure A9. Supplementary data 2 ..................................................................................................... 136
LIST OF ILLUSTRATIONS

Illustration 1. Schematic Diagram of eNOS Structure ............................................................... 6
Illustration 2. Proposed model for eNOS activation in endothelial cells. ................................. 12
Illustration 3. Main structural features of caveolae and Cav-1.................................................. 15
LIST OF ABBREVIATIONS

4HNE  4-hydroxy-2-nonenal
ApoE  Apolipoprotein E
BAECs  Bovine Aortic Endothelial Cells
bp  base pairs
β-gal  β-galactosidase
Cav-1  Caveolin-1
Cav-1 CSD  Cav-1 Scaffolding Domain
cGMP  cyclic guanosine monophosphate
CPE  Cytopathic Effect Assay
DMEM  Dulbecco’s Modified Eagle Medium
DTT  Dithiothreitol
E.Coli  Escherichia Coli
eNOS  Endothelial Nitric Oxide Synthase
ERK  Extracellular signal-related kinase
F92  Phenylalanine 92
FBS  Fetal Bovine Serum
FMN  Flavin mononucleotide
FAD  Flavin adenosine dinucleotide
GTP  Guanosine Triphosphate
GST  Glutathione-S-Transferase
HA  Hemagglutinin
HEK  Human Embryonic Kidney
HSP90  Heat Shock Protein 90
HMW  High Molecular Weight
IPTG  Isopropyl β-D-1-thiogalactopyranoside
LB  Luria Broth
MBS  Mes-Buffered Saline
MOI  Magnitude of Infection
NADPH  Nicotinamide adenine dinucleotide phosphate
NGS  Normal goat serum
NO  Nitric Oxide
O2−  Superoxide Anion
PFU  Plaque Forming Units
PMSF  Phenylmethanesulfonylfluoride
PCR  Polymerase Chain Reaction
PBS  Phosphate Buffered Saline
PAGE  Polyacrylamide gel electrophoresis
SOD  Superoxide Dismutase
s-GC  soluble-guanylate cyclase
T90  Threonine 90
T91  Threonine 91
VEGF  Vascular endothelial growth factor
ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr Pascal Bernatchez, for his guidance and support, as well as my supervisory committee – Dr Darryl Knight, Dr Ismail Laher and Dr Casey Van Breeman, for their time and invaluable input throughout my project. I would also like to give special thanks to my lab members – Carol Yu, Soraya Utokarparch, Andy Trane and Cleo Leung and my family members for their support and understanding. In addition, I offer my enduring gratitude to the faculty, staff and students at the Heart and Lung Institute of St. Paul’s Hospital, Vancouver BC, as well as the Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia. Lastly, I would like to thank the Canadian Institute of Health Research, for providing me with a Master’s training award.
Chapter 1. Introduction
1.1. Scope of the thesis

Endothelial function is highly dependent on the availability of a critical mediator, known as Nitric Oxide (NO). Indeed, a common factor in most cardiovascular diseases is endothelial dysfunction, which is characterized by the reduced bioavailability of NO. Therefore, a better understanding of the factors that regulate NO synthesis clearly warrants further investigation. Endothelial nitric oxide synthase (eNOS) is the enzyme constitutively expressed in endothelial cells responsible for the production of NO. eNOS activity is positively regulated by multiple factors, however, one of the few proteins that bind to and negatively regulates eNOS under basal conditions is Caveolin-1 (Cav-1), the major coat protein of plasma membrane caveolae (small lipid-enriched invaginations). Previous work has confirmed that this inhibition is mediated by the phenylalanine 92 (F92) residue of the Cav-1 scaffolding domain, and mutation of this amino acid to alanine, the mutant known as F92A Cav-1, fails to inhibit eNOS-derived NO release. Furthermore, preliminary data indicates that expression of high levels of F92A Cav-1 can unexpectedly increase basal NO release. Thus, the current thesis will explore the possible mechanisms behind the protective effects of F92A Cav-1 and how modulation of the eNOS/Cav-1 system can be beneficial for increasing NO bioavailability in disease settings.

1.2. The endothelium and regulation of vascular tone

The endothelium is the innermost layer of the blood vessel, which is not only a vital boundary between the blood and underlying tissue, but also an important regulator of vascular tone. A single layer of endothelial cells that coat the endothelium are responsible for the maintenance of vascular 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. In turn, these vasoactive substances modify the contractile state of vascular smooth muscle cells, located in the middle layer of the blood vessel. Of these vasoactive substances released by the endothelium, NO has emerged as one of the most critical mediators of endothelial function and will be the focus of the current work.

1.3. The discovery of NO

In 1980, the pioneering work of Furchgott and Zawadski demonstrated that acetylcholine-induced relaxation of blood vessels was strictly dependent on the presence of endothelial cells. 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. This substance was subsequently identified as NO, a highly versatile gaseous molecule, which has generated a tremendous amount of interest in the cardiovascular field.

1.4. Physiological roles of NO

Since the discovery of NO, multiple studies have revealed the many physiological functions of this mediator. Primarily, NO has been well established as the most potent endogenous vasodilator, which is attributed to its capacity to directly induce smooth muscle cell relaxation. NO diffuses from the endothelium to the vascular smooth muscle cell, where it then interacts with and activates soluble-guanylate cyclase (s-GC), an enzyme that catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). In turn, cGMP is the principal second messenger responsible for mediating the vasodilatory
effects of NO, mainly by reducing intracellular Ca\(^{2+}\) concentration resulting in smooth muscle cell relaxation\(^{13}\). The ability of NO to effectively regulate vascular tone and consequently blood flow, lends credence to its pivotal role in blood pressure regulation.

Endogenous NO is also an anti-inflammatory autacoid, due to its ability to inhibit platelet aggregation, monocyte adhesion and vascular smooth muscle cell proliferation\(^{14-17}\). Accordingly, NO contributes to preserving the non-thrombogenic and anti-adhesive surface of the endothelium, allowing it to maintain the integrity of the vasculature and prevent the formation of unnecessary blood clots that would compromise blood fluidity. Furthermore, NO is an essential mediator in vascular endothelial growth factor (VEGF)-induced angiogenesis\(^{18}\), which is the formation of new blood vessels from pre-existing ones.

Although the above-mentioned physiological roles of NO are mostly a result of increased cGMP activity, several studies have reported that some of these actions of NO can occur in a cGMP independent manner. For example, NO-induced vasorelaxation can partly be brought about by activation of calcium-dependent potassium channels\(^{19, 20}\). In addition, VEGF-induced vascular permeability is brought about by increased synthesis of NO by downstream phosphorylation events\(^{21}\).

Lastly, it is important to add that in the central, peripheral and enteric nervous system, NO is considered a non-classical neurotransmitter\(^{22}\), which regulates gastrointestinal, respiratory and genitourinary tract functions\(^{23}\). Given that NO plays an important role physiologically, its synthesis and regulation will be discussed in the following sections.

**1.5. NO synthesis**

NO is synthesized by a class of enzymes known as nitric oxide synthases (NOS), which
catalyze the oxidation of the amino acid, L-arginine, to NO and L-citrulline. The NOS family of enzymes consists of three known isoforms: NOS I or neuronal NOS (nNOS) is predominantly found in neuronal tissues, NOS II or inducible NOS (iNOS) is induced by numerous cell types during inflammation and NOS III or endothelial nitric oxide synthase (eNOS) is constitutively expressed in vascular endothelial cells\textsuperscript{24}. Since the current work is focused on endothelium-derived NO, the structure and regulation of eNOS will be described in greater detail.

### 1.6. eNOS structure

eNOS is a homodimeric enzyme consisting of two domains, which are the N-terminal oxygenase domain and the C-terminal reductase domain linked by the Calmodulin (CaM) regulatory domain\textsuperscript{25}. Molecular cloning of the eNOS cDNA has identified binding sites for substrates and cofactors required for eNOS function located in these domains (Illustration 1). The N-terminal oxygenase domain binds the substrate, L-arginine, an essential redox cofactor, tetrahydropterin (BH\textsubscript{4}), and the heme prosthetic group, whilst the C-terminal reductase domain binds to the redox cofactors: flavin mononucleotide (FMN), flavin adenosine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH)\textsuperscript{26}. During NO synthesis, electrons donated by NADPH are transferred via FMN and FAD from the reductase domain to the oxygenase domain, where it interacts with the heme prosthetic group and BH\textsubscript{4} to catalyze the reaction of oxygen and L-arginine to produce NO and L-citrulline\textsuperscript{24}. This reaction is highly dependent on Ca\textsuperscript{2+} binding to CaM, in response to increases in intracellular Ca\textsuperscript{2+} concentration upon shear stress and agonist stimulation, resulting in the interaction between the Ca\textsuperscript{2+}/CaM complex and eNOS\textsuperscript{27}. More importantly, eNOS is only catalytically active as a dimer and not a monomer, with BH\textsubscript{4} being critical for dimer formation\textsuperscript{28}. 
**1.7. eNOS “uncoupling”**

In the event of L-arginine or BH₄ depletion, electron flow is disrupted and instead of NO, eNOS generates superoxide anion (O₂⁻), which is a detrimental oxidative stress mediator, often resulting in cellular damage⁹, ¹⁰. This phenomenon, often described as “eNOS uncoupling” has attracted much attention in the pathogenesis of vascular diseases, especially atherosclerosis. Moreover, O₂⁻ reacts rapidly with NO, forming peroxynitrite, a cytotoxic molecule, which has additional detrimental effects on vascular function¹¹. Because NO participates in scavenging O₂⁻, the reaction between NO and O₂⁻ further contributes to the reduction in NO bioavailability¹². Several in vivo studies on humans and rodents have demonstrated that vascular disease, such as hypertension and atherosclerosis can be reversed upon L-arginine and BH₄ supplementation, thereby specifically implicating the importance of substrate and co-factor availability in maintaining proper eNOS function¹³-¹⁵.

Other important sources of O₂⁻ in the endothelium are electron-donating enzymes such as NADPH oxidase, xanthine oxidase and mitochondrial electron transport chain enzymes, which reduce molecular oxygen to O₂⁻ and can participate in reducing NO bioavailability. In
addition, initial O₂ production by NADPH oxidase can trigger further uncoupling of eNOS resulting in the increased incidence of vascular diseases.

1.8. The link between eNOS-derived NO and vascular disease

It is without a doubt that NO plays an unequivocal role in the maintenance of vascular homeostasis. In fact, a risk factor that precedes nearly all known cardiovascular diseases is characterized by a reduction in NO bioavailability, which subsequently leads to impaired endothelium function. This risk factor, aptly named endothelial dysfunction, has become an independent clinical diagnostic tool for various cardiovascular diseases, including hypertension and atherosclerosis. A classic feature of patients exhibiting endothelial dysfunction is the attenuation of endothelium-dependent vasodilation in response to acetylcholine. Indeed, several human clinical studies have demonstrated the correlation between NO bioavailability and the presence of cardiovascular diseases.

Furthermore, genetic evidence from in vivo studies has validated the importance of eNOS-derived NO in the prevention of vascular diseases. Genetic deletion of eNOS in mice results in higher blood pressures, development of left ventricular hypertrophy and significant reduction in growth-factor induced angiogenesis compared to their wild type counterparts. Another relevant model to illustrate the significance of eNOS-derived NO is the Apolipoprotein E (ApoE) knockout mouse, which is genetically pre-disposed to develop atherosclerosis when placed on a high fat diet. Crossbreeding of the ApoE knockout mice with eNOS knockout mice increased severity of atherosclerotic lesions as compared to ApoE knockout mice. Unexpectedly, over-expression of eNOS specifically in the endothelium of the ApoE/eNOS double knockout mice also resulted in exacerbated atherosclerosis associated with lower NO
production and enhanced $\text{O}_2^-$ synthesis$^{45}$. Hence, these studies provide compelling data that proper eNOS regulation is required for its protective activity.

1.9. eNOS regulation

Due to its profound importance in vascular homeostasis, many studies have investigated the key mechanisms that regulate eNOS activation. The main eNOS regulatory mechanisms can be subdivided into 4 main categories: transcriptional regulation, enzyme phosphorylation, proper subcellular compartmentalization and protein-protein interactions.

1.9.1. Transcriptional regulation of eNOS

Stimuli that increase the transcriptional activity of the eNOS gene are either able to induce transcription, stabilize eNOS mRNA or both. Increased transcription of the enzyme in turn, results in sustained activation of eNOS-dependent activities$^{46}$. For instance, sheer stress activates the extracellular signal-regulated kinases (ERK1/2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which bind to the promoter region of the eNOS gene, thereby upregulating eNOS expression$^{47}$. In addition, sheer stress has been implicated in modulating eNOS mRNA stability. Growth factors, including VEGF, stimulate transcription and activity of eNOS$^{48,49}$. A result of increased transcription is augmented NO production, which then decreases eNOS transcription as part of a negative feedback mechanism. On the contrary, there are stimuli that decrease eNOS gene transcription, including the inflammatory mediator, tumour necrosis factor-alpha (TNF-α)$^{50}$, and high concentrations of oxidized low density lipoprotein (LDL), both of which are upregulated during atherosclerosis$^{46}$. 
1.9.2. Phosphorylation

Protein phosphorylation is a post-translational modification that has been commonly accepted to regulate eNOS activity. Indeed, several consensus sequence sites for protein kinases have been identified on the enzyme. The protein kinase Akt is activated and phosphorylated downstream of sheer stress and VEGF-induced signaling. In turn, Akt phosphorylates eNOS on serine residues 1177 (human) and 1179 (bovine), thereby increasing eNOS activity. In fact, over-expressing the activated form of Akt in endothelial cells augments basal NO release, whereas, over-expression of the activation-deficient Akt mutant attenuates NO release in response to VEGF. Moreover, mutation of the serine 1179 to alanine, inactivates and the phosphorylation site and diminishes Akt-dependent NO release. Therefore, sheer stress and VEGF-induced NO production are highly dependent on the phosphorylation status of eNOS. Phosphorylation sites that reduce the catalytic activity of eNOS have also been characterized. In the absence of Ca\(^{2+}\)/CaM, AMP-activated kinase phosphorylates eNOS at threonine 495 and inhibits eNOS activity. Lastly, phosphorylation of another tyrosine residue of eNOS at an unknown site has been shown to downregulate eNOS activity by about 50%.

1.9.3. Subcellular localization of eNOS

In 1992, Pollock et al deduced that eNOS was a membrane-associated protein attributed to N-myristoylation of the enzyme. N-myristoylation is a post-translational protein modification, which involves the attachment of myristic acid, a fatty acid, to an amide group at the N-terminal of eNOS. Through the use of non-myristoylated mutant eNOS, it was first demonstrated that this fatty acid modification was necessary for Golgi membrane association and subsequently NO release. Later, it was observed that N-myristoylation was required 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\textsuperscript{58}.

A series of in vitro and immunofluorescence studies carried out by William Sessa’s group further identified that cysteine palmitoylation enabled eNOS to target to specialized plasma membrane microdomains, known as caveolae\textsuperscript{59}. Functionally, caveolae localization was found to optimize the ability of efficient NO production from eNOS, as demonstrated by attenuated NO release by palmitoylation-deficient eNOS mutants\textsuperscript{59,60}.

### 1.9.4. Protein-protein interactions

There are several eNOS-associated proteins that have been characterized to modulate the function of the enzyme, however, heat shock protein 90 (HSP90), Ca\textsuperscript{2+}/CaM and Cav-1 are recognized as the most important regulators of eNOS activity. The interactions between the three proteins and how they modulate eNOS function will be discussed in this section.

HSP90 belongs to the heat shock protein family and is highly abundant in the cytoplasm of eukaryotic cells\textsuperscript{61}. HSP90 is involved in the proper folding of synthesized proteins and is an integral part of the signal transduction events in cells\textsuperscript{62}. In unstimulated endothelial cells, HSP90 was found in a complex with eNOS\textsuperscript{63,64}. Immunoprecipitation studies have demonstrated an increased interaction of HSP90 and eNOS when exposed to VEGF and sheer stress, which occurs in a rapid time frame consistent with the induction of stimulus\textsuperscript{63}. The increased association of HSP90 and eNOS was also correlated with enhanced activity of the enzyme\textsuperscript{63}, thereby HSP90 is considered an allosteric modulator of the enzyme.

CaM is an ubiquitiously expressed Ca\textsuperscript{2+}-binding protein that regulates a variety of intracellular Ca\textsuperscript{2+}-dependent enzymes, including eNOS\textsuperscript{65}. Binding of the Ca\textsuperscript{2+}/CaM complex to
the enzyme is essential for eNOS catalytic activity. Indeed, an increase or depletion of intracellular Ca\(^{2+}\) concentration results in the enhancement or attenuation of eNOS activity respectively\(^66\). Another important feature of Ca\(^{2+}\)/CaM-induced increase in eNOS activity is the ability of the complex to dissociate eNOS from Cav-1, a protein that negatively regulates eNOS activity.

As mentioned previously, for optimal NO release, localization of eNOS to caveolae microdomains is essential. Ironically, under basal conditions, eNOS binds to and is inhibited by Cav-1, the major coat protein of caveolae\(^2\). Upon agonist (VEGF, acetylcholine)-induced increase in intracellular Ca\(^{2+}\) and shear stress-induced phosphorylation, eNOS is activated by recruiting large amounts of Ca\(^{2+}\)/CaM and HSP90\(^67\). Recruitment of Ca\(^{2+}\)/CaM and HSP90 facilitate the dissociation of eNOS from Cav-1, resulting in efficient stimulus-response coupling to effectively produce NO\(^67\). In fact, the inhibition of eNOS by Cav-1 has been shown to be competitive and completely reversed by Ca\(^{2+}\)/CaM, suggesting a mutually exclusive relationship between Ca\(^{2+}\)/CaM and Cav-1\(^64, 68\). Furthermore, low amounts of HSP90 bound to eNOS basally, facilitates the dissociation of eNOS from Cav-1 by lowering the concentration of Ca\(^{2+}\)/CaM required to activate the enzyme\(^64\). A proposed model for eNOS activation in caveolae is illustrated in Illustration 2.
Under basal conditions, eNOS is kept in a “less active” state by binding to Cav-1. Activation of eNOS occurs by stimuli, (sheer stress, agonists), which cause an increase in intracellular Ca$^{2+}$ and subsequent recruitment of co-factors and phosphorylation of the enzyme. Following which, eNOS is dissociated from Cav-1 and efficient NO production occurs.

The complexities of the Cav-1 and caveolae organelle system in modulating eNOS 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.
1.10. Caveolae and lipid rafts as key regulators of eNOS function

Caveolae are cholesterol and sphingolipid-enriched plasma membrane invaginations, structurally identified by their flask-shaped morphology. These 50-100nm plasma membrane microdomains, although originally observed in the epithelium by electron microscopy\(^69\), are now found in a variety of different cell types such as myocytes and fibroblasts, and are particularly abundant in endothelial cells\(^70\).

Caveolae belong to a subset of plasma membrane microdomains, known as lipid rafts, which are also highly enriched in cholesterol and sphingolipids. The lipid composition of these domains facilitates tight packing through interactions with their fatty acyl chains, giving rise to a highly liquid-ordered phase as compared to the surrounding plasma membrane, which is mainly composed of phospholipids\(^71\). The characteristic properties of lipid rafts and caveolae are a) resistance to solubilization by detergents at 4\(^\circ\)C and b) low density due to their high lipid to protein ratio\(^71\). Based on these properties, detergent and detergent-free methods involving sucrose gradients have been developed to isolate these domains and the proteins associated with them from bulk plasma membrane proteins. A distinguishable feature of caveolae, which allows its separation from non-caveolae lipid rafts, is the presence of the structural proteins, known as caveolins\(^72\).

1.11. Caveolins are structural proteins of caveolae

The caveolae coat proteins, caveolins, which consists of three isoforms, namely Cav-1, Cav-2 and Cav-3, govern the structural integrity of caveolae. The three isoforms differ in their expression pattern in different cell types. Cav-1 and Cav-2 are abundant in non-muscle caveolae-rich cells, such as endothelial cells, whereas Cav-3 expression is limited to vascular
smooth muscle cells, cardiomyocytes and skeletal muscle cells \textsuperscript{73-75}. In mice, genetic ablation of any of the caveolin genes is not lethal, suggesting the presence of compensatory mechanisms for these caveola\textsuperscript{e} organelles. However, specific knockout of Cav-1 and Cav-3, but not Cav-2, results in the complete loss of caveola\textsuperscript{e}, stressing the importance of these proteins in the caveola\textsuperscript{e} organelle assembly\textsuperscript{76}. Moreover, expression of Cav-1 in cells that lack caveolins gives rise to the formation of invaginated caveola\textsuperscript{e}\textsuperscript{77}. Cav-2 has been found to be a positive regulator of Cav-1 during organelle biogenesis\textsuperscript{78}. Since the predominant isoform of endothelial cell caveola\textsuperscript{e} is Cav-1, our discussion will be limited to its role in caveola\textsuperscript{e} organelle biogenesis and contribution to the physiological functions of caveola\textsuperscript{e}.

\textbf{1.12. Structure of Cav-1}

Cav-1 is a small 22kDa membrane-associated protein, which forms a hairpin loop through the membrane, consisting of two cytoplasmic N- and C-terminal domains and a membrane-spanning domain\textsuperscript{79}. Membrane attachment of Cav-1 is attributed to the Cav-1 scaffolding domain (CSD), which is part of the N-terminal and comprises of amino acids 82-101 (Illustration 3). The role of Cav-1 CSD in membrane attachment is substantiated by the use of truncation mutants of Cav-1 without the CSD, which were found to be soluble\textsuperscript{80}.

In addition to membrane attachment, there are two unique properties of Cav-1, which are believed to mediate the process of caveola\textsuperscript{e} organelle assembly. Firstly, Cav-1 is a cholesterol binding protein and palmitoylation at residues 143 and 156 of the C-terminal domain is required for cholesterol binding\textsuperscript{81, 82}. This property is assumed to be important in caveola\textsuperscript{e} organelle synthesis because cholesterol-depleting drugs, such as methyl-\textbeta-cyclodextrin, disassemble caveola\textsuperscript{e} organelles\textsuperscript{72}. Secondly, Cav-1 has the ability to auto-assemble into high
molecular weight (HMW) oligomeric complexes ranging from 200-400kDa, containing about 14-16 Cav-1 monomers. Following synthesis in the endoplasmic reticulum, Cav-1 monomers undergo homo-oligomerization, which is mediated by the Cav-1 CSD\textsuperscript{80, 83} and hetero-oligomerization with Cav-2\textsuperscript{84}. A second round of post-translational oligomerization between the C-terminal domains occur while trafficking to the plasma membrane\textsuperscript{85}. This oligomerization process is believed to be responsible for driving organelle biogenesis and maintaining the “flask-shape” invagination of caveolae\textsuperscript{72}.

Illustration 3. Main structural features of caveolae and Cav-1

Caveolae are “flask shaped” invaginations of the plasma membrane comprising of the structural protein Cav-1. Cav-1 (22kDa) consists of an N- and C-terminal facing the cytoplasm and a trans-membrane domain embedded in the membrane bilayer. The Cav-1 CSD is responsible for binding cholesterol binding, membrane attachment and oligomerisation, all of which contribute to caveolae biogenesis.
1.13. Physiological roles of caveolae and caveolins

Caveolae and caveolins have been implicated in various cellular functions, including cholesterol transport and homeostasis, calcium signaling, intracellular trafficking of molecules, endocytosis and transcytosis\textsuperscript{81}. However, the heavy involvement of caveolae and Cav-1 in signal transduction has been a topic of intense current investigation. Fractionation studies have revealed that caveolae are rich in signal transduction molecules, thereby concentrating various receptors, proteins and their co-factors and substrates, implicating these organelles in cell signaling and protein-protein interaction events through spatial regulation\textsuperscript{86}. These signaling molecules are primarily regulated by Cav-1, in particular by interacting with the Cav-1 CSD. Some examples of signaling molecules localized in caveolae are G-protein coupled receptors, G proteins, tyrosine kinase receptors, ERK pathway signaling molecules and fatty acylated proteins such as eNOS and Src family of kinases\textsuperscript{87}. Moreover, Cav-1 oligomers serve to concentrate and compartmentalize signaling molecules within the caveolae organelle\textsuperscript{83}.

1.14. Caveolae and caveolins in the endothelium

Caveolae are highly abundant in the vascular endothelium, comprising 95\% of cell surface vesicles and about 15\% of cell volume\textsuperscript{88}. The fundamental role that caveolae, and in particular Cav-1, play in vascular regulation is best described in Cav-1 knockout mice. Although viable, these mice exhibit various cardiopulmonary abnormalities, including loss of myocardial function, cardiac hypertrophy and atherosclerosis\textsuperscript{89-92}. Mechanistically, these abnormalities arise due to dysregulated NO synthesis, increased cellular proliferation and enhanced vascular permeability\textsuperscript{93}. The generation of Cav-1 knockout mice with the specific re-expression of Cav-1 in the endothelium rescued the cardiopulmonary defects observed in Cav-1 knockout mice\textsuperscript{93},
further substantiating the importance of endothelium-specific Cav-1 in cardiovascular regulation. With the understanding that Cav-1 is important in vascular regulation, extensive amount of research has shown that the predominant role of Cav-1 in the endothelium is to modulate eNOS-derived NO synthesis.

1.15. Cav-1 regulation of eNOS-derived NO

Considering eNOS is negatively regulated by Cav-1, it is not surprising that Cav-1 knockout mice have elevated eNOS activity and enhanced responses to acetylcholine-induced endothelium-dependent vasodilation, the opposite being true for mice that over-express Cav-1 in the endothelium, which exhibit impaired NO-mediated functions\(^\text{89, 94}\). On the contrary, despite being a protective mediator of vascular function, enhanced NO levels in Cav-1 knockout mice still result in adverse cardiovascular phenotypes. This is due to abnormal eNOS activity and dysregulated NO synthesis because treatment with a pharmacological eNOS inhibitor improves cardiac function\(^\text{95}\). Therefore, Cav-1 serves to maintain eNOS in a tonic inhibitory state, preventing hyperactivation of the enzyme. Furthermore, caveolae localization critically places eNOS in an environment rich in its substrates (L-arginine), co-factors (HSP90, CaM) and receptors (VEGF-receptor).

1.16. Direct interaction of Cav-1 and eNOS

Binding assays performed with Glutathione-S-Transferase (GST)-linked Cav-1 deletion mutants, revealed that binding to and inhibition of eNOS was attributed to the putative Cav-1 CSD\(^2\). Indeed, intracellular delivery of a synthetic cell-permeable peptide (Antennapedia; AP) fused to the Cav-1 CSD, the peptide known as AP-Cav, was able to inhibit NO release in vitro, attenuate acetylcholine-induced vasodilation, behave as an anti-inflammatory and anti-
tumour molecule by reducing microvascular permeability in vivo, all of which in an eNOS-dependent manner\textsuperscript{96-98}, further confirming the biological target of the Cav-1 CSD is eNOS. A study by Bernatchez et al further mapped the inhibitory domain by testing truncated mutants of AP-Cav on eNOS-dependent biological activities\textsuperscript{3}. It was first observed that the eNOS inhibitory domain was located in the hydrophobic amino acid sequence 89-95 of the Cav-1 CSD. Subsequently, mutation of each amino acid in this hydrophobic region to an alanine, a technique known as alanine scanning, identified that threonine 90, 91 (T90, 91), and especially phenylalanine 92 (F92) are crucial for eNOS inhibition\textsuperscript{3}. Compelling in vitro data clearly shows that the mutant F92A Cav-1 failed to block NO release in vitro as compared to WT Cav-1 (Figure 1A)\textsuperscript{3}. In addition, we have collected preliminary data showing that expression of F92A Cav-1 at high levels can unexpectedly increase basal NO release (Figure 1B).

These data have advanced our knowledge on the amino acids involved in Cav-1 mediated eNOS inhibition, however, the mechanisms behind them are less understood and deserve further investigation. Moreover, understanding the mechanisms by which F92A Cav-1 mediates its positive effect on basal NO release could provide us with the necessary tools to modify the interaction between Cav-1 and eNOS specifically in settings of endothelial dysfunction and cardiovascular diseases, where NO bioavailability is comprised.
Figure 1. Mutant F92A Cav-1 does not block eNOS-derived NO release

(A) Human embryonic kidney (HEK) cells were transfected with βgal (lane 1), eNOS (lane 2), eNOS and WT Cav-1 (lane 3) and eNOS and F92A Cav-1 (lane 4) and NO release was quantified using a chemiluminescence NO-specific analyzer. WT Cav-1 inhibited NO release, while F92A Cav-1 restored NO release to control levels. (Adapted from Bernatchez et al, PNAS, 2005)

(B) Preliminary data in endothelial cells, demonstrating increased NO release upon high expression of F92A Cav-1. Endothelial cells were infected with low (MOI=5) and high (MOI=20) doses of WT and F92A Cav-1 adenoviruses and NO release was quantified. At high levels of expression, F92A Cav-1 was able to increase NO release substantially, whereas WT Cav-1 had no effect on NO release.
Chapter 2. Research Overview
2.1. Rationale

Cardiovascular diseases are one of the leading causes of morbidity and mortality worldwide and research has shown that a common factor in most cardiovascular diseases arises due to an impaired endothelium, a condition known as endothelial dysfunction\textsuperscript{37}. This phenomenon is well characterized by the reduction in the bioavailability of a protective mediator of endothelial cell activity, called NO. Another feature of endothelial dysfunction is the up-regulation of endothelial cell oxidative stress, mostly mediated by $O_2^-$. Moreover, scavenging of $O_2^-$ by NO further contributes to the reduction in NO bioavailability\textsuperscript{32}. eNOS is the enzyme capable of synthesizing NO and $O_2^-$ in physiological and pathological settings, respectively\textsuperscript{29, 30}. Therefore, therapies that up-regulate eNOS-derived NO release and down-regulate $O_2^-$ release/oxidative stress in settings of endothelial dysfunction are therapeutically relevant and clearly warrant further investigation.

Cav-1, the major coat protein of caveolae organelles (small invaginations at the plasma membrane), is one of the few proteins known to bind to eNOS and inhibit eNOS-derived NO release in resting endothelial cells. Cav-1 binding to and inhibition of eNOS is attributed to the Cav-1 CSD (amino acids 82-101)\textsuperscript{2}. Previously, Dr Bernatchez and his colleagues have shown that mutation of the phenylalanine 92 residue of the Cav-1 CSD to alanine, the mutant known as F92A Cav-1 loses the ability to inhibit eNOS\textsuperscript{3}. Furthermore, preliminary data suggests that expression of high levels of F92A Cav-1 in various eNOS-expressing cells can in fact increase NO release. A possible explanation would be that F92A Cav-1 prevents eNOS binding to and inhibition by endogenous Cav-1 leading to increased eNOS activity. However, Cav-1 is known to mediate many other biological activities in endothelial cells, it is therefore possible that F92A Cav-1’s effect on NO release might be attributable to a change in the basic biochemical
properties of Cav-1. For instance, Cav-1 (22kDa) targets to caveolae, where it auto-assembles to form HMW oligomers (>250kDa), which are essential for caveolae organelle biogenesis and caveolae-mediated signal transduction events. In addition, Cav-1 behaves as a scaffolding protein, concentrating and compartmentalizing various signaling molecules. Hence, the current work aims to investigate the possible mechanisms by which F92A Cav-1 exerts a positive effect on NO release.

2.2. Hypothesis

Having the knowledge that F92A Cav-1 cannot inhibit eNOS activity, we hypothesize that the F92A mutation does not interfere with the other biological activities of Cav-1.

2.3. Specific aims

Aim 1: Determine if the capacity to target to caveolae and auto-assemble to form HMW oligomers is retained in F92A Cav-1.

Aim 2: Compare the ability of WT and F92A Cav-1 to bind eNOS.

Aim 3: Determine if F92A Cav-1 preserves eNOS subcellular targeting and co-localization with Cav-1.

Aim 4: Investigate if F92A Cav-1-induced increase in NO release might be a result of decreased O$_2^-$ production from eNOS as compared to WT Cav-1.
Chapter 3. Methods And Materials
3.1. **Cell culture**

For in vitro experiments, Bovine Aortic Endothelial Cells (BAECs) were isolated from bovine aortas, characterized by their cobblestone morphology and eNOS expression and cultured for 5 to 15 passages. COS cells and Human Embryonic Kidney Cells (HEK; ATCC Inc) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen), supplemented with 5-10% Fetal Bovine Serum (FBS; Hyclone) and 1X penicillin/streptomycin (Sigma Chemicals). Cells were placed in a humidified incubator at 37°C with 7% CO₂ to ensure optimal growth conditions.

3.2. **Cell transfection and infection**

In order to over-express WT and F92A Cav-1 *in vitro*, cells were either transfected with hemagglutinin (HA)-tagged versions of human WT and F92A Cav-1 cDNA plasmids or infected with adenoviruses encoding for WT Cav-1 (myc tag) and F92A Cav-1 (HA-tag). The adenoviruses coding for WT and F92A Cav-1 are referred to as AdWT Cav-1 and AdF92A Cav-1, respectively. 48 hours after transfection or infection, cells were utilized or lysed for experiments with the appropriate buffer.

3.2.1. **Cell transfection**

COS cells were seeded in a 100mm² cell culture dish (BD Biosciences) a day prior to cell transfection. Before transfection, cells should have reached a 70-80% confluency to ensure high transfection efficiency. Optimem (Gibco, Invitrogen) was used as the transfection media and Lipofectamine 2000 (Invitrogen) was the lipid-based vehicle reagent⁹⁹. For transfection, WT and F92A Cav-1 plasmids (1ug of DNA concentration each) were added to optimem and
allowed to mix for 5 minutes at room temperature. At the same time Lipofectamine 2000 was also added to optimem and allowed to mix for 5 minutes. Following which, the optimem + DNA solution and the optimem + Lipofectamine 2000 solution were allowed to mix for an additional 30 minutes at room temperature. The transfection media was then added to cells and allowed to incubate for 6 hours in a humidified incubator at 37°C with 7% CO₂. After the 6-hour treatment, the transfection media was replaced with DMEM containing 5% FBS and the cells were allowed to grow for an additional 48 hours to ensure high expression of the plasmids.

3.2.2. Cell infection

BAECs and HEK cells were seeded in 100mm² cell culture dishes (for fractionation experiments) and 24 well plate (for superoxide measurement assays). BAEC and HEK cells were grown to 80 and 100% confluency, respectively. At the time of infection, media was removed from the dish and replaced with fresh DMEM containing 5% FBS and 1X penicillin/streptomycin. AdWT and AdF92A Cav-1 were then added to the plates to a low magnitude of infection (MOI) of 5 and a high MOI of 20 depending on the assay being performed. MOI was calculated as follows:

\[
\text{MOI} = \frac{\text{volume added (ml)} \times \text{Plaque-forming Unit (PFU)/ml}}{\text{Number of cells/ml}}
\]

The method used in order to calculate PFU, which is the amount of viral particles per milliliter (ml), will be discussed in Section 3.2.4.

3.2.3. Amplification of AdWT Cav-1 and AdF92A Cav-1

Virus amplification was performed with HEK cells using a protocol adapted from the University of Iowa Virus Amplification Facility. HEK cells were grown to confluency in 15
150mm$^2$ cell culture dishes. Once confluent, the cells were infected with viral stock and were collected when they reached 50% viability. Cells were then harvested by centrifugation and the virus was released through 3 freeze-thaw cycles, with 30-second vortexing between the cycles. Lysates were then spun down again at 4000rpm for 10 minutes at 4°C to remove cell membranes and the adenovirus was purified by centrifugation using two sequential cesium chloride gradients. The supernatant, containing the adenovirus, was laid on top of a cesium chloride gradient in an ultracentrifuge tube as followed:

- 0.66mL of 1.5g/mL Tris pH 7.9
- 4mL of 1.35g/mL Tris pH 7.9
- 4mL of 1.25g/mL Tris pH 7.9

The first spin at 28,000rpm at 12°C was performed for 6 hours, after which the cloudy floating band formed was removed and put on top of a second gradient for an overnight spin. The band was isolated and subsequently desalted and stabilized by dialysis against 10mM Tris HCl at pH 7.9 at room temperature for 45 minutes, 10mM Tris HCl at pH 7.9 at 4°C for 1 hour, and finally against 10mM Tris HCl pH 7.9 and 1mM MgCl$_2$ at 4°C for 45 minutes.

### 3.2.4. Cytopathic effect assay (CPE assay) to determine adenovirus virulence

The cytopathic effect (CPE) assay was performed to determine the titre of adenovirus in order to maintain a comparable number of viral particles throughout experiments. Briefly, on day 1, 50µl of DMEM supplemented with 10% FBS is added to each well of a sterile 96-well plate (BD Biosciences). The virus was then diluted 1:10,000 and added to the first well and further diluted 1:2 across 23 wells. The experiment was done in triplicate (24 wells/set) with an uninfected well in each set to serve as a negative control to ensure accuracy in determining cell lysis. Following which, a 50µl of HEK cells suspension (1 X 10$^6$ cells/ml) was added to each
well. The cells were then monitored once daily under a light microscope for the next 9 days and are fed with DMEM supplemented with 10% FBS on day 3, 6 and 9. Finally on day 10, cell toxicity is examined and the numbers of wells with lysed cells were counted. The viral titre can then be calculated using Equation 1.

\[
\text{Titre} = 3^n x 40 x 10^5 \text{ PFU/ml}
\]

where \( n \) is the number of wells showing cell death.

### 3.3. Western blot analysis

Proteins were separated by SDS/Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was performed as per normal. The primary antibodies used were rabbit polyclonal anti-Cav-1 (Santa Cruz Biotechnologies), rabbit polyclonal anti-GST (Santa Cruz Biotechnologies), mouse monoclonal anti-eNOS (Zymed, Invitrogen), mouse monoclonal anti-myc (Santa Cruz Biotechnologies), rat monoclonal anti-HA (Roche Applied Science) and rabbit polyclonal β-Cop (ABR). The secondary antibodies used were goat anti-mouse 788 (Invitrogen), goat anti-rabbit 698 (Invitrogen) and goat anti-rat 788 (Invitrogen). The ODYSSEY Infrared Imaging System (Licor) was used for fluorescence detection of proteins and quantification of protein intensity by computer-assisted linear densitometry.

### 3.4. Isolation of caveolae/lipid rafts by sucrose gradient fractionation

In order to determine if F92A Cav-1 was able to target to and localize in caveolae, sucrose gradient fractionation was performed in order to isolate caveolae/lipid rafts from cytosolic proteins. Infected BAECs and transfected COS cells (two 100mm\(^2\) cell culture dishes each) were washed twice with ice-cold Phosphate-Buffered Saline (PBS) and lysed in a non-
detergent buffer comprising of 500mM sodium carbonate (Na₂CO₃; pH 11)/ 3.2mg/ml protease inhibitors (Roche Applied Science)/ 2mM phenylmethanesulfonylfluoride (PMSF; Fluka Chemicals). Cellular aggregates were dissociated by sonication (three 10-second bursts) and the solubilized lysate was then adjusted to 45% sucrose by adding 2ml of 90% sucrose prepared in Mes buffered saline (MBS: 25 mM Mes, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. The solution was mixed well by pipetting up and down and incubated on ice for 2 hours. A 5–35% discontinuous sucrose gradient was then formed above (6ml of 35% sucrose/2 ml of 5% sucrose; both prepared in MBS containing 500mM Na₂CO₃) and subjected to centrifugation at 28,000 rpms for 16–20 h at 4°C in an SW28 rotor (Beckman Instruments). A light-scattering whitish band confined to the 5–35% sucrose interface was observed that contained caveolin-associated proteins but excluded most other cellular proteins. Twelve equal 1ml fractions were taken out and Western blot analysis was carried out. Cav-1 was used as a marker for caveolae/lipid rafts while HSP90 was used as a marker for bulk cytosolic proteins.

3.5. Determination of protein molecular weight by velocity gradient centrifugation.

As mentioned in the introduction, endogenously Cav-1 is present in cells as a HMW oligomer. Therefore in order to determine the native molecular weight of F92A Cav-1, velocity gradient centrifugation was performed, a technique that separates proteins according to molecular weight. Infected BAEC and HEK Cells (two confluent 100mm² dishes) were washed with ice-cold PBS and lysed with 500ul of MBS containing 60mM β-octyl glucopyranoside/1.6mg/ml protease inhibitors/ 1mM PMSF. Cellular aggregates were dissociated by sonication (three 10-s bursts) and the solubilised lysate was then loaded on top of a 5-45% linear sucrose
gradient prepared in MBS/60mM β-octyl glucopyranoside (12ml) and subjected to ultracentrifugation at 28,000rpm for 16-20 hours at 4°C. Following centrifugation, thirteen 1ml fractions were collected from the gradient and Western blot analysis performed. In order to calibrate the molecular weight of the proteins from each fraction, velocity gradient centrifugation was also carried out on 5 established molecular weight standards; thyroglobullin (670kD), appoferritin (480kD), catalase (250kD), bovine serum albumin (BSA; 66kD) and carbonic anhydrase (29kD), all purchased from Sigma Aldrich.

3.6. Glutathione-S-Transferase (GST) pull-down experiments

The effect of the F92A mutation on Cav-1’s ability to bind to eNOS was assessed through GST pull-down experiments. GST and GST Cav (comprising Cav-1 amino acids segment 61-101) plasmids were provided by Dr. Michael Lisanti's laboratory. GST F92A Cav was designed and generated as follows; firstly, the F92A Cav cDNA was synthesized and amplified by Polymerase Chain Reaction (PCR), following which, the F92A Cav cDNA was inserted into the GST vector (pGEX-4T-3, GE Healthcare), resulting in a recombinant plasmid that can be transformed in bacterial cells for protein purification.

3.6.1. F92A Cav-1 cDNA synthesis by PCR

In order to generate the F92A Cav-1 cDNA, PCR was performed using the GST Cav cDNA as a template. Forward and Reverse primers which included the F92A mutation were designed (Table 1) and synthesized (Sigma Genosys).
Table 1: Forward and reverse primer sequences for F92A Cav PCR reaction.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Melting Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST Cav Forward</td>
<td>ATTATTGGATCCGATGACGTGGTCAAGATTG</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>ACTTTGAAG</td>
<td></td>
</tr>
<tr>
<td>GST F92A Cav Reverse</td>
<td>CACGATGCGGCCGCTCGAGTCGACCCCGGAA  TTCTTAGCGGTAAAAACCAGTATTGGTCACA  GTGCGGGGTGAAGCTGGCC</td>
<td>80°C</td>
</tr>
</tbody>
</table>

A 25µl reaction volume consisting of 0.25µl of GST Cav cDNA, 0.25µl of Forward Primer (5pmol), 0.25µl of Reverse Primer (5pmol), 0.25µl dNTP (10mM), 2.5µl 10X Taq buffer, 0.5µl Taq DNA polymerase (250 units), 2µl MgCl₂ (25mM) and 19µl water were combined together in a 0.5ml PCR tube, mixed well and placed in a thermocycler (BioRad) to allow for amplification of the target DNA (Table 2).

Table 2: Temperature and duration cycles for PCR. *indicates repeated for 35 cycles

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>1.5</td>
</tr>
<tr>
<td>2*</td>
<td>94</td>
<td>0.5</td>
</tr>
<tr>
<td>3*</td>
<td>56</td>
<td>0.5</td>
</tr>
<tr>
<td>4*</td>
<td>72</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>Forever</td>
</tr>
</tbody>
</table>
The contents of the PCR reaction were then run on a 1.5% agarose gel containing GelRed™ (Invitrogen) in order to visualize the PCR products. A single band was observed at approximately 180 base pairs (bp) and the band was excised from the gel and purified using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

3.6.2. Cloning of F92A Cav-1 PCR product and transformation into competent cells

Once the PCR product has been excised from the gel and purified, it is cloned into an expression vector. For this purpose, we used the TOPO® Cloning Kit (Invitrogen), which is specific for Taq-amplified DNA products. Briefly, TOPO cloning employs the use of a dual purpose enzyme, DNA topoisomerase I, which functions both as a restriction enzyme and ligase, allowing cleavage of the DNA product at the 5’-(C/T)CCTT-3′ site and re-ligation by forming a covalent bond with the phosphate group of the 3’-thymidine. To clone, 2.5µl of PCR product was combined with 1µl salt solution, 1µl of TOPO vector and 5.5ul of water and was allowed to incubate for 5 minutes at room temperature. Once the ligation reaction was complete, the recombined vector can be transformed into *Escherichia coli* (*E. coli*).

For transformation, the recombinant vector was heat shocked with *E.coli* JM109 CaCl₂ competent cells. Briefly, an aliquot of JM109 cells was thawed on ice, and separated into 100 µL portions in pre-chilled Eppendorf tubes (Fisher) to which the recombined TOPO vector with the F92A Cav-1 insert was added, blended by swirling gently before incubating on ice for 30 minutes. The tubes were then heat shocked in a 42°C water bath for 45 seconds to allow maximal transformation efficiency to occur and subsequently chilled on ice for 2 minutes. The transformed cells were then plated onto Luria Broth (LB) agar plates supplemented with ampicillin (100 µg/ml) and incubated overnight at 37°C. The transformed cells that harbour the
recombinant vector with the ampicillin resistant gene were able to grow and produce separate colonies.

3.6.3. Overnight culturing and isolation of DNA plasmids from bacterial plasmids

Seven colonies from the plate were picked out and added to individual 14 ml Falcon tubes containing 5ml of LB media supplemented with 100µg/ml ampicillin. The bacterial plasmids were allowed to propagate overnight at 37°C in a shaking incubator (250rpm). The DNA plasmids were isolated from the cells using the Promega Wizard® Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions.

3.6.4. Digestion and linearization of DNA using restriction enzymes

The recombinant DNA plasmid was digested with Bam HI and Not I restriction enzymes (both from Invitrogen), in order to extract the F92A Cav insert. Following the overnight digestion, the reaction products were run on a 1.5% agarose gel in order to separate the F92A Cav insert from the TOPO vector. The F92A Cav insert was excised from the gel and purified using the Qiaquick gel extraction kit as per manufacturer’s instructions.

3.6.5. Ligation and transformation of F92A Cav-1 with GST vector

Using a 1:2 vector:insert ratio, a 20 µL reaction containing the GST (pGEX-4T-3) vector and F92A Cav insert with T4 DNA ligase enzyme (Invitrogen) were combined in a 1.5ml Eppendorf tube and allowed to ligate for 1 hour at room temperature. Following which, transformation into E.Coli JM109 competent cells and isolation of GST F92A Cav plasmid was performed as described above in Section 3.6.2 and 3.6.3. An aliquot of the GST F92A Cav plasmid was stored at -20°C for the maintenance of the plasmid.
3.6.6. Sequencing, re-transformation and re-culturing of GST plasmids

Once the GST F92A Cav plasmids were isolated, it was submitted to the University of British Columbia’s Nucleic Acid Protein Service (NAPS) Unit for sequencing. The most accurate sequence of GST F92A Cav was then chosen. Next, the GST, GST Cav and GST F92A Cav plasmids were re-transformed into *E.Coli* BL21 (Amersham Biosciences) CaCl₂ competent cells. The BL21 bacterial cell line is highly recommended for expression of GST fusion proteins. Briefly, an aliquot of BL21 cells was thawed on ice, and separated into three 100µL portions in pre-chilled Eppendorf tubes, followed by the addition of 3µL of the GST fused plasmids to each tube separately. The transformation, heat-shock and plating of GST fused plasmids were performed at described in Section 3.6.2. Following which, colonies were picked from each plate expressing GST, GST Cav and GST F92A Cav and re-cultured in LB media overnight. An aliquot of the bacterial culture was removed and stored in a 50% glycerol solution at -80°C and the remaining overnight culture of *E.Coli* BL21 GST fused plasmids were utilized for protein purification.

3.6.7. Purification of GST and GST-fused proteins

The overnight culture (approximately 4.5ml) of *E.Coli* BL21 containing plasmids encoding GST, GST Cav or GST F92A Cav was added to 500ml of LB media supplemented with 100µg/ml ampicillin. The resulting culture was then grown in a 37°C incubator, constantly shaking for 1-2 hours until an Absorbance 600 (A₆₀₀) of 0.3 is reached. Expression of fusion proteins was then induced with the addition of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) and incubated for an additional hour at 34.5°C with constant shaking at 220rpm. Following which, the bacteria were harvested by centrifugation at 7,000 x g for 15 minutes at
4°C. The bacterial pellet corresponding to 250ml of bacterial culture was then resuspended in 10ml of STE (pH8; 50mM Tris-Cl, 150mM NaCl, 5mM EDTA pH8) buffer on ice. The solution was again subjected to centrifugation at 3,000 x g for 10 minutes at 4°C. The pellet was again resuspended in 10ml of STE buffer, followed by the addition of 1mM PMSF and Lysozyme (100µg/ml; Sigma). After incubation for 10 minutes on ice, 5mM of Dithiothreitol (DTT; Sigma) and 1.5% of N-laurylsarcosyl (Sigma) was added and the cells were homogenized for 2 minutes on ice using a Dounce Homogenizer. These steps facilitate the solubilisation of GST fused proteins prior to the removal of E.Coli cell wall and outer membrane components, which was achieved through the addition of Triton X-100 (Sigma) to a final concentration of 2% followed by high speed centrifugation (13,000 x g) for 30 minutes at 4°C. The supernatant was collected and the pellet was saved separately. During each step of the purification process, a small aliquot was kept for Western blot analysis.

3.6.8. Binding of purified GST fused proteins to glutathione beads

The purified GST fused protein supernatant (1.5ml) was added to 200µl of 50% slurry containing Immobilized Glutathione beads (ThermoScientific). After 1 hour of rotating at 4°C, the beads were pelleted by centrifugation (1,200 x g) for 5 minutes and washed extensively (three-5 minute intervals) with STE buffer containing 1% Triton X-100. A small aliquot of GST, GST Cav and GST F92A Cav beads were separated by SDS/Page followed by Coomassie staining for protein detection and quantification.

3.6.9. Binding experiments with BAEC lysates and recombinant eNOS

To determine binding of GST Cav and GST F92A Cav to eNOS, a GST pulldown
assay using BAEC lysates, which are an excellent source of eNOS, and pure recombinant eNOS, was performed. BAECs were grown to confluency in a 100mm² cell culture dish with DMEM supplemented with 5% FBS (1 confluent dish per reaction). Once confluent, cells were lysed in a STET buffer (pH 8; 50mM Tris-Cl, 150mM NaCl, 1mM EDTA pH 8, 1% Triton X-100) containing 1.6mg/ml protease inhibitors and 1mM PMSF on ice. Cellular aggregates were dissociated by Dounce homogenization (20 strokes) and rotated for 1hr at 4°C. Following which, cellular membranes were removed by centrifugation at 13,000 x g for 10 minutes at 4°C. The supernatant was then removed, sonicated (three 10-s bursts) and distributed equally (500ul) into 3 Eppendorf tubes consisting of beads containing GST alone, GST-Cav or GST F92A-Cav (~80ul packed volume with equal protein concentration). As GST is usually more highly concentrated than GST Cav and GST F92A Cav, the beads volume for GST is compensated by the addition of blank glutathione beads. The reaction was then allowed to incubate overnight at 4°C, rotating end-over-end. After binding, the beads were washed extensively (three 5-minute intervals) at 4°C with wash buffer containing 50mM Tris-Cl (pH 7.4), 125mM NaCl, 1mM EDTA and 1mM EGTA.

As mentioned earlier in the introduction, eNOS is a lipid-modified enzyme; therefore to ensure that binding to Cav is occurring independently of these modifications, in vitro interactions of GST Cav and GST F92A Cav were examined with recombinant eNOS (Cayman Chemicals). This recombinant eNOS is isolated from a Baculovirus overexpression system. A reaction mixture (500ul per reaction) of recombinant eNOS (0.2mg/ml) diluted in the binding buffer (50mM Tris-Cl, pH 7.4/ 20% glycerol) was added to 3 Eppendorf tubes consisting of beads containing GST alone, GST-Cav or GST F92A-Cav (~80ul packed volume with equal protein concentration). The reaction mixture was then incubated for 2 hrs at 4°C, rotating end-
over-end. After binding beads were washed extensively (five 5-minute intervals) with a high salt wash buffer (50mM Tris-Cl, pH 7.7, 400mM NaCl and 1mM EDTA).

Proteins that bound to GST and GST fused Cav Beads were eluted by boiling in 1X SDS sample buffer and separated by SDS/PAGE. Western Blot analysis was then performed with anti-GST (Sigma) and anti-eNOS (Zymed, Invitrogen) antibodies to detect and quantify eNOS binding.

**3.7. Co-localization of Cav-1 proteins with eNOS by immunocytofluorescence**

Immunofluorescence staining was performed on BAECs infected AdWT and AdF92A Cav-1. Coverslips were placed into each well of a 12-well plate and coated with gelatin. BAECs were then seeded on the gelatin-coated coverslips and infected (MOI of 10) as described in Section 3.2.2. 48 hours post infection, cells were washed twice with PBS and fixed with 3% Paraformaldehyde (400µl per well) for 10 minutes at room temperature. The cells were then washed thoroughly twice with PBS and permeabilized in 0.1% Triton X-100 (400µl per well). This permeabilization step is necessary to allow antibodies to permeate the cell and recognize their antigen. After washing extensively three times with PBS, non-specific binding was then eliminated by incubating the cells with blocking solution, consisting of 0.1% BSA (Sigma-Aldrich) and 10% Normal Goat Serum (NGS; Invitrogen) for 2 hours at room temperature. Following which, the blocking buffer is removed and primary antibodies (1:100 dilutions in the blocking solution) and equal concentration non-immune IgG controls were added and allowed to incubate overnight at 4°C. For cells that were infected with WT Cav-1, mouse monoclonal anti-eNOS and rabbit monoclonal anti-myc (Cell Signalling Technology) were used as primary antibodies, while normal mouse and rabbit IgG (Santa Cruz
Biotechnologies) served as non-immune controls. On the other hand, for cells infected with F92A Cav-1, anti-eNOS and rat polyclonal anti-HA were used as primary antibodies, while mouse and rat IgG (Serotec) served as non-immune controls. An additional set was also incubated with blocking solution overnight to serve as a secondary antibody control. Following overnight incubation with primary antibodies and IgG controls, cells were washed three times with PBS and incubated with secondary antibodies (1:2500 dilutions in blocking solution) for an additional hour at room temperature. WT Cav-1 infected BAECs were incubated with anti-mouse Alexa Fluor 488 (Invitrogen) and anti-rabbit Alexa Fluor 568 (Invitrogen), while F92A Cav-1 infected BAECs were incubated with anti-mouse Alexa Fluor 488 and anti-rat Cy5 (Jackson ImmunoResearch Laboratories). After washing three times with PBS, the coverslips were then mounted on glass slides (Fisherbrand) with Gelvatol/DAPI (Sigma), which is a fluorescent dye for labeling cellular nuclei. The images were then captured with Leica Inverted Fluorescence microscope with Confocal Scanner and co-localization of the Cav-1 with eNOS were analyzed by the Volocity® software (PerkinElmer). Images were quantified using the Manders colocalization coefficients\textsuperscript{100}, which basically accounts for the ratio of colocalized pixels (with eNOS) to the total pixel intensity for either WT or F92A Cav-1.

### 3.8. Superoxide anion release quantification

In order to detect and quantify WT and F92A Cav-1’s effect on $O_2^-$ production in unstimulated BAECs, the Cytochrome C Reduction Assay adapted from Dikalov et al\textsuperscript{101} (with modifications) was performed. Ferricytochrome c receives an electron from $O_2^-$ and is reduced to ferrocytochrome c, which results in increased absorbance specifically at 550nm whereas absorbance at 540nm and 560nm are unaltered, allowing us to quantify the difference and
estimate \( \text{O}_2^- \) production (Figure 4). However, ferricytochrome c can be reduced by other electron donating molecules and ferrocytochrome c can be reoxidized by oxidants such as \( \text{H}_2\text{O}_2 \). For this reason, the reaction mixture has to include catalase (to catalyze the decomposition of \( \text{H}_2\text{O}_2 \)) and has to carried out in the presence and absence of superoxide dismutase (SOD; an inhibitor of \( \text{O}_2^- \)) in order to accurately quantify \( \text{O}_2^- \)-induced signal (Figure 2). Another important point to note is that the assay is light-sensitive, therefore preparation of solutions and incubations with cells were carried out in the dark.

BAECs were seeded in two 24-well plates (BD Biosciences) and infected (MOI of 5 and 20) as described in Section 3.2.2. An additional set of cells were also infected with adenovirus encoding for \( \beta \)-galactosidase (Ad\( \beta \)-gal), which serves as a control virus. 48 hours after infection, cells were washed twice with PBS and incubated with a PBS (containing \( \text{Ca}^{2+}/\text{Mg}^{2+} \))-based buffer (150\( \mu \)l per well) containing 50\( \mu \)M 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 well/tube) and placed on ice. Immediately, the cells were incubated for an additional 30 minutes at 37°C with a PBS (containing \( \text{Ca}^{2+}/\text{Mg}^{2+} \))-based buffer (150\( \mu \)l per well) containing 50\( \mu \)M 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 reducing molecule present in the cellular system. The post-incubation supernatant containing SOD was then collected from cells and the measurement process is repeated. The second set of OD values
would represent reduction of ferricytochrome c by any reducing molecule other than $\text{O}_2^-$. Therefore, to calculate the amount of $\text{O}_2^-$ produced from each well, Equations 2 and 3 were used:

*Equation 2.*

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

*Equation 3.*

$$\text{O}_2^- = (\Delta \text{OD } 550\text{nm without SOD} - \Delta \text{OD } 550\text{nm with SOD}) / 21.1 \text{mmol/L} \cdot \text{cm}^{-1}$$

where $\Delta$ represents change in absorbance at 550nm and 21.1 mmol/L $\cdot$ cm$^{-1}$ is the extinction coefficient for $\text{O}_2^-$. In this regard the value for $\text{O}_2^-$ is in mmol/L.

To validate the specificity of the assay, we treated BAECs for 3 hours with either duroquinone$^{102}$, an exogenous superoxide donor, or 4-Hydroxy-2-nonenal (4HNE), an eNOS uncoupling agent$^{103}$, which served as positive controls.
Figure 2. Detection of $O_2^-$ using the Cytochrome C Reduction Assay

3.9. Statistical analysis

Data are represented as mean ± standard error of the mean (S.E.M.). Statistical comparisons were done using GraphPad PRISM® Version 4.0 (GraphPad Software). Non-Parametric t-tests were performed and data was considered significant if $P<0.05$. Uncoupled eNOS can produce $O_2^-$, which can cause the reduction of ferricytochrome c to ferrocytochrome c and this reaction is inhibited by SOD. Any other enzyme can directly reduce ferricytochrome c and is resistant to SOD. Therefore SOD is added to the reaction to differentiate between the two pathways. Ferrocytochrome c alters absorbance at 550nm in a specific manner, but does not affect absorbance at 540nm and 560nm. To prevent ferrocytochrome c to be reoxidized to ferricytochrome c, catalase is added to the reaction.
Chapter 4. Results
4.1. WT and F92A Cav-1 target similarly to caveolae/lipid rafts

The ability of F92A Cav-1 to target to and localize in caveolae was assessed through sucrose gradient fractionation, a technique that separates cellular components based on their cholesterol content. Caveolae/Lipid Rafts and caveolae-associated proteins are rich in cholesterol, thereby rendering them less dense and are observed as a single floating band across the sucrose gradient\textsuperscript{72, 104}. In contrast, cytosolic proteins that are not high in cholesterol-rich environments are denser and present at the bottom of the gradient. Because caveolae/lipid rafts are resistant to detergent solubilization, conventionally this method involved extraction of cells using mild detergents. However, in our case we chose to employ a more recently developed technique, known as the detergent-free method for purifying caveolar membranes\textsuperscript{105}. This detergent-free method was preferred over the conventional method because it retained the morphological structure of the membrane and closely resembled the composition of the membrane to the time it was extracted from the cell, which included preserving the interactions of caveolae-resident proteins\textsuperscript{105}, such as eNOS.

In our experiments, the lysates from BAECs, infected with AdWT Cav-1 and AdF92A Cav-1, were subjected to sucrose gradient fractionation and protein content in each fraction was assessed through Western blot analysis. Western blots in Figure 3A show protein content in each of the twelve fractions. Cav-1 and HSP90 were used as controls, as Cav-1 is a marker for caveolae-enriched microdomains and detected in lighter fractions 2 to 5, whereas β-Cop is a bulk cytosolic protein marker distinctly present in fractions 9 to 12. The Western blots and quantification of protein intensity using densitometry (graph above) showed that both WT and F92A Cav-1 followed a similar separation pattern that was comparable to endogenous Cav-1. Furthermore, our experiments reveal that about 72% and 66% of WT and F92A Cav-1,
respectively was enriched in the caveolae-enriched microdomains (fractions 2 to 5), thereby suggesting that F92A Cav-1 is able to target to and localize in caveolae-enriched microdomains.

Endothelial cells have high endogenous Cav-1 levels; therefore a deficiency in the ability of the mutant Cav-1 to target to caveolae could potentially be compensated by endogenous Cav-1. In order to address this potential issue, we performed the experiment using a reconstituted cell system (COS cells), which have low endogenous Cav-1 levels. **Figure 3B** shows Western blots and quantification of protein content in each fraction. Similar to the results observed in BAECs, COS cells transfected with WT and F92A Cav-1 plasmids, demonstrate that about 63% and 55% of WT and F92A Cav-1 respectively, target to caveolae enriched microdomains. Collectively, these data confirm that F92A Cav-1 is indeed similar to WT Cav-1 with respects to targeting to caveolae.
Isolation of caveolae from BAECs infected with AdWT (myc tag) and AdF92A Cav-1 (HA tag) at an MOI of 25 by sucrose gradient fractionation. Fractions were collected from the top, separated by SDS/Page (12.5% acrylamide) and proteins were detected by anti Cav-1, anti-β-Cop, anti-myc and anti-HA primary antibodies. WT and F92A Cav-1 like endogenous Cav-1 targeted to low density caveolae fractions (2 to 5). β-Cop is a bulk cytosolic protein marker present in fractions 9 to 12 and excluded from low-density fractions. Quantification (graph above) of the amount of protein (black: Cav-1, red: WT Cav-1 and green: F92A Cav-1) in each fraction was determined by densitometry and expressed as total protein present.
Isolation of caveolae from COS cells transfected with HA-tagged versions of WT and F92A Cav-1 (1µg each) by sucrose gradient fractionation. Fractions were collected from the top, separated by SDS/Page (12.5% acrylamide) and detected by anti-HA primary antibodies. WT and F92A Cav-1 like endogenous Cav-1 targeted to low-density caveolae fractions (2 to 5) with a small amount in the cytosol. Quantification (graph above) of the amount of protein (red: WT Cav-1 and green: F92A Cav-1) in each fraction was determined by densitometry and expressed as total protein present.
4.2. *WT and F92A Cav-1 auto-assemble to form HMW oligomers (>250kDa)*

Another unique property of Cav-1, which is essential for caveolae organelle biogenesis, is the ability to oligomerize with itself, and to a lesser extent with Cav-2, to form HMW oligomeric complexes. The ability of F92A Cav-1 to form HMW oligomers was determined through velocity gradient centrifugation, a technique that uses a linear sucrose gradient to separate proteins according to their native molecular weight. Upon subjection to centrifugation, heavier proteins push through to the bottom of the sucrose gradient, encountering liquid of increasing density and viscosity, whilst light proteins that are unable to overcome liquid density and viscosity, remain in the lighter fractions on top.

First, we calibrated the size-based protein separation from the gradient using established molecular weight standards. For this purpose, purified carbonic anhydrase (29kDa), BSA (66kDa), catalase (250kDa), apoferritin (480kDa) and thyroglobullin (670kDa) were each subjected to velocity gradient centrifugation, analyzed by coomassie staining and quantified using densitometry. **Figure 4** represents protein analysis of the molecular weight standards, which exhibit a linear protein distribution with the maximum amount of Carbonic Anhydrase, BSA, Catalase, Apoferritin, Thyroglobullin at fractions 2, 3, 6, 7 and 8, respectively.

**Figure 5A** shows Western blots and protein quantification of BAEC lysates subjected to velocity gradient centrifugation. Our results reveal that endogenous Cav-1 migrates as HMW complex of about 250-480kDa with peak fractions at 6 and 7, further confirming that Cav-1 is present as a HMW oligomeric complex in vitro. Similarly, 62 and 60% of WT and F92A Cav-1 respectively, were also predominantly enriched in fractions 6 and 7. Interestingly, despite being a mere 22kDa protein, there were no Cav-1 monomers detected for endogenous, WT and F92A Cav-1. Again, since high levels of endogenous Cav-1 could compensate for any deficiency in
the oligomerization capability of the mutant, the experiment was repeated using a reconstituted cell system (HEK cells). Results from HEK cells complemented the data from BAECs, where majority of WT (66%) and F92A (61%) Cav-1 is predominantly enriched in fractions 5 to 7 (Figure 5B), further substantiating that the F92A mutation does not alter the oligomerization capabilities of Cav-1 significantly.

**Figure 4. Velocity gradient centrifugation determines native protein size**

![Protein Size](image)

Carbonic anhydrase (29kDa), BSA (66kDa), Catalase (250kDa), Apoferritin (470kDa) and Thyroglobulin (670kDa) were subjected to velocity gradient centrifugation to determine which fractions the proteins migrated at. Proteins were separated by SDS/Page (5-12.5% acrylamide) and stained by Coomassie Blue. Densitometry analysis of the amount of protein in each fraction was determined. Protein density of carbonic anhydrase (black), BSA (blue), catalase (green), apoferritin (purple) and thyroglobulin (light blue) peaked at fractions 2,3,6,7 and 8, respectively.
Figure 5. WT and F92A Cav-1 auto-assemble to form HMW oligomers

BAECs were infected with AdWT (myg tag) and AdF92A Cav-1 (HA tag) at an MOI of 25 and subjected to velocity gradient centrifugation for determination of oligomeric capacity. Fractions were collected from the top, separated by SDS/Page (12.5% acrylamide) and detected by anti Cav-1, anti-myc and anti-HA primary antibodies. WT and F92A Cav-1, like endogenous Cav-1, are predominantly enriched in fractions 6-8, which correspond to >250kDa in size. Quantification (graph above) of the amount of protein (black: Cav-1, red: WT Cav-1 and green: F92A Cav-1) in each fraction was determined by densitometry and expressed as total protein present.
HEK cells were infected with AdWT (myg tag) and AdF92A Cav-1 (HA tag) at an MOI of 15 and subjected to velocity gradient centrifugation for determination of oligomeric capacity. Fractions were collected from the top, separated by SDS/Page (12.5% acrylamide) and detected by anti-myc and anti-HA primary antibodies. WT and F92A Cav-1 are predominantly enriched in fractions 5-7, which correspond to > 200kDa in size. Quantification (graph above) of the amount of protein (red: WT Cav-1 and green: F92A Cav-1) in each fraction was determined by densitometry and expressed as total protein present.
4.3. F92A Cav-1 retains the ability to bind eNOS

The F92 residue of the Cav-1 CSD is responsible for eNOS inhibition; however, previous work has established that the amino acids that determine eNOS binding also reside in the Cav-1 CSD\textsuperscript{3}. Therefore, it was pertinent to examine if F92A Cav-1 could retain the ability to bind to eNOS. For this specific aim, the GST fusion protein pull-down assay was performed, as it is an excellent tool for the detection of protein-protein interactions. In this case GST was fused to a Cav protein segment (amino acids 61-101), which included the Cav-1 CSD. GST (negative control), GST Cav and GST F92A Cav plasmids were designed and generated (Figure A1). The recombinant plasmids were then expressed in BL21 bacterial cells, amplified and purified onto glutathione beads. Amplification of GST fusion proteins from bacterial lysates gives rise to a large amount of cellular material that needs to be removed in the purification process, thereby ensuring quality expression of proteins on glutathione beads with minimal degradation products that could potentially produce artifacts during the binding process. Figures 6A and 6B showed the purification steps and protein expression on glutathione beads. GST migrates as a single product at 25kDa, whereas GST Cav and GST F92A Cav migrate as a single product at 31kDa, all three were purified with minimal degradation products.

Once the glutathione beads were purified, incubation with solubilised eNOS from BAEC lysate was performed to determine binding. The Western blot in Figure 7A shows that there is equal and specific binding of eNOS to GST Cav and GST F92A Cav, whereas minimal binding occurred with GST alone. These results eliminate the possibility of unspecific binding to GST alone and demonstrate that binding of eNOS is solely attributed to the Cav portion of the GST fused protein. Furthermore, the binding of GST F92A Cav to eNOS was comparable to GST Cav, suggesting that the F92A substitution in Cav did not alter the binding to eNOS.
With the knowledge that in endothelial cells, eNOS is N-myristoylated, cysteine palmitoylated and found in protein complexes, it was important to perform binding experiments with pure recombinant eNOS. Figure 7B demonstrates that GST Cav and GST F92A Cav had equal association with recombinant eNOS. These results confirm that Cav interacts with eNOS via a direct protein-protein interaction without the need for the eNOS protein to be modified by the addition of fatty acid groups or be present as part of a protein complex. More importantly, the data convincingly shows that the F92A mutation of Cav does not interfere with Cav-1’s interaction with eNOS.
Figure 6. GST, GST Cav and GST F92A Cav purification to glutathione beads

(A) Steps involved in purification of GST, GST Cav and GST F92A Cav from bacterial cells to glutathione beads. An aliquot from each step was mixed with SDS sample buffer and separated by SDS/Page (7.5-12.5% acrylamide). Blots in the upper panel are ponceau stains showing all protein products and the lower panel represent Western blots with anti-GST antibody showing specific GST fused products. (B) Coo massie stained gel of pure GST, GST Cav and GST F92A Cav beads. The proteins migrate as a single product with very minimal degradation products.
Figure 7. eNOS binds equally to GST Cav and GST F92A Cav

BAEC lysate was incubated with GST, GST Cav and GST F92A Cav beads overnight at 4°C. Following incubation, beads were washed; samples were eluted in 1X SDS sample buffer and separated by SDS/Page (7.5-12.5% acrylamide). The membrane was stained with ponceau (top) prior to probing with anti-eNOS and anti-GST primary antibodies. The ponceau stain shows a large number of proteins bound to GST Cav (31kDa) and GST F92A Cav (31kDa), but not to GST (25kDa) alone. Western blot (W.B.) analysis (bottom) revealed equal and specific binding of eNOS (150kDa) to GST Cav and GST F92A Cav. There was minimal unspecific binding of eNOS to GST alone. The graph (right) quantifies amount of eNOS bound to each protein as a percentage of eNOS bound to GST Cav.
Recombinant eNOS was incubated with GST, GST Cav and GST F92A Cav beads for 2 hours at 4°C. Following incubation, beads were washed; samples were eluted in 1X SDS sample buffer and separated by SDS/Page (7.5-12.5% acrylamide). The membrane was stained with ponceau (top) prior to detection by anti-eNOS and anti-GST primary antibodies. Western blot (W.B) analysis (bottom) revealed equal and specific binding of eNOS to GST Cav (31kDa) and GST F92A Cav (31kDa). There was minimal unspecific binding of eNOS to GST alone (26kDa). The graph (right) quantifies amount of eNOS bound as a percentage of eNOS bound to GST F92A Cav.
4.4. F92A Cav-1 does not alter sub-cellular targeting and co-localization with eNOS

With the knowledge that eNOS is a Golgi- and caveolae-resident protein, which also co-localizes with Cav-1, further investigation was necessary to determine if F92A Cav-1 altered eNOS subcellular targeting and co-localization with Cav-1. For this reason, isolation of caveolae by sucrose gradient fractionation to determine eNOS subcellular targeting and immunofluorescence to visualize co-localization of eNOS with Cav-1 was performed on BAECs infected with AdWT Cav-1 and AdF92A Cav-1. Western blots from Figure 8A show eNOS localization in both caveolae and bulk cytosolic protein fractions, as previously reported in the literature\textsuperscript{57, 60}. Over-expression of F92A Cav-1 preserved eNOS localization in both cellular compartments, as indicated by the similar distribution pattern (graph above) of eNOS in both WT and F92A Cav-1 over-expressing BAECs.

Figure 8B displays confocal images of the Cav-1 proteins (red), eNOS (green) and the overlap between the two proteins (right; merged panel) in BAECs infected with AdWT Cav-1 and AdF92A Cav-1 and stained with the appropriate antibodies. eNOS was localized in the perinuclear region. Similarly, WT and F92A Cav-1 protein had distinct perinuclear staining with diffuse peripheral membrane staining. The merged panel on Figure 8B reveals a high degree of co-localization between eNOS and the Cav-1 proteins (yellowish-orange stain). Non-immune IgG control (Inset) and secondary antibody control (data not shown) had no staining, validating the specificity of antibodies used. These confocal images do not entirely represent eNOS and Cav-1 localization in BAEC due to cell culture artifacts (discussion in Chapter 5). Nonetheless, quantification of the images reveal that 92±6% of WT Cav-1 and 84±3% of F92A Cav-1 are co-localized with eNOS, clearly demonstrating the similarity between the WT and F92A Cav-1.
Collectively, these data confirm that over-expression of F92A Cav-1 does not mislocalize eNOS as well as alter the compartmentalization of the enzyme in native endothelial cells.

**Figure 8. F92A Cav-1 preserves eNOS subcellular localization**

BAECs were infected with AdWT Cav-1 and AdF92A Cav-1 and subjected to sucrose gradient fractionation to determine eNOS localization. Fractions were collected from the top, separated by SDS/Page (7.5 - 12.5% acrylamide) and detected by anti Cav-1, anti-eNOS, anti-myc and anti-HA primary antibodies. Western blot (bottom) shows eNOS targets to low-density caveolae fractions (2 to 5) and to bulk cytosolic protein fractions (9 to 12). In BAECs that were over-expressed with F92A Cav-1, eNOS still retains the ability to target to low-density caveolae fractions (2 to 5) and to bulk cytosolic protein fractions (9 to 12). The graph above shows eNOS protein intensity in each fraction determined by densitometry and expressed as a percentage of total eNOS intensity. eNOS distribution is similar for BAECs over-expressing both WT and F92A Cav-1.
WT and F92A Cav-1 co-localization with eNOS determined by Immunofluorescence. BAECs were infected with AdWT Cav-1 (myc-tag) and Ad F92A Cav-1 (HA-tag). Cells were fixed, incubated with anti-myc (upper panel; red), anti-HA (lower panel; red) and anti-eNOS (both panels; green) primary antibodies and mounted on glass slides with media containing nuclear dye DAPI (blue). Right shows merged images (indicated in orange/yellow) with DAPI staining. The graph below quantifies colocalization of eNOS and WT or F92A Cav-1 using Manders Colocalization (Volocity software), which accounts for colocalized pixels (indicated in orange/yellow staining) as a percentage of total WT or F92A Cav-1 staining (indicated in red). Data is represented as mean ± SEM, p=0.1, NS= non-significant.
4.5. **Over expression of F92A Cav-1 decreases $O_2^-$ release in endothelial cells**

In endothelial cells, uncoupled eNOS has been shown to be a contributor of $O_2^-$ production\[^{29}\] and there are many factors that could cause a switch in the production of NO to $O_2^-$. Furthermore, a decrease in eNOS-derived NO production could be due to an upregulation of $O_2^-$ release. Consequently, we evaluated extracellular $O_2^-$ release in confluent BAECs infected with WT and F92A Cav-1 using the Cytochrome C Reduction assay.

Validation of the technique was achieved through performing the Cytochrome C Reduction assay on established positive controls\[^{102, 103}\]. Duroquinone was chosen because it is a superoxide generator and would therefore allow us to assess if our technique was capable of detecting high amounts of $O_2^-$ generated. In addition, BAECs were also treated with 4-HNE, a BH\(_4\) depleting agent that causes uncoupling of eNOS, hence allowing us to confirm that our assay was capable of specifically detecting eNOS-derived $O_2^-$. As expected, both duroquinone and 4-HNE produced a dose-dependent increase in basal $O_2^-$ production (**Figures 9A and 9B**), thereby confirming the validity of our method.

Control BAECs (infected with β-gal) produced 99.05 ± 20.4 nmol $O_2^-$/million cells. This was used as a baseline and subtracted from values obtained from BAECs infected with WT and F92A Cav-1. As shown in **Figure 10**, at a low MOI of 5, treatment with WT and F92A Cav-1 yielded similar results (99.05± 20.4 vs 95.32 ± 12.7 nmol $O_2^-$/million cells), whereas at a higher MOI of 20, there was a significant difference (P < 0.05) between basal $O_2^-$ production for WT and F92A Cav-1 infected cells (179.62 ± 22.53 vs 5.2 ± 29.35 nmol $O_2^-$/million cells), indicating that F92A Cav-1 could increase NO production by modulating $O_2^-$ release. BAECs express a high amount of endogenous Cav-1, therefore eNOS is maximally inhibited by Cav-1 under basal conditions and as a result a low dose of WT and F92A Cav-1 virus has minimal effect.
However, upon treatment with a high dose of the virus, WT and F92A Cav-1 are over-expressed and their respective differences in $O_2^\cdot$ release is larger. The Western blot in Figure 10 represents protein expression in BAECs with different treatments. eNOS protein expression remains consistent regardless of viral load, which indicates that a difference in $O_2^\cdot$ production is not due to differential protein expression. However, WT and F92A Cav-1 protein expression increase with the viral load added, demonstrating over-expression of target protein.

An important point to mention is that endothelial cells do not produce high levels of basal $O_2^\cdot$, therefore the low concentration of $O_2^\cdot$ makes quantitative measurement of $O_2^\cdot$ in endothelial cells, a difficult task\textsuperscript{106, 107}. The Cytochrome C Reduction assay is a well-adapted technique that has been widely used for measuring $O_2^\cdot$ in cultured endothelial cells\textsuperscript{107, 108}. This technique detects extracellular $O_2^\cdot$ and was chosen because the specificity of the signal could be accurately determined with the use of exogenous applied SOD, which does not permeate the cell membrane\textsuperscript{107}. However, like every assay developed to quantify $O_2^\cdot$ concentration, the Cytochrome C Reduction assay has some weaknesses. In endothelial cells, the low levels of $O_2^\cdot$ produced means we are working within the lower detection limits of the assay. This would result in the amplification of tiny errors, such as pipetting and weighing of reagents, during the calculation process\textsuperscript{101}. As a result of this variability, a high n number was included to minimize error and ensure consistency of results. In addition, our assay used acetylated ferricytochrome c, which enhances the specificity of the assay by decreasing the direct electron transfer by mitochondrial enzymes\textsuperscript{106}. Lastly, the endothelial source of $O_2^\cdot$ could not be identified because addition of oxypurinol, apocynin and N-Nitro-L-Arginine Methyl Ester, which inhibit xanthine oxidase, NADPH oxidase and eNOS respectively, have been found to inaccurately increase baseline absorbance levels\textsuperscript{101}.  

59
Figure 9. Validation of the Cytochrome C Reduction Assay

BAECs were treated with (A) Duroquinone (10-100µM), which was first diluted in DMSO and then DMEM, and (B) 4-HNE (15-35µM), which was first diluted in 100% ethanol and then DMEM, for approximately 3 hours. The vehicle control for Duroquinone and 4-HNE was 0.1% DMSO and 0.1% ethanol diluted in media respectively. After treatment, O$_2^-$ release was quantified by Cytochrome C Reduction assay. Both agents increased basal O$_2^-$ release from BAECs in a dose dependent manner. (n=3-8,* p<0.05 vs control).
Figure 10. F92A Cav-1 decreases $O_2^-$ release in endothelial cells as compared to WT Cav-1

BAECs were infected with Adβgal, AdWT and AdF92A Cav-1 for 48 hours (Low MOI=5 and High MOI = 20). Following which, basal $O_2^-$ release was evaluated by cytochrome c reduction assay and values were subtracted from βgal control. The Western blots show protein expression for each condition. F92A Cav-1 (MOI=20) decreased basal $O_2^-$ release as compared to WT Cav-1 (MOI=20). (n=7-10,* p<0.05)
Chapter 5. Discussion
Previous studies have established that Cav-1 inhibits basal eNOS-derived NO release through a direct protein-protein interaction between eNOS and the Cav-1 CSD\textsuperscript{2, 3}. However, a single amino acid substitution of the Cav-1 CSD, the mutant known as F92A Cav-1, is not only able to restore basal NO release but also increases the activity of the enzyme\textsuperscript{3}. The major finding of the current study is that despite their differential effects on eNOS activity, WT and F92A Cav-1 are similar with respects to localization in caveolae and auto-assembling to form HMW oligomers, both of which are properties essential for caveolae organelle biogenesis. Moreover, F92A Cav-1 retains the ability to bind to eNOS and does not alter eNOS subcellular localization, indicating that F92A Cav-1 may behave as an eNOS/Cav-1 antagonist. In addition, our results indicate for the first time that F92A Cav-1 is able to decrease basal O\textsubscript{2} release in comparison to WT Cav-1, which could explain F92A Cav-1’s positive effect on NO release. By characterizing F92A Cav-1, our data describes a novel approach to increase NO bioavailability, which can be beneficial in settings of endothelial dysfunction. Having these data in mind, this chapter will aim to discuss the methodology used in our study, the relevance of our data with regards to literature and the potential implications of our study.

5.1. Caveolae disruption: increased or decreased eNOS activation?

A plethora of research has studied the effects of caveolae disruption, either by genetic ablation of Cav-1 or with the use of cholesterol-binding agents, such as methyl-\(\beta\)-cyclodextrin and oxidized LDL. In light of the inhibitory action of Cav-1 on eNOS activity, treatment of aortic preparations with cholesterol depleting agents unpredictably results in attenuation of acetylcholine-induced endothelium dependent relaxation accompanied with reduced caveolae number\textsuperscript{109, 110}. Moreover, these results are inconsistent with those obtained
from Cav-1 knockout models, which exhibit enhanced eNOS activity\textsuperscript{89}. A possible explanation for this discrepancy is associated with Cav-1 protein levels. In Cav-1 knockout mice, there is a complete absence of Cav-1 protein, whereas upon cholesterol depletion, internalization of caveolae organelles leads to translocation of Cav-1\textsuperscript{111}. Therefore, Cav-1 protein levels remain unchanged and eNOS is inhibited by increased interaction with Cav-1 present. In support of Cav-1 protein levels in determining eNOS activity, a study by Brouet et al demonstrated that treatment of aortic endothelial cells with statins reduced Cav-1 protein levels and increased NO-dependent angiogenesis\textsuperscript{112}. Another explanation is the possible loss of acetylcholine receptors associated with cholesterol depletion, which could lead to a reduction in acetylcholine-induced NO release. These studies strongly suggest that alterations in caveolae organelles and Cav-1 protein levels could have a deep impact on eNOS/Cav-1 regulation.

In the current study, we demonstrated that F92A Cav-1 increased NO release without interfering with the basic biochemical properties of Cav-1. In particular, F92A Cav-1 does not interfere with membrane attachment and oligomerization processes, both of which could have explained the increased eNOS activity observed with F92A Cav-1. This finding is pivotal because altering the Cav-1 and caveolae dynamics could have other acute downstream effects.

### 5.2. Dissociation of the eNOS inhibition domain and caveolae formation

As mentioned in the introduction, in addition to negatively regulating eNOS, the Cav-1 CSD is known to mediate the processes involved in caveolae assembly, particularly membrane attachment and oligomerization. The observation that the F92A substitution in the Cav-1 CSD impairs its eNOS blocking activity without interfering with the two properties of
Cav-1 that are trademarks of caveolae organelle formation, supports the concept that the CSD consists of many subdomains. This indicates that within the Cav-1 CSD, there are other domains that participate in proper membrane attachment and oligomerization process. This finding is significant because a major drawback that arises with modulating Cav-1 signaling, is that genetic deletion of Cav-1 is directly linked to the loss of caveolae organelles, therefore making it difficult to dissociate between caveolae and Cav-1 dependent signaling. Our data are the first to show that it is possible to dissociate Cav-1’s ability to form oligomers and subsequent caveolae formation from its signaling activities, in particular its regulation of eNOS-derived NO release. Although caveolae are non-essential to life, they are important organelles in maintaining cellular physiology due to their role in endocytosis, cholesterol transport and signal transduction. Consequently, our data show that modulation of Cav-1’s interaction with eNOS can occur while preserving the other functions of caveolae.

5.3. Protein-protein detection: co-immunoprecipitation vs GST pulldown assay for binding quantification.

Protein-protein interactions are largely detected either by co-immunoprecipitation or GST pulldown assays. Co-immunoprecipitation employs the use of an antibody to immunoprecipitate the target protein, at the same time co-precipitating the interacting proteins. This technique is extremely useful in predicting physiological interactions in vivo, however there are some pitfalls that are associated with it. For example, co-immunoprecipitations are highly dependent on the specificity of the antibody, in that way increasing the chance of unspecific binding. Also, co-immunoprecipitation does not allow the use of pure recombinant 'baits' or 'target', which routinely leads to misinterpretation of protein complexes. Another
important point to note is that with co-immunoprecipitation, although interactions between proteins may seem direct, other proteins could be involved in the interaction.

In our study, we used highly purified GST-F92A Cav, which is not endogenously expressed. Therefore, a GST pulldown assay was chosen in order to study the direct molecular interaction between Cav, F92A Cav and eNOS, through the use eNOS found as native protein complexes in endothelial cells and pure recombinant eNOS. In this case, the GST tag was already fused to the protein of interest, which circumvented the need for an antibody and generated more specific interactions.

5.4. F92A Cav-1: a potential eNOS/Cav-1 antagonist?

The current work provides direct evidence that F92A Cav-1 has retained its eNOS binding abilities. Accordingly, since F92A Cav-1 does not alter the biochemical properties of Cav-1, it may be convenient to assume that F92A Cav-1 behaves similarly to an eNOS/Cav-1 “antagonist”, mediating it’s positive effect on eNOS-derived NO release by preventing endogenous Cav-1 from binding to and inhibiting eNOS. In line with this, Gratton et al demonstrated the mutually exclusive relationship between Cav-1 and the CaM on eNOS signaling with the use of GST pulldown assays. In their study, GST Cav-1 was bound to eNOS prior to the addition of exogenous recombinant CaM to the complex. Addition of CaM resulted in decreased association of eNOS from Cav-1 in a dose dependent manner. With this data in mind, to confirm the antagonistic potential of F92A Cav-1, GST pulldown assays with peptides derived from the Cav-1 CSD with the F92A mutation could be carried out in order to test if F92A Cav peptide could displace the GST Cav and eNOS complex.

To take it a step further, one could define the kinetics of binding by performing
radiolabelled ligand binding studies. In this case, GST-eNOS would be the constant factor and radiolabelled Cav-1 protein would be the tracer ligand. Bound radiolabelled counts can be measured using a scintillation counter. Addition of increasing concentration of unlabelled F92A Cav-1 could then potentially displace the radiolabelled Cav-1 and reduce counts. The equilibrium dissociation of F92A Cav-1 can then be calculated by using the Scatchard plot analysis of bound and unbound levels of radiolabelled Cav-1, allowing the assessment of antagonist potential of F92A Cav-1.

5.5. Similar eNOS binding to WT and F92A Cav-1: evidence of a secondary eNOS binding domain

Mounting evidence has shown that Cav-1 and eNOS interact via a direct protein-protein interaction, and it is this binding that causes eNOS inhibition by Cav-1. In particular, using truncation mutants of Cav-1, it has been demonstrated that eNOS binding to and inhibition of eNOS is mediated by the Cav-1 CSD. Another interesting aspect of our data is that we show within the Cav-1 CSD, there exists two separate entities that modulate eNOS, namely the eNOS binding domain and the eNOS inhibitory domain. The eNOS inhibitory domain has been previously identified as F92 with T90 and T91 also contributing to this inhibition. The eNOS binding domain in Cav-1 has not been elucidated yet, however, amino acids 82-88 of the Cav-1 CSD, appear to compete with Cav-1 for eNOS in co-immunoprecipitation experiments. By accurately characterizing the eNOS-binding domain, one could then determine if this domain overlaps with the Cav-1 segment that mediates caveolae formation. Thus in doing so, it could be possible to inactivate the Cav-1 signaling site (in this case eNOS binding) by either deletion or alanine substitutions and preserve the caveolae function and formation abilities of this Cav-1.
mutant. This can serve as a valuable model to better understand the molecular pathways involved in Cav-1 signaling, independent of caveolae functions.

5.6. Cav-1 interacts with various signaling molecules

As mentioned in the introduction, caveolae participate in signal transduction, predominantly by housing a large number of receptors, proteins and signaling molecules. In caveolae, these resident proteins interact with and are modulated by Cav-1. GST Cav-1 pulldown assays and isolation of caveolae followed by mass spectrometric analysis of proteins bound have led to the identification of various caveolae resident and Cav-1 interacting proteins. One example of a protein first identified in endothelial cell caveolae by these methods is myoferlin, subsequently found to be a key regulator in VEGF receptor signaling and caveolae-mediated endocytosis.

In regards to eNOS, our study strongly indicates that F92A Cav-1 can still bind and preserve caveolae localization of eNOS. However, a frequent question that comes to mind is if F92A Cav-1 alters the binding and mislocalization of other proteins. The definite answer to this question would be to perform mass-spectrometric analysis and compare proteins bound to WT Cav-1 and F92A Cav-1. Nonetheless, ponceau stains of bound complexes from our GST Cav and GST F92A Cav binding assays demonstrate similar protein bands, indicating that F92A Cav-1 would not significantly alter proteins binding to Cav-1.

5.7. Differences in intracellular Cav-1 localization: a possible cell culture artifact

It is a well-established fact eNOS is a Golgi- and caveolae-resident protein, which is
constantly palmitoylated and depalmitoylated, thereby facilitating trafficking of the protein to and from caveolae\textsuperscript{57, 59, 60}. This palmitoylation cycle regulates the amount of eNOS present in caveolae\textsuperscript{59}. On the other hand, Cav-1 is predominantly localized to plasma membrane caveolae, with a small pool residing in the Golgi\textsuperscript{116}. Using immunofluorescence techniques, multiple studies have examined the localization of eNOS and Cav-1 in cell culture as well as in aortic preparations. In line with literature, an elegant study by Garcia-Cardena et al showed a high degree of co-localization between eNOS and Cav-1 at the plasma membrane, while a large pool of eNOS was still present in the Golgi region of cultured bovine lung microvascular endothelial cells (BLMVEC)\textsuperscript{59}. In the current work, we showed a high degree of eNOS/WT Cav-1 and eNOS/F92A Cav-1 co-localization in cultured BAECs, however, predominantly in the perinuclear Golgi regions with additional diffuse membrane staining. Our results are not completely supportive of the aforementioned study, but artifacts in cell culture conditions and differences in caveolae organelle number between vascular beds can explain the differences observed. BAECs have been shown via electron microscopy studies to retain approximately ten times less caveolae than BLMVEC, where a significant amount of eNOS and Cav-1 can be easily detected on the plasma membrane\textsuperscript{59, 117}. In contrast, eNOS is minimally expressed in plasma membrane regions of BAECs cultured in identical conditions\textsuperscript{57}. Moreover, caveolae number in cultured endothelial cells is markedly less than the endothelium in vivo with significant differences observed across vascular beds\textsuperscript{117}. Hence, it is likely that the amount of Cav-1 and eNOS present in caveolae can be underestimated and is dependent on culture conditions (in vivo vs in vitro) and the vascular bed from which the endothelial cells are isolated. Collectively, these reasons explain the differences in eNOS and Cav-1 localization observed between studies.
Our data are however consistent with a previous study by Bernatchez et al, which revealed that F92A substitution does not alter Cav-1’s co-localization with eNOS as compared to WT Cav-1 in a reconstituted cell system\(^3\).

**5.8. Superoxide production: a factor in determining NO bioavailability**

The current study provides evidence for the first time that F92A Cav-1 decreases basal endothelial cell O\(_2^-\) release as compared to WT Cav-1. This observation is pivotal as a decrease in O\(_2^-\) production could explain F92A Cav-1’s positive effect on NO release. The balance between synthesis and degradation of NO determines NO bioavailability. NO synthesis is predominantly dependent on the activity of eNOS. On the other hand, inactivation of NO may occur by its reaction with oxyhaemoglobin in erythrocytes and more importantly through its reaction with O\(_2^-\), resulting in the formation of peroxynitrite. The reaction between NO and O\(_2^-\) is extremely rapid and occurs in a diffusion-limited manner, therefore increases in O\(_2^-\) levels can diminish NO levels quickly. Thus, the increase in O\(_2^-\) release in cells over-expressing WT Cav-1, could explain the associated reduction in NO levels, while the opposite balance occurs in cells over-expressing F92A Cav-1. However, due to the complexities involved in detecting O\(_2^-\), we are not certain of the source responsible for O\(_2^-\) production. In spite of this, knowing the predominant role of Cav-1 in the endothelium is to regulate eNOS, it is highly possible that eNOS is responsible for the differences observed in O\(_2^-\) release between WT and F92A Cav-1.

**5.9. Other cellular mechanisms that could rationalize F92A Cav-1 positive effect on NO release**

Considering F92A Cav-1 was shown to behave drastically different than WT Cav-
1 towards eNOS activity, our initial hypothesis examined several eNOS-dependent and independent mechanisms of F92A Cav-1 mediated positive effect on NO release. However, due to the dynamic cellular regulation of eNOS and the intricacies of the caveolae/Cav-1 system, there are other possible mechanisms responsible for this phenomenon that are yet to be explored.

Structurally, the only difference between WT Cav-1 and the mutant F92A Cav-1 is the mutation of a single amino acid phenylalanine to an alanine. The phenylalanine residue consists of an aromatic benzene ring that is absent from an alanine residue. A model for Cav-1 mediated inhibition of eNOS proposes that binding of the Cav-1 CSD to eNOS interferes with the reductase function of the enzyme by preventing proper electron flux from the oxygenase domain\textsuperscript{118}. The aromatic benzene ring of the phenylalanine residue is able to accept and stabilize electrons, thereby disrupting electron flow and mediating inhibition of eNOS\textsuperscript{3}. However, the lack of crystallography studies on the Cav-1 structure makes it impossible to further assess this potential mechanism.

Emerging evidence has shed light on a significant new role for Cav-1 in regulating BH\textsubscript{4} synthesis\textsuperscript{119}. The study by Peterson et al was the first to demonstrate that GTP cyclohydrolase I (GTPCH I), the rate limiting enzyme for BH\textsubscript{4} production in endothelial cells, is present in caveolae microdomains and co-localizes with Cav-1\textsuperscript{119}. Functionally, over-expression of Cav-1 in vitro has been shown to exert a negative control on GTPCH I activity. This is supported by data in Cav-1 knockout mice, which exhibit increased GTPCH I activity accompanied with augmented BH\textsubscript{4} levels\textsuperscript{119}. By inhibiting GTPCH I activity, Cav-1 reduces the availability of the essential co-factor, BH\textsubscript{4}, required for NO synthesis from eNOS. Since F92A Cav-1 is able to increase NO and decrease O\textsuperscript{2−} basally, both of which are crucially dependent on BH\textsubscript{4} availability to eNOS, it is possible that F92A Cav-1 may modulate GTPCH I activity,
although this was not investigated.

5.10. Clinical implications of the study

It is without a doubt that NO contributes significantly to cardiovascular homeostasis. Reduced NO availability is involved in various aspects of cardiovascular pathophysiology, including hypertension, atherosclerosis, angina and angiogenesis, among many others. Therapies that upregulate NO bioavailability have proven effective against some cardiovascular diseases. For instance, nitroglycerin, a compound that is converted to NO by mitochondrial enzymes is routinely prescribed to patients with angina, manifested from coronary artery disease\textsuperscript{120}. Oral supplementation of L-arginine and BH\textsubscript{4} has been successful in improving endothelial function in animal models and patient studies, particularly in hypertensive settings\textsuperscript{34, 35, 121, 122}.

Our study contributes to the growing field of nitric oxide research by providing a novel approach to increase NO bioavailability by modulating the protein-protein interaction between eNOS and Cav-1. Furthermore, Cav-1 has proven to be a critical component of normal physiology, substantiated by increased incidence of cardiovascular pathology linked to genetic deletion of Cav-1. This stresses the importance of maintaining caveolae organelle structure and integrity. In addition, another cardiovascular risk factor associated with decreased eNOS activity and subsequent endothelial dysfunction is aging. Recent evidence has shown a decrease in eNOS activity accompanied with an increase in intracellular Cav-1 protein levels\textsuperscript{123}. In support of this, our own unpublished data demonstrates a striking age-related increase in eNOS/Cav-1 interaction and attenuation of eNOS activity. Thus, by characterizing F92A Cav-1, we have demonstrated that it is able to increase NO in vitro and in vivo, whilst preserving the integrity of
caveolae. As a result, we may have identified the first eNOS/Cav-1 antagonist, a therapeutically relevant target to increase NO bioavailability in settings of endothelial dysfunction. Consequently, the structure of F92A Cav-1 can be used as a ‘template’ for screening chemical compounds that mimic the interaction between eNOS and Cav-1, which can then lead to the modification of these compounds to prevent inhibition of eNOS.
Chapter 6. Conclusions And Future Directions
In conclusion, our study was aimed at comparing the molecular and pharmacological characteristics of WT and F92A Cav-1, in an attempt to understand the primary functional difference to rationalize their drastically contrasting effects on eNOS activity and NO release. Our findings indicate that F92A Cav-1 is still able to localize in caveolae microdomains and retain the ability to auto-assemble and form HMW oligomers, both of which are essential factors in driving caveolae organelle biogenesis. In endothelial cells, over-expressing F92A Cav-1 did not alter subcellular localization of eNOS to caveolae, a property that is important for optimal NO release in response to agonists and sheer stress. Moreover, we show that WT and F92A Cav-1 co-localize with eNOS in a similar manner. Another important component of the study was demonstrating that F92A Cav-1 could still bind to eNOS as effectively as WT Cav-1, without negatively regulating its activity. With respects to these findings, we deduced that F92A Cav-1 could potentially be an eNOS/Cav-1 antagonist by preventing endogenous Cav-1 from binding to and inhibiting eNOS. Our study is also the first to provide evidence that F92A Cav-1 decreases basal $O_2^-$ release as compared to WT Cav-1 in endothelial cells. This finding is significant because NO-mediated scavenging of $O_2^-$ plays a role in reducing NO levels.

The negative regulation of eNOS by Cav-1 has received a lot of attention in the past decade. These findings have contributed to the field of eNOS/Cav-1 research by identifying and characterizing the eNOS inhibitory domain in Cav-1, thereby providing a novel approach to improve eNOS activity and target NO bioavailability.

The next steps of the study would be to apply the in vitro data to an in vivo setting. A good animal model to test the efficacy of a cell-permeable peptide version of F92A Cav-1 would be the ApoE knockout mouse model for atherosclerosis. As mentioned, NO
contributes to the pathophysiology of atherosclerosis. Therefore, atherosclerotic lesions could be quantified and compared in mice treated with vehicle or the F92A Cav-1 peptide. Moreover, it is hoped that this study would pave the way for a breakthrough in caveolae research, in which “knock-in” transgenic models can be developed expressing mutant Cav-1 with intact caveolae but inactivated eNOS signaling sites. This will allow us to better differentiate Cav-1 dependent cell signaling pathways from caveolae functions.
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APPENDICES

APPENDIX 1

Figure A1. Summary of steps involved in GST F92A Cav generation

Electrophoresis analysis of amplified F92A Cav from PCR (top) and digested recombinant TOPO vector + F92A Cav (bottom) showed a single band at expected size of 180bp and 120bp, respectively.
**APPENDIX 2**

Figure A2. eNOS dimer/monomer interaction in BAECs infected with AdWT Cav-1 and F92A Cav-1

BAECs were infected with Ad WT Cav-1 and Ad F92A Cav-1 at different concentrations for 48 hours. Following which, cells were lysed and loaded onto a low temperature SDS/Page gel. Samples were loaded with B-Me and without boiling, to ensure eNOS dimer structure (220kDa) was preserved. An eNOS monomer control (130kDa) sample that was boiled was added as well. Both WT and F92A Cav-1 did not alter eNOS dimer/monomer formation.
APPENDIX 3

Pascal Bernatchez*, Arpeeta Sharma*, Ethan Marin, Philip M. Bauer, Takahisa Murata, and William C. Sessa
Increased eNOS-derived nitric oxide release and vasodilation through delivery of mutant non-inhibitory caveolin-1 scaffolding domain
(Manuscript in Preparation)

*Both authors contributed equally to the work
Increased eNOS-derived Nitric Oxide release and vasodilation through delivery of mutant non-inhibitory Caveolin-1 scaffolding domain

Pascal Bernatchez¹*, Arpeeta Sharma¹*, Ethan Marin², Philip M. Bauer², Takahisa Murata³, and William C. Sessa².

Running title: Increased NO release via regulated eNOS/Cav-1 interaction

¹The James Hogg Research Centre, Heart + Lung Institute at St. Paul’s Hospital, Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, St. Paul's Hospital, Vancouver, BC, Canada, ²Department of Pharmacology and Vascular Biology & Therapeutics Program, Yale University School of Medicine, New Haven, CT, and ³Department of Veterinary Pharmacology, School of Agriculture and Life Sciences, University of Tokyo, Tokyo, Japan. * Denotes equal contribution

Authors for Correspondence:

Pascal Bernatchez
Providence Heart + Lung Institute
St. Paul’s Hospital, James Hogg Research Centre, 1081 Burrard st, room 166
Vancouver (BC) Canada, V6Z 1Y6
Phone : 604-682-2344 x66060
Fax : 604-806-9274
e-mail : pbernatc@interchange.ubc.ca

William C. Sessa
Department of Pharmacology and Program in Vascular Cell Signaling & Therapeutics
Yale University School of Medicine
10 Amistad Street, Room 437 P.O. Box 208089, New Haven, CT 06520
Telephone: 203-737-2213
Fax: 203-737-2290
e-mail: william.sessa@yale.edu
Abstract

Endothelial Nitric Oxide synthase (eNOS)-derived Nitric Oxide (NO) is well known to be instrumental in the regulation of vascular tone, and reduced NO release is associated with a variety of cardiovascular pathologies. Caveolin-1 (Cav-1), the main coat protein of caveolae, is highly expressed in endothelial cells (EC), and its scaffolding domain (CAV; a.a. 82-101) is a well-described eNOS inhibitor. Using the cell permeable peptide Cavtratin (AP-CAV) and full-length Cav-1 protein, we have identified the CAV F92 residue as the main amino acid responsible for inhibiting eNOS-derived NO release, providing an accurate characterization of the eNOS inhibitory domain of CAV.

Herein, we show that a Cavtratin peptide and Cav-1 protein both containing the F92 amino acid substitution to Alanine have retained their eNOS binding ability and can unexpectedly increase NO release in eNOS-expressing cells. Moreover, a Cavtratin peptide containing the F92A mutation can lower blood pressure in vivo and decrease KCl and phenylephrine-induced vasoconstriction by up to 80% in aortic vessels isolated from aged-mice. This effect was not observed in vessels from aged Cav-1-null or eNOS-null mice, or young mice, providing evidence of a Cav-1-, eNOS- and age-dependent effect. Mechanistically, biochemical and GST pulldown assays show that F92A Cav-1/CAV mutants can bind eNOS and prevent its inhibition by endogenous Cav-1. Together, these data raise the concept of specifically regulating eNOS binding to endogenous Cav-1 to increase NO release and might lead to new therapeutic avenues for cardiovascular disease.
Introduction

Nitric Oxide (NO) is well known to be one of the most potent endogenous vasodilator and a critical mediator of cardiovascular function. Under physiological conditions, NO is synthesized by the endothelial nitric oxide synthase (eNOS), a dynamically regulated enzyme highly expressed in vascular endothelial cells (EC). In addition to being a key endogenous vasodilator, eNOS-derived NO is well known to possess anti-inflammatory and atheroprotective properties, and significantly contributes to basal vascular tone and integrity. Homozygous deletion of the eNOS gene causes obvious alterations in cardiovascular homeostasis, such as increase in blood pressure\textsuperscript{124, 125}, abnormal vascular tone and increased susceptibility to atherosclerosis\textsuperscript{126}, further substantiating the broad spectrum of eNOS-dependent NO activities. Furthermore, a reduction in NO synthesis and bioavailability leads to a condition known as endothelial dysfunction, which is an independent clinical risk factor in pathological settings of hypertension and atherosclerosis.

For its proper regulation, eNOS is targeted to plasmalemmal microdomains known as caveolae through N-myristoylation and cysteine palmitoylation\textsuperscript{60}, ensuring its appropriate subcellular localization. Caveolae are cholesterol-rich microdomains, also highly expressed in the vascular endothelium and are important cardiovascular signaling platforms in part through their ability to sequester various signaling molecules. Only when localized in caveolae is eNOS critically placed into an environment rich in its substrates, cofactors, and regulators\textsuperscript{60}. Ironically, under basal conditions, eNOS is tonically inhibited by Caveolin-1 (Cav-1), the major coat protein of caveolae\textsuperscript{2, 127}.

Cav-1 is essential for caveolae organelle biogenesis, as homozygous inactivation of Cav-1 results in complete loss of caveolae in vivo and in vitro, leading to abnormal eNOS regulation and alterations in cardiovascular homeostasis\textsuperscript{76, 89} which demonstrates the importance of this
protein to both organelle formation and EC signaling. This is attributable to Cav-1’s inherent ability to bind cholesterol\textsuperscript{93} and auto-assemble to form high molecular weight homo-oligomers scaffolds, causing its apparent molecular weight to increase from 21kDa as a monomer to >350kDa\textsuperscript{83,128}, an essential step in caveolae formation. Functional mapping of the Cav-1 protein has revealed that membrane attachment, oligomerisation, as well as eNOS binding and inhibition are all mediated by the Cav-1 scaffolding domain (CAV), comprising of amino acids 82-101\textsuperscript{80,83}. Upon stimulation with agonists, such as acetylcholine, and mechanical forces, the inhibitory clamp of Cav-1 is relieved and efficient NO production occurs. We showed that inhibition of eNOS NO release by Cav-1 can be mimicked by fusing CAV to the Antennapedia (AP) cellular internalization sequence, a cell-permeable synthetic peptide known as AP-CAV or Cavtratin\textsuperscript{96,97}. Moreover, we demonstrated that CAV threonine 90,91 (T90,91) and in particular phenylalanine 92 (F92), are responsible for eNOS inhibition\textsuperscript{3}. The triple inactivation of T90, 91 and F92 of AP-Cav by alanine substitution, the peptide known as AP-Cav-3PM or CavNoxin, as well as the single point mutant F92A Cav-1, could not inhibit eNOS activity in various in vitro and in vivo assays\textsuperscript{3}.

In this report, we show that non-inhibitory F92A Cav-1 and CavNoxin can unexpectedly increase NO release in eNOS-expressing cells and lead to vasodilation ex vivo and lower blood pressure in vivo. Use of reconstituted cell systems and genetically modified mice reveals that F92A Cav-1 and CavNoxin effects are Cav-1 and eNOS-dependent. Mechanistic experiments suggest that the F92A substitution in CAV does not affect its eNOS binding properties, and that F92A Cav-1 and CavNoxin increase NO release specifically by preventing eNOS binding to endogenous (inhibitory) Cav-1. In view of the fact that basal NO release is a critical mediator of vascular homeostasis, understanding the relationship between eNOS and these mutant forms of
Cav-1 will provide valuable insights into modulating this system to increase NO release in settings of endothelial dysfunction.
Methods

Cell Culture: Bovine aortic endothelial cells (BAEC) below passage 15, COS cells and human embryonic kidney (HEK) cells were cultured in DMEM (Invitrogen) supplemented with 5% FBS and 1X penicillin/streptomycin in a humidified incubator at 37°C with 7% CO₂ as described.

Cell Transfection and Infection: Cell transfection was carried out as previously described³. Plasmid amounts were normalized to ensure equal DNA quantities. Cell proteins were isolated 48 hours later for experiments with the appropriate buffer. For cell infection, semi-confluent BAEC grown in 100mm tissue culture dish were infected with adenoviruses encoding for WT Cav-1 (myc-tag) and F92A Cav-1 (HA-tag). Cell proteins were isolated 48 hours later for experiments.

Isolation of Caveolae/Lipid Rafts and Nitric Oxide accumulation: Isolation of Caveolae/Lipid Rafts was performed by a detergent-free fractionation method as previously described¹¹⁵. Treatment of cells and quantification of NO release by chemiluminescence (GE; Boulder, CO) were performed as previously described³.

Velocity gradient centrifugation: Cells (two confluent 100mm dishes) were lysed with 500ul of Mes-buffered saline (MBS; 25mM Mes, pH6.5/0.15M NaCl)/60mM β-octyl glucoside. Cellular aggregates were dissociated by sonication (three 10-s bursts) and the solubilised material was then loaded on top of a 5-45% continuous sucrose gradient prepared in
MBS/60mM β-octyl glucoside (12ml) and centrifuged at 28,000rpm for 16-20 hours. Following centrifugation, twelve 1ml fractions were collected from the gradient and western blot analysis performed. To better estimate the molecular mass of the proteins from the gradient, velocity gradient centrifugation was carried out on 5 molecular weight standards; Thyroglobullin (670kD), Appoferritin (480kD), Catalase (250kD), BSA (66kD) and carbonic anhydrase (29kD).

**Purification of GST-Caveolin-1 fusion proteins (WT and F92A Cav-1) and in vitro interactions:** The caveolin fusion protein expression was performed as described previously. To determine binding of GST-Cav-1 and GST-F92A Cav-1 to eNOS, a GST pulldown assay using BAEC lysates was performed. Cells were lysed in a STET buffer (50mM Tris-Cl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100) containing 1X protease inhibitors and 1mM PMSF. The samples were Dounce homogenized (20 strokes) and tumbled for 1hr at 4°C. Following which, samples were centrifuged for 10 minutes at 13,200 rpm at 4°C, sonicated (three 10-s bursts) and incubated overnight at 4°C with beads containing GST alone, GST-Cav or GST F92A-Cav-1 (∼ 80ul packed volume) in a total volume of 500ul. After binding, the beads were washed (three 5-minute intervals) with was buffer containing 50mM Tris-Cl (pH 7.4), 125mM NaCl, 1mM EDTA and 1mM EGTA. Furthermore, in vitro interactions of GST Cav-1 and GST F92A Cav-1 with recombinant eNOS (Cayman Chemicals) was performed, as previously described for 2 hrs at 4°C in binding buffer (50mM Tris-Cl, pH 7.4 in 20% glycerol). After binding beads were washed (five 5-minute intervals) with a high salt wash buffer (50mM Tris-Cl, pH 7.7, 400mM NaCl and 1mM EDTA). Beads were eluted by boiling in 1X SDS sample buffer and subjected to SDS/PAGE- gel electrophoresis followed by western blotting with anti-GST and anti-eNOS antibodies.
**Immunofluorescence:** Immunofluorescence experiments were performed on BAECs infected with WT and F92A Cav-1 adenoviruses. BAECs were seeded in gelatin-coated coverslips and infected as described above. Forty-eight hours after infection start, immunofluorescence staining was performed as previously described$^3$ and visualized by confocal microscopy.

**Vascular reactivity assays and whole mount immunostaining:** All animal experiments were approved by the Yale University and University of Tokyo Animal Care committees. WT and eNOS-null mice were from Charles River or JAX, respectively, whereas Cav-1 KO mice were bred in-house as previously described$^93$. Long-term husbandry was done at Yale University; mice were fed normal chow diet. Isolation and pretreatment of aortic rings, as well as vascular reactivity assays were performed as previously described$^{94,96}$. Whole mount immunostaining was performed on mouse pulmonary arteries as previously described$^{93}$.

**Blood pressure measurements:** Blood pressure measurements were performed in non-anesthetized aged mice using the Coda-4 system. Mice were pre-conditioned for two weeks in regular chambers. Blood pressure was determined at baseline, and at one hour following intraperitoneal injection of either vehicle peptide or varying concentrations of CavNoxin peptide$^{93}$. This procedure was performed over 4 consecutive weekdays. Mean changes in BP following peptide injection are presented.
Statistical analysis. Data are presented as mean ± standard of the mean and considered statistically different if P < 0.05 by Analysis of Variance followed by Dunnet’s t test.
Results

F92A Cav-1 increases NO release in eNOS expressing cells: Having previously made the observation that CAV-derived peptides lacking F92 can mediate biological activities, we sought to determine if F92A Cav-1 can increase NO release in native EC. For this purpose, an adenovirus coding for F92A Cav-1 was designed and amplified. Infection of confluent EC with a previously described adenovirus encoding for WT Cav-1 (Myc-tagged; 10 or 50 MOI) did not decrease basal NO release (Fig. 1C), which indicated that the abundant endogenous Cav-1 expression in EC is sufficient to completely inhibit basal eNOS release. In contrast, infection with a F92A Cav-1 encoding adenovirus (HA-tagged) caused a 1.6-fold increase in basal NO release in confluent cultured EC (Fig. 1A). Western blotting against total Cav-1 protein showed a moderate increase in total Cav-1 levels with both viruses, although their respective expression was confirmed by anti-Myc or -HA blotting (bottom blots). HSP90 was used as a loading control. Correlation between nitrite release and tagged Cav-1 expression is shown (Fig. 1B).

To determine if this increase in NO is derived from eNOS and not other NOS isoforms, we used a reconstituted cell line that releases NO only when transfected with plasmids expressing a NOS isoform. As expected, transfection with an eNOS encoding plasmid increased basal (unstimulated) NO release (Fig. 1C). Co-transfection of eNOS with increased amounts of WT Cav-1 plasmids caused a dose-dependent decrease in NO release because of low endogenous levels of Cav-1 (Fig. 1D, linear correlation). In contrast, co-transfection of eNOS with increasing amounts of F92A Cav-1 caused a dose-dependent increase in NO release as high as 2.1-fold above eNOS-only expressing cells (Fig. 1C). Western blot analysis indicated that eNOS levels were unchanged by WT or F92A Cav-1 co-transfection. Since this reconstituted cell system does not express iNOS or nNOS, this indicated that F92A Cav-1 increases eNOS-
derived NO release.

_CavNoxin increases NO release in vitro and decreases vascular tone in vivo._ We have previously shown that inactivation of AP-CAV T90, 91 and F92 residues, resulting in the peptide know as AP-Cav-3PM or _CavNoxin_, caused a complete loss of its eNOS inhibitory activity in vitro and in vivo following agonist stimulation. Since point mutation of Cav-1 F92 residue to an alanine unexpectedly increases basal NO release, we tested the possibility that CavNoxin could increase basal non-stimulated NO release in vitro. Incubation of cultured EC with CavNoxin’s vehicle peptide alone (control condition) for 6 h (0.1-20 uM caused a weak, non-dose-dependent increase in basal NO release (Fig. 2A). Similar pretreatment with Cavtratin (AP-CAV; 10 uM) did not cause an increase in NO release, whereas pretreatment with CavNoxin (0.1-20 uM) caused a dose-dependent (up to 1.9-fold) increase in NO release compared to vehicle treated control cells (Fig. 2A.)

Since Cavtratin was shown to block inflammation and tumor growth in vivo when delivered IP, we sought to test whether CavNoxin’s effect on EC NO release could decrease blood pressure in vivo. IP injection of non-anesthetized aged mice (1 year old) with vehicle peptide (same molar ratio as CavNoxin) caused no significant decreases in systolic, diastolic or mean pressure (Fig. 2B, top). In contrast, similar injection of aged mice with CavNoxin (0.1, 1, 4 mg/kg) caused a significant decrease in all three blood pressure parameters at the two highest doses (Fig. 2B, bottom).

To gain insight into the mechanism of action of CavNoxin on blood pressure, vessels from aged mice where isolated, treated overnight with peptides (10 uM) under organ culture conditions and mounted on a myograph. During preconstrictions with KCl (50 mM) in the
continuous presence of ibuprofen (prostacyclin synthesis inhibitor) we observed that CavNoxin-treated vessels showed a 49% decrease in contractility (Fig. 2C) compared to vehicle-treated vessels originating from the same aorta. Dose-response curves revealed that the contractile response to PE ($10^{-9} - 10^{-4}$ M) is abrogated by up to 85% in CavNoxin-treated vessels (Fig. 2D). Together, this suggests that CavNoxin effect on contractility is agonist-independent. Pretreatment with eNOS inhibitor l-NAME rescued PE-induced constriction by approximately 70-80%, indicating a mostly NO-dependent effect of CavNoxin (data not shown). As expected, CavNoxin-treated vessels showed little vasodilation in response to Ach ($10^{-9} - 10^{-4}$ M) since they mostly do not preconstrict to PE, a pre-requisite for Ach vasodilation, whereas vehicle-treated vessels showed greatly increased PE preconstruction followed by a more typical Ach-induced vasodilation (Fig. 2E).

Finally, we have already shown that peptide AP-CAV-A (CAV amino acids 82-88) was the shortest peptide we tested that did not contain inhibitory amino acid F92 but showed an effect on eNOS/Cav-1 interaction. Having the knowledge that CavNoxin vasodilatory effect is NO dependent and that decreased eNOS binding to inhibitory Cav-1 results in NO production, AP-Cav-A could display similar vasodilatory activity as CavNoxin. Overnight treatment of vessels from aged mice with AP-Cav-A resulted in similar decrease in KCl- and PE-induced compared with CavNoxin (Fig. 2F,G), supporting the concept that CavNoxin increases NO release by modulating eNOS and Cav-1 interaction.

*Genetic evidence supporting the role of Cav-1, eNOS and aging in CavNoxin-induced vasodilation.* Having the knowledge that CAV-derived peptide lacking F92 can regulate eNOS/Cav-1 interactions and increase NO release, we hypothesized that the 85% decrease in
vascular tone induced by CavNoxin is Cav-1 and eNOS dependent. To test this hypothesis, we challenged aortic rings isolated from aged Cav-1 or eNOS-null mice with CavNoxin as described above. In both Cav-1 and eNOS-null mice, an overnight pretreatment with CavNoxin did not decrease KCl or PE-induced constriction (Fig. 3A, B) compared to vehicle. These data provide genetic evidence about the direct role of Cav-1 and eNOS in CavNoxin activity. Moreover, the decrease in contractility induced by CavNoxin in vessels from aged mice was not observed in vessels from young (2 months old) mice under similar KCl and PE-induced contractility (Fig. 3C), providing evidence of an age-dependent effect.

CAV F92A substitution does not modulate Cav-1 biochemical properties. In an attempt to better understand how F92A Cav-1 increases NO release, we determined whether the F92A substitution affects the main biochemical properties of Cav-1 involved in caveolae formation and signaling. For this purpose, we performed sucrose fractionation and velocity gradient centrifugation to assess Cav-1’s ability to target to cholesterol-enriched microdomains (CEM) and form high molecular weight oligomers, respectively. In Cav-1-rich EC, infection with adenoviruses encoding for WT and F92A Cav-1 revealed that about 72% and 66% of WT and F92A Cav-1 respectively were found in CEM fractions 2 to 5, and both followed closely the separation pattern of endogenous Cav-1 (Fig. 4A). Since high levels of endogenous Cav-1 could potentially rescue a defect in F92A Cav-1 localization, we used a reconstituted cell system with low endogenous Cav-1 levels. Transfection with WT and F92A Cav-1 plasmids showed that about 63% and 55% of WT and F92A Cav-1 respectively, localized in CEM (Fig. 4B), confirming that F92A substitution did not affect Cav-1 localization in CEM. In addition, velocity gradient centrifugation, a technique that separates protein according to their native
molecular weight revealed that about 62% of WT Cav-1 and 60% of F92A Cav-1 were found in fraction 6 to 8 (Fig. 4C), corresponding to approximately 250kDa to 600kDa oligomers (see molecular weight standards, Supplemental Figures 1A), which was identical to endogenous Cav-1. In a low endogenous Cav-1-expressing system, WT and F92A Cav-1 were present in high molecular weight fractions 5-8 (66% and 61% respectively) (Fig. 4D), further substantiating the fact that despite being a mere 21kDa protein, Cav-1 is present in vitro as a high molecular weight oligomer (>250kDa) and that the F92A substitution does not interfere with the oligomerization process. Collectively, these data suggests that F92A Cav-1 alter the basic biochemical properties of Cav-1 that lead to caveolae organelle formation.

Inactivation of Cav-1’s eNOS inhibitory domain (F92A) does not impair eNOS binding. Although F92A substitution impairs CAV’s ability to inhibit eNOS3, other amino acids could potentially mediate CAV’s binding to eNOS. Accordingly, we examined if F92A Cav-1 retained the ability to interact with eNOS. We generated glutathione beads coated with GST, GST-CAV (61-101) and GST-F92A-CAV (61-101) proteins and incubated them with solubilised eNOS from EC lysates. Incubation of EC lysate with GST-CAV and GST-F92A-CAV but not GST alone, resulted in equal and specific binding of eNOS (Figure 5A). Furthermore, we tested if this binding occurred independently of lipid modifications of the eNOS protein by using purified eNOS. As expected, incubation with purified eNOS resulted in the specific interaction of eNOS with GST-CAV and GST-F92A-CAV (Fig. 5B). Together, our data indicate that non-inhibitory F92A Cav-1 binds eNOS similarly to endogenous WT Cav-1, and provide evidence that the increased in eNOS activity we report (Fig. 1) is attributable to F92A Cav-1’s ability to ‘antagonize’ eNOS binding to endogenous inhibitory WT Cav-1.
Lastly, we sought to determine if eNOS subcellular targeting to caveolae and co-localization with Cav-1 remained intact with the F92A substitution. We isolated CEM to determine eNOS subcellular localization and visualized eNOS co-localization with Cav-1 by immunofluorescence in EC that overexpressed WT and F92A Cav-1. eNOS was found to be enriched in both CEM and cytosolic protein fractions of WT Cav-1-expressing cells, which is typical of this lipid-modified enzyme that targets to Golgi membranes and caveolae (Supplemental Fig. 1C). Overexpression of F92A Cav-1 resulted in the similar distribution pattern of eNOS (Supplemental Fig. 1C). Also, localization of eNOS with WT or F92A Cav-1 in EC via immuno-fluorescence shows that eNOS, WT (Figure 5C, upper panel) and F92A Cav-1 (Figure 5C, lower panel) have similar perinuclear staining. The colocalization of both WT and F92A Cav-1 with eNOS followed a similar distribution pattern (Fig. 5C, merged images), and quantification revealed that 92% of WT Cav-1 and 84% of F92A Cav-1 colocalizes with eNOS although no statistical difference was observed (Supplemental Figure 1), signifying that the F92A substitution does not mislocalize Cav-1 or eNOS. Collectively, these data suggests that F92A Cav-1 is able to increase eNOS activity and NO release without modulating the main biochemical properties of Cav-1, which are crucial to caveolae organelle formation and function.

*Aging causes eNOS redistribution in blood vessels.* Aging causes numerous changes in blood vessels although an unexpected one was the lack of effect of CavNoxin in vessels isolated from young mice (Fig. 1I). Since CavNoxin vasodilatory effect cannot be observed in eNOS-null mice, indicating an eNOS-dependent mechanism, we hypothesized that profound changes in eNOS localization can be observed between young and old vessels. Whole mount immunostaining of eNOS\textsuperscript{93} in pulmonary arteries from young and old mice (approximately 12 months
old; n=4 each) revealed profound differences in eNOS staining pattern and localization. In young vessels, most of eNOS (green channel) was found highly concentrated in the perinuclear region characteristic of the Golgi apparatus as we previously showed (white arrows)\textsuperscript{93}, with additional diffuse (weak) eNOS staining in the cytosol (Fig. 5D, left and Supplemental Fig. 2A). However, 12-month old mice showed very limited perinuclear staining and much increased cytosol staining (Fig. 5D, right and Supplemental Fig. 2A) that contrasted with the staining observed in young vessels. Nuclei are shown in blue. These data highlight changes in eNOS in old vs young vessels, and lends credence to the age-dependent effect of CavNoxin in vessels ex vivo.
Discussion

Mounting evidence show that eNOS-derived NO release is highly regulated by Cav-1 through a direct interaction with CAV. However, little is known about the extent by which eNOS is inhibited by Cav-1 under physiological, non-stimulated conditions. This intriguing problem is a result from well-documented observations that genetic inactivation of the Cav-1 locus is directly linked to loss of caveolae organelles i.e. removal of Cav-1 control of eNOS cannot be dissociated from caveolae assembly. Herein, we show that the F92A Cav-1 mutant has retained its eNOS binding and caveolae-forming properties, and can prevent eNOS from binding to endogenous Cav-1, leading to a sustained increased in eNOS-derived NO release under basal conditions. Moreover, specific modification of an eNOS inhibitor (Cavtratin) resulted in a cell-permeable peptide called CavNoxin which was capable of increasing NO release, decreasing blood pressure in vivo and promoting vasodilation ex vivo. eNOS is specifically expressed in EC, which confirms that CavNoxin effect is endothelium and eNOS-dependent and not mediated through direct SMC vasodilation. CavNoxin effect on eNOS-derived NO release was age-dependent which can be rationalized by profound changes in eNOS distribution pattern in vessels from aged vs young mice. Hence, our data depict unexpected biological activities of F92A Cav-1 and reveal that CAV signaling towards eNOS is independent of its caveolae forming abilities. Most importantly, we describe a novel approach to increase eNOS activity in an age-dependent manner.

Caveolin-1 scaffolding domain: signaling vs caveolae assembly.

Caveolae, although non-essential to life, are important organelles in maintaining cellular integrity because of their role in endocytosis, cholesterol transport and signal transduction. In
addition to negatively regulating eNOS-derived NO release, CAV is known to be critical for caveolae assembly. CAV has been reported to be involved in proper membrane attachment of Cav-1 and participating in the oligomerization process\textsuperscript{80, 83} both of which give rise to regulated formation of the caveolae organelle. By modulating a single amino acid (F92) in the CAV region, interfering with CAV membrane attachment and oligomerization processes was a possibility, which would have led to the disruption of caveolae organelle formation and possibly explain the resultant increase in eNOS activity similarly to that observed in Cav-1-null animals. On the contrary, our biochemical analysis data clearly demonstrate that the mutant F92A Cav-1 is able to preserve localization to caveolae and its ability to form high molecular weight oligomers. Furthermore, F92A Cav-1 was effectively able to bind eNOS and preserve the subcellular localization of the enzyme in cultured EC. Hence, a single point mutation of CAV was able to abolish the inhibitory activity on eNOS (signaling) without altering the other relevant biological activities of Cav-1 (caveolae formation). With these data in mind, to our knowledge we are the first to suggest the possibility of dissociating CAV’s ability to form oligomers and subsequent caveolae organelles from its signaling activities, in particular its regulation of eNOS-derived NO release. This finding is of importance since a major drawback that arises with the genetic ablation of Cav-1 is the loss of both caveolae and Cav-1 dependent signaling.

**Aging and eNOS regulation.**

Aging is well known to impair endothelial function by causing profound changes in eNOS regulation and NO release. *In vitro*, cultured EC at high passages show decreased expression and activity of eNOS as well as increased eNOS/Cav-1 interaction\textsuperscript{123}. Furthermore,
compelling data from in vivo studies have demonstrated that vessels isolated from aged rodents have dramatically increased eNOS association with Cav-1\textsuperscript{129} and decreased eNOS dimer formation\textsuperscript{130}, all of which contribute to the age-related decline in eNOS function. Our data showing a highly selective effect of CavNoxin in vessels from aged mice further support the increasing role of Cav-1 in down-regulating eNOS function in settings of aging. Since we show that Cav-1 and eNOS are essential for mediating the vasodilatory effects of CavNoxin in these vessels, our data suggest that differences in eNOS regulation between young vs old vessels likely explain the age-specific effect of CavNoxin.

The findings documenting altered eNOS localization in aged vs young vessels, as demonstrated by our whole mount immunostaining of eNOS in young and aged vessels, further documents change in eNOS signaling in settings of aging. These data shed a new light on the many mechanisms involved in decreased eNOS signaling that are associated with aging. Consequently, inactivating the eNOS inhibitory domain of Cav-1 whilst preserving caveolae organelle formation, could prove to be highly relevant in settings of endothelial dysfunction and cardiovascular diseases.
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**Figure Legends**

**Figure A3: F92A Cav-1 increases Nitric Oxide release in eNOS-expressing cells.**

A) Infection of BAEC with F92A Cav-1 encoding adenoviruses increases nitrite accumulation. Confluent BAEC were infected with low (5 MOI) and high (50 MOI) amounts of adenovirus. Accumulation in culture media was performed for 16h and quantified by NO-specific chemiluminescence. Expression of WT and F92A Cav-1 was confirmed by Western blot analysis against Myc- and HA-tag. Data are individual nitrite accumulation per 1x10^6 BAEC, performed in duplicate. * P<0.001 compared to control adenovirus-infected BAEC.  

B) Linear correlation depicting increased nitrate accumulation in cells expressing F92A Cav-1 vs no change in cells expressing WT Cav-1 as a function of viral Cav-1 expression (nitrate accumulation per 1x10^6 cells as a function of arbitrary units of tag expression).  

C) F92A CA-1 increases nitrate accumulation in cells transfected with eNOS, whereas transfection with WT Cav-1-encoding plasmids decreases nitrate accumulation. Cells were transfected with increasing doses of plasmids both HA tagged. eNOS expression levels were unchanged by transfection with Cav-1 plasmids. Individual values are shown. Performed in triplicate. * P<0.001 compared to eNOS expressing cells.  

D) Linear correlation depicting increased and decreased NO release in cells transfected with F92A or WT Cav-1, respectively. Data are expressed as in A, B.

These data indicate that F92A Cav-1 is capable of not only abolishing the inhibitory activity of Cav-1 but is also able to increase basal eNOS-derived NO release.

**Figure A4: CavNoxin increases NO release and decreases vascular tone.**

A) Incubation of BAEC with increasing concentrations of CavNoxin increases basal NO release (12 h) compared with vehicle peptide, whereas incubation with AP-Cav inhibits NO release. Nitrate release from untreated cells was quantified and used as background value. N=4 in duplicate.  

B) IP injections
of CavNoxin dose-dependently decreases systolic, diastolic and mean blood pressure in trained, unanesthetized aged mice. C, D) Overnight pretreatment of aortic rings isolated from aged mice with CavNoxin under organ culture conditions decreases KCl and PE-induced constriction. Vessels were mounted on a myograph, and tension is shown (baseline tension is 9.8 mN). N=8, cumulative data shown. E) Ach shows little vasodilatory activity in vessels pretreated with CavNoxin and pre-constricted with PE (EC\textsubscript{50} dose) since they are near fully relaxed in the presence of PE. N=6, cumulative data shown. F, G) Overnight treatment with a truncated version of CavNoxin (AP-Cav-A) shows similar vasodilatory activity on KCl and PE-induced constriction vessels from aged mice. N=6, cumulative data shown. * P<0.05 compared to vehicle peptide condition.

Treatment with CavNoxin, which contains the inactivated NO signaling domain, shows dose dependent decreases in blood pressure and enhanced endothelium-dependent vasodilation, both of which are important NO-dependent signaling activities.

**Figure A5:** CavNoxin has no effect in aortic rings isolated from aged Cav-1 or eNOS-null mice, or young WT mice. A-C) Vessels isolated from aged Cav-1- or eNOS-null mice or young (2 months old) WT mice were pretreated with CavNoxin or vehicle peptides and challenged with agonists as described if Figure 2. N=4-5 animals per group, with 4 rings per animal, cumulative data shown.

Mechanistically, these data show that CavNoxin’s effect is not observed in young mice, eNOS and Cav-1 knockouts, strongly suggesting its activity is eNOS, Cav-1 and age-specific.

**Figure A6:** F92A and WT Cav-1 show similar biochemical properties. A) BAECs were infected with adenoviruses encoding for WT (myc tag) and F92A Cav-1 (HA tag) at an MOI of 25 and subjected to sucrose gradient fractionation 48 hours later. Bottom: Immunoblot analysis
from fractions collected. Fractions 2 to 5 represent low-density caveolae/lipid raft (CEM/LR) fractions (marker Cav-1), whereas HSP90 was used as a bulk cytosolic protein marker (fractions 9-12). Top: Quantification of the amount of protein in each fraction was determined by densitometry and expressed as a percentage of total protein present. B) Expression of WT and F92A Cav-1 plasmids (HA tagged) in a reconstituted cell system with low endogenous Cav-1 levels and subjected to sucrose fractionation and Western blotting as in (A). C) BAECs were infected as described in (A), lysed and subjected to velocity gradient centrifugation to compare their oligomer-forming ability. Fractions were collected from the gradient and immunoblot analysis was carried out cytosol. Bottom: Immunoblot analysis and quantification from fractions reveal that endogenous Cav-1, WT and F92A Cav-1 are predominantly expressed in fractions 6-8 at an estimated molecular mass of 300-600kDa, with no detectable amounts of monomeric Cav-1. Molecular weight markers are indicated. Top: Quantification graph. (D) Expression of WT and F92A Cav-1 in a low endogenous Cav-1 reconstituted cell system as described in B. Samples were processes as described in C. All experiments were performed in triplicate, typical data shown.

F92A Cav-1 is able to preserve the ability to target to cholesterol-rich caveolae microdomains and auto-assemble to form high molecular weight oligomers, similarly to WT Cav-1, both of which are important factors in driving caveolae biogenesis.

**Figure A7: CAV F92A substitution does not affect eNOS binding and Cav-1 localization, and confirms changes in eNOS localization.** A, B) BAEC lysates or recombinant eNOS were incubated with beads coated with GST alone, GST Cav (62-101) or GST F92A Cav (62-101), washed and the amount of eNOS binding as a protein complex (A) or direct protein-protein interaction (B) was determined by immunoblotting analysis. Bottom panel: pulldown of eNOS
and similar amounts of GST, GST Cav and GST F92A Cav. Top Panel: Quantification of eNOS binding using linear densitometry. (C) CAV F92A substitution does not affect its co-localization with eNOS. BAECs were infected as described in Figure 4, cells were fixed and immunofluorescence using antibodies against myc (upper panel; red), HA (lower panel; red) and eNOS (both panels; green) was performed. Right shows merged images. Nuclei were visualized with DAPI (blue). Typical cells are shown. (D) Aging is associated with altered eNOS localization. Whole mount staining of pulmonary arteries isolated from 2- and 12-month old WT mice. eNOS and nuclei are shown in the green and blue channels, respectively. Arrows indicate high eNOS expression in perinuclear regions.

F92A Cav-1 still retains the ability to bind to eNOS and preserves its subcellular localization in caveolae microdomains. This suggests that F92A Cav-1 could potentially behave as an eNOS/Cav-1 antagonist, preventing endogenous Cav-1 from binding and inhibiting eNOS.

**Supplemental Figure 1:** A) Molecular Weight standards used for Velocity Gradient Centrifugation calibration. Carbonic anhydrase (29kDa), BSA (66kDa), Catalase (250kDa), Appoferritin (470kDa) and Thyroglobullin (670kDa) were subjected to velocity gradient centrifugation to determine which fractions the proteins were migrated at. Bottom: Coomassie stained SDS/Page gels of protein distribution in fractions. Top: Densitometry analysis of the amount of protein in each fraction. B) Western blots showed highly purified glutathione beads coated with GST, GST Cav and GST F92A Cav free of bacterial contamination. C) Sucrose Fractionation on BAECs lysates infected with AdWT Cav-1 and AdF92A Cav-1 showed similar eNOS distribution in low-density CEM/LR fractions (2 to 5) and bulk cytosolic protein fractions (9 to 12). In BAECs that were overexpressed with F92A Cav-1, eNOS retained its ability to target to low-density CEM/LR fractions and to bulk cytosolic protein fractions. Top panel:
eNOS protein intensity in each fraction determined by densitometry and expressed as a percentage of total eNOS intensity. eNOS distribution is similar for BAECs overexpressing both WT and F92A Cav-1. (D) Quantification of eNOS and WT or F92A Cav-1 colocalization from images captured in Figure 5C were calculated using the Manders colocalization formula, which accounts for colocalized pixels (indicated in orange/yellow staining) as a percentage of total WT or F92A Cav-1 staining (indicated in red). No statistical difference were observed (n=5 cells per conditions).

**Supplemental Figure 2:** Individual samples showing in situ whole mount immuno-staining in pulmonary arteries of 2- and 12-month old mice (n=4 each). eNOS (green) and nuclear expression (DAPI; blue) are shown. eNOS localization is predominantly in the perinuclear Golgi region in 2 month old mice, but is shifted to the cytosol in 12 month old mice. eNOS localization differs in an age-specific manner. In young mice, eNOS is mainly localized to the perinuclear Golgi region, whereas, in aged mice, eNOS is uniformly distributed in the cytosol. Therefore, it is highly possible that reduced NO bioavailability associated with aging could be due to increased interaction with Cav-1.
Figure A3. F92A Cav-1 increases basal NO release

**Figure A3**

**Panel A**
- Graph showing nitrite release (pMol/10^6 BAEC) with different treatments.
- Comparison between Unstimulated BAEC, AdF92A Cav-1, AdWT Cav-1, Low MOI, Hi MOI.
- * indicates significant difference.

**Panel B**
- Graph showing nitrite release (pMol/10^8 cells) with AdWT or AdF92A Cav-1 overexpression.
- Linear relationship with MOI values.
- AdWT Cav-1 and AdF92A Cav-1 treatments.

Unstimulated BAEC

Nitrite release (pMol/10^6 BAEC)

AdF92A Cav-1

AdWT Cav-1

Low MOI

Hi MOI

HSP90

β-Gal
Figure A4. CavNoxin increases eNOS activity and NO-dependent activities
Figure A5. CavNoxin’s effect is eNOS and Cav-1 specific

A

12-months old Cav-1 KO mice

15
14
13
12
11
10
9

Tension (mN)

AP
AP-Cav 3PM
KCI

-9
-8
-7
-6
-5
-4

PE (log M)

AP
AP-Cav 3PM

12-months old eNOS KO mice

26
22
18
14
10

Tension (mN)

AP
AP-Cav 3PM
KCI

-9
-8
-7
-6
-5
-4

PE (log M)

AP
AP-Cav 3PM
Figure A6. F92A Cav-1 does not alter biochemical properties of Cav-1

(A) BAEC
Sucrose gradient (5% - 45%)

(B) Reconstituted
Sucrose Fractionation (5% - 45%)

(C) BAEC
Cav-1 Homo-oligomer size

(D) Reconstituted
Cav-1 Homo-oligomer size
Figure A7. F92A Cav-1 still binds eNOS and retains its subcellular localisation
Figure A8. Supplementary data 1

A

B

C

D

BAEC

Sucrose Fractionation (5% - 45%)

% Colocalised Pixels / Total Pixel Intensity

WT Cav-1  F92A Cav-1
Figure A9. Supplemental data 2

A

2-months old WT 1
2-months old WT 2
2-months old WT 3
2-months old WT 4

12-months old WT 1
12-months old WT 2
12-months old WT 3
12-months old WT 4

- eNOS
- DAPI