Endothelial Cell Response to Simvastatin and Fluid Wall Shear Stress: An Investigation into Statin-Shear Stress Interactions

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To my husband Jan who always believed in me

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ABSTRACT

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (also known as statins) are widely prescribed to patients with hypercholesterolemia to mitigate the risk for a cardiovascular event. These drugs effectively reduce cardiovascular related morbidity and mortality by inhibiting the biosynthesis of cholesterol, which consequently reduces blood cholesterol levels. In addition to lowering cholesterol, there is also ample evidence that statins have pleiotropic or non-lipid lowering benefits, which include improving endothelial cell function and reducing vascular inflammation. To date, the influence of statins on endothelial cell biology has been conducted primarily under static conditions. The lack of a hemodynamic environment in current *in vitro* models may be limiting our understanding of the non-lipid lowering effects of statins. The work presented in this thesis reports the combined effects of simvastatin and shear stress (steady, non-reversing pulsatile and oscillating) on the expression of select markers of endothelial cell function. Specifically, we evaluated Kruppel-like factor 2 (KLF2), endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM) expression which are associated with maintaining an anti-inflammatory and anti-thrombotic blood vessel. We also examined the expression of inflammatory proteins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which allow attachment and migration of leukocytes across the blood vessel. Our results show that simvastatin potentiates the tumor necrosis factor α (TNFα)-induced increase in VCAM-1 and ICAM-1 mRNA in static culture. This potentiating effect of simvastatin was eliminated by unidirectional laminar shear stress. We also report that simvastatin and unidirectional steady or pulsatile shear stress separately enhances KLF2, eNOS and TM expression. Combining both simvastatin and shear stress (steady or non-reversing pulsatile) resulted in an additive increase in KLF2, eNOS and TM mRNA at low statin concentrations. At higher simvastatin concentrations, a synergistic increase in gene expression was detected. On the other hand,

oscillating shear stress impaired endothelial cell response to simvastatin at lower statin concentrations. Our results show that shear stress can alter endothelial cell response to statin drugs *in vitro*. These findings demonstrate the importance of including hemodynamic stimuli when modeling the effects of statins on endothelial cell gene expression.

RÉSUMÉ

Les inhibiteurs de l'enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) réductase (aussi nommés statines) sont largement prescrits à des patients souffrant d'hypercholestérolémie afin de diminuer le risque de problèmes cardiovasculaires. Ces drogues réduisent efficacement les taux de morbidité et de mortalité reliés aux maladies cardiovasculaires en empêchant la biosynthèse de cholestérol résultant en une réduction du taux de cholestérol sanguin. Outre la diminution du taux de cholestérol, plusieurs autres effets secondaires ont été observés incluant l'amélioration de la fonction des cellules endothéliales et la diminution de l'inflammation vasculaire. À ce jour, l'influence des statines sur les cellules endothéliales *in vitro* a été principalement étudiée dans des conditions statiques. Le travail présenté dans cette thèse porte sur l'expression de certains gènes importants pour la fonction des cellules endothéliales suite à l'application simultanée de simvastatine et de forces de cisaillement (constantes, pulsatiles et oscillatoires). Plus précisément, nous avons évalué l'expression du facteur de type Krüppel KLF2, de l'oxyde nitrique synthétase endothéliale (eNOS) et de la thrombomoduline (TM), lesquels sont reliés à l'inflammation et la thrombose des vaisseaux sanguins. Nous avons aussi étudié l'expression des protéines pro-inflammatoires (molécule d'adhésion des cellules vasculaires) VCAM-1 et (molécule d'adhésion intra-cellulaire) ICAM-1. Nos résultats démontrent que la simvastatine amplifie l'effet du facteur nécrosant des tumeurs alpha (TNFα) pour des conditions statiques. Cependant, l'application de forces de cisaillement élimine l'augmentation de VCAM-1 et ICAM-1 par la simvastatine. Nous avons aussi observé que la simvastatine et les forces de cisaillement appliquées séparément augmentent l'expression de KLF2, eNOS et TM. L'application simultanée de simvastatine à de faibles concentrations et de forces de cisaillement (constantes ou pulsatiles) a augmenté l'expression de KLF2, eNOS et TM de manière additive. À des concentrations supérieures de simvastatine, une augmentation synergétique a été observée. Par contre, l'application de forces de

cisaillement oscillatoires a diminué l'effet de la simvastatine utilisée à de faibles concentrations. Nos résultats démontrent que des forces de cisaillement peuvent changer l'effet des statines sur les cellules endothéliales *in vitro*. Par conséquent, il est important d'inclure la stimulation hémodynamique lors de l'étude des effets des statines sur les cellules endothéliales dans des modèles *in vitro*.

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CONTRIBUTION OF AUTHORS

In this section the roles each author had in the preparation of the four manuscripts presented in this thesis is described.

Article 1 - Concentration and Time Effects of Dextran Exposure on Endothelial Cell Viability, Attachment and Inflammatory Marker Expression in Vitro

Contributions:

Leonie Rouleau – gathered, analyzed and interpreted the proliferation, attachment, adhesion, protein expression and some of the mRNA data, collected the viscosity and density data, wrote the manuscript, and replied to reviewers.

Joanna Rossi – assisted in the experimental design, gathered samples with Leonie Rouleau, developed the quantitative real-time polymerase chain reaction (qRT-PCR) protocol, performed and interpreted some of the qRT-PCR experiments, helped write the manuscript and responded to reviewers.

Richard L. Leask – contributed to the experimental design and revised the manuscript.

Article 2 - Laminar Shear Stress Prevents Simvastatin-induced Adhesion Molecule Expression in Cytokine Activated Endothelial Cells

Contributions:

Joanna Rossi – designed and performed the flow experiments, gathered the protein and RNA, developed the qRT-PCR protocol, performed the qRT-PCR and Western blot analyses, analyzed the data, wrote the paper and responded to reviewers.

Leonie Rouleau – developed the three dimensional tubular endothelial cell culture models, troubleshooted the majority of the Western blot protocol, contributed to the design of experiments and assisted in editing the final manuscript.

Alexander Emmott – helped in the experimental set-up and gathering some of the protein samples.

Jean-Claude Tardif – provided clinical insight and helped develop the study.

Richard L. Leask – was involved in the planning of experiments, in data analysis and in editing the paper for publication.

Article 3 - Effect of Simvastatin on Kruppel-like Factor2, Endothelial Nitric Oxide Synthase, and Thrombomodulin Expression in Endothelial Cells under Shear Stress

Contributions:

Joanna Rossi – designed and performed the flow experiments, gathered the protein and RNA, developed the qRT-PCR protocol, performed the qRT-PCR and Western blot analyses, analyzed the data, wrote the paper and responded to reviewers.

Leonie Rouleau – developed the three dimensional tubular endothelial cell culture models, troubleshooted the majority of the Western blot protocol, contributed to the design of experiments and assisted in editing the final manuscript.

Jean-Claude Tardif – provided clinical insight and helped develop the study.

Richard L. Leask – was involved in the planning of experiments, in data analysis and in editing the paper for publication.

Article 4 - Differential Response of Endothelial Cells to Simvastatin when Conditioned with Steady, Non-Reversing Pulsatile or Oscillating Shear Stress

Contributions:

Joanna Rossi – designed and performed all experiments, developed the qRT-PCR protocol, troubleshooted the Western blot protocol, interpreted the results and wrote the manuscript.

Paul Jonak – developed and validated the Labview program to run the pulsatile and oscillatory flow experiments.

Leonie Rouleau – developed the majority of the Western blot protocol, assisted in the design of experiments and editing the manuscript.

Lisa Danielczak – helped in the cell culture, extracted cells from the models for mRNA and protein analysis, provided technical support and edited the manuscript.

Jean-Claude Tardif – provided clinical insight and helped develop the study.

Richard L. Leask – assisted in the design of experiments and editing the manuscript.

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4. Rouleau, L., Rossi, J., Leask, R.L. Concentration and Time Effects of Dextran Exposure on Endothelial Cell Viability, Attachment and Inflammatory Marker Expression *in Vitro*. *Annals of Biomedical Engineering* 38(4), 1451-1462, 2010.
5. Rouleau, L., Rossi, J., Leask, R.L. The Response of Human Aortic Endothelial Cells in a Stenotic Hemodynamic Environment: Effect of Duration, Magnitude and Spatial Gradients in Wall Shear Stress. *Journal of Biomechanical Engineering* (in press). DOI: 10.1115/1.4001217

CHAPTER 1: INTRODUCTION

Atherosclerosis, a disease in which the large and medium-sized arteries harden and narrow, is the leading cause of death in the developed world [1]. The disease begins by damage to the endothelium, a monolayer of cells that lines the inner wall of all blood vessels. Russell Ross first hypothesized in 1973 that the endothelial cell "response to injury" initiates atherosclerosis [2, 3]. It is now known that endothelial cells are vital to the health of the artery and dysfunction leads to chronic inflammation, lipid accumulation in the intima, lumen narrowing and potentially plaque rupture. Despite the many systemic risk factors associated with atherosclerosis and coronary heart disease such as low-density lipoprotein (LDL) cholesterol, smoking, and hypertension, atherosclerotic lesions tend to develop in regions of disturbed blood flow where the wall shear stress patterns are complex, such as bifurcations, branch points and curvatures [4, 5]. There is ample evidence that mechanical forces such as wall shear stress can "injure" or cause endothelial cell dysfunction and play a fundamental role in atherogenesis [6-8].

Clinical studies have demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, effectively reduce the progression of atherosclerosis and the incidence of cardiovascular events [9-11]. Statins achieve this by inhibiting HMG-CoA reductase, the rate limiting enzyme in the synthesis of cholesterol. Decreased cholesterol biosynthesis reduces hepatic production of LDL and upregulates LDL receptors, both of which contribute to lowering plasma LDL levels [12-14]. However, the benefits of statins are now believed to extend beyond LDL reduction. There is ample evidence, both clinical and experimental, to suggest that statins have pleiotropic, or cholesterol independent benefits which include, improving or restoring endothelial function, enhancing the stability of atherosclerotic plaques, and reducing inflammation, oxidative stress and smooth muscle cell proliferation [15-18].

To date, the influence of statins on endothelial cell biology has been conducted primarily under static conditions. Indeed few studies have investigated statin-shear stress interactions on endothelial cell gene expression. Given that shear stress is a well established modulator of endothelial cell phenotype, it is of significant interest to evaluate the effects of statins on endothelial cells under flow conditions. Of particular interest is to investigate whether shear stress alters statin-induced changes in endothelial cell gene expression. Knowledge of these potential interactions will provide a better understanding of the non-LDL lowering benefits of statins observed in the clinic.

CHAPTER 2: OBJECTIVES

The research question asked is does wall shear stress alter endothelial cell response to statin therapy? The underlying hypothesis of this thesis is that phenotypic changes in endothelial cells due to shear stress can modulate their response to statins. To investigate this hypothesis, the biological response of endothelial cells to statin drugs was evaluated and compared under static and dynamic stimulation. The endothelial cells were exposed to three types of shear stress: steady, non-reversing pulsatile and oscillating. Using simvastatin as a model of statin drugs, the following objectives were identified:

1. Determine whether it would be appropriate to use dextran as an agent to increase the viscosity of the cell perfusion medium by evaluating the effects of dextran on endothelial cell viability, gene expression and leukocyte adhesion.
2. Assess the separate effects of steady laminar wall shear stress and simvastatin on endothelial cell expression of pro-inflammatory (VCAM-1 and ICAM-1) and atheroprotective (KLF2, eNOS and TM) genes with and without cytokine stimulation. Determine whether the combined effect (shear stress and simvastatin) produces an additive, synergistic or antagonistic induction of these genes.
3. Investigate the effect of temporal gradients in shear stress and reversing flow in conjunction with simvastatin on endothelial cell expression of KLF2, eNOS, and TM.
4. Determine whether inhibition of HMG-CoA reductase is the mechanism in which statins alter gene expression in endothelial cells conditioned with shear stress.
5. Verify whether mevalonate influences endothelial cell response to shear stress.

Evaluating the interactions between wall shear stress and statin drugs provides a more physiologically realistic model, and will improve our understanding of the non-lipid lowering effects of statins.

CHAPTER 3: LITERATURE REVIEW

3.1. Cardiovascular System

3.1.1. Physiology

The cardiovascular system is a pump and tubing structure that supplies blood, nutrients and oxygen to all the cells in the body and removes the byproducts of metabolism. It is one of the main biotransport systems. The others are the lymphatic system and the urinary and digestive tract. The heart circulates blood to the pulmonary arteries for gas exchange in the lungs, to itself through the coronary arteries, and to the rest of the body via the aorta. The cells of all tissues in the body depend on a constant and unobstructed supply of blood, without which they would die or become severely damaged. Blocked or obstructed arteries can manifest as a stroke, myocardial infarction (heart attack), pulmonary embolism or peripheral vascular disease.

3.1.2. Artery Wall Structure

The artery wall can be broken down into three principal layers: the intima, media, and adventitia [19]. The intima is the innermost layer, which contains the endothelium, a one cell thick layer of longitudinally oriented endothelial cells that is supported by a basement membrane (Figure 3-1). Endothelial cells are in direct contact with blood and maintain a smooth surface for blood to flow over. The media is located below the intima between the internal and external elastic lamina. The media layer is composed principally of circumferentially oriented smooth muscle cells, elastin, and collagen [19]. In muscular arteries such as the coronary arteries, the contraction and relaxation of smooth muscle cells regulate blood vessel diameter. The outermost layer is the adventitia which consists mainly of

collagen, connective tissue, with few fibroblasts, and smooth muscle cells. Fibroblasts synthesize collagen and the extracellular matrix and are important in wound healing. The adventitia and part of the media layer contains vasa vasorum; small blood vessels that supply oxygen and nutrients to the outer layers of the vessel wall.

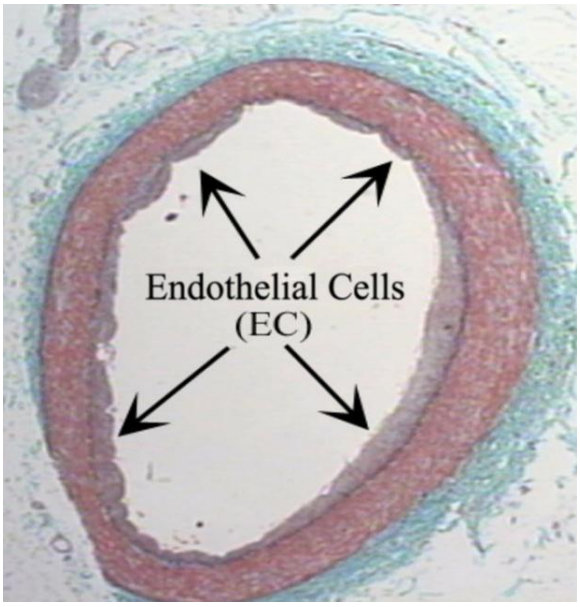

Figure 3-1: Structure of a normal muscular artery. Taken with permission from Richard L. Leask.

3.1.3. Endothelium

The endothelium lines all blood vessels and is vital to vascular homeostasis. Endothelial cells have many functions including regulating vascular tone (contraction/dilation of blood vessels), inflammation, cell migration and proliferation, and blood coagulation [20]. Under normal conditions these cells express a gene profile (or phenotype) that maintains a vasoactive, anti-thrombotic and anti-inflammatory surface. Dysfunctional endothelial cells, on the other hand, have impaired vascular reactivity and express genes that promote inflammation, cell proliferation and migration, and thrombosis. This section provides a general overview of the four principle functions of the vascular endothelium.

Vascular Tone

In the body, blood pressure can be increased or decreased by the constriction and dilation of blood vessels, respectively. Endothelial cells regulate vascular tone by synthesizing either vasodilators or vasoconstrictors, which cause smooth muscle cells in the media layer to either relax or contract [21, 22]. Nitric oxide (NO) and prostacyclin (PGI_2) are powerful vasodilators, whereas endothelin-1 (ET-1) and angiotensin-II (A_{II}) are important vasoconstrictors.

NO is synthesized by three isoforms of the enzyme nitric oxide synthase (NOS). In endothelial cells, NO is synthesized primarily by NOSIII (also known as endothelial nitric oxide synthase (eNOS)) through the conversion of L-arginine to L-citrulline [23, 24]. The other isoforms of nitric oxide synthase, NOSI and NOSII, are predominantly located in nerves and inflammatory cells, respectively. NOSII, also known as inducible NOS (or iNOS), is upregulated by endothelial cells at sites of inflammation. NO not only is a potent vasodilator, but also reduces inflammation [25], smooth muscle cell proliferation [26] and platelet aggregation [27]. As a result of its many protective functions, NO is considered an anti-atherogenic molecule and is one of the most important indicators of endothelial cell function. Injured endothelial cells generally display reduced NO bioavailability [21, 28].

PGI_2, another potent vasodilator, is synthesized in part from the enzyme cyclo-oxygenase (COX). COX converts arachidonic acid to prostaglandin G_2 (PGG_2). The same enzyme modifies PGG_2 into prostaglandin H_2 (PGH_2). Subsequently, PGH_2 is converted to PGI_2 by prostacyclin synthetase (PGIS). Two isoforms of COX exists: COX-1 and COX-2. COX-1 is constitutively expressed by endothelial cells, whereas COX-2 is produced at sites of inflammation. Both NO and PGI_2 are co-released by endothelial cells and additively dilate blood vessels [29]. Similar to NO, PGI_2 inhibits platelet aggregation and is considered to have cardioprotective functions.

Vasoconstriction is achieved in part through the vasoconstrictor, ET-1 which is produced by endothelial cells, smooth muscle cells, and fibroblasts [30]. ET-1 is translated as a pre-pro ET-1 polypeptide that is modified into pro-ET-1 (or big ET-1) by a series of enzymes and finally converted to active ET-1 by ET converting enzyme-1. ET-1 interacts with ET receptors ET_A and ET_B via paracrine and autocrine signalling. High expression of ET-1 is associated with endothelial cell dysfunction and vascular disease.

The production of A_{II} also contributes to blood vessel constriction, which consequently raises blood pressure [31]. Circulating angiotensin I (A_I) in plasma is converted into A_{II} by angiotensin-converting enzyme (ACE) [32, 33]. ACE is found in plasma, endothelial cells, and in the heart, kidneys and brain [33]. A_{II} exerts its vasoconstrictive effects by binding to AT_1 receptors [33]. ACE inhibitors (ACE-I) and AT_1 receptor blockers (ARBs) have been developed to treat hypertension by reducing A_{II}-induced vasoconstriction [33-35].

Inflammation

Endothelial cells are also important regulators of vascular inflammation. Under pro-inflammatory conditions, endothelial cells express on their surface selectins (E-selectin and P-selectin) and cell adhesion molecules (VCAM-1, ICAM-1, and PECAM-1). These molecules attract circulating leukocytes in blood to roll, firmly attach, and transmigrate across the endothelium and into the vessel wall (Figure 3-2) [36]. Specifically, P-selectin and E-selectin are involved in the initial tethering and rolling of leukocytes onto the endothelium [36]. Intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) mediate firm adhesion [37] and platelet endothelial cellular adhesion molecule (PECAM-1) allow leukocytes to migrate across the vessel [38]. Inflammation is important in protecting the body against infection however, chronic inflammation into the artery wall leads to vascular diseases such as atherosclerosis [5, 39].

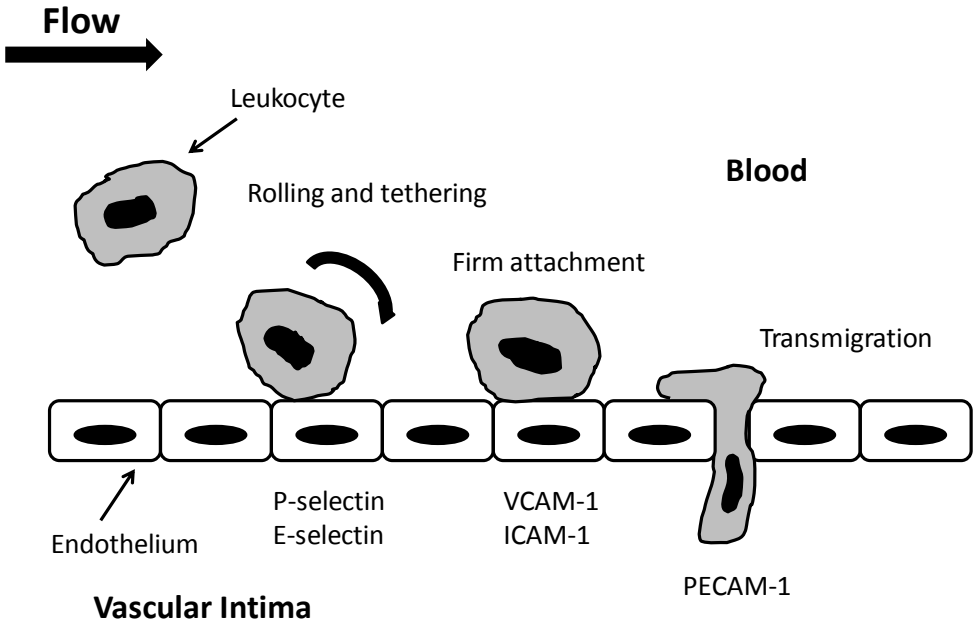

Figure 3-2: Leukocyte-endothelial cell interactions. Adapted from Blankenberg et al. (2003) Adhesion Molecules and Atherosclerosis [40]

Cell Proliferation and Migration

Endothelial cells are also essential regulators of cell proliferation and migration, largely through the generation and release of NO. The liberation of NO from the endothelium suppresses abnormal vascular smooth muscle cell proliferation [26, 41-43] and migration [43-46]. The excessive proliferation and migration of smooth muscle cells occurs following vascular injury and contributes to the progression of atherosclerosis [47]. Stimulants of cell proliferation and migration processes include, platelet derived growth factor (PDGF), A_{II} and some pro-inflammatory cytokines (MCP-1, IL-1β, TNFα and IL-8) [48-54], which are present at the site of vascular injury.

Blood Coagulation, Thrombosis and Fibrinolysis

Blood coagulation is an important defence mechanism of the body. It is triggered when the vessel wall is disrupted and blood is exposed to extravascular tissue. To stop further bleeding, the wound site is quickly plugged with fibrin and activated platelets. The clot is later dissolved by the fibrinolytic system [55]. The coagulation cascade is initiated when blood comes into contact with tissue factor (TF), present on the surface of extravascular cells [56]. TF binds to factor VII and subsequently activates factor X which produces the procoagulant protein, thrombin [57]. Thrombin promotes blood coagulation by converting fibrinogen into fibrin [58]. Thrombin also has many other effects on the coagulation cascade including platelet activation and amplifying the coagulation system via feedback mechanisms [56].

Endothelial cells play a vital role in the regulation of thrombosis, blood coagulation, and fibrinolysis by synthesizing and releasing activators and inhibitors of these processes [55]. Under normal conditions, the endothelium maintains an anti-thrombotic, anti-coagulant and pro-fibrinolytic surface [20]. Accordingly, in a functional state the endothelial cell discourages platelet activation and aggregation, anti-coagulant mechanisms are favoured and any formed blot clots can be quickly dissolved through fibrinolysis. Disruption of this natural balance promotes the formation and stability of blood clots contributing to vascular diseases such as atherosclerosis.

Anti-coagulant mechanisms are promoted in part through the expression of thrombomodulin on the endothelial cell surface. Thrombomodulin forms a complex with thrombin which activates the anticoagulant protein C [59, 60]. Activated protein C is an inhibitor of the coagulation cascade as it degrades factor Va and factor VIIIa via proteolytic cleavage, preventing the production of thrombin [59]. Consequently, by inhibiting thrombin formation via activation of protein C, thrombomodulin exerts anticoagulant properties. In addition, fibrinolysis is facilitated in healthy endothelial cells through the expression of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator

(uPA), which convert plasminogen into plasmin [61, 62]. Plasmin degrades fibrin into soluble degradation products, allowing blood clots to break down [55]. An important inhibitor of tPA and uPA activity is plasminogen activator inhibitor-1 (PAI-1) [63]. Overexpression of PAI-1 can impair the fibrinolytic activity of endothelial cells and potentially increase the risk for an atherothrombotic event [20, 64, 65].

3.2. Atherosclerosis

3.2.1. General Overview

Atherosclerosis is a chronic inflammatory disease affecting the large and medium-sized arteries. The disease is characterized by the hardening and narrowing of the arteries as a result of plaque build-up in the blood vessel walls. Atherosclerosis mainly affects the intima with minimal damage to the media and almost no effect on the adventitia. The progressive hardening and narrowing of blood vessels can lead to abnormal blood flow and rupture of the formed plaque, which can partially or completely block the artery. The obstruction of blood flow deprives cells of oxygen and nutrients which can result in severe tissue damage or even tissue death. Plaque rupture in the coronary arteries can lead to angina or myocardial infarction (heart attack), while blockage of arteries to the brain and legs can cause strokes and gangrene, respectively.

3.2.2. Atherosclerotic Prone Regions

The diet, genetic, and lifestyle risks factors associated with atherosclerosis [66] cannot explain how the lesions tend to form at focal locations in the vascular network. Plaques are inclined to develop at sites where blood flow is disturbed, such as bifurcations, branch points or curvatures [67-72]. The geometry of these regions can cause complex flow dynamics including flow separation, recirculation, reattachment and significant temporal and spatial shear stress gradients [73]. In contrast, cells exposed to unidirectional shear stress are often

protected from forming plaques [74-76]. It is now well establish that hemodynamic factors, in particular wall shear stress, play a central role in the initiation and progression of atherosclerotic plaques [4].

3.2.3. Endothelial Cell Dysfunction - Response to Injury Hypothesis

The initiating event in the formation of atherosclerotic lesions is believed to be "injury" to the endothelial cells [3, 77]. External environmental factors can cause endothelial cell injury and alter the gene expression profile from an anti-inflammatory, anti-thrombotic and pro-fibrinolytic phenotype to a state that facilitates inflammation, lesion growth, and plaque rupture [78]. Endothelial cell "injury" or "dysfunction" can be caused by local stimuli namely biomechanical, as well as systemic factors including LDL cholesterol, smoking, hypertension, oxidative stress, diabetes, homocysteine, and infectious microorganisms [5]. It is believed that disturbed shear stress in conjunction with systemic risk factors causes a pro-atherogenic phenotype in endothelial cells.

The exact sequence of events leading to the development of atherosclerostic lesions is not precisely understood. The classical description involves LDL, however it is now understood that there are many manifestations of the disease [79, 80]. It is known that dysfunctional endothelial cells allow for the enhanced permeability of LDLs and inflammatory cells into the artery wall. LDLs that traverse the endothelium and deposit in the intima can be modified by oxidative stresses into oxidized LDL (oxLDL). These oxLDL are taken up by macrophages and become what is termed 'foam cells'. Macrophages continue to take up oxLDL regardless of the amount they have initially consumed and eventually become necrotic due to the high lipid content. This is the beginning of the formation of a fatty streak in the intima. Foam cell generation stimulates the production and release of cytokines such as tumor necrosis factor alpha (TNFα), which promotes an inflammatory reaction resulting in the recruitment of more monocytes from the bloodstream into the intima [1]. As monocytes accumulate in

the intima, more and more foam cells are produced, which enhances the growth of the lipid core in the artery wall. Further lesion growth can cause smooth muscle cells to migrate into the intima and encapsulate the lipid core in collagen, forming a fibrous cap [5]. The fibrous cap essentially stabilizes the plaque by walling off the lipid core from the blood stream [81]. Growth of the lesion can lead to stenosis (lumen narrowing) and potentially thrombosis (blood clot formation). These processes can partially or completely obstruct blood flow, which may cause tissue ischemia or infarction.

3.3. Blood Rheology

Whole blood is a suspension composed of approximately 55% formed elements (cells) and 45% plasma (liquid fraction). The formed elements consist of red blood cells, white blood cells (i.e. leukocytes), and platelets, while plasma contains water (92%), plasma proteins (7%), and other solutes (1%). At low shear rates blood can behave as a non-Newtonian fluid, whereby red blood cells cross-link and form rouleaux. As shear rate increases the rouleaux stacks break apart and the fluid approaches Newtonian behavior [82, 83]. Blood flow in most vessels in the body with the exception of small capillaries (< 0.5 mm) is often approximated to display Newtonian behaviour with a viscosity of 3.5 to 4 centipoise (cP) [82, 84].

3.4. Endothelial Cell Response to Hemodynamic Forces

It is now well established that endothelial cells respond to the local hemodynamic environment by altering their gene expression profile and cell morphology. Located at the interface between the vessel wall and blood, endothelial cells experience three types of biomechanical forces: cyclic strain (stretch), hydrostatic pressure, and wall shear stress [85]. It has been shown that endothelial cells respond to all three forces [86-88], however, wall shear stress is studied most. The general observation is that endothelial cells located in relatively

straight regions of the vascular tree and exposed to high unidirectional blood flow express a phenotype that is conducive to maintaining normal endothelial cell function and is an environment that protects against the formation of atherosclerotic lesions (i.e. athero-protective hemodynamics) [6, 74]. In contrast, endothelial cells that experience complex wall shear stress patterns, express genes that support inflammation and plaque growth (i.e. athero-prone hemodynamics) [74, 85].

3.4.1. Wall Shear Stress

Wall shear stress is the frictional force exerted by the flow of fluid over a surface and is commonly presented in units of dynes/cm^2 when describing blood flow in arteries and veins. Shear stress acts tangential to the surface in the direction of blood flow. Wall shear stress is commonly in the range of 1 – 6 dynes/cm^2 in veins and 10 – 70 dynes/cm^2 in arteries [4]. For a Newtonian, incompressible fluid under steady fully developed 1D flow, shear stress (τ) is proportional to the shear rate (dv/dr) with dynamic viscosity (μ) as the proportionality constant.

$$\tau = \mu \frac{dv}{dr} \qquad (1)$$

Fully developed steady laminar flow through a straight tube produces a parabolic velocity profile with maximum velocity at the center and zero at the wall. Consequently, shear stress is maximum at the wall and minimum in the center. The Hagen-Poiseuille solution can be used to relate wall shear stress to the volumetric flow rate (Q), equation 2.

$$\tau = \frac{4\mu Q}{\pi r^3} \qquad (2)$$

3.4.2. *In Vitro* Models

Most of our knowledge on endothelial cell response to shear stress has come from *in vitro* cell culture models such as the cone-plate viscometer or the parallel-plate flow chamber (Figure 3-3). These flow devices enable the analysis of endothelial cell response to controlled levels of shear stress and have provided abundant information on flow induced changes in endothelial cell morphology [89, 90], function [74, 91, 92], and gene expression [93, 94]. However, blood flow in arteries is much more complex than steady laminar flow due to the pulsatile nature of the cardiac cycle, the tortuous geometry of blood vessels, and changes in vessel diameter as a result of dilation/contraction. Consequently, endothelial cells *in vivo* are exposed to complex temporal and spatial gradients in fluid wall shear stress [95, 96]. Temporal gradients are defined as the change in shear stress at a given location over time. On the other hand, spatial gradients are the difference in shear stress between two adjacent points at a given time [73]. Several groups have modified the cone-plate viscometer or the parallel-plate flow chamber to examine endothelial cell response to temporal [97, 98] and spatial [99, 100] gradients in wall shear stress. For instance, Nagel et al introduced a rectangular bar in the flow field of a cone-plate viscometer to create reversing flow immediately after the perturbation [99]. Laminar shear stress was re-established further downstream. This study showed that endothelial cell gene expression was different between reversing flow and laminar flow. Specifically, endothelial cells exposed to reversing flow induced the expression of a number of transcription factors, including nuclear factor-κB (NF-κB), early growth response-1 (Erg-1), and activator protein-1 (AP-1) [99]. Himburg et al. studied the response of endothelial cells to different frequencies in a parallel-plate flow chamber [101]. They observed that 1 Hz reversing (7.5 ± 15 dynes/cm^2) or oscillating (0 ± 15 dynes/cm^2) flow repressed the expression of VCAM-1 relative to steady flow and that the effect was reversed at 2 Hz. These results suggest that atherosclerotic lesion development is potentially greater at areas in the vasculature exposed to reversing flow at higher frequencies than the normal heart rate.

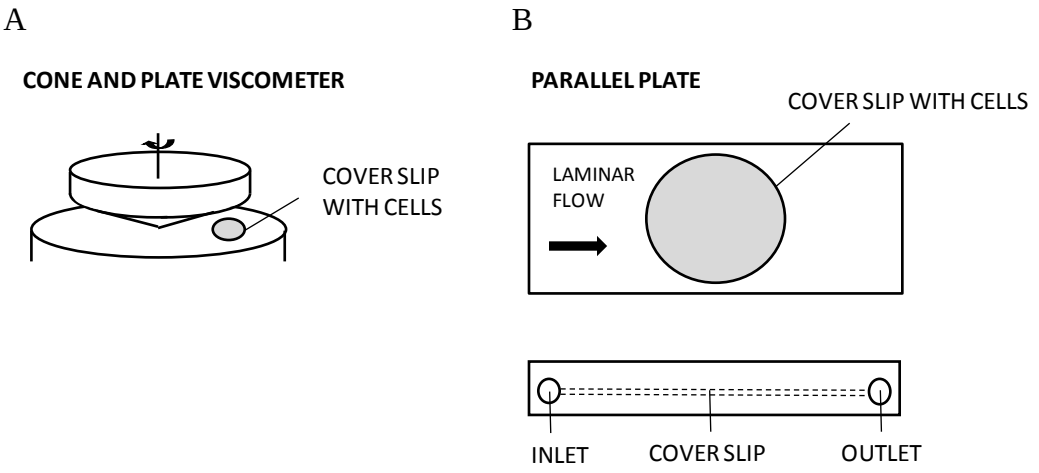

Figure 3-3: Schematic diagrams of a (A) cone-plate viscometer redrawn from Dewey et al (1981) The Dynamic Response of Vascular Endothelial Cells to Fluid Shear Stress [90] and (B) parallel-plate flow chamber redrawn from Levesque et al (1985) The Elongation and Orientation of Cultured Endothelial Cells in Response to Shear Stress [89]

3.4.3. Mechanotransduction in Endothelial Cells

Although it is well known that endothelial cells respond to mechanical stimuli, the mechanism in which the cells sense the hemodynamic environment in which they are exposed to and transduce these forces into a biochemical signal is not well understood. Endothelial cells may sense mechanical forces through mechanosensors that in turn trigger signalling pathways within the cell. There are potentially multiple pathways in which the endothelial cells sense and transduce the mechanical environment. Proposed sensors of mechanical forces include ion channels, growth factor receptors, G proteins, calveolae, adhesion proteins, the cytoskeleton, the glycocalyx, and primary cilia [102-105]. The signalling pathways triggered by mechanosensors activate transcription factors, including NF-κB, AP-1, Kruppel-like factor 2 (KLF2), and Erg-1 promoting the transcription of a variety of genes [93, 99, 106]. Transcription factors are proteins that bind to the promoter region of specific genes, facilitating the transcription of the gene to RNA. Interestingly, Ohura et al, reported using DNA microarray analysis that fluid shear stress modulated roughly 3% of the 5600 genes evaluated [94].

The upregulation/downregulation of many genes by wall shear stress has been extensively examined [107]. Some of the most commonly studied include eNOS, KLF2, TM, E-selectin, NF-κB, VCAM-1 and ICAM-1. The following sections briefly describe the reported effects of wall shear stress on the regulation of the aforementioned genes.

KLF2

KLF2 has been identified as a shear responsive transcription factor that is strongly upregulated by steady and pulsatile flow [93]. Comparison of the two flow profiles revealed that unidirectional pulsatile shear stress enhanced KLF2 mRNA levels to a greater extent than steady flow [93, 108], suggesting the importance of temporal shear stress gradients. High expression of KLF2 is considered to impart atheroprotective properties to endothelial cells since this transcription factor is reported to upregulate numerous genes vital to maintaining vascular homeostasis. TM [91, 109] and eNOS [91, 109, 110] are among the vasoprotective genes shown to be regulated by KLF2. Indeed, Dekker et al. demonstrated that lentivirus-mediated overexpression of KLF2 in human umbilical vein endothelial cells (HUVEC) resulted in enhanced eNOS and TM protein levels compared to controls [91]. Consistent with these findings, Lin et al demonstrated that knockdown of KLF2 with small interfering RNA (siRNA) reduced eNOS and TM protein below control levels [109].

Analysis of human iliac arteries by *in situ* hybridization revealed higher KLF2 mRNA levels at the inner wall of the bifurcation where wall shear stress is typically laminar and high [108]. In contrast, endothelial cells exposed to low shear stress at the outer wall of the iliac bifurcation demonstrated substantially less KLF2 mRNA levels and neointimal thickening was detected. The outer walls of bifurcations are characteristically high probability areas for initial atherosclerotic plaque formation. Consequently, low endothelial expression of KLF2 may

partially explain why lesions form preferentially in regions of low/disturbed shear stress.

NF-κB

Leukocyte infiltration and accumulation in the vascular intima represents one of the early stages in atherogenesis. This process may involve transcription factor NF-κB, which is known to upregulate the expression of several pro-inflammatory genes [111, 112]. In unstimulated cells, NF-κB is attached to an inhibitory component, IκB and the entire complex is located in the cytoplasm. Upon stimulation, IκB becomes phosphorylated and degrades [113]. Subsequently, the liberated p50/p65 subunits of NF-κB translocate from the cytoplasm to the cell nucleus. In the nucleus, the p50/p65 subunits bind to the promoter region of several pro-inflammatory genes including ICAM-1, VCAM-1, and E-selectin, stimulating their transcription [112, 114-117]. Activation of NF-κB is a vital element in inflammation and is believed to contribute to atherogenesis. Known activators of NF-κB include fluid shear stress, cytokines, lipopolysaccharide (LPS), oxidized low density lipoproteins (LDLs) and oxidative stress [111, 117-119].

Laminar shear stress (12 dynes/cm^2) has been reported to increase NF-κB DNA binding within 30 min of the onset of flow and reached maximum levels at 60 min [106]. Similarly, Mohan et al. showed that high laminar shear stress (16 dynes/cm^2) transiently enhanced NF-κB DNA binding to a maximum at 30 min [120]. However, long-term (24 h) exposure to high shear stress reduced NF-κB DNA binding to less than static controls. It was also observed that under low shear stress (2 dynes/cm^2), NF-κB DNA binding increased with time and was maintained higher than static cells at 24 h. In another study, Nagel et al. showed that endothelial cells exposed to low reversing flow expressed greater NF-κB activation than cells under steady flow and in static culture [99]. The differential regulation in NF-κB activation between high uniform shear stress and low/reversing flow may help to explain the focal nature of lesion formation at

sites where blood flow is disturbed. Indeed, *in vivo* studies have shown that activated NF-κB and the expression of NF-κB inducible genes are highly expressed at lesions prone regions of the vasculature, whereas much lower levels are detected in atherosclerosis resistant areas [113, 121, 122].

eNOS

Laminar shear stress has been shown in numerous studies to increase eNOS mRNA and protein expression [123-130] The mechanism responsible for superior eNOS expression from shear stress may be a combination of increased *de novo* transcription and enhanced mRNA stability [131]. Using nuclear run-on analysis, Davis et al showed that laminar shear stress increased eNOS gene transcription within the first hour of flow, but then quickly returned to control levels. However, a prolonged increase in eNOS mRNA stability was observed. Interestingly, Weber et al. and demonstrated a link between 3' poly(A) tail length and shear stress on enhanced eNOS mRNA stability [132]. They demonstrated that an increase in shear stress magnitude and duration lengthened the eNOS 3' poly(A) tail and enhanced the stability of the transcripts.

Ziegler et al. compared eNOS mRNA levels in endothelial cells exposed to low oscillating shear stress, representative of atheroprone regions of the vasculature, with higher unidirectional pulsatile flow [125]. They reported that non-reversing pulsatile flow enhanced eNOS mRNA, whereas oscillating flow was comparable to static conditions. A similar observation was made by Cicha et al., whereby less eNOS protein was detected in cells conditioned with oscillating shear stress compared to laminar flow [133]. These studies support the hypothesis that disturbed shear stress can cause flow induced endothelial cell dysfunction through reduced NO bioavailability.

TM

Reported effects of shear stress on endothelial cell expression of TM are somewhat inconsistent. The majority of studies have shown that unidirectional shear stress increases TM mRNA and proteins in a time and magnitude dependent manner [127, 128, 134, 135]. Given that TM is an atheroprotective gene through its anti-coagulant properties, these results are consistent with the hemodynamic hypothesis for the focal nature of atherosclerotic plaques, whereby regions exposed to high shear stress are more protected from developing lesions. However, there have been some reports that shear stress reduces TM expression [136, 137]. Gosling et al. showed that TM protein levels decreased when the human saphenous vein was treated with arterial levels of shear stress for 45 and 90 min [137]. This study presents a potential mechanism for occlusion of saphenous vein bypass grafts, however does not represent the response of native arterial endothelial cells to shear stress. Malek et al. found that TM mRNA and protein levels in bovine aortic endothelial cells decreased with increasing magnitude and duration of laminar shear stress [136].

ICAM-1 and VCAM-1

ICAM-1 and VCAM-1 are cell adhesion molecules expressed on the surface of endothelial cells. Both ICAM-1 and VCAM-1 are involved in the firm arrest of leukocytes onto the vessel wall following initial rolling and tethering mediated by selectins. ICAM-1 is basally expressed in endothelial cells, whereas VCAM-1 is only present upon stimulation. Both of these cell adhesion molecules are greatly upregulated in the presence of cytokines such as TNFα and IL-1β [138, 139]. The expression of these cells adhesion molecules in vascular endothelial cells may contribute to early lesion formation by promoting the migration of inflammatory cells into the intima [140]. Both VCAM-1 and ICAM-1 are expressed in lesion prone areas of the vasculature before atheroma formation, as well as in established plaques [121, 141]. *In vivo* animal studies have identified VCAM-1 as

having a more dominant role in early atherogenesis than ICAM-1 [121, 142]. Given that VCAM-1 knockout mice do not survive, VCAM-1 domain 4-deficient (D4D) mice ($Vcam1^{D4D/D4D}$) were developed to elucidate the role of VCAM-1 in the initiation of atherosclerosis. In $Vcam1^{D4D/D4D}$ mice, VCAM-1 mRNA and protein expression was 2-8% of the wild type. It was observed that deficiency in VCAM-1 not ICAM-1 reduced early lesion area size in LDL receptor (LDLR) knockout mice ($ldlr^{-/-}$) when fed a proatherogenic diet [142]. Moreover, VCAM-1 is expressed exclusively in lesion susceptible areas and established atheroma, whereas ICAM-1 is present in both lesion prone and protected regions [121]. These results suggest that the regional expression differences of VCAM-1 observed *in vivo* may be a response to shear stress.

Numerous *in vitro* cell culture experiments have shown that uniform laminar flow reduces VCAM-1 mRNA and protein expression [135, 143-145]. Few studies have observed no effect of laminar flow on VCAM-1 expression [146, 147]. In contrast, laminar flow has been found to increase ICAM-1 mRNA and protein [8, 145-148]. Sampath et al. found that the increase in ICAM-1 from laminar shear stress (15 dynes/cm^2) was transient, whereby the protein and mRNA returned to basal levels or less, respectively after prolonged exposure to flow [145]. Similarly, Topper et al. observed that laminar flow (10 dynes/cm^2) increased ICAM-1 mRNA above control levels at 1 and 6 h after the onset of flow and then were reduced to less than basal levels at 24 h [148].

Endothelial cells respond differently to disturbed and uniform flow. In contrast to steady shear stress, oscillating or disturbed flow has been shown to increase both VCAM-1 and ICAM-1 expression [149-151]. These results may explain the higher expression of VCAM-1 in lesion prone regions.

E-Selectin

Selectins mediate the initial rolling and tethering of leukocytes on the endothelium. These interactions are weak and reversible. There are three known selectins: L-selectin, P-selectin and E-selectin. L- and E-selectins are expressed in leukocytes and endothelial cells, respectively. P-selectin is found in both platelets and endothelial cells [40]. E-selectin is present at very low levels under basal conditions, but is strongly induced by cytokines and lipopolysaccharide [118]. Its expression may also be modulated by shear stress. Sampath et al. found that laminar shear stress (10 dynes/cm^2) slightly decreased E-selectin mRNA [145]. Tsou et al. observed that low wall shear stress (2 to 4 dynes/cm^2) upregulated E-selectin, while high shear stress (> 8 dynes/cm^2) reduced expression below control levels [152]. Conversely, some studies reported that steady flow had no effect on E-selectin mRNA and protein [8, 147].

3.5. Statin Drugs

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (also known as statins) are a class of lipid-lowering drugs that are given to patients with hyperlipidemia, a major risk factor for heart disease. These drugs have been shown in the clinic to greatly reduce cardiovascular related morbidity and mortality [9-11, 153-155]. Many different types of statins are presently approved for clinical use. These include simvastatin, lovastatin, mevastatin, rosuvastatin, fluvastatin, cerivastatin, pitavastatin, atorvastatin, and pravastatin. The aforementioned statins are lipophilic with the exception of pravastatin and rosuvastatin [156-158]. They all competitively inhibit HMG-CoA reductase preventing the synthesis of mevalonate, a precursor to cholesterol and cholesterol intermediate synthesis (Figure 3-4).

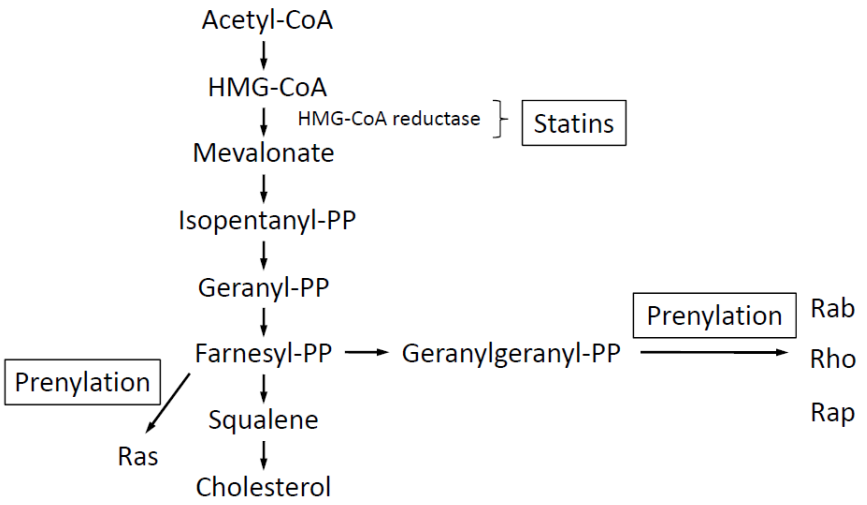

Figure 3-4: Cholesterol biosynthesis. Adapted from Rikitake and Liao (2005) Rho GTPases, Statins and Nitric Oxide [159]

3.5.1. Cholesterol Reduction

Statins reduce cholesterol levels by inhibiting HMG-CoA reductase, the rate limiting enzyme in the synthesis of cholesterol. By preventing the biosynthesis of cholesterol, statins decrease hepatic production of LDL and upregulate LDL receptors resulting in lower concentrations of circulating LDL in the bloodstream [160]. These drugs can reduce circulating LDL levels anywhere from 19% to 60% depending on the dose, patient, and type of statin [13]. In addition to lowering LDL cholesterol, statins have been shown to increase high density lipoproteins (HDLs) and reduce serum triglyceride levels [158]. Consequently, by altering serum LDL, HDL, and triglyceride levels, statins can slow down the progression of atherosclerotic plaques and reduce the incidences of cardiovascular events [14].

3.5.2. Non-lipid Lowering Effects

Although originally designed to lower cholesterol levels, the benefits of statins are believed to extend beyond reducing plasma LDL. This hypothesis stems from the repeated observation that the benefits of statins in the clinic cannot be explained by cholesterol reduction alone [155, 161]. For instance, simvastatin improved endothelial cell function in patients before a noticeable reduction in serum cholesterol levels [162]. In another study, it was shown that patients given pravastatin had a lower risk for a cardiovascular event than the placebo group despite similar LDL cholesterol levels [153]. The potential pleiotropic effects of statins were also demonstrated in a study that compared endothelial cell function in patients given a 4 week treatment of simvastatin or ezetimibe [163]. Ezetimibe is a drug that lowers plasma LDL by inhibiting cholesterol absorption in the intestine [164]. In this study they found that endothelial cell function was improved with simvastatin but not with ezetimibe despite a similar reduction in cholesterol levels [163].

The mechanism in which statins achieve these proposed non-lipid lowering effects is not completely understood, however, ample evidence attribute these beneficial effects to inhibiting the function of small GTP-binding proteins (also known as GTPases) such as Ras, RhoA, Rac1, and Cdc42 [159, 165, 166]. Inhibition of HMG-CoA reductase by statins prevents the synthesis of mevalonate a precursor to isoprenoid intermediates farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Figure 3-4). GGPP and FPP post-translationally modify a number of intracellular signaling molecules including Ras, RhoA, Rac1, and Cdc42. Prenylation of these small GTPases by FPP or GGPP is required for their proper membrane localization and activation [167, 168]. These proteins have a wide range of cellular activities including the activation of certain transcription factors (e.g. NF-κB), the generation of reactive oxygen species, and the regulation of cell morphology, adhesion, proliferation and motility [167, 169-172]. The GTPases remain inactive in the cytoplasm in the absence

of cellular GGPP and FPP, providing a potential mechanism for the pleiotropic effects of statins.

3.5.3. Effect of Statins on Endothelial Cells

Statins have been shown to modulate endothelial cell gene expression in a mechanism that is independent of lowering lipid levels. Genes reported to be modulated by statins include KLF2, eNOS, TM, E-selectin, P-selectin, VCAM-1, ICAM-1, endothelin-1, PAI-1, and tPA [59, 128, 173-187]. These genes are involved in various biochemical processes such as inflammation, vasoreactivity, thrombosis, and fibrinolysis. Consequently, statins have the potential to impact the progression of vascular diseases such as atherosclerosis independent of reducing blood LDL levels through its effects on gene expression in endothelial cells.

Several different studies have shown that statins significantly increase KLF2 [15, 128, 180, 188], eNOS [128, 175, 182, 183], TM [59, 128, 175, 178, 179], and tPA [176] expression in cultured endothelial cells in a concentration and time dependent manner. The effect can be completely abrogated when the statin is co-treated with mevalonate [59, 180, 183]. Consequently, the mechanism in which statins modulate the expression of KLF2, eNOS, TM, and tPA is through inhibition of HMG-CoA reductase and mevalonate synthesis. A closer look into the cholesterol biosynthesis pathway has revealed that inhibition of GGPP but not FPP is the dominant mechanism in which statins alter endothelial cell gene expression [15, 59, 179, 183]. RhoA, Rac1 and Cdc42 are prenylated by GPP, while FPP prenylates Ras. Indeed, it has been demonstrated in many studies that statins modulate endothelial cell gene expression mainly through inhibition of small GTPases of the Rho family (Rac1/Cdc42) [59, 178, 183].

The effect of statins on selectins and cell adhesion molecules is controversial and contradictory. Several studies have shown that statins potentiated the TNFα-induced increase in E-selectin, VCAM-1 and ICAM-1

mRNA and/or protein expression [189-192], whereas other groups have reported that statins decreased their expression [16, 193-195]. On the other hand, there is also evidence that statins had no effect on the TNFα-induced increase in ICAM-1 [185, 196, 197]. The wide variability in results may be due to differences in cell lines, type of statin, concentration, quantification method, and the duration of statin and cytokine stimulation. Interestingly, Landsberger et al. reported that cerivastatin increased VCAM-1 and ICAM-1 mRNA levels and degraded IκB in TNFα-stimulated endothelial cells in a concentration dependent manner [184]. However, it was also observed that cerivastatin increased the levels of soluble VCAM-1 and ICAM-1 protein, but reduced their surface expression [184]. They proposed that the reduction in VCAM-1 and ICAM-1 surface expression by cerivastatin is a result of increased protein shedding. Chung et al. found that although lovastatin had no effect on the TNFα-induced increase in ICAM-1, it reduced the effect of interferon-γ (IFN-γ) [196]. Similarly, Takeuchi et al. observed that cerivastatin suppressed the LPS-induced increase in ICAM-1, but not TNFα [185]. As a result of the large variation in reported effects, further studies are required in more physiologically realistic conditions to elucidate the effect of statins on endothelial cell expression of selectins and cell adhesion molecules under cytokine stimulation. This would provide a better understanding of the mechanism in which statins reduce vascular inflammation independent of lowering serum LDL levels.

3.5.4. Effect of Statins on Leukocytes

In addition to targeting endothelial cells, statins have also been shown to affect leukocytes. Yoshida et al. found that pre-treating monocytes (U937 cell line) with cerivastatin for 48 h significantly reduced their firm adhesion onto activated endothelial cells under steady flow conditions (shear stress = 1.0 and 2.0 dynes/cm^2, for 10 min) [198]. The effect was attributed in part to a downregulation in integrin adhesion molecules. In another study by the same group, Hiraoka et al. reported a significant inhibition of monocyte conversion from rolling to stable

adhesion under fluid flow (shear stress = 1.0 dyne/cm^2, for 10 min) when monocytes (THP-1 cell line) were pre-incubated with pitavastatin [199]. The effect was abolished when monocytes were simultaneous treated with mevalonic acid. The altered interaction between monocytes and endothelial cells by pitavastatin was attributed to a modification in MCP-1 mediated conversion of THP-1 cells from rolling to stable adhesion through a RhoA-dependent pathway. Islam et al showed that treatment of LPS stimulated U937 cells with atorvastatin significantly reduced their attachment to cultured endothelial cells by decreasing the expression of VLA-4 and CD11b [200].

It has also been shown that statins can alter leukocyte-endothelial cell interactions independent of inhibition of HMG-CoA reductase. Studies have demonstrated that statins inhibit lymphocyte-function-associated antigen-1 (LFA-1) interactions with ICAM-1 by binding to the inserted (I) domain, which locks the integrin in an inactive state [201-203]. This interferes with the firm adhesion step in the leukocyte-adhesion cascade, providing another mechanism for the pleiotropic effects of statins.

3.6. Interaction between Wall Shear Stress and Statins on Endothelial Cell Function

It is now well known that both wall shear stress and statin drugs can modulate endothelial cell gene expression. Moreover, shear stress and statins alter the expression of many of the same genes. At present, the literature available on endothelial cell response to statins under flow conditions is limited. Knowledge of potential shear stress-statin interactions is important in order to better understanding of the non-lipid lowering effects of these drugs in patients. Recently, Ali et al. (2009) showed that preconditioning HUVEC with laminar shear stress (12 dynes/cm^2) followed by the addition of atorvastatin and then continuing the culture under flow conditions produced a synergistic increase in the cytoprotective enzyme heme oxygenase-1 (HO-1) [204]. Conversely, oscillating

shear stress (1 Hz, ± 5 dynes/cm^2) impaired the ability of atorvastatin to increase HO-1 expression. This study was the first to show the presence of shear stress-statin interactions, whereby steady laminar flow enhanced endothelial cell responsiveness to statins and oscillating flow impaired it.

Few studies have examined the effect of statins on endothelial cell expression of inflammatory markers such as P-selectin, E-selectin, VCAM-1 and ICAM-1 under flow [195, 197]. Eccles et al. (2008) reported that pre-conditioning endothelial cells with 1 µM simvastatin or fluvastatin for 6 h followed by steady flow (1.1 dynes/cm^2) reduced P-selectin and E-selection mediated neutrophil-endothelial cell interactions. The effect of the statins was completely abrogated when co-treated with mevalonate. These results support the hypothesis that statins may have anti-inflammatory properties, potentially by interfering with leukocyte-endothelial cell interactions through inhibition of HMG-CoA reductase. Similarly, Cicha et al. (2009) showed that 1 µM simvastatin reduced the non-uniform shear stress and TNFα-induced increase in VCAM-1 protein and monocyte adhesion [195].

3.7. Summary

The benefits of statins in the clinic cannot be explained by cholesterol reduction alone. This has led to the hypothesis that statins may have non-lipid lowering or pleiotropic effects on vascular cells. It has been shown in numerous studies that statin drugs can modulate gene expression in endothelial cells and interfere with leukocyte-endothelial cell interactions. Presently, the response of endothelial cells to statins under flow is limited. Specifically, knowledge of whether fluid shear stress alters endothelial cell response to statins is minimal. Evaluation of the potential shear stress-statin interactions would provide a better understanding of the effects of statins on the endothelium *in vivo* and enhance our understanding of the non-LDL cholesterol lowering benefits of these drugs. The work presented in this thesis is my attempt to provide more insight into shear stress-statin interactions.

CHAPTER 4: CONCENTRATION AND TIME EFFECTS OF DEXTRAN EXPOSURE ON ENDOTHELIAL CELL VIABILITY, ATTACHMENT, AND INFLAMMATORY MARKER EXPRESSION IN VITRO

4.1. Preface

Prior to evaluating the effect of statins on endothelial cells under flow, we investigated various methods of achieving physiologically relevant shear stress with our flow loop. Many published *in vitro* studies on endothelial cell response to fluid wall shear stress have used dextran to increase the viscosity of cell growth medium in order to better match physiological parameters. However, it is unknown whether endothelial cells respond to the presence of dextran in the culture medium. Specifically, we asked the question does dextran alter endothelial cell viability, gene expression and neutrophile attachment. Knowledge of the potential effects of dextran on endothelial cells will reduce the possibility for misleading or confounding results. In this study, we found that dextran has a non-negligible effect on cultured endothelial cells. In particular, the conclusions drawn from the effect of fluid shear stress on endothelial cell gene expression are different depending on whether time-matched dextran containing static controls are used. This article will aid in the design of future flow experiments and help prevent artifacts. Indeed, we decided to reduce the diameter of our models to achieve physiologically relevant shear stress levels in the studies with statins rather than potentially confound the results with dextran. The article is published in *Annals of Biomedical Engineering Vol. 38, No. 4 (2010) pp. 1451-1462.*

4.2. Article 1

Concentration and Time Effects of Dextran Exposure on Endothelial Cell Viability, Attachment and Inflammatory Marker Expression *in Vitro*

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4.3. Abstract

Dextran is commonly used to alter growth medium rheological properties for *in vitro* flow experiments in order to match physiological parameters. Despite its acceptance in literature, few studies have examined dextran effects on cells. In this study, we investigated changes in endothelial cell function due to dextran, under static and flow conditions, in a concentration and time dependent manner. Dextran increased endothelial cell viability, decreased their ability to attach to culture plates and decreased leukocyte adhesion to endothelial cells. Under static conditions, dextran increased protein and mRNA expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in a concentration and time dependent manner and caused the nuclear translocation of NF-κB. Steady laminar wall shear stress modulated the effects of dextran on ICAM-1, VCAM-1 and NF-κB expression in straight/tubular *in vitro* models. When the expression was normalized to their respective time matched static dextran control, it did not affect the ability to detect changes caused by shear on the mRNA expression of ICAM-1 and VCAM-1. This study demonstrates that dextran can alter endothelial cell function and therefore, caution is advised and time matched dextran controls are necessary when using dextran for dynamic cell studies.

4.4. Introduction

Hemodynamic forces have been linked to atherosclerosis and thus, many studies have examined the response of endothelial cells to flow. There is strong evidence that shear stress represents a source of endothelial injury and can contribute to the initiation and progression of atherosclerosis. *In vitro* devices such as the parallel plate flow chamber and the cone-plate viscometer have been developed to study, in a controlled manner, the relationship between wall shear stress and endothelial cell function. Studies using these models have provided abundant information on changes in endothelial cell cytoskeleton, uptake of macromolecules, channel activations, release of pro-inflammatory molecules and vasoactive substances [5,14,17,18,22,35,36,49]. The adhesion of blood components to endothelial cells in response to mechanical stimuli has also been examined to better understand the role of inflammation in cardiovascular diseases [39,50].

Performing *in vitro* flow experiments requires the use of growth medium which is much less viscous than blood. Although non-dimensionalization can be used to overcome these property differences, limitations in pump flow rate or in device geometry make it necessary or desirable to alter the growth medium viscosity. Dextran has often been used for this as it is believed to have little side effects [5,12,13,37,46]. It has been used in other applications because of its effects on blood components [6,7]. Clinically, low molecular weight dextran (MW 40,000) is used to expand blood volume and reduce blood clotting [15,51], hence reducing risks of thrombosis and as an anticoagulant [3,9,15,21,29,51]. Dextran has been shown to inhibit platelet aggregation at lower concentrations (MW 10,000 – 40,000) [7,23,53]. Considering the known effects of dextran on blood components, it is surprising that very few studies have examined its effects on endothelial cells and compared it to results without dextran [52]. In this study, we report the temporal and concentration effects of dextran on endothelial cell viability and attachment to plates, leukocyte adhesion to endothelial cells and endothelial cell inflammatory marker expression under static and flow conditions.

4.5. Methods

4.5.1. Cell culture

Human abdominal aortic endothelial cells (HAAECs) derived from a 20 year old male were purchased from ATCC (American Type Culture Collection, CRL-2472) and expanded up to passage 5. Cells were cultured in endothelial cell growth medium (Promocell, C-22010, C-39215), supplemented with 10% fetal bovine serum (Invitrogen, Gibco, 26140-079) and 1% penicillin streptomycin (Invitrogen, Gibco, 15140-122) in tissue culture flasks coated with 0.1% pig gelatin (Sigma-Aldrich, G2500). At confluency, cultures were rinsed with phosphate buffered saline solution (PBS) and harvested with 0.25% Trypsin-EDTA (Invitrogen, 25200-072).

4.5.2. Modifications of cell culture medium using dextran

The viscosity of the cell culture medium was altered with dextran (MW 135,000) (Sigma-Aldrich, D4876), as used in previous studies [37]. Dextran powder was weighed and dissolved in cell culture medium. The solutions were allowed to degass and solubilize before filtering through a polyethersulfone membrane with a pore size of 0.22 µm (Fisher Scientific, 09-761-11). The solutions were stored at 4°C for up to one month. The viscosity was verified by rheological testing with a double gap rheometer (Bohlin, Model CVO 120 HR) at a constant temperature of 37°C. Measurements were taken over a range of shear rates to verify the Newtonian behavior of the solutions. Density was measured using a pycnometer (Fisher Scientific, 3-247).

4.5.3. Cell viability assay

The effect of dextran (0 - 17.5% w/w) on endothelial cell viability was measured by a MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide, Sigma-Aldrich, M5655). HAAECs were plated in

96-well cell culture plates at a density of 2 x 10^5 cells/cm^2 and left to adhere overnight in unaltered medium. The medium was then replaced with growth medium containing dextran and incubated at 37°C. After 24 hrs, 10% v/v MTT (5 mg/mL) was added to the wells and incubated for 2 hrs. Afterwards, 100% v/v of detergent (0.01 M HCl, 10% SDS) was added to lyse the cells and solubilize the colored crystals. After 2 hrs incubation at 37°C, the absorbance was measured at 570 nm in a Benchmark Plus plate reader (Bio-Rad) [34].

4.5.4. Endothelial cell attachment assay

The effect of the presence of dextran (0 - 17.5% w/w) on the attachment of endothelial cells to 96-well cell culture plates was also evaluated by seeding cells in the wells in growth medium containing dextran at a concentration of 2 x 10^5 cells/cm^2. Cells were left to adhere for 24 hrs, after which the wells were washed with PBS before the MTT assay was performed to quantify the number of viable cells that attached.

4.5.5. Straight/tubular in vitro models

The effects of dextran on leukocyte adhesion to cultured endothelial cells and endothelial cell inflammatory marker expression in response to shear stress were tested in straight/tubular cell culture models. These models have been used in previous studies to evaluate the effect of wall shear stress and statins on endothelial cell response and leukocyte adhesion [19,40-42]. The models were built by pouring a non-toxic, transparent and compliant material, Sylgard184™ in an acrylic mould with a centered stainless steel rod (3.175 mm inner diameter) and connectors. Once the silicone was cured, the rod was removed to create the three dimensional *in vitro* model. The models were hydrophilized using 70% sulphuric acid for 45 minutes, boiled in sterile deionized water for 30 minutes and coated with 40 μg/mL fibronectin in deionized water (Sigma-Aldrich, F0895) overnight

at 37°C on a rotator at 8 rpm (Labquake Rotor, Series 1104, Barnstead/Thermolyne). Solutions were aspirated and the models washed with media once before cells were seeded (1 mL, 1 x 10^5 cells/cm^2) at a density of 1 x 10^6 cells/mL (± 2 x 10^5 cells/mL) (Beckman Coulter, Canada). The models containing the cell suspension were attached to a rotator for even coverage of ECs on the surface. Growth medium was changed the day after seeding. Cells were incubated for a total of 48 hrs, i.e. when a confluent monolayer had been established, before the experiments were performed.

4.5.6. Leukocyte adhesion assay

The effect of dextran (0 & 6.7 % w/w) was tested on leukocyte adhesion to endothelial cells under static conditions in the previously described *in vitro* models. An acute promyelocytic leukaemia cell line (NB4 cells) was used to quantify the adherence of leukocytes to non-stimulated and tumor necrosis factor alpha (TNF-α) stimulated endothelial cells. NB4 cells were maintained in T75-flasks as a suspension culture at 2x10^5 - 1x10^6 cells/mL in RPMI 1640 medium with 2 mM L-glutamine (Hyclone, SH3002701), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, 26140-079) and 1% penicillin streptomycin (Invitrogen, 15140-122). For differentiation into granulocytes, cells were stimulated for 48 hrs in the presence of 10^{-6} M all-*trans*-retinoic acid (ATRA) (Sigma R2625). Tumor necrosis factor alpha (TNF-α) was used for 24 hrs at 10 ng/mL (GF 023, Chemicon) in order to create an inflamed endothelium. NB4 cells were allowed to adhere in static conditions, at a concentration of 5x10^5 cells/mL, on endothelial cells in the straight/tubular *in vitro* models for 1 hr, after which non adherent cells were removed by washing the models with unaltered growth medium three times. These experiments allowed the study of the adhesive properties of NB4 cells on ECs in static conditions by manually counting the number of NB4 cells adhered from light microscope images (10X).

4.5.7. Static conditions

The effect of dextran concentration on cell expression under static conditions was studied in 6-well plates at a seeding concentration of 1 x 10^5 cells/cm^2. Cells were grown for 24 hrs in standard medium and incubated for 24 hrs with modified growth medium at different concentrations (0 - 17.5% w/w). Cells were gathered and protein and mRNA analysis were performed. The effect of incubation time (0 – 24 hrs) was tested under static conditions in the *in vitro* models at a concentration of 6.7 % w/w, as it increases medium viscosity to 3.5 cP, similar to blood viscosity.

4.5.8. Flow experiment

The three dimensional *in vitro* models were used to study the response of endothelial cells, under different dextran concentrations (0, 7 & 14%), to long term (24 hrs) unidirectional laminar wall shear stress exposure. The effect of incubation time was tested using a dextran concentration matching blood viscosity (6.7 %) and using time matched dextran containing static controls. The flow loop consisted of individual reservoirs, silicone tubing, flow dampeners and an 8-rollers peristaltic pump head with a programmable drive (Ismatec, ISM 404 and ISM 732) used to produce a steady flowrate. The resulting flow parameters are listed in Table 4-1 and Table 4-2. Dextran concentrations were varied between 0 and 14% w/w. The entire flow loop was located in at 37°C incubator with 5% CO_2. After the perfusion, the cells were gathered from the models with 0.25% Trypsin/EDTA for western blotting or quantitative RT-PCR or fixed in 1% paraformaldehyde and stored in glycerol:PBS (1:1, v/v) at 4°C for immunofluorescent staining.

Table 4-1: Hemodynamic data concerning the RNA expression analysis (10 dyn/cm^2)

Dextran concentration (%)	0	7	14
Density (kg/m^3)	998.2	1021.4	1046.3
Viscosity (cP)	0.975	3.778	10.692
Flowrate (mL/min)	193.36	49.90	17.63
Re	1323	90	12
Shear rate (s^{-1})	1025.6	264.7	93.5
Shear stress (dyne/cm^2)	10	10	10

Table 4-2: Hemodynamic data concerning the protein expression analysis for a dextran concentration of 6.7% (5 and 10 dyn/cm^2)

Dextran concentration (%)	6.7	
Density (kg/m^3)	1021.0	
Viscosity (cP)	3.438	
Flowrate (mL/min)	27.42	54.83
Re	55	110
Shear rate (s^{-1})	145.4	290.8
Shear stress (dyne/cm^2)	5	10

4.5.9. Quantitative real-time PCR

Cells were gathered with 0.25% Trypsin/EDTA (Invitrogen) and total RNA was extracted using RNeasy spin columns (Qiagen) with DNase I (Qiagen) on column digestion. Total RNA was quantified by absorbance measurements at 260 nm. First-strand complementary DNA (cDNA) was synthesized with 0.5 µg total RNA, random hexamers (Applied Biosystems) and MultiScribe^{TM} reverse transcriptase (Applied Biosystems) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Real-time PCR reactions were carried out in Power SYBR® Green PCR Master Mix (Applied Biosystems) with

QuantiTect Primer Assays (Qiagen) and performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The housekeeping gene was β-Actin and the ΔΔCt method was used for relative quantification of gene expression.

4.5.10. Protein analysis and western blotting

Proteins were gathered and lysed in RIPA Lysis Buffer (50mM Tris-HCl (pH 6.8), 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Sigma P8340), using cycles of freezing and thawing. Cell debris were discarded from proteins by centrifugation at 16,000g for 15 minutes at 4°C. After quantification using the Bicinchoninic acid assay, proteins were resuspended in loading buffer (12mM Tris-HCl, 10% glycerol, 0.4% SDS and 0.02% Bromophenol blue) and boiled for 5 minutes. Proteins (2 μg) were loaded on a 7% polyacrylamide gel (30% w/v, 1:29.2) in a Mini Protean III apparatus. The proteins were transferred on Polyvinylidene Fluoride (PVDF) membranes in a Transblot apparatus. The membranes were incubated for 30 minutes at room temperature in phosphate buffer saline (PBS) containing 5% nonfat dry milk in 0.1% Tween-20 to block nonspecific protein binding and incubated overnight at 4°C in primary antibody (Santa Cruz biotechnology) specific for β-actin (1:200, sc-47778, used as an internal control), ICAM-1 (1:100, sc-8439) and VCAM-1 (1:100, sc-8304) in antibody dilution buffer (0.05% Tween-20, 2% Milk in PBS). After three washes in PBST (0.1% Tween-20 in PBS), secondary antibodies were incubated for 1.5 hr at room temperature (1:10,000). The membranes were washed and detection accomplished with the enhanced chemiluminescence method (Pierce) and X-Ray films (X-Omat, Kodak).

4.5.11. Immunofluorescent staining and confocal imaging

Endothelial cells fixed *in situ* in 1% paraformaldehyde were stained for ICAM-1 and VCAM-1 expression as well as NF-κB translocation. Cells were washed with PBS and blocked for 30 minutes at room temperature in PBS containing 2% normal donkey serum (NDS, Jackson Immunoresearch, 017-000-121) and 0.2% Triton X-100 (Sigma, T8787). Primary antibodies (Santa Cruz biotechnology) against ICAM-1 (1:100, sc-8439), VCAM-1 (1:100, sc-20069) and NF-κB (1:500, sc-372) diluted in 1% NDS and 0.05% Triton-100 were incubated overnight at 4°C. After three washes in PBS, secondary antibodies were incubated for 1 hr at room temperature (Invitrogen, donkey Alexa Fluor 488 anti-rabbit IgG (A21206) and Alexa Fluor 555 anti-mouse IgG (A31570), dilution 1:600). Models were cut and mounted in glass bottom dishes (MatTek) using 0.2% Dabco/Glycerol (Sigma D2522, R6513, 1:5). Cells were examined under a laser scanning confocal microscope (LSM 510, Zeiss), using Argon (488 nm) and HeNe1 (543 nm) lasers for excitation of the fluorochromes and a 32x/0.4 A-Plan objective (Zeiss). Maximum intensity projections were produced from Z series and secondary antibody controls were performed.

4.5.12. Statistical analysis

Statistical analysis was performed by nonparametric tests using GraphPad Prism^{TM} software. Mean values were compared using t-tests, one-way and two-way analysis of variance (ANOVA) followed by a Bonferroni post tests with a 95% confidence interval. P values less than 0.05 were considered statistically significant.

4.6. Results

4.6.1. Cell culture medium properties

The changes in growth medium viscosity and density as a function of dextran concentration were measured for a dextran molecular weight of 135,000. Viscosity increases rapidly with an increase in concentration whereas density increases linearly, Figure 4-1. In all cases, the growth medium was Newtonian over the measured shear rates (25-1000 s^{-1}) (data not shown).

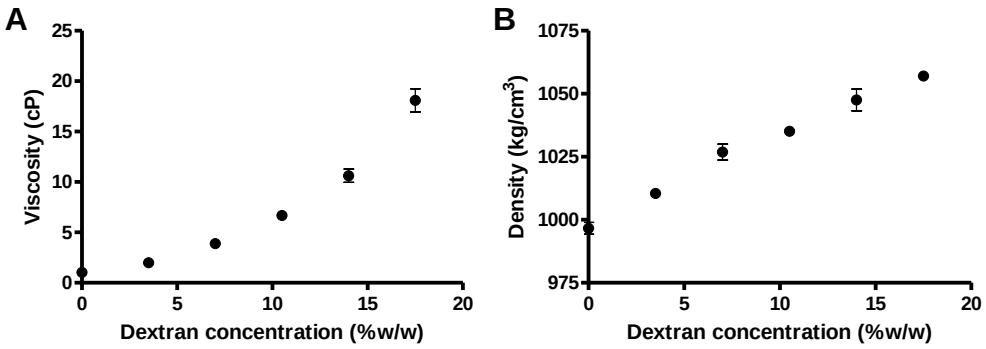

Figure 4-1: Growth media viscosity (A) and density (B) for different dextran concentrations varying between 0 and 17.5% w/w (MW 135,000) (mean ± standard deviation, n=6).

4.6.2. Endothelial cell viability

The effect of dextran on cell viability was quantified. Dextran appeared to be non-toxic after 24 hrs. High dextran concentrations significantly increased cell viability (10.5%, 14% and 17.5% w/w, $P<0.01$, $P<0.001$, $P<0.001$ respectively, one-way ANOVA, Bonferroni post tests) when compared to cells grown in unaltered growth medium, Figure 4-2A.

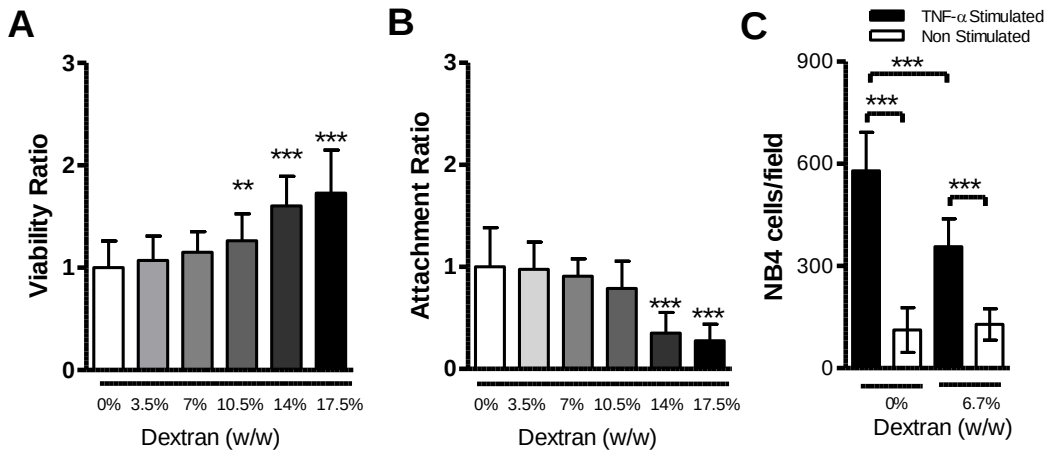

Figure 4-2: Effect of dextran on endothelial cell viability (A) and attachment to culture plates (B), observed in 96-well plates, as quantified by the viability and attachment ratio respectively, both compared to the unaltered medium, and NB4 cell adhesion to TNF-α and non-stimulated endothelial cells (C) at different concentrations in the *in vitro* models. Significant differences are indicated with respect to the unaltered medium, 0% w/w dextran (** P<0.01, *** P<0.001) (mean ± standard deviation, n=4).

4.6.3. Cell attachment

Endothelial cell attachment to plates was significantly altered by the presence of dextran in the growth medium, Figure 4-2B. A significant reduction in cell attachment was observed at higher dextran concentrations (14% and 17.5% w/w, P<0.001, one-way ANOVA, Bonferroni post tests) as measured by the MTT assay.

4.6.4. Leukocyte adhesion

Leukocyte adhesion to endothelial cells under static conditions was quantified. Stimulation of endothelial cells with TNF-α significantly increased leukocyte adhesion in the presence or absence of dextran with respect to non-stimulated cells (P<0.001, one-way ANOVA, Bonferroni posts tests). Very few NB4 cells adhered to non-stimulated endothelial cells and no statistical difference

was seen with the addition of dextran, Figure 4-2C. However, dextran (6.7% w/w) significantly decreased leukocyte adhesion to TNF-α stimulated endothelial cells ($P<0.001$, one-way ANOVA, Bonferroni posts tests).

4.6.5. Inflammatory marker expression

Changes in cell adhesion molecule expression after 24 hrs of dextran exposure were observed qualitatively by immunofluorescent staining and western blotting as well as quantitatively by mRNA analysis using RT-PCR. Confocal imaging showed noticeable ICAM-1 and VCAM-1 upregulation as well as translocation of transcription factor NF-κB to the nucleus at high (≥ 7% w/w) dextran concentrations, Figure 4-3A. Increasing amounts of ICAM-1 and VCAM-1 protein were observed visually by western blotting with increased dextran concentration, Figure 4-3B. In addition, mRNA analysis showed significant upregulation of VCAM-1 and ICAM-1 with higher dextran concentrations (≥ 10.5% w/w). The greatest increase was observed at 17% w/w dextran, whereby VCAM-1 and ICAM-1 mRNA levels were 20- and 15-fold greater than unstimulated cells respectively ($P<0.001$, one-way ANOVA, Bonferroni posts tests), Figure 4-3C.

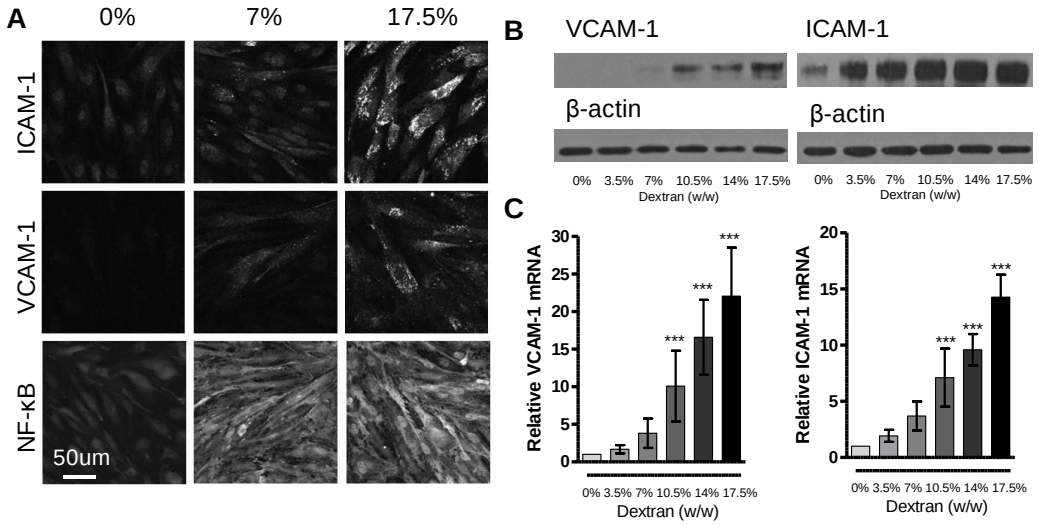

Figure 4-3: Effect of dextran concentration on the expression of cell adhesion molecules (ICAM-1 and VCAM-1) and the translocation of transcription factor NF-κB observed by confocal microscopy (A). Protein and mRNA expression as determined by *w*estern blotting (B) and quantitative RT-PCR (C) (mean ± standard error, n=3). Experiments were performed under static conditions in 6-wells plates for 24 hrs (*** $P<0.001$, with respect to the 0% w/w dextran control).

4.6.6. Time dependent effects of dextran under static conditions

The temporal difference in the inflammatory marker expression due to the presence of dextran was also investigated. Cells were incubated for 0, 0.5, 2, 6, 12 or 24 hrs in straight/tubular *in vitro* models. The endothelial cell expression of VCAM-1 and ICAM-1 after exposure to dextran (6.7% w/w, $\mu = 3.5$ cP) over a 24 hrs time period is presented in Figure 4-4. Significant increases in VCAM-1 and ICAM-1 mRNA levels were found at times greater than 6 hrs compared to the initial baseline levels ($P<0.05$, two-way ANOVA, Bonferroni posts tests). ICAM-1 mRNA levels continued to be significantly elevated after 24 hrs of exposure to dextran, whereas the upregulation in VCAM-1 dropped at this time point. No significant differences in VCAM-1 and ICAM-1 expression were detected in unaltered medium (0% w/w dextran) over the 24 hrs time frame.

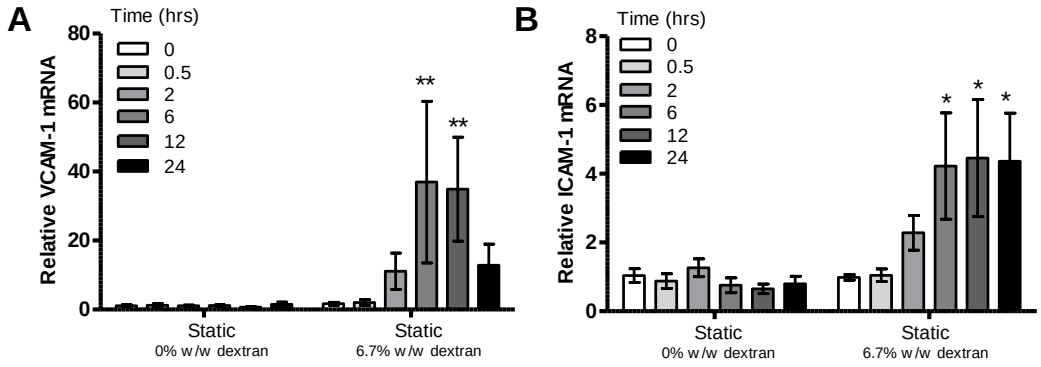

Figure 4-4: Time dependent effects of dextran (6.7% w/w) on mRNA expression of cell adhesion molecules (ICAM-1 (A) and VCAM-1(B)) as determined by quantitative RT-PCR (mean ± standard error, n=3). Experiments were performed in the *in vitro* models under static conditions for various time periods between 0 and 24 hrs. Statistically significant differences between the dextran stimulated and the initial (0 hrs time point) cells are indicated (*P<0.05, **P<0.001).

4.6.7. Combined dextran and hemodynamic effects

The effect of dextran on inflammatory marker expression was evaluated after 24 hrs of steady flow (τ = 5 and 10 dyn/cm^2). Confocal analysis showed a greater expression and nuclear translocation of transcription factor NF-κB in endothelial cells exposed to dextran than to unaltered growth medium under both static and flow conditions (10 dyn/cm^2), Figure 4-5A. As seen with western blotting and confocal microscopy, without dextran, cell adhesion molecules are basally expressed at low levels. ICAM-1 is visibly upregulated with increasing wall shear stress magnitude after 24 hrs of flow, whereas results concerning VCAM-1 showed no clear upregulation with wall shear stress magnitude. In the presence of dextran (6.7% w/w, μ = 3.5cP) their expression followed the same trends, Figure 4-5B&C.

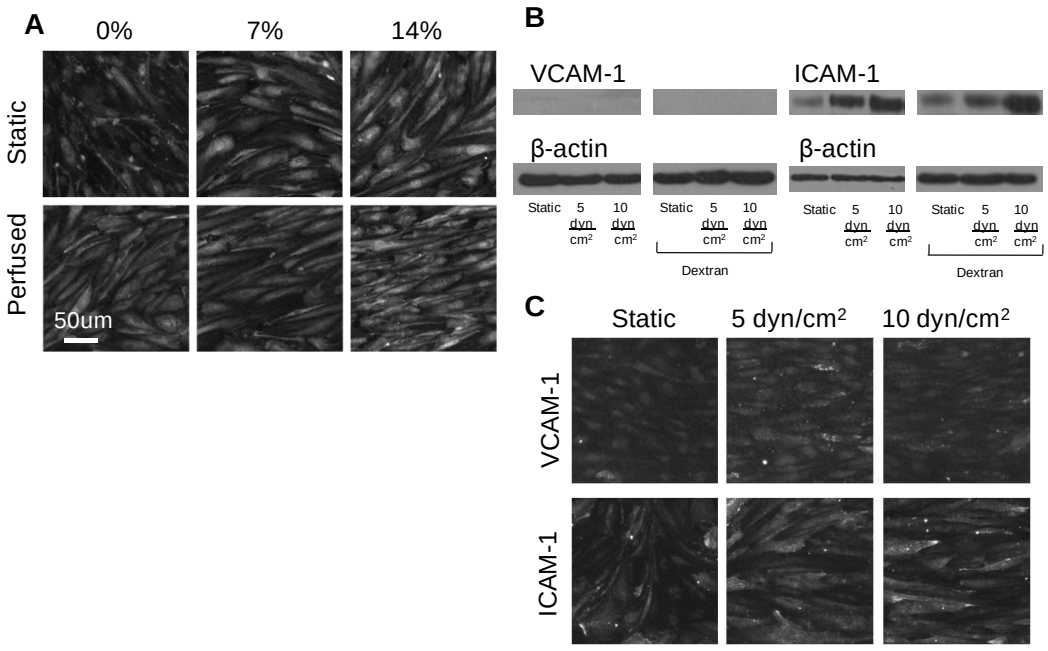

Figure 4-5: Combined dextran and hemodynamics effects in the *in vitro* models. Translocation of transcription factor NF-κB determined by confocal microscopy, at dextran concentrations of 0, 7 and 14% w/w at a mean wall shear stress of 10 dyn/cm^2 after 24 hrs (A). Protein expression of cell adhesion molecules measured by western blotting (B) and by confocal microscopy (C) under mean entrance wall shear stresses of 5 and 10 dyn/cm^2 after 24 hrs of exposure to 6.7% w/w dextran.

Analysis of VCAM-1 and ICAM-1 mRNA confirmed that increasing dextran concentration significantly upregulated VCAM-1 and ICAM-1 ($P<0.001$ and $P<0.001$, two-way ANOVA) after exposure to a wall shear stress of 10 dyn/cm^2, Figure 4-6A. However, by 24 hrs of steady flow, the expression of both VCAM-1 and ICAM-1 mRNA are significantly lowered by shear compared to the static controls ($P<0.001$ and $P<0.001$, two-way ANOVA) despite the increase seen at the protein level. When the mRNA levels under flow are normalized to the dextran static controls, the concentration of dextran does not appear to affect the fold change in either VCAM-1 or ICAM-1 ($P=0.9956$ and $P=0.7404$, two-way ANOVA), Figure 4-6B.

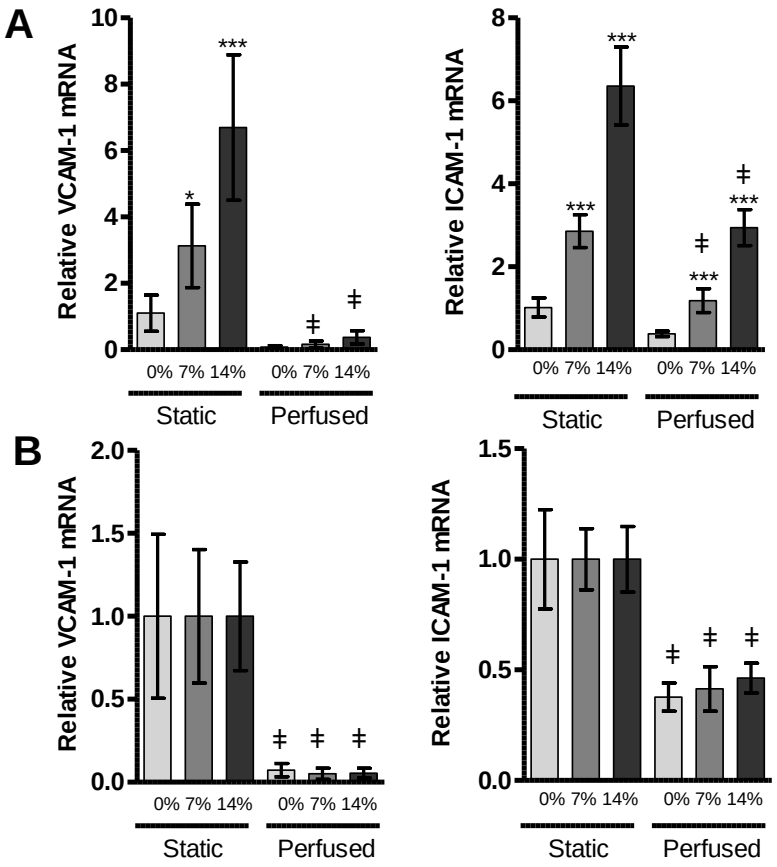

Figure 4-6: Dextran concentration effects under perfusion at 10 dyn/cm^2 after 24 hrs in the *in vitro* models. Expression of cell adhesion molecules analyzed by quantitative RT-PCR (mean ± standard error, n=3). The data was either normalized to the static control containing no dextran (A) or to the respective dextran containing static control (B). (* P<0.05, *** P<0.001, with respect to the 0% control, ǂ P<0.001 with respect to the static controls).

4.6.8. Time dependent effect of shear and dextran

Time matched controls were used to normalize the data obtained in perfusion studies. Endothelial cells in straight/tubular *in vitro* models were extracted and normalized either to the unaltered static control or to the time matched dextran containing static control, Figure 4-7. Expression levels did not vary in time in the static control containing no dextran for both VCAM-1 and ICAM-1 (two-way ANOVA, P>0.05) (data not shown). When the unaltered

medium is used to normalize the mRNA expression, a significant increase in VCAM-1 and ICAM-1 is observed after 6 hrs of flow exposure ($P<0.05$, two-way ANOVA). This increase is followed by a decrease in the mRNA of these genes at 12 and 24 hrs. On the other hand, markedly different results are obtained if the time matched static dextran controls are used, with significant decreases ($P<0.05$, t-tests), Figure 4-7B. When time matched dextran static controls are used no significant increase is observed due to shear stress and mRNA levels are lower after 24 hrs of flow exposure than initially.

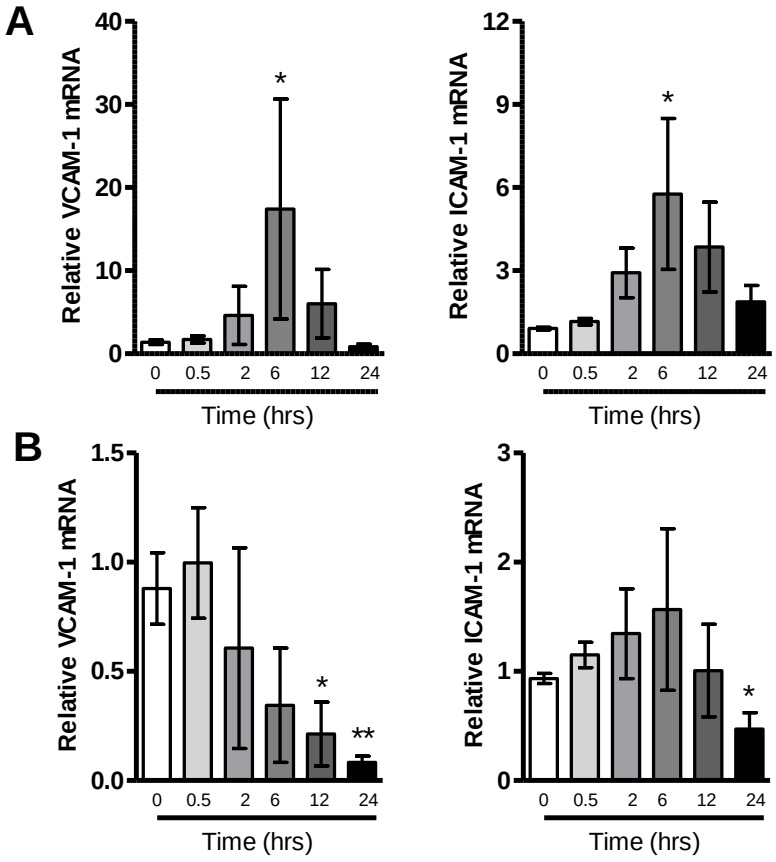

Figure 4-7: Dextran temporal effects under perfusion at 18 dyn/cm^2, in the *in vitro* models, on VCAM-1 and ICAM-1 mRNA expression analyzed by quantitative RT-PCR (mean ± standard error, n=3). The data was normalized either to the initial static control containing no dextran (A) or to the respective time matched dextran static control (B). (* $P<0.05$).

4.7. Discussion

Much of our knowledge of atherosclerosis and thrombosis has come from *in vitro* endothelial cell culture studies in defined geometries and many have used dextran to alter the rheologic properties of the growth medium [4,5,11-13,17,22,24,35-37]. In endothelial cell hemodynamic studies, dextran is often used to help match values of blood viscosity and density. Despite the known effects of dextran on cells and its use in the isolation of blood components, it is often assumed in endothelial cell hemodynamic studies that the effects of neutral dextran are negligible [5,17,35,36]. Indeed, very few studies have examined the specific effects of dextran in a concentration and time dependent manner, under static and flow conditions and presented results obtained with time matched dextran containing static controls.

Dextran not only increases medium viscosity and density [45,50] but also osmolality. In this work, we chose a dextran molecular weight in the range used in perfusion dynamic cell studies (MW 135,000) [12,13,32,37,44,49], and concentrations up to 17.5%, above the range normally used which varies from 1% w/w [16,17] and 9.5% w/w [12,13,37]. Molecular weights up to 2 million and concentration up to 30 % w/w have been shown to have a Newtonian behaviour [1,8,43,48]. The viscosity values we obtained compare well with literature data, Figure 4-1. We did not measure the osmolality of the dextran containing growth medium. However, since no decrease in cell viability or noticeable morphological changes were observed, it is likely that increase in osmolality was minimal. Indeed, concentrations up to 10% have been reported to minimally alter osmolality [17,20], dextran concentrations of 5% changing osmolality less than 10% [39].

Once the rheological properties were quantified, we examined cell viability and observed a significant increase with increasing dextran concentration, Figure 4-2A. An increase in cell viability has not been noted in other studies. Very few studies have examined the concentration effects of dextran on viability. Wechezak *et al.* [52] found no significant difference in static

culture, indicating that there might not be mitogenic or cytotoxic effects on endothelial cells. However, they showed that dextran increased subconfluent endothelial cell survival under shear, with less severe cell loss when they were exposed to shear stress. It has also been documented that adding 5% w/w uncharged dextran to growth medium does not affect cell viability [31], integrity or detachment [30]. The reason of the increased cell viability we observed could be extrapolated from the increase in cell survival observed.

Dextran is usually used to increase the viscosity of the perfusate in flow experiments; hence it is rarely used during EC attachment to the substrate. However, in an attempt to better understand the reduction in neutrophil adhesion in static conditions, we tested the attachment of ECs to cell culture plates in the presence of dextran. Dextran decreased endothelial cell attachment at concentrations ≥14% (Figure 4-2B). At these concentrations, the solution was also difficult to transfer. Consequently, the observed decrease in cell attachment could result from limitations in the ability of cells to disperse and settle in the wells. In order to tests whether dextran could be used in experiments including neutrophils, static adhesion assays were performed. We found that neutrophil adhesion to TNF-α stimulated endothelial cells was significantly reduced in growth medium containing 6.7% dextran, Figure 4-2C, similar to the results obtained from EC attachment to plates. It is not believed that this reduction is a result of altered endothelial cell adhesion molecule and/or leukocyte ligand expression since static mRNA and protein levels of ICAM-1 and VCAM-1 progressively increased in time (> 2 hrs) with increasing dextran concentration, Figure 4-3 and Figure 4-4. In agreement with our results, Termeer *et al.* found that lymphocyte (T cells, TK-1) adhesion to endothelial cells (eEnd.2) decreased with increasing dextran concentration for both non-stimulated and TNF-α stimulated cells [47]. It is possible that the reduction in adhesion is a physical effect whereby dextran interferes with the ability of cells to settle because of changes in growth medium density and osmolality [6,38]. Dextran is used in the isolation of blood components [7,26], and a similar phenomena could explain the reduction seen

in endothelial cell attachment to plates and the results obtained for NB4 cell adhesion to endothelial cells.

Dynamic endothelial cell studies are often used to evaluate the link between hemodynamics and inflammation. We investigated the effect of dextran on transcription factor NF-κB and cell adhesion molecules, VCAM-1 and ICAM-1. Transcription factor NF-κB (p65 subunit) is involved in inflammation. It is typically translocated from the cytoplasm to the nucleus upon activation by cytokines or following the onset of flow. It is associated with the subsequent upregulation of VCAM-1 and ICAM-1 in endothelial cells, which are involved in the attachment of leukocytes to the vessel wall [10,11,24,25,35]. Under static conditions, we found that dextran, in a concentration dependent manner, translocated NF-κB to the nucleus and increased VCAM-1 and ICAM-1 expression at the protein and mRNA levels after 24 hrs of exposure, Figure 4-3. This was followed by an evaluation of the time dependent increase in VCAM-1 and ICAM-1 mRNA levels due to 6.7% w/w dextran exposure, Figure 4-4. A significant increase in VCAM-1 and ICAM-1 mRNA was detected after 6 hrs. The increase in ICAM-1 was maintained up to the 24 hr time point, whereas the upregulation in VCAM-1 decreased after 12 hrs. These results suggest that dextran can produce an inflammatory endothelial phenotype. The discrepancy between levels observed in Figure 4-3 and Figure 4-4 could be explained by the differences in the cell environment in terms of coating and substrate stiffness. Very few studies have thoroughly examined the effects of dextran on endothelial cell inflammatory marker expression under static conditions and to our knowledge none in a time dependent manner.

After obtaining these results, we thought it was important to evaluate the effects of dextran under flow, as it is used to increase growth medium viscosity in perfusion experiments. We have not attempted to determine the effect of dextran on endothelial cell attachment and neutrophil adhesion under flow as a clear effect was observed in static conditions. Although the strength of attachment was not

directly tested, we observed no significant detachment of the cells at wall shear stresses up to 20 dynes/cm^2 and most dynamic studies use lower values than this. Also, no studies to our knowledge use dextran in adhesion assays and most studies perform endothelial cell function studies using dextran. Hence, this work would allow us to determine if dextran could confound the effects of shear stress on endothelial cell expression in perfusion assays. As indicated in Table 4-1 and Table 4-2, the required flow rate to achieve a fixed wall shear stress varies, as dextran alters the rheological properties of the perfusate. In order to verify the effects of dextran, the flow rate was adjusted in order to match the wall shear stress and the expression of cells was quantified. Under steady laminar flow, NF-κB showed increased expression as well as nuclear translocation in the dextran treated cells when compared to the untreated cells (0% dextran), Figure 4-5A. Under increased wall shear stress levels (5 and 10 dyn/cm^2), protein expression, as observed using western blotting and confocal imaging, was increased for ICAM-1 in the presence and absence of dextran after 24 hrs of perfusion in a wall shear stress magnitude dependent manner. However, results showed no clear difference for VCAM-1, Figure 4-5B&C.

Indeed, Tsuboi *et al.* examined ICAM-1 at the protein level at 4 hrs of exposure and found a WSS magnitude dependence [49]. Nagel *et al.* [35] and Morigi *et al.*[33] observed an increased protein expression in a time-dependent manner, at a wall shear stress at 10 dyn/cm^2 and 8 dyn/cm^2 respectively, with a sustained expression at 48 hrs. Hence, the upregulation of ICAM-1 noticed at the protein level in a wall shear stress magnitude dependent manner above 5 dyn/cm^2 is in agreement with previous studies that have used dextran. VCAM-1 protein levels were less affected. Ando *et al.* [2] found that the decrease in VCAM-1 protein expression was wall shear stress magnitude dependent at 6 hrs [28]. Nagel *et al.* [36] found that VCAM-1 was not stimulated at 10 dyn/cm^2 for 4 hrs, however, their results show a slight increase of cell surface expression at 24 hrs. The medium composition, the experimental design and the cell line used in these studies differ, hence potentially altering the measured response.

We also observed that dextran increased VCAM-1 and ICAM-1 mRNA in a concentration dependent manner under flow and that wall shear stress decreased their expression, Figure 4-6A. Consequently, VCAM-1 and ICAM-1 mRNA levels were lower under flow than under static conditions at 24 hrs after the onset of flow. When normalized to the matched dextran containing static control, dextran does not affect the conclusions that would be drawn on the wall shear stress effects, Figure 4-6B. These results suggest that time matched static containing controls are necessary to separate wall shear stress and dextran effects on the expression of cell adhesion molecules in endothelial cells.

In our time dependent study, different conclusions would be drawn when evaluating the mRNA levels of VCAM-1 and ICAM-1 as a result of wall shear stress exposure depending on the controls used. Normalization of mRNA data with respect to the initial static control containing no dextran would suggest a significant increase in mRNA expression at 6 hrs, for both VCAM-1 and ICAM-1, Figure 4-7A, comparable to other studies [2,28,35,49]. However, when compared to the time matched dextran containing static control (6.7% w/w), a significant decrease is observed, Figure 4-7B. Nagel *et al.* [35] observed mRNA levels of ICAM-1 and VCAM-1, using 1% w/w dextran (MW ~500 000, 1.74 cP). They found that shear stress induced in a time dependent increase in ICAM-1, independent of wall shear stress magnitude. No significant differences were observed for VCAM-1 in this study. In another study, Ando *et al.* [2] observed that VCAM-1 mRNA levels decreased in response to low wall shear stress levels (1.5 dyn/cm^2). They determined that this response was shear stress specific and not affected by the shear rate. Similarly, another study found that increasing levels of shear stress further decreased VCAM-1 expression [28]. However, it is not clear, whether the above mentioned studies used time matched dextran static controls. As a result, it is difficult to separate the changes due to dextran from effects of flow and to relate their work to our own study.

Dextran supplied by commercial sources clearly affects cell inflammatory expression. This could be partly due to contamination of the product with bacterial lipopolyssaccharides known to activate the inflammatory NF-κB pathway in endothelial cells [27]. The molecular weight which was chosen is one of the most used in flow perfusion studies. When a higher molecular weight is used, a lower concentration is required to achieve the same viscosity, potentially affecting the inflammatory markers differently. Hence, other perfusate additives and molecular weight of dextran could have been used; however, none have been fully validated to our knowledge. Cell lines were used and steady flow was tested, although it is known that different types of flow can influence endothelial cells and the source of a cell significantly changes their phenotype. In this work, no attempt was made to understand why a reduction in adhesion occurs. We hypothesize that this reduction is due to an increase in density of the media and an inability of the cells to properly settle on the surface. It is possible that there is an active effect of the dextran on the cells. However, this would have to be a very fast response and we saw no evidence of inflammatory marker changes within the 1hr adhesion assay period. The effect of dextran on the strength of attachment was not evaluated and could vary between different surfaces. However, we do not believe these differences affect the conclusions of our study, as our objective was to identify in the same conditions the effect of dextran.

In conclusion, we found that dextran has a non negligible effect on endothelial cell viability, attachment and inflammatory marker expression, as well as leukocyte adhesion. Dextran concentration and duration of exposure had a significant effect on inflammatory marker expression both under static and flow conditions. This work suggests that it is prudent to use time matched dextran containing static controls when evaluating wall shear stress effects in dynamic endothelial cell experiments in order to limit confounding conclusions by the response of cells to dextran.

4.8. Acknowledgements

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CHAPTER 5: LAMINAR SHEAR STRESS PREVENTS SIMVASTATIN-INDUCED ADHESION MOLECULE EXPRESSION IN CYTOKINE ACTIVATED ENDOTHELIAL CELLS

5.1. Preface

The previous paper was used to help design our statin-shear stress interaction experiments. Given that dextran was found to alter endothelial cell gene expression, we decided not to use it in our flow experiments with statins. Instead, physiologically relevant shear stress levels were achieved by reducing the diameter of our tubular cell culture models from 3.175 mm to 2 mm and perfusing them with unaltered cell growth medium. In this study we asked the question, does laminar shear stress alter the inflammatory response of endothelial cells to statins? In addition to lowering serum LDL levels, statins are also suspected to have anti-inflammatory properties. These anti-inflammatory effects may be a result of reducing cell adhesion molecule expression in endothelial cells, which are involved in the extravasation of immune cells into the vascular intima. At present, the literature available on the effects of statins on VCAM-1 and ICAM-1 expression in cultured endothelial cells is inconsistent and controversial. In addition, limited information is available on their expression by statins in endothelial cells conditioned with shear stress, which is more representative of *in vivo* conditions. In this study we evaluated, the effects of simvastatin on VCAM-1 and ICAM-1 expression in endothelial cells under different magnitudes of steady laminar shear stress (0, 1.25, 12.5 and 25 dynes/cm^2). We observed that shear stress eliminated the statin-induced increase in cell adhesion molecule expression detected in static culture. Consequently, the effects of simvastatin on VCAM-1 and ICAM-1 expression are not conserved when dynamic stimulation is applied to the cells. This study demonstrates the importance of including shear stress when

modeling *in vitro* the effects of statins on endothelial cell function. This paper will be published in the European Journal of Pharmacology DOI: 10.1016/j.ejphar.2010.09.016.

5.2. Article 2

Laminar Shear Stress Prevents Simvastatin-induced Adhesion Molecule Expression in Cytokine Activated Endothelial Cells

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Abstract

In addition to lowering cholesterol, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, have been shown to modulate gene expression in endothelial cells. The effect of statins on cell adhesion molecule expression is unclear and largely unexplored in endothelial cells exposed to shear stress, an important regulator of endothelial cell function. In this study, the effect of simvastatin on vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression was evaluated in human abdominal aortic endothelial cells (HAAEC) conditioned with various levels of laminar wall shear stress with or without tumor necrosis factor alpha (TNFα). As expected, TNFα alone greatly enhanced both VCAM-1 and ICAM-1 mRNA and protein. In static culture, simvastatin potentiated the TNFα-induced increase in VCAM-1 and ICAM-1 mRNA but not total protein at 24 h. Mevalonate, a precursor to cholesterol biosynthesis, eliminated the effect of simvastatin. Exposure of endothelial cells to elevated levels of laminar shear stress during simvastatin treatment prevented the potentiating effect of simvastatin on cell adhesion moledule mRNA. A shear stress of 12.5 dynes/cm^2 eliminated the increase in VCAM-1 by simvastatin, while 25 dynes/cm^2 was needed for ICAM-1. We conclude that simvastatin enhances VCAM-1 and ICAM-1 gene expression in TNFα-activated endothelial cells through inhibition of HMG-CoA reductase. High levels of laminar shear stress prevented the upregulation of VCAM-1 and ICAM-1 by simvastatin suggesting that an induction of cell adhesion molecules by statins may not occur in endothelial cells exposed to shear stress from blood flow.

5.3. Introduction

Atherosclerosis is a chronic inflammatory disorder that begins with endothelial cell dysfunction (Ross, 1993). Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expressed by activated endothelial cells mediate the firm adhesion of leukocytes to the vessel wall (Nakashima et al., 1998). The recruitment of inflammatory cells into the vascular intima contributes to the progression and instability of atherosclerotic plaques (Hansson, 2005; Stary et al., 1995).

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are potent inhibitors of cholesterol biosynthesis and have been shown to effectively reduce the progression of atherosclerosis and the incidence of cardiovascular events (Downs et al., 1998; Nissen et al., 2006; Shepherd et al., 1995). In addition to their lipid lowering properties, it is now believed that statins also have pleiotropic or cholesterol independent benefits. Statins have been shown in numerous studies to modulate endothelial cell gene expression in a mechanism that is independent of lowering serum cholesterol levels (Laufs and Liao, 1998; Masamura et al., 2003; Sen-Banerjee et al., 2005).

Several groups have investigated the effect of statins on cell adhesion molecule expression in endothelial cells and conflicting results have been reported (Bernot et al., 2003; Chung et al., 2002; Dimitrova et al., 2003; Eccles et al., 2008; Kim et al., 2007; Sadeghi et al., 2000; Schmidt et al., 2002; Takeuchi et al., 2000; Zapolska-Downar et al., 2004). The discrepancies between studies can be partially explained by differences in cell lines, type of statin, concentration, method of quantification, and the duration of statin and cytokine stimulation. Most studies have been done in static culture and only a few groups have evaluated cell adhesion molecule expression by statins in endothelial cells under fluid shear stress (Cicha et al., 2009; Eccles et al., 2008). The importance of shear stress as a regulator of endothelial cell function and phenotype is well established (Dekker et al., 2002; Resnick and Gimbrone, 1995). However, it is not well

known how biomechanical stimuli and statin drugs interact. Knowledge of shear stress-statin interactions will provide a better understanding of statin therapy. Recently, Eccles et al., (2008) showed that pre-treating endothelial cells with simvastatin reduced neutrophil-endothelial cell interactions during flow (1.1 dynes/cm^2). Similarly, Cicha et al. (2009) reported that simvastatin reduced VCAM-1 protein levels and monocyte adhesion in endothelial cells exposed to non-uniform shear stress and TNFα. Ali et al. (2009) were the first to show that shear stress altered endothelial responsiveness to statin drugs. They observed enhanced heme oxygenase-1 expression by atorvastatin in endothelial cells conditioned with laminar shear stress and impairment with oscillatory shear stress.

In this study, we evaluated whether statin-induced changes in cell adhesion molecule expression is affected by laminar shear stress. The mRNA and protein expression of VCAM-1 and ICAM-1 in human abdominal aortic endothelial cells (HAAEC) under static and varying levels of laminar shear stress were compared. The effect was also tested under cytokine induced increase by TNFα. The study shows that shear stress strongly affects cell adhesion molecule expression by simvastatin and is thus important to consider when evaluating the response of endothelial cells to statin drugs.

5.4. Materials and Methods

5.4.1. Reagents

Recombinant human tumor necrosis factor alpha (TNFα) was purchased from Peprotech (Rocky Hill, NJ). The inactive lactone form of simvastatin was obtained from Sigma-Aldrich (Oakville, Canada) and reconstituted in DMSO to a concentration of 25 mM. The active open acid form was obtained by adding 300 µl of 0.1 M NaOH to 100 µl of the 25 mM simvastatin in DMSO solution and incubating at 50°C for 2 h. The solution was neutralized with 0.1 M HCl to a pH of 7.2 and diluted to 0.5 mM with phosphate buffered saline (PBS). The solution

was then sterilized by filtration through a 0.22 µm polyvinylidene fluoride (PVDF) membrane in a laminar flow cabinet and diluted to working concentrations in PromoCell endothelial cell growth medium (C-22010, PromoCell, Heidelberg, Germany). Mevalonic acid lactone was purchased from Sigma-Aldrich (Oakville, Canada).

5.4.2. Cell Culture

Human abdominal aortic endothelial cells (HAAEC) derived from a 20 year old male were obtained from American Type Culture Collection (ATCC, CRL-2472) and grown in PromoCell endothelial cell growth medium (C-22010, PromoCell, Heidelberg, Germany) containing 10% fetal bovine serum, 1% penicillin-streptomycin, 2% fetal calf serum, 0.4% endothelial cell growth supplement, 0.1 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast growth factor, 22.5 µg/ml heparin, and 1 µg/ml hydrocortisone. HAAEC were cultured on 0.1% pig gelatin (Sigma-Aldrich, Oakville, Canada) pre-coated tissue culture flasks. Cell culture medium was refreshed every second day. Fifth passage cells were gathered from culture flasks with 0.25% Trypsin/EDTA (Invitrogen, Burlington, Canada), diluted in growth medium and counted with a cell counter (Z2^{TM} Coulter$^{®}$ Particle Count and Size Analyzer, Beckman Coulter). HAAEC were seeded at a density of 1.25 x 10^6 cells/ml into sterile three dimensional tubular cell culture models (2 mm in diameter) made of Sylgard 184^{TM} silicone elastomer (Dow Corning, Midland, MI) which were pre-coated with a sterile fibronectin solution from human plasma (40 µg/ml) in deionized water (Sigma-Aldrich, Oakville, Canada). A more detailed description of the silicone tubular models is described elsewhere (Farcas et al., 2009; Rossi et al., 2010; Rouleau et al., 2010). The models were placed on a rotator (Labquake Rotor, Series 1104, Barnstead/Thermolyne) for even coverage of endothelial cells on the tubular surface in a humidified incubator (37°C, 5% CO_2). Cell growth medium was changed the following day.

5.4.3. Flow Experiment

HAAEC grown to confluence in the three dimensional tubular cell culture models (described in the previous section) were connected to a closed perfusion loop consisting of vented media reservoirs, flow dampeners, an 8-channel peristaltic pump, and silicone tubing. The HAAEC were preconditioned for 6 h with steady laminar flow at a calculated wall shear stress of 1.25, 12.5 or 25 dynes/cm^2 with 10 ng/ml TNFα where indicated. This range in wall shear stress was chosen to evaluate the effect of shear stress magnitude. The response of endothelial cells in vitro to wall shear stress is most commonly study in the range of 5-20 dyne/cm^2 as it is representative of the mean shear stress in various parts of the arterial system, specifically the coronary arteries. Both laminar shear stress and TNFα were introduced simultaneously. Without stopping the perfusion, control vehicle (0.06% v/v DMSO in cell culture medium), simvastatin or simvastatin with mevalonate were added to the flow loop to reach concentrations of 0.1, 1 or 10 µM simvastatin and 200 µM mevalonate. Static controls were run in parallel. Twenty four hours after the addition of simvastatin, the models were disconnected from the perfusion loop and the cells were gathered with 0.25% Trypin/EDTA (Invitrogen, Burlington, Canada).

5.4.4. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNeasy spin columns (Qiagen, Mississauga, Canada) with on-column DNaseI digestion (Qiagen, Mississauga, Canada) following the manufacturer's instructions. RNA concentration and purity was measured by absorbance readings at 260 and 280 nm in a UV-Visible spectrophotometer. RNA (0.5 µg) was reversed transcribed into complementary DNA (cDNA) with MultiScribe^{TM} reverse transcriptase (Applied Biosystems, Foster City, CA), random primers (Applied Biosystems, Foster City, CA) and

RNase inhibitor (Applied Biosystems, Foster City, CA) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Real-time quantitative PCR was achieved in a ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, CA) with QuantiTect Primer Assays (β-Actin QT01680476, VCAM-1 QT00018347, and ICAM-1 QT00074900, Qiagen, Mississauga, Canada), and Power SYBR® green PCR master mix (Applied Biosystems, Foster City, CA) under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The data was normalized to the endogenous control, β-Actin, using the ΔΔCt method.

5.4.5. VCAM-1 and ICAM-1 Protein Analysis by Western Blot

The cell pellet was lysed by sonication for 30 min in RIPA lysis buffer (50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (Fisher Scientific, Ottawa, Canada) with protease inhibitor cocktail (Sigma-Aldrich, Oakville, Canada). Cell debris was separated from lysates by centrifugation at 16000 x g for 15 min. Protein concentration was determined by the Bicinchoninic acid (BCA) assay method (Pierce, Rockford, IL). Three or four µg of protein was separated on 7% SDS-PAGE gels in a Mini-PROTEAN Tetra Cell (Biorad, Mississauga, Canada) and transferred onto PVDF membranes in a Trans-Blot Cell (Biorad, Mississauga, Canada). The membranes were probed with rabbit polyclonal anti-VCAM-1 (1:100, sc-8304) or mouse monoclonal anti-ICAM-1 (1:100, sc-8439) antibodies overnight at 4°C in PBS containing 2% non-fat dried milk and 0.05% Tween 20. Membranes were also probed with mouse monoclonal anti-glyceraldehyde phosphate dehydrogenase (GAPDH, 1:200, sc-32233) as a loading control. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were washed 4 times in PBST (0.1% Tween 20 in PBS) and then probed with goat anti-mouse (1:10000, Biorad, Mississauga, Canada) or goat anti-rabbit secondary antibodies (1:10000, Biorad, Mississauga, Canada) conjugated to horseradish peroxidase in PBS containing 2%

non-fat dried milk and 0.05% Tween 20. The membranes were rinsed 4 times in PBST and detection of the immunoreactive bands was achieved with the SuperSignal® West PICO Chemiluminescent Substrate (Pierce, Rockford, IL) and photographic film paper (X-Omat™ LS film, Kodak, Sigma-Aldrich, Oakville, Canada). Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, Maryland) gel analyzer tools on scanned films from at least three separate experiments.

5.4.6. Statistical Analysis

The mRNA data is presented as the mean ± S.E.M. for 3 or 4 separate experiments. Statistically significant differences were determined by t-tests or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test using GraphPad Prism 5.0 software (Graphpad, San Diego, CA). A p-value less than 0.05 was considered statistically significant.

5.5. Results

5.5.1. Laminar shear stress reduces VCAM-1 and ICAM-1 mRNA in endothelial cells

The effect of laminar shear stress on VCAM-1 and ICAM-1 mRNA was evaluated by exposing HAAEC to various levels of laminar shear stress (0, 1.25, 12.5 and 25 dynes/cm^2) for 30 h. The samples were normalized relative to the unstimulated static control. We observed that after 30 h, as little as 1.25 dynes/cm^2 laminar shear stress significantly decreased VCAM-1 and ICAM-1 mRNA compared to static control cells ($P<0.001$ and $P<0.05$, respectively, one-way ANOVA, Bonferroni's multiple comparison test), Figure 5-1A and B. The highest shear stress (25 dynes/cm^2) produced the greatest decrease in VCAM-1 and ICAM-1 mRNA, whereby the levels were 9- and 2.5-fold less than static

cells, respectively (P<0.001 and P<0.01, respectively, one-way ANOVA, Bonferroni's multiple comparison test).

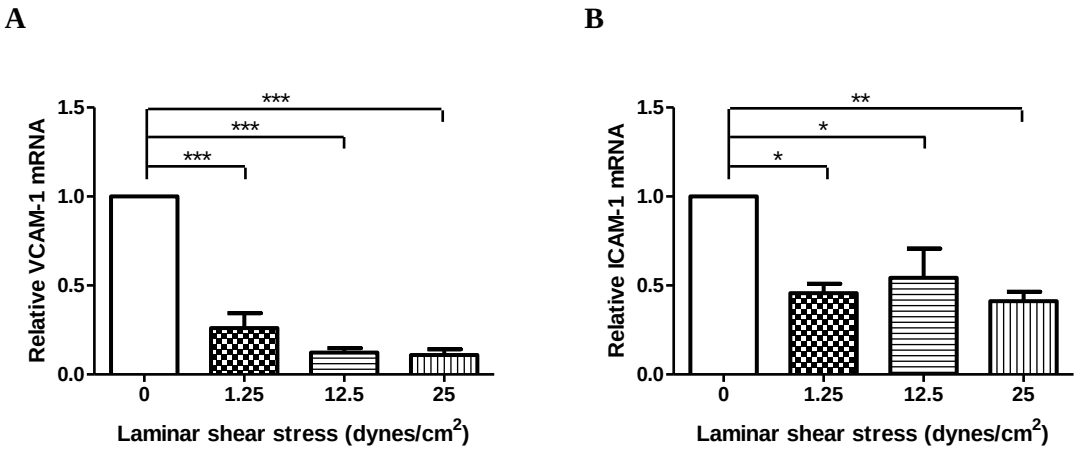

Figure 5-1: Effect of laminar shear stress on VCAM-1 (A) and ICAM-1 (B) mRNA. HAAEC were exposed to various levels of laminar shear stress (0, 1.25, 12.5 or 25 dynes/cm^2) for 30 h. The data is normalized to static controls. The values are means ± S.E.M. for n=3 or 4 separate experiments. Statistically significant differences are marked: *P<0.05, ** P<0.01, ***P<0.001.

5.5.2. Laminar shear stress reduces VCAM-1 mRNA in TNFα treated endothelial cells

The effect of laminar shear stress on VCAM-1 and ICAM-1 mRNA was then evaluated in TNFα activated endothelial cells. HAAEC were treated with laminar shear stress and 10 ng/ml TNFα for 30 h. Both laminar shear stress and TNFα were introduced at the same time. As expected, TNFα strongly induced the expression of VCAM-1 and ICAM-1 mRNA in static culture (P<0.001 and P<0.05, respectively, t-test), Figure 5-2A and B. A significant decrease in VCAM-1 mRNA was observed with a laminar shear stress value of 1.25 dynes/cm^2 (P<0.05, one-way ANOVA, Bonferroni's multiple comparison test) in TNFα treated cells (Figure 5-2C). At 25 dynes/cm^2, the greatest reduction in VCAM-1 mRNA was detected whereby the levels were 5-fold less than static controls (P<0.01, one-way ANOVA, Bonferroni's multiple comparison test).

Hence, both under the influence of TNFα and in its absence, a significant downregulation in VCAM-1 mRNA was found with laminar shear stress. laminar shear stress did not significantly reduce ICAM-1 mRNA, however, a trend toward decreasing ICAM-1 mRNA with increasing laminar shear stress was observed (Figure 5-2D).

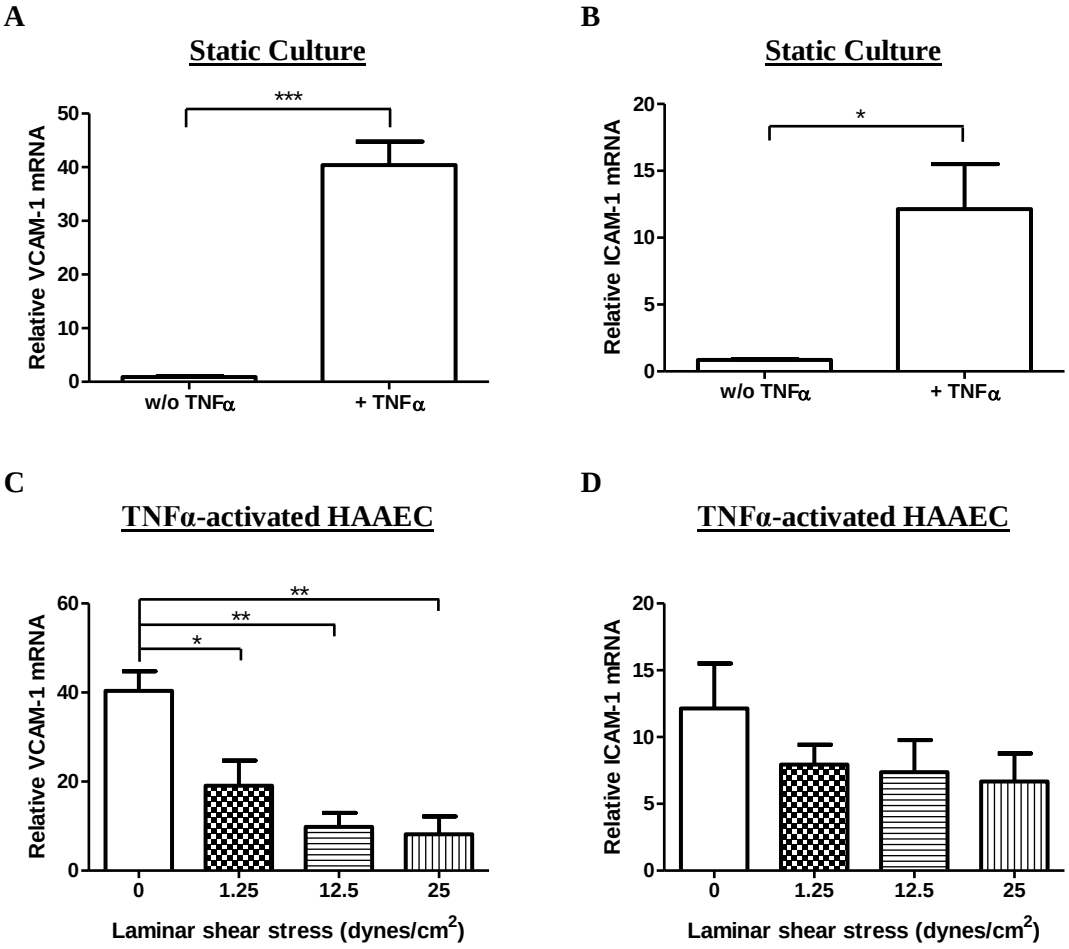

Figure 5-2: Effect of TNFα and laminar shear stress on VCAM-1 (A and C) and ICAM-1 (B and D) mRNA expression. *A and B:* HAAEC were stimulated with TNFα (10 ng/mL; 30 h) in static culture. *C and D:* HAAEC were treated with TNFα (10 ng/ml) and laminar shear stress (0, 1.25, 12.5 or 25 dynes/cm^2) for 30 h. Both TNFα and laminar shear stress were introduced simultaneously. All data was normalized relative to the unstimulated static cells. Values are expressed as

means ± S.E.M. (n=3 or 4 separate experiments). Statistically significant differences are labelled: *P<0.05, **P<0.01, ***P<0.001.

5.5.3. Laminar shear stress reduces VCAM-1 and ICAM-1 protein in TNFα treated endothelial cells

HAAEC were stimulated with either laminar shear stress alone or a combination of laminar shear stress and 10 ng/ml TNFα for 30 h. Laminar shear stress and TNFα were introduced at the same time. Total protein was gathered and analyzed by Western blot for VCAM-1 and ICAM-1. The basal constitutive levels of VCAM-1 and ICAM-1 protein are shown in lane 1 in Figure 5-3. As expected, TNFα strongly induced the expression of VCAM-1 and ICAM-1 protein (P<0.01 for VCAM-1 and P<0.001 for ICAM-1, one-way ANOVA, Bonferroni's multiple comparison test), Figure 5-3. Laminar shear stress (25 dynes/cm^2) significantly decreased VCAM-1 protein levels in TNFα stimulated endothelial cells (P<0.01, one-way ANOVA, Bonferroni's multiple comparison test), Figure 5-3A. The decrease in VCAM-1 protein by laminar shear stress under TNFα stimulation is consistent with the observed reduction in VCAM-1 mRNA (Figure 5-2C). Laminar shear stress slightly reduced ICAM-1 protein levels under TNFα stimulation, however statistical significance was not observed. The reduction in ICAM-1 protein by laminar shear stress in the presence of TNFα is in agreement with the observed trend in decreasing ICAM-1 mRNA (Figure 5-2D). It was also observed that laminar shear stress (25 dynes/cm^2) reduced ICAM-1 protein below basal levels (lane 2, Figure 5-3B), which is consistent with the decrease in ICAM-1 mRNA observed with increasing laminar shear stress (Figure 5-1B). However, statistical significance in ICAM-1 protein between unstimulated and laminar shear stress treated cells was not observed by densitometry analysis.

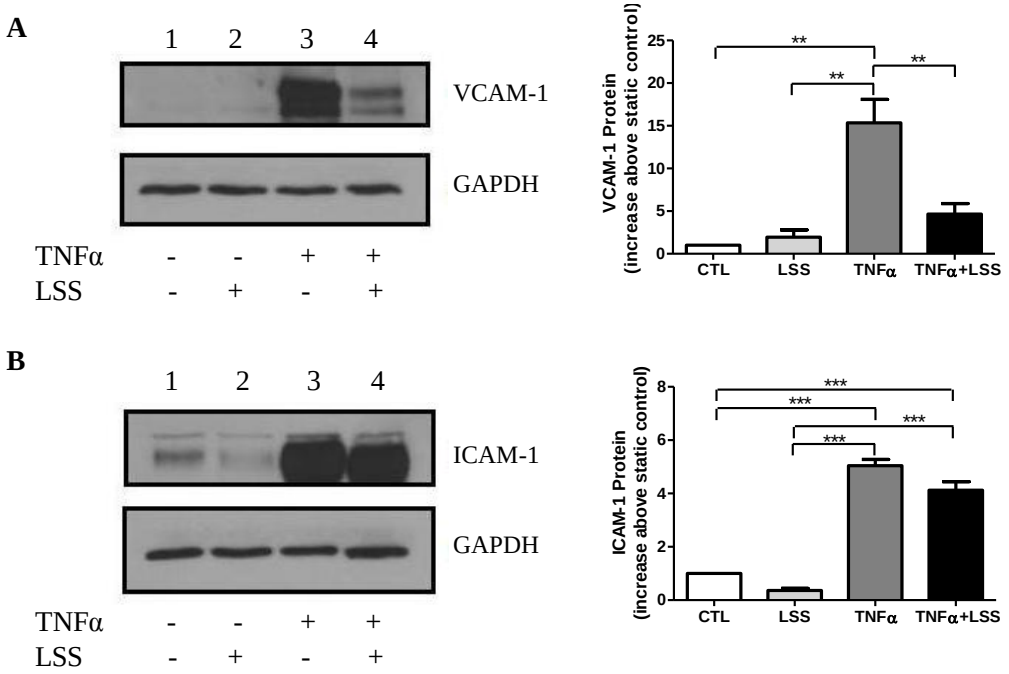

Figure 5-3: Total VCAM-1 (A) and ICAM-1 (B) protein in HAAEC after exposure to TNFα (10 ng/mL) and/or laminar shear stress (25 dynes/cm^2) for 30 h. TNFα and laminar shear stress were introduced at the same time. Four µg of protein was separated on 7% SDS-PAGE gels. Densitometry analysis was done on Western blots from 3 separate experiments. Statistically significant differences are labelled (**P<0.01, ***P<0.001). LSS: Laminar shear stress.

5.5.4. Induction of ICAM-1 mRNA by simvastatin is abolished by laminar shear stress

The influence of simvastatin on VCAM-1 and ICAM-1 expression in endothelial cells was evaluated in static culture and under increasing levels of laminar shear stress (1.25, 12.5 and 25 dynes/cm^2), Figure 5-4. In static culture, 10 µM simvastatin slightly increased ICAM-1 mRNA (< 2-fold increase compared to the static control, P<0.05, one-way ANOVA, Bonferroni's multiple comparison test) (Figure 5-4B). No significant difference was observed after co-incubation of 10 µM simvastatin with 200 µM mevalonate when compared to untreated cells. Exposure of the endothelial cells to laminar shear stress (1.25, 12.5 or 25 dynes/cm^2) eliminated the increase in ICAM-1 mRNA by simvastatin.

No significant increase in VCAM-1 mRNA by simvastatin was observed (Figure 5-4A).

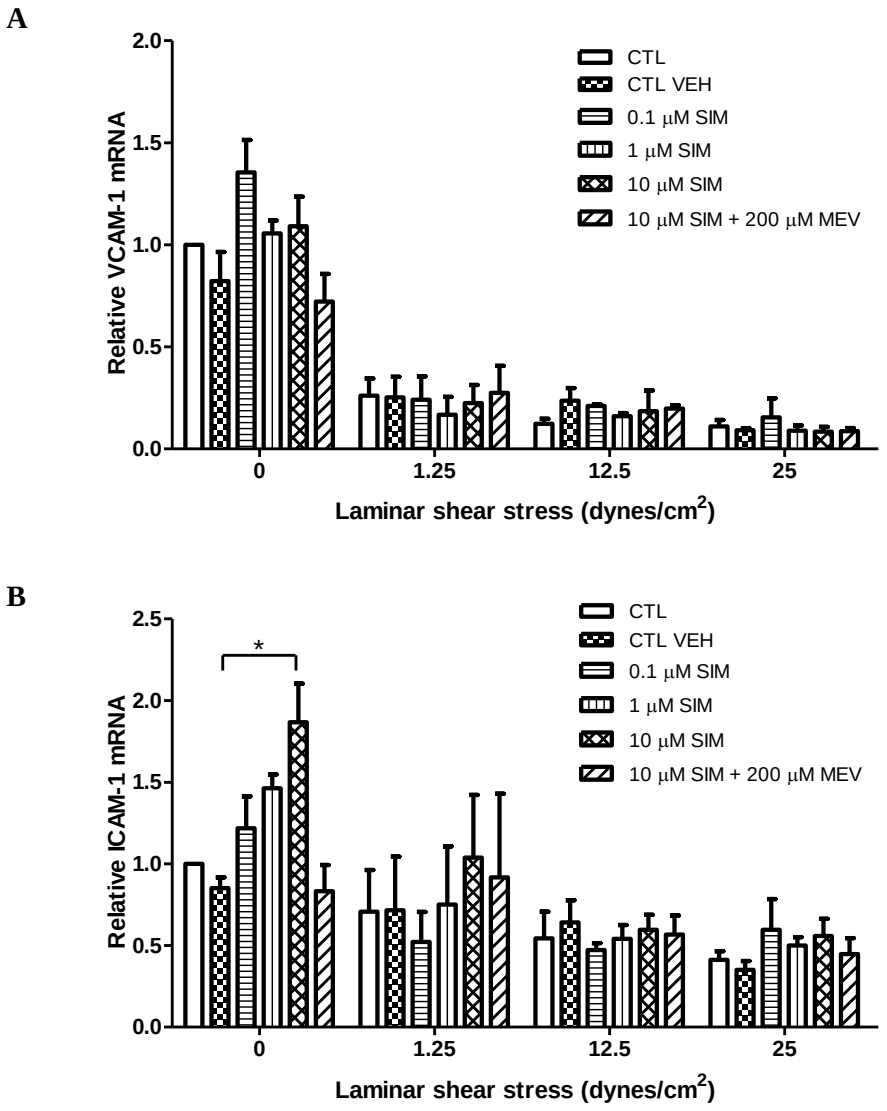

Figure 5-4: Effect of simvastatin on VCAM-1 (A) and ICAM-1 (B) mRNA in endothelial cells in static culture and under laminar shear stress. HAAEC were pre-conditioned for 6 h with various levels of laminar shear stress (0, 1.25, 12.5 or 25 dynes/cm^2). Afterwards, vehicle (DMSO), simvastatin or simvastatin with mevalonate were added to the perfusion and the flow experiment was continued for 24 h. The data is normalized relative to the unstimulated static control. The values are means ± S.E.M. for n=3 or 4 experiments. Statistically significant differences are marked: * $P<0.05$. Legend: CTL = unaltered cell culture medium, CTL VEH = 0.06% *v/v* DMSO in cell culture medium, SIM = simvastatin, MEV = mevalonate.

5.5.5. Potentiating effect of simvastatin on VCAM-1 and ICAM-1 mRNA by simvastatin in TNFα stimulated endothelial cells is eliminated by laminar shear stress

The effect of simvastatin on VCAM-1 and ICAM-1 expression in TNFα activated endothelial cells was then evaluated. HAAECs were pre-conditioned with laminar shear stress and 10 ng/ml TNFα for 6 h prior to the addition of simvastatin. The culture was continued under laminar shear stress for 24 h after simvastatin was added. Static controls were run in parallel. Treatment of the endothelial cells with 1 or 10 μM simvastatin potentiated the effect of TNFα, resulting in higher levels of VCAM-1 and ICAM-1 mRNA ($P<0.05$ and $P<0.001$, respectively, one-way ANOVA, Bonferroni's multiple comparison test), Figure 5-5. The increase in VCAM-1 and ICAM-1 mRNA by simvastatin was reduced with increasing laminar shear stress. The enhancing effect of simvastatin was completely eliminated at 12.5 dynes/cm^2 for VCAM-1 and 25 dynes/cm^2 for ICAM-1 (Figure 5-5). Co-incubation with 200 μM mevalonate abolished the effect of simvastatin in static culture and under each laminar shear stress investigated.

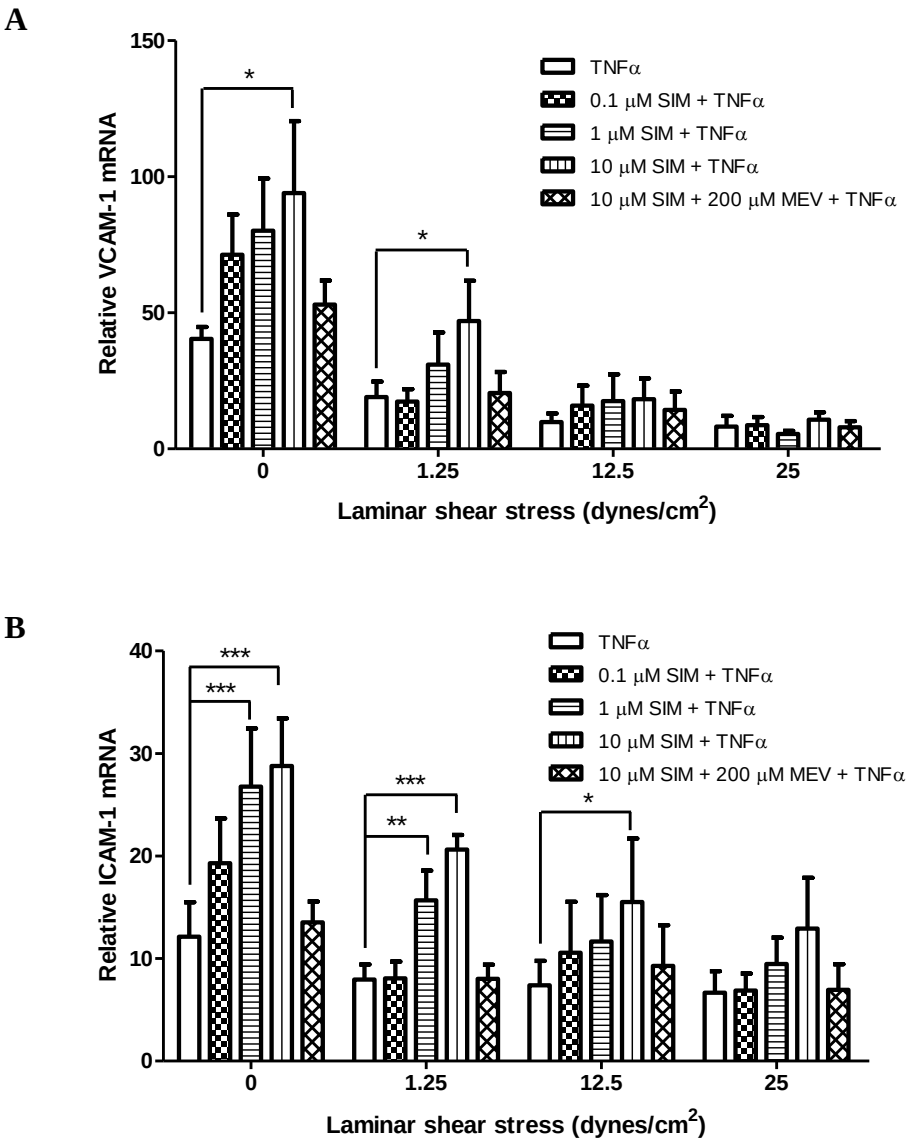

Figure 5-5: Effect of simvastatin on VCAM-1 (A) and ICAM-1 (B) mRNA in laminar shear stress and TNFα pre-treated endothelial cells. HAAEC were pre-conditioned with various levels of laminar shear stress (0, 1.25, 12.5 or 25 dynes/cm^2) with 10 ng/ml TNFα for 6 h. Laminar shear stress and TNFα were introduced simultaneously. Afterwards, simvastatin or simvastatin with mevalonate were added to the perfusion and the flow experiment was continued for 24 h. The data is normalized relative to the unstimulated static control. The values are means ± S.E.M. for n=3 or 4 experiments. Statistically significant differences between TNFα only and simvastatin+TNFα or simvastatin+mevalonate+TNFα are marked: * P<0.05, ** P<0.01, ***P<0.001. Legend: SIM = simvastatin, MEV = mevalonate.

5.5.6. Simvastatin does not potentiate the TNFα-induced increase in VCAM-1 and ICAM-1 protein

The effect of TNFα and simvastatin on VCAM-1 and ICAM-1 protein under static conditions is shown in Figure 5-6. HAAEC were pre-treated with 10 ng/ml TNFα for 6 h. After 6 h, the culture medium was replaced with fresh medium containing simvastatin with or without 200 μM mevalonate and 10 ng/ml TNFα. Total protein was extracted 24 h after the addition of simvastatin and analyzed by Western blot for VCAM-1 and ICAM-1. Simvastatin alone had no effect on VCAM-1 and ICAM-1 protein levels at 24 h (lane 2, Figure 5-6). Treatment of endothelial cells with TNFα resulted in a large increase in both VCAM-1 and ICAM-1 protein ($P<0.05$ for VCAM-1 and $P<0.001$ for ICAM-1, one-way ANOVA, Bonferroni's multiple comparison test), Figure 5-6 (lane 3). Simvastatin with or without mevalonate did not alter the induction of VCAM-1 and ICAM-1 protein by TNFα (lanes 4-7, Figure 5-6). As a result, the potentiating effect of simvastatin on VCAM-1 and ICAM-1 expression observed in mRNA was not observed in total protein at this time point. The effect of TNFα and simvastatin on VCAM-1 and ICAM-1 protein under laminar shear stress (25 dynes/cm^2) is presented Figure 5-7. We observed that VCAM-1 and ICAM-1 protein levels were higher with TNFα stimulation. Similar to static culture, it was found that simvastatin with or without mevalonate had no effect on the TNFα-induced increase in VCAM-1 and ICAM-1 protein (lanes 4-7, Figure 5-7).

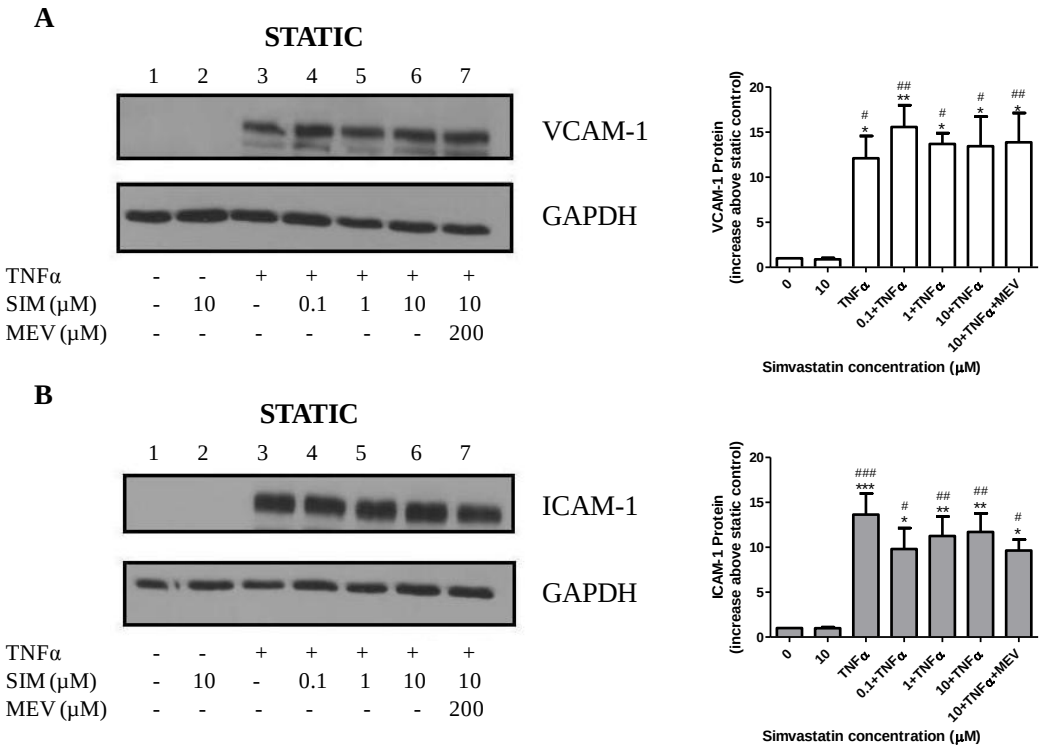

Figure 5-6: Total VCAM-1 (A) and ICAM-1 (B) protein in HAAEC after treatment with simvastatin for 24 h in static culture. HAAEC were pre-treated with 10 ng/ml TNFα for 6 h where indicated. After this initial pre-conditioning period, the culture medium was replaced with medium contatining simvastatin (0.1, 1 or 10 μM) with or without 200 μM mevalonate and 10 ng/ml TNFα. The culture was continued under static conditions for 24 h. Three μg of protein was separated on 7% SDS-PAGE gels. Densitometry analysis was done on Western blots from 3 or 4 separate experiments. Statistically significant differences are labelled (with respect to 0 μM simvastatin *P<0.05, **P<0.01, ***P<0.001; with respect to 10 μM simvastatin #P<0.05, ##P<0.01, ###P<0.001).

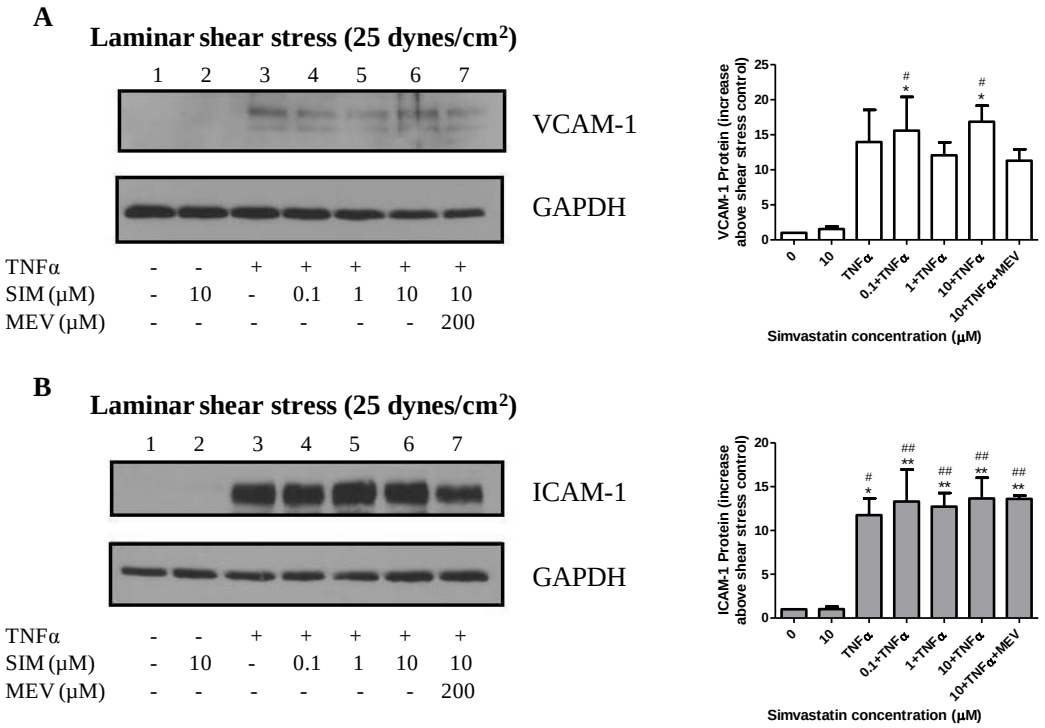

Figure 5-7: Total VCAM-1 (A) and ICAM-1 (B) protein in HAAEC after treatment with simvastatin for 24 h under laminar shear stress (25 dynes/cm^2). HAAEC were pre-conditioned with laminar shear stress (25 dynes/cm^2) for 6 h with 10 ng/ml TNFα where indicated. Simvastatin with or without mevalonate was added to the perfusion to reach concentrations of 0.1, 1 or 10 μM simvastatin and 200 μM mevalonate. The culture was continued under laminar shear stress for 24 h. Three μg of protein was separated on 7% SDS-PAGE gels. Densitometry analysis was done on Western blots from 3 or 4 separate experiments. Statistically significant differences are labelled (with respect to 0 μM simvastatin *P<0.05, **P<0.01; with respect to 10 μM simvastatin #P<0.05, ##P<0.01).

5.6. Discussion

Evidence exists that statins can potentially improve endothelial cell function by modulating gene expression (Laufs and Liao, 1998; Sen-Banerjee et al., 2005; Shi et al., 2003). However, the effect of statins on endothelial cell expression of VCAM-1 and ICAM-1 in TNFα-treated cells is unclear (Bernot et al., 2003; Chung et al., 2002; Dimitrova et al., 2003; Eccles et al., 2008; Kim et al., 2007; Sadeghi et al., 2000; Schmidt et al., 2002; Takeuchi et al., 2000; Zapolska-

Downar et al., 2004) and unknown for endothelial cells exposed to varying magnitudes of laminar shear stress. Our findings show that laminar shear stress reduces VCAM-1 and ICAM-1 mRNA and protein in untreated and TNFα stimulated endothelial cells, Figure 5-1, Figure 5-2 and Figure 5-3. These results are in agreement with other groups who have reported that laminar shear stress reduces the expression *in vitro* of inflammatory markers such as VCAM-1, MCP-1, and E-Selectin (Bergh et al., 2009; Brooks et al., 2002; Butcher and Nerem, 2007; Chiu et al., 2004; Tsao et al., 1996; Yamawaki et al., 2003) and decreases the adhesion of leukocytes onto endothelial cells (Ando et al., 1994; Sampath et al., 1995; Walpola et al., 1995). ICAM-1 mRNA and surface expression has been shown to transiently increase with laminar shear stress and then return to basal levels for protein within 24 h and below basal levels for mRNA within 6 h (Sampath et al., 1995).

Unexpectedly, we observed that simvastatin enhances the TNFα-induced expression of VCAM-1 and ICAM-1 mRNA, Figure 5-5. Simvastatin, however, had no detectable effect on total cellular VCAM-1 and ICAM-1 protein levels at the time point investigated (24 h of simvastatin treatment), Figure 5-6. The inability to detect changes in protein levels with simvastatin treatment may be due to timing and sensitivity of the detection method. Other studies have reported that statins increased surface VCAM-1 and ICAM-1 protein in TNFα stimulated endothelial cells using ELISA, flow cytometry, and confocal microscopy (Bernot et al., 2003; Dunoyer-Geindre et al., 2005; Schmidt et al., 2002). The upregulation in VCAM-1 and ICAM-1 mRNA by simvastatin observed in static culture are in opposition to the well documented clinical benefits of statins (Ray and Cannon, 2004). For instance statins have been shown in the clinic to exhibit anti-inflammatory properties independent of lowering serum cholesterol levels as determined by a decrease in the inflammatory marker C-reactive protein (CRP), an improvement of endothelial cell function and a reduction of transplant-associated arteriopathy (Jain and Ridker, 2005; Ridker et al., 2001; Shirakawa et al., 2007). In this study, we found that elevated levels of laminar shear stress

completely eliminated the induction of VCAM-1 and ICAM-1 mRNA by simvastatin (Figure 5-5). A laminar shear stress of 12.5 dynes/cm^2 was sufficient to prevent the increase in VCAM-1 by simvastatin, while 25 dynes/cm^2 was needed for ICAM-1. This suggests that unidirectional laminar shear stress has the dominating effect on cell adhesion molecule expression. As a result, the protective effects of unidirectional laminar shear stress on endothelial cells counteract the ability of simvastatin to increase cell adhesion molecule mRNA. The pleiotropic anti-inflammatory properties of statins observed in the clinic may be a result of increasing the expression of atheroprotective genes such as endothelial nitric oxide synthase (eNOS), Kruppel-like factor 2 (KLF2), thrombomodulin, and heme oxygenase-1 (HO-1) in endothelial cells (Ali et al., 2007; Ali et al., 2009; Sen-Banerjee et al., 2005) rather than altering cell adhesion molecule expression.

The effects of statins on VCAM-1 and ICAM-1 expression reported in the literature are contradictory. Several groups have reported that statins potentiated the effect of TNFα resulting in enhanced ICAM-1 and VCAM-1 mRNA and protein levels (Bernot et al., 2003; Dunoyer-Geindre et al., 2005; Schmidt et al., 2002), whereas other studies have demonstrated that statins decreased the induction of VCAM-1 and ICAM-1 mRNA and/or protein by TNFα (Cicha et al., 2009; Kim et al., 2007; Zapolska-Downar et al., 2004). On the other hand, there have also been reports that statins had no effect on the TNFα-induced increase in ICAM-1 mRNA and protein (Chung et al., 2002; Eccles et al., 2008; Takeuchi et al., 2000). From the wide range of reported effects, it appears that cell adhesion molecule expression by statins is greatly influenced by the experimental conditions. The studies differ in the cell line used, type of statin, concentration, method of quantification, and the duration of statin and cytokine stimulation.

Few studies have evaluated the effect of statins on VCAM-1 and ICAM-1 expression in endothelial cells during flow. Eccles et al (2008) found that simvastatin had no effect on ICAM-1 protein expression in static culture and failed to alter ICAM-1 mediated neutrophil-endothelial cell interactions under 1.1

dynes/cm^2 unidirectional laminar flow (Eccles et al., 2008). We also saw no detectable change in VCAM-1 and ICAM-1 protein levels from statin treatment, although we did observe an increase in cell adhesion molecule mRNA. Cicha et al (2009) observed that treatment of human umbilical vein endothelial cells (HUVEC) with simvastatin during non-uniform shear stress in a simplified model of an arterial bifurcation with TNFα stimulation significantly reduced VCAM-1 protein expression and the adhesion of monocytes compared to non-simvastatin treated cells (Cicha et al., 2009). The differences between these results and our study may be explained by differences in cell lines, the type of shear imposed on the endothelial cells (non-uniform shear stress vs unidirectional laminar shear stress) and the duration of TNFα stimulation (2 h vs 30 h). To the best of our knowledge, our study is the first to evaluate the effect of statins on cell adhesion molecule expression in endothelial cells exposed to different magnitudes of unidirectional laminar shear stress. We have shown that laminar shear stress can reduce the potentiating effect of simvastatin on VCAM-1 and ICAM-1 mRNA in TNFα-activated endothelial cells. These results show the importance of including shear stress when modeling the *in vivo* response of endothelial cells to statins.

The increase in cell adhesion molecule mRNA expression by simvastatin was abrogated when co-incubated with mevalonate, whose formation is catalyzed by the enzyme HMG-CoA reductase in the cholesterol biosynthesis pathway. These results suggest that the induction of VCAM-1 and ICAM-1 mRNA by simvastatin is dependent on the inhibition of mevalonate synthesis. By inhibiting HMG-CoA reductase, statins not only prevent cholesterol synthesis but also the synthesis of cholesterol intermediates geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). Small GTPases including Rho, Rac, and Ras have diverse cellular effects including the generation of reactive oxygen species, activation of pro-inflammatory pathways and regulation of cell proliferation, motility and shape (Liao and Laufs, 2005). These small GTPases require post translational modification by GGPP or FPP for proper membrane translocation and activation (Liao and Laufs, 2005). Consequently, the cellular activity of the GTPases can be

significantly reduced by statins. In agreement with our results, Bernot et al. (2003) showed that supplying mevalonate or GGPP eliminated the simvastatin-induced upregulation of VCAM-1 and ICAM-1 surface expression in TNFα-activated endothelial cells (Bernot et al., 2003).

5.7. Conclusion

Our findings show that simvastatin potentiates the TNFα-induced increase in VCAM-1 and ICAM-1 mRNA but not total protein through inhibition of HMG-CoA reductase. Elevated laminar shear stress (12.5 or 25 dynes/cm^2) prevented the enhancing effect of simvastatin on cell adhesion molecule mRNA. These results show that an induction in VCAM-1 and ICAM-1 expression seen in static culture can be overcome by shear stress and may explain why increased inflammation due to statin therapy is generally not observed in the clinic.

5.8. Acknowledgments

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CHAPTER 6: EFFECT OF SIMVASTATIN ON KRUPPEL-LIKE FACTOR2, ENDOTHELIAL NITRIC OXIDE SYNTHASE, AND THROMBOMODULIN EXPRESSION IN ENDOTHELIAL CELLS UNDER SHEAR STRESS

6.1. Preface

In the previous study, we showed that laminar shear stress can alter the inflammatory response of endothelial cells to statins. In this study we hypothesized that laminar shear stress could also alter KLF2 and some of its downstream genes eNOS and TM, It is well established that both laminar shear stress and statins modulate endothelial cell gene expression. In fact, shear stress and statins impact many of the same genes including KLF2, eNOS and TM. However, limited information is available on endothelial cell response when both stimuli are combined, which is more representative of *in vivo* conditions. In this paper, we have shown that shear stress and simvastatin separately enhance KLF2, eNOS and TM mRNA and combining the two stimuli resulted in an overall additive increase. This study demonstrates that the effects of statins on KLF2, eNOS and TM expression observed in static culture are conserved when endothelial cells are conditioned with laminar shear stress. Our work further supports the hypothesis that statins have pleotropic effects and may help in the design of new therapies. The manuscript is published in Life Sciences 87 (2010) 92-99.

6.2. Article 3

Effect of Simvastatin on Kruppel-like Factor2, Endothelial Nitric Oxide Synthase and Thrombomodulin Expression in Endothelial Cells under Shear Stress

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6.3. Abstract

Aims: Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) and fluid wall shear stress have been reported to modulate the expression of genes related to inflammation, blood coagulation, thrombosis, and vascular constriction in cultured endothelial cells. In this study, we investigated the combined effect of laminar shear stress (LSS) and statins on endothelial cell gene expression. **Main methods:** Kruppel-like factor 2 (KLF2), endothelial nitric oxide synthase (eNOS), and thrombomodulin (TM) mRNA and protein expression was evaluated in human abdominal aortic endothelial cells (HAAEC) treated with simvastatin (0.1, 1 or 10 μM) at various levels of LSS (0, 1.25, 12.5 or 25 dynes/cm^2). **Key findings:** As expected, simvastatin and LSS separately enhanced KLF2, eNOS, and TM mRNA expression. The combination of simvastatin and LSS resulted in significantly higher mRNA levels of all three genes compared to cells treated with LSS only. The highest KLF2, eNOS, and TM mRNA levels were detected at 10 μM simvastatin and 25 dynes/cm^2. Under these conditions, eNOS and TM protein levels were also elevated. Combining LSS and simvastatin produced an overall additive increase in KLF2, eNOS, and TM mRNA. Treatment of the endothelial cells with 10 μM simvastatin and 200 μM mevalonate completely eliminated the effect of simvastatin. **Significance:** Our results suggest an additive increase in KLF2, eNOS, and TM expression when simvastatin and LSS are combined. These results may help to explain the proposed non-lipid lowering benefits of statins observed in the clinic.

6.4. Introduction

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are a class of cholesterol lowering drugs that are widely prescribed to treat hypercholesterolemia. Although originally designed as inhibitors of cholesterol biosynthesis, statins are now believed to also have non-lipid lowering effects. The benefits of these drugs in the clinic cannot be explained by cholesterol reduction alone (Jain and Ridker 2005; Weitz-Schmidt 2002). For instance, despite similar reduction in cholesterol, benefits are seen earlier in patients taking statins than other cholesterol lowering therapies (Vaughan et al. 1996; Weitz-Schmidt 2002).

It has been proposed that the non-lipid lowering effects of statins may be in part due to improved endothelial cell function (Beckman and Creager 2006). Previous studies have reported that statins can modulate the expression of genes related to vascular constriction, coagulation, thrombosis, and inflammation in cultured endothelial cells (Ali et al. 2007; Essig et al. 1998; Laufs et al. 2002; Ohkita et al. 2006; Schmidt et al. 2002). Statins have been shown to increase the expression of atheroprotective genes such as Kruppel-like factor 2 (KLF2) (Ali et al. 2007; Parmar et al. 2005; Sen-Banerjee et al. 2005) and its downstream targets endothelial nitric oxide synthase (eNOS) (Laufs et al. 1998; Laufs and Liao 1998; Morikawa et al. 2002) and thrombomodulin (TM) (Masamura et al. 2003; Morikawa et al. 2002; Shi et al. 2003; van Thienen et al. 2006). Both eNOS and TM are essential in maintaining an anti-thrombotic and anti-inflammatory endothelium.

To date, the effect of statins on gene expression in cultured endothelial cells has been primarily limited to static culture and only a few studies have incorporated the effect of blood flow (Ali et al. 2009; Cicha et al. 2008a; Cicha et al. 2008b; da Silva et al. 2007). This is particularly important since fluid mechanical stimuli are significant regulators of endothelial phenotype and function (Chatzizisis et al. 2007; DeBakey et al. 1985; Malek et al. 1999). In fact,

unidirectional laminar shear stress (LSS) has been shown to modulate many of the same genes as statins including KLF2, eNOS, and TM (Bergh et al. 2009; Dekker et al. 2006; Lin et al. 2005; Uematsu et al. 1995). Recently, Ali et al. (2009) showed that human umbilical vein endothelial cells (HUVEC) were more responsive to atorvastatin when pre-conditioned with LSS (12 dynes/cm^2) for 12 h (Ali et al. 2009). They reported that heme oxygenase-1 (HO-1) induction by atorvastatin was increased by LSS and impaired by oscillatory flow. This study was the first to show that biomechanical signaling affects the response of endothelial cells to statins (Ali et al. 2009). HO-1 was used as a model, however, it is unknown whether this synergistic increase in gene expression also occurs for other genes.

In this study we sought to determine whether the combination of LSS and statins produces a synergistic, additive or antagonistic induction of KLF2 and some of its downstream target genes eNOS and TM. This would provide further information on whether the physiological environments in which endothelial cells are exposed to can affect statin pleiotropy.

6.5. Materials and Methods

6.5.1. Materials

Simvastatin (S6196) and mevalonic acid lactone (M4667) were purchased from Sigma-Aldrich (Oakville, Canada). Inactive simvastatin lactone was reconstituted in DMSO to a concentration of 25 mM and converted to the active form by alkaline hydrolysis in 0.1 M NaOH at 50°C for 2 h. Afterwards, the pH was adjusted to 7.4 with 0.1 M HCl and the solution was diluted to 0.5 mM in phosphate buffer saline (PBS). The solution was then sterilized by filtration through a 0.22 µm PVDF membrane in a laminar flow cabinet and diluted to

working concentrations in PromoCell endothelial cell growth medium (C-22010) purchased from PromoCell (Heidelberg, Germany).

6.5.2. Cell Culture

Human abdominal aortic endothelial cells (HAAEC) purchased from American Type Culture Collection (ATCC, Manassas, VA, CRL-2472) were cultured on 0.1% pig gelatin (Sigma-Aldrich, Oakville, Canada) pre-coated tissue culture flasks in PromoCell endothelial cell growth medium (C-22010, PromoCell, Heidelberg, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Burlington, Canada), 1% penicillin-streptomycin (Invitrogen, Burlington, Canada), and PromoCell supplement mix (C-39215, PromoCell, Heidelberg, Germany) containing 0.4% endothelial cell growth supplement/heparin, 0.1 ng/mL epidermal growth factor, 1 µg/mL hydrocortisone, 1 ng/mL basic fibroblast growth factor, and 2% fetal calf-serum. The cells were incubated at 37°C (5% CO_2) with growth media changes every 48 h.

Three dimensional tubular cell culture models (inner diameter = 2 mm) were formed out of Sylgard^{TM} 184 silicone elastomer (Dow Corning, Midland, MI), a transparent and non-toxic material. These models are described in more detail elsewhere (Farcas et al. 2009; Rouleau et al. 2010a; Rouleau et al. 2010b). The tubular models were prepared for cell culture by hydrophilizing the inner surface with 70% sulphuric acid (Fisher Scientific, Whitby, Canada). After hydrophilization, the models were sterilized in boiling deionized water for 30 min. The models were allowed to cool to room temperature and the lumen was then coated with a sterile fibronectin solution from human plasma (40 µg/mL) (F0895, Sigma-Aldrich, Oakville, Canada) overnight at 37°C. To ensure equal fibronectin surface coverage, the model was rotated continuously on a rotator at 8 rpm (Labquake Rotor, Series 1104, Barnstead/Thermolyne). HAAEC (passage 5) were harvested with a 0.25% Trypsin/EDTA solution (Invitrogen, Burlington, Canada)

from culture flasks, resuspended in PromoCell cell culture medium and counted with a cell counter (Z2^{TM} Coulter$^{®}$ Particle Count and Size Analyzer, Beckman Coulter Canada, Mississauga, Canada). Cell concentration was adjusted with growth medium and recounted. HAAEC were seeded in each model at a density of 1.25 x 10^6 cells/mL and grown to confluence (approximately 48 h) on a rotator at 8 rpm (Labquake Rotor, Series 1104, Barnstead/Thermolyne) in a 37°C incubator (5% CO_2). The cell culture media was changed every 24 h.

6.5.3. Flow Experiment

Two days after seeding, the models were connected to a closed perfusion loop consisting of vented media reservoirs, flow dampeners, an 8-channel programmable peristaltic pump (Ismatec Pump, Cole-Parmer Canada, Montreal, Canada), and silicone tubing (Cole Parmer Canada, Montreal, Canada). A schematic of the flow loop is presented in Figure 6-1. The viscosity of the cell culture medium was measured to be 0.975 cP at 37°C using a double gap rheometer (Bohlin, Model CVO 120 h) (Rouleau et al. 2010b). From the measured viscosity and model radius, the following equation was used to calculate the volumetric flow rate (Q) required to obtain a desired wall shear stress in a straight rigid tube.

$$Q = \frac{\tau \pi r^3}{4\mu}$$

Where τ is wall shear stress, r is the radius and μ is the dynamic viscosity. The Reynold's number was maintained well under 2000 and theoretical entrance length calculations were done to ensure that the flow was fully developed. Previous work by our group has validated the developed nature of the flow using photochromic molecular flow visualization in the tubular cell culture models (Rouleau et al. 2010a). Confluent HAAEC were pre-conditioned with steady

laminar flow (LSS = 1.25, 12.5 or 25 dynes/cm^2) for 6 h in growth medium. Without stopping the perfusion, control vehicle (0.06% v/v DMSO in cell growth medium), simvastatin or simvastatin with mevalonate were added to the perfusion media to reach concentrations of 0.1, 1 or 10 µM simvastatin and 200 µM mevalonate. The endothelial cells were preconditioned for 6 h prior to the addition of simvastatin to minimize the effect of the acute response of endothelial cells to flow (Chien 2007; Sampath et al. 1995) and to create a more realistic model of *in vivo* conditions given that the endothelium is under the influence of flow before treatment with statins. The flow experiment was continued for 24 h after the addition of simvastatin and mevalonate. Static controls were run in parallel. These cells were treated with the indicated concentrations of simvastatin with or without 200 µM mevalonate for 24 h in the absence of flow.

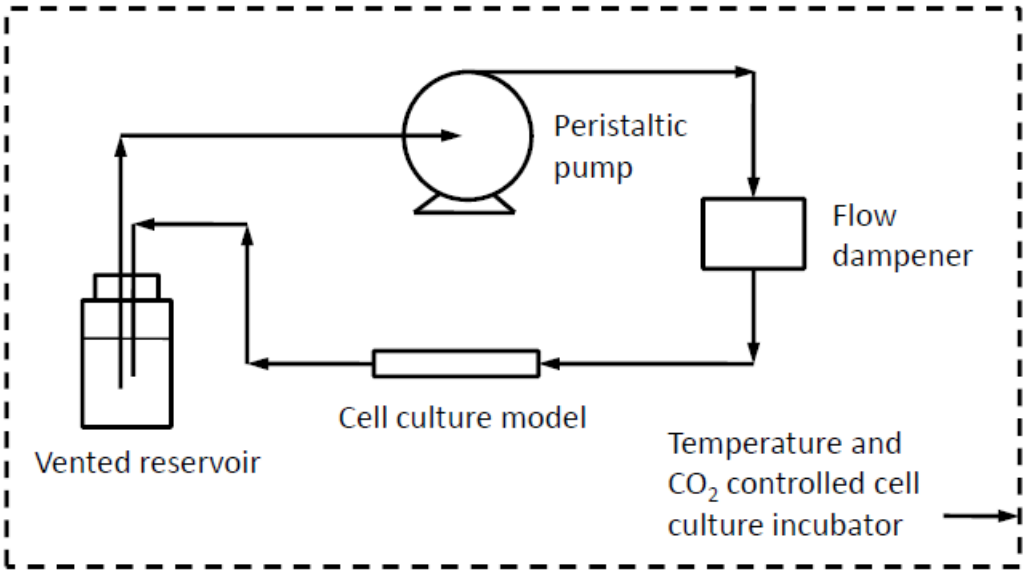

Figure 6-1: Schematic representation of the flow system. The entire set-up was located in a humidified chamber at 37°C with 5% CO_2.

The concentrations of simvastatin selected for this study range from clinically realistic to substantially higher than the endothelium would be exposed to *in vivo*

as clinical plasma levels vary from 10 to 34 ng/mL (0.03 to 0.08 µM) (Bellosta et al. 2004). The 0.1 to 10 µM simvastatin concentration range was selected to detect changes in endothelial cell gene expression in a relatively short time frame (24 h statin treatment).

6.5.4. Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

HAAEC were gathered from the models with 0.25% Trypsin/EDTA (Invitrogen, Burlington, Canada). Total RNA was extracted using RNeasy spin columns (Qiagen, Mississauga, Canada) with on column DNase I digestion (Qiagen, Mississauga, Canada). Total RNA was quantified by absorbance measurements at 260 nm. First-strand complementary DNA (cDNA) was synthesized with 0.5 µg total RNA, random hexamers (Applied Biosystems, Foster City, CA) and MultiScribe™ reverse transcriptase (Applied Biosystems, Foster City, CA) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Real-time PCR reactions were carried out in Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) with QuantiTect Primer Assays (β-Actin QT01680476, KLF2 QT00204729, eNOS QT00089033, TM QT00199920, Qiagen, Mississauga, Canada) and performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems, Foster City, CA) under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The housekeeping gene was β-Actin and the ΔΔCt method was used for relative quantification of gene expression.

6.5.5. eNOS and TM Protein Analysis by Western Blot

HAAEC cells were gathered with trypsin, washed twice in PBS and stored in RIPA lysis buffer (50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1% NP-40, 0.5%

sodium deoxycholate, 0.1% SDS (Fisher Scientific, Whitby, Canada)) with protease inhibitor cocktail (Sigma-Aldrich, Oakville, Canada) at -80°C. Lysis of the cell pellet was achieved by sonication for 30 min and the lysate was separated from the cell debris by centrifugation (16,000 g, 15 min). The Bicinchoninic acid (BCA) assay method (Pierce, Rockford, IL) was used to determine protein concentration. Proteins were separated on 7% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were immunoblotted with mouse monoclonal anti-eNOS (1:300, BD Transduction Laboratories, Mississauga, Canada), mouse monoclonal anti-TM (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal anti-glyceraldehyde phosphate dehydrogenase (GAPDH, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight at 4°C. The membranes were washed in PBST (0.1% Tween 20 in PBS) 4 times and probed with goat anti-mouse (1:10000, Biorad, Mississauga, Canada) secondary antibodies. The blots were washed 4 times in PBST and the immunoreactive bands were detected with SuperSignal® West PICO Chemiluminescent Substrate (Pierce, Rockford, IL). Densiometric analyses were performed using ImageJ (National Institutes of Health, Bethesda, Maryland) gel analyzer tools. Western blotting experiments were performed repeatedly (n=3), the x-ray films were developed, scanned, and the image file (.tiff) was used.

6.5.6. Statistical Analysis

Data is presented as the mean ± standard error (S.E.M.) for each test condition. A minimum of 3 independent repeats were analyzed. The effect of simvastatin and LSS was evaluated with a one-way or two-way analysis of variance (ANOVA) with Bonferroni post-tests. One-sample t-tests were used to determine whether the combined statin and LSS effects were significantly different from the sum of the mean individual responses. A p-value less than 0.05 was considered significant. All analysis was performed with GraphPad Prism™ 5.0 software (GraphPad, San Diego, CA).

6.6. Results

6.6.1. LSS increases KLF2, eNOS, and TM mRNA

The effect of different magnitudes of LSS on the expression of KLF2, eNOS, and TM was verified, Figure 6-2. The data is presented as fold increases over static control cells. As expected, LSS significantly enhanced KLF2, eNOS, and TM mRNA with the greatest increase observed at the highest shear stress ($P<0.01$, one-way ANOVA). At 25 dynes/cm^2, KLF2, eNOS, and TM mRNA expression were 4.5, 5.6 and 3.6 -fold greater than static controls, respectively.

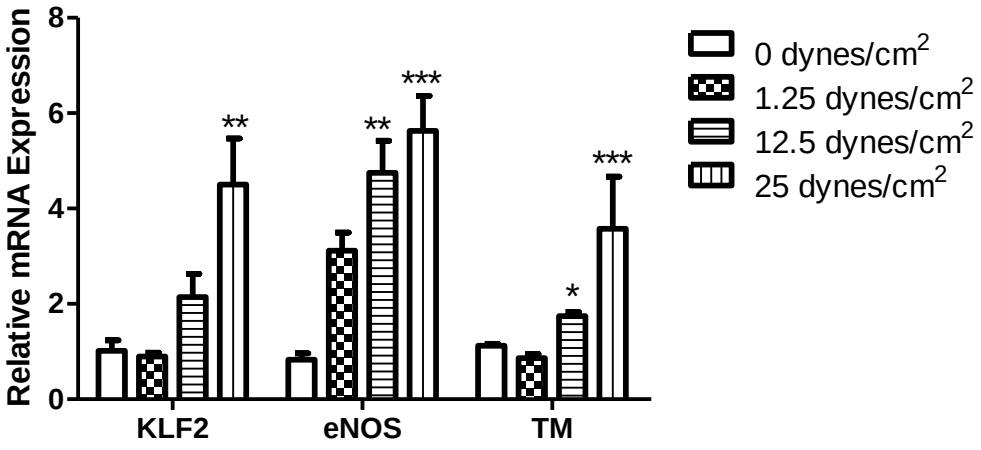

Figure 6-2: Effect of LSS on KLF2, eNOS, and TM mRNA expression. HAAEC were exposed to various levels of LSS (0, 1.25, 12.5 or 25 dynes/cm^2) for 30 h. The data was normalized to the static control by the ΔΔCt method. The values are means ± S.E.M. for n=3 or 4 separate experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

6.6.2. Simvastatin increases KLF2, eNOS, and TM Expression

The effect of simvastatin on KLF2, eNOS, and TM mRNA levels in endothelial cells under static culture is shown in Figure 6-3A. Simvastatin

significantly enhanced KLF2, eNOS, and TM mRNA levels in a concentration dependent manner ($P<0.05$, one-way ANOVA) to a maximum of 5.4, 3.9 and 3.4 -fold increase over the control cells, respectively. As expected, the induction of KLF2, eNOS, and TM by simvastatin was completely eliminated upon co-incubation with 200 µM of mevalonate, a precursor to cholesterol and isoprenoid intermediate synthesis. The effect of simvastatin on eNOS and TM protein levels was evaluated by Western blot analysis, Figure 6-3B. Similar to the mRNA results, eNOS and TM protein increased in a dose-dependent manner with the highest levels observed at 10 µM simvastatin. This dose dependent increase in protein is evident in the densiometry analysis, however, statistical significance was not achieved. Appropriate antibodies for KLF2 protein analysis were not available and therefore no protein data is presented for KLF2.

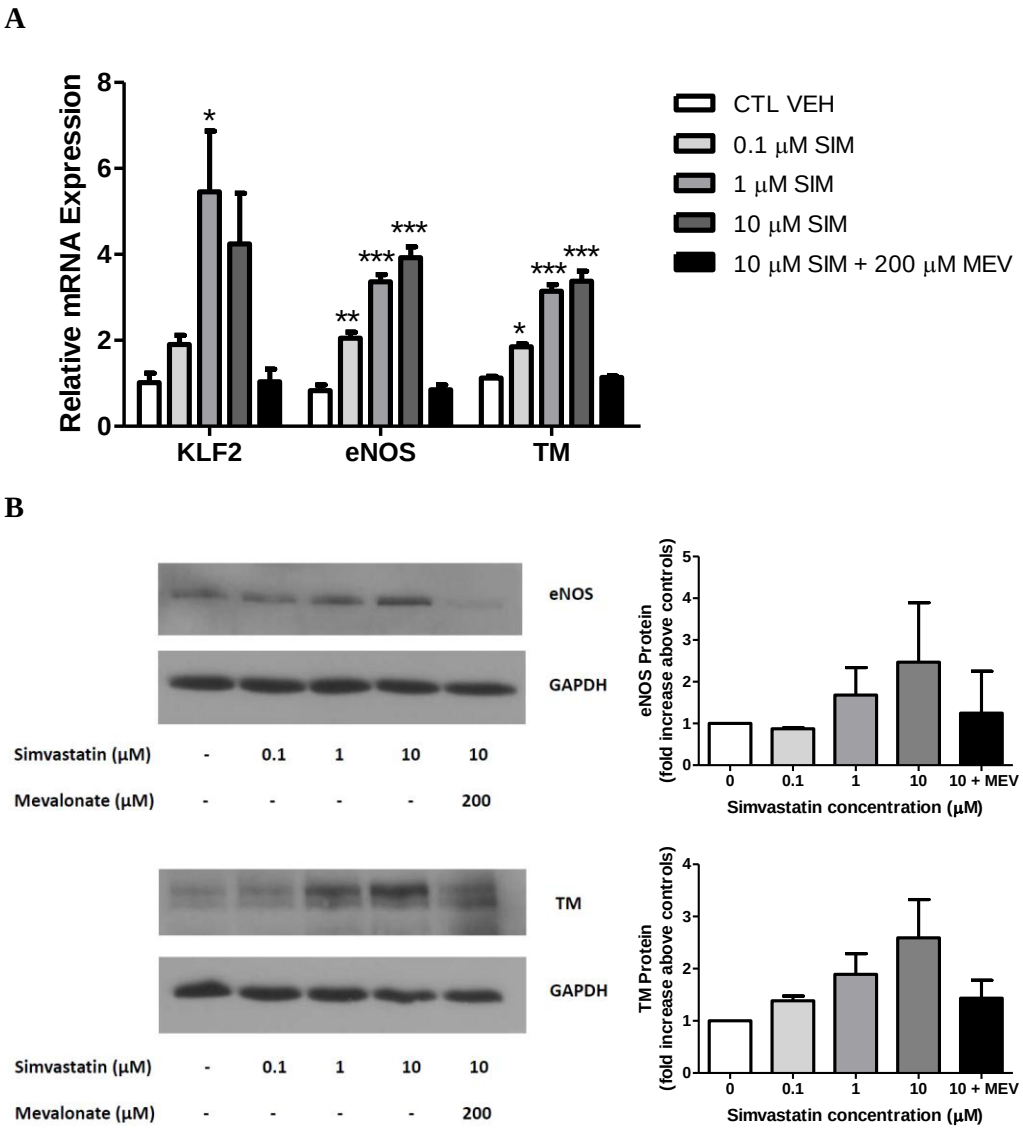

Figure 6-3: Effect of 24 h simvastatin treatment on KLF2, eNOS, and TM mRNA (A) and eNOS and TM protein expression (B) in HAAEC under static culture. The cells were treated with either control vehicle (CTL VEH: 0.06% v/v DMSO), simvastatin (0.1, 1 or 10 μM) or 10 μM simvastatin with 200 μM mevalonate for 24 h. A: The mRNA data was normalized to the unstimulated static control. The values are means ± S.E.M. for n=3 or 4 separate experiments (*p<0.05, **p<0.01, and ***p<0.001). SIM: Simvastatin; MEV: Mevalonate. B: Protein analysis was done by Western blot using 8 μg of total protein separated on 7% SDS-PAGE gels. The data is presented as the mean ± S.E.M. for 3 separate experiments.

6.6.3. Simvastatin Increases KLF2, eNOS, and TM mRNA Levels in Endothelial Cells Exposed to LSS

The effect of simvastatin on KLF2, eNOS, and TM mRNA levels in endothelial cells under dynamic stimulation is shown in Figure 6-4. Both LSS and simvastatin had a significant effect on all genes (two-way ANOVA). Pre-conditioning endothelial cells with LSS for 6 h followed by the addition of simvastatin and continuing the culture for 24 h under LSS significantly elevated the mRNA expression of KLF2, eNOS, and TM above levels achieved by LSS alone, Figure 6-4. A combination of LSS at 25 dynes/cm^2 and 10 μM simvastatin produced the highest KLF2, eNOS, and TM mRNA levels of 11.7, 13.1 and 10.3 - fold increase above static controls, respectively. Co-incubation of 10 μM simvastatin with 200 μM mevalonate under LSS completely eliminated the effect of simvastatin. As shown by Western blot analysis, LSS at 25 dynes/cm^2 elevated eNOS and TM protein levels above static controls, Figure 6-5. Similar to the mRNA results, the combination of LSS (25 dynes/cm^2) and simvastatin (10 μM) generated higher eNOS and TM protein levels than LSS alone. Densiometry analysis on multiple samples revealed statistical significance between the static expression of eNOS and LSS alone or with 10 μM simvastatin ($P<0.05$, one-way ANOVA). Trends indicating greater eNOS and TM protein levels with combined LSS and 10 μM simvastatin compared to LSS alone were observed, however, statistical significance was not seen, Figure 6-5.

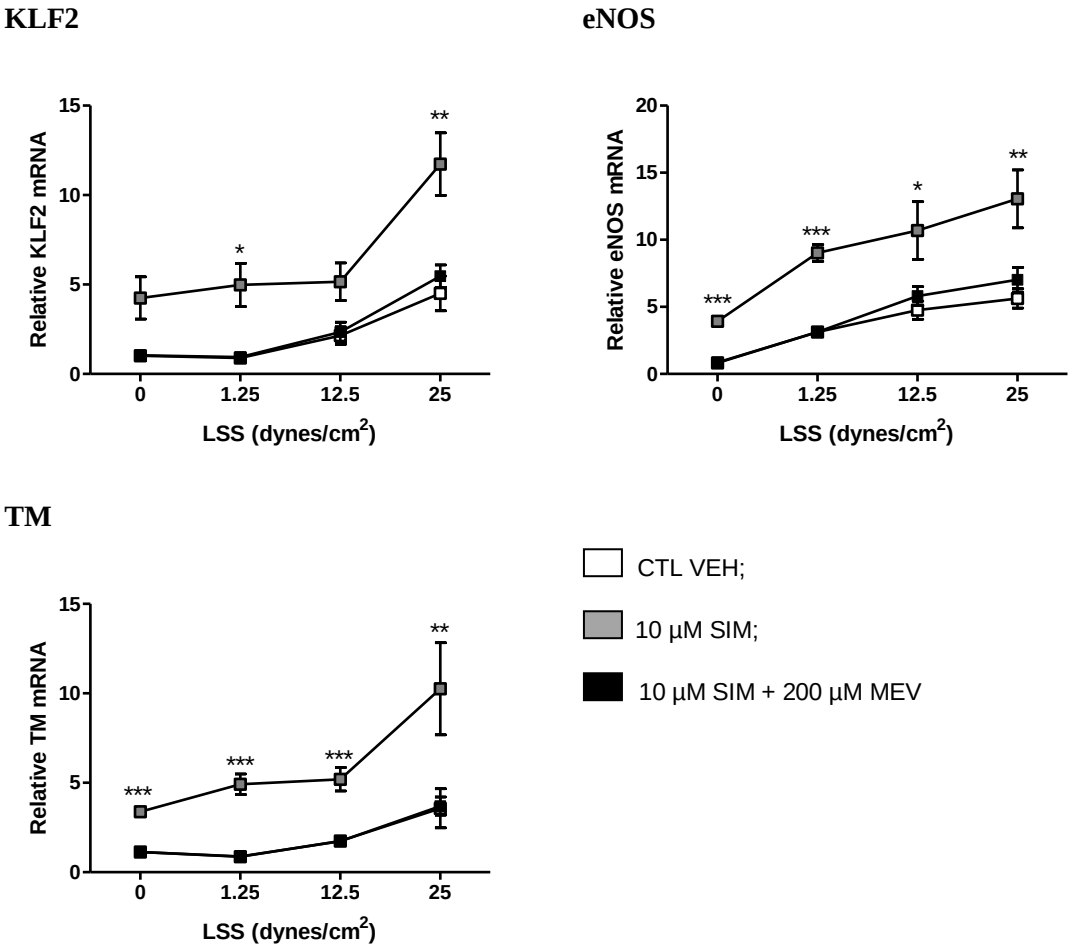

Figure 6-4: Effect of 24 h simvastatin treatment on KLF2, eNOS, and TM mRNA in HAAEC exposed to various levels of LSS (0, 1.25, 12.5, or 25 dynes/cm^2). The cells were preconditioned with LSS for 6 h and then either control vehicle, simvastatin or simvastatin with mevalonate was added to the perfusion media to reach 10 µM of simvastatin and 200 µM of mevalonate. The data was normalized to the unstimulated static control. The values are means ± S.E.M. for n=3 or 4 separate experiments. Statistically significant differences between 10 µM simvastatin and control cells are labeled (*p<0.05, **p<0.01, ***p<0.001). CTL VEH: Control vehicle; SIM: Simvastatin; MEV: Mevalonate.

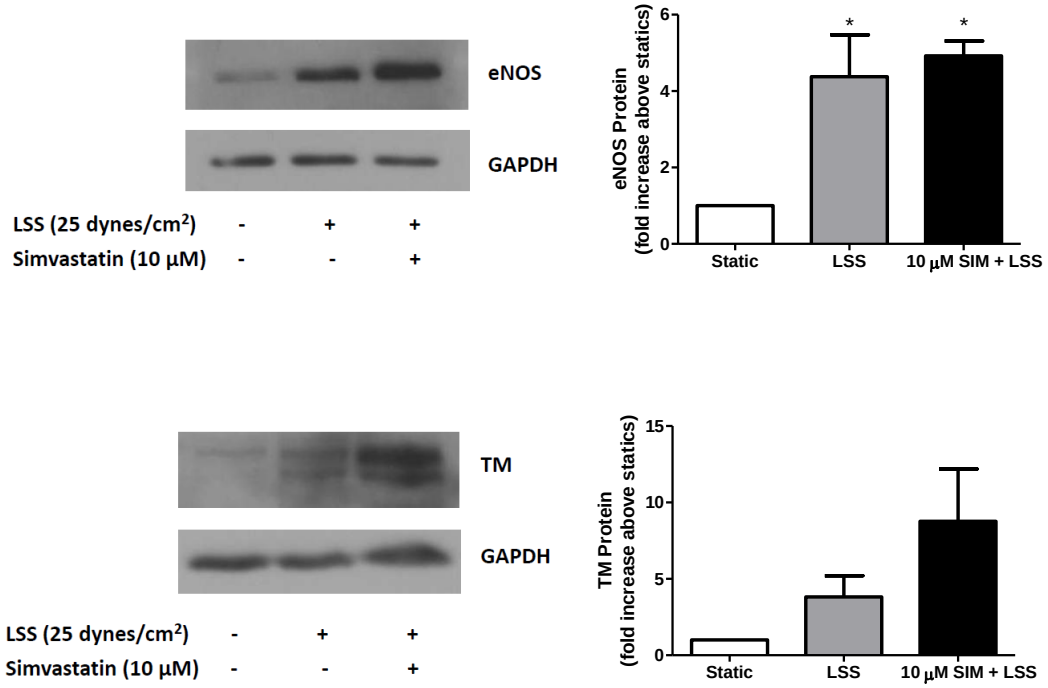

Figure 6-5: Effect of 25 dynes/cm^2 LSS and 10 µM simvastatin on eNOS and TM protein levels. Protein analysis was done by Western blot using 8 µg of total protein separated on 7% SDS-PAGE gels. The data is presented as the mean ± S.E.M. for 3 separate experiments.

6.6.4. Combination of Simvastatin and LSS Produces an Additive Increase in KLF2, eNOS, and TM mRNA

Following the observation that the combination of LSS and simvastatin produces a greater increase in KLF2, eNOS, and TM mRNA, we then evaluated whether this effect was additive or synergistic, Figure 6-6. This was determined by comparing the observed response of simvastatin with LSS to the arithmetic sum of the mean individual responses (predicted response). At lower simvastatin concentrations (0.1 and 1 µM) the observed value is generally comparable to the predicted response for all genes, except for TM at 0.1 µM simvastatin. At 0.1 µM simvastatin with 25 dynes/cm^2 LSS, the observed response was significantly less than the predicted response for TM ($P<0.05$, one sample t-test). Consequently, an

antagonistic response was observed under these conditions for TM. At high simvastatin concentration (10 μM), the combination of simvastatin and 25 dynes/cm^2 LSS resulted in a response that was slightly greater than the predicted value. Although t-test analysis between the observed and predicted response demonstrated that there were no significant differences at 10 μM simvastatin. Consequently, our results suggest that both LSS and simvastatin increase KLF2, eNOS, and TM mRNA separately and combining the two stimuli results for the most part in an additive increase in all three genes.

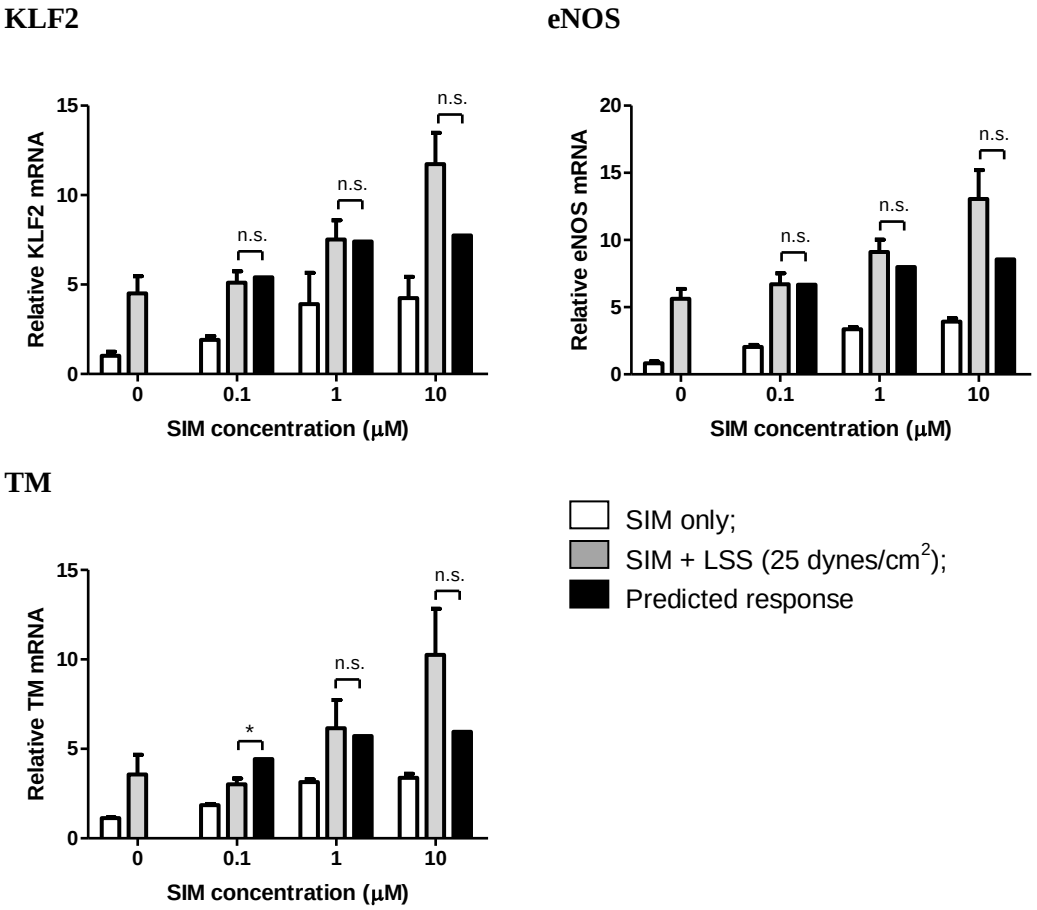

Figure 6-6: Additive increase in KLF2, eNOS, and TM mRNA detected when LSS and simvastatin are combined. HAAEC were pre-conditioned with LSS (25 dynes/cm^2) for 6 h. Afterwards, simvastatin (0.1, 1 or 10 μM) was added and the flow experiment was continued for 24 h. The predicted response represents the level of mRNA expected if the individual effects of LSS and simvastatin are added (Predicted Response = Relative increase due to simvastatin + Relative increase due to LSS – Baseline (1)). The data was normalized to the unstimulated static control. The values are means ± S.E.M. for n=3 or 4 separate experiments. SIM: Simvastatin. (*P<0.05 and n.s.: not significant).

6.7. Discussion

As a result of their location at the inner artery wall, endothelial cells are in direct contact with blood and are thus subjected to fluid mechanical forces including hydrostatic pressures, cyclic stretch, and fluid wall shear stress (Chien 2007; Garcia-Cardena and Gimbrone 2006). These hemodynamic forces, in

particular wall shear stress, are essential for maintaining proper endothelial cell function (Davies 1995; Dewey et al. 1981; Garcia-Cardena et al. 2001; Gimbrone et al. 2000). Wall shear stress has been shown in numerous studies to alter endothelial cell morphology and the expression of genes related to inflammation, blood coagulation, thrombosis and vascular constriction (Dekker et al. 2002; Dewey et al. 1981; Korenaga et al. 1997; Levesque and Nerem 1985; Nagel et al. 1994). The shear-responsive transcription factor KLF2 has been associated with the induction of the vasoprotective genes eNOS and TM (Fledderus et al. 2007; SenBanerjee et al. 2004). It has also been shown that statin drugs such as lovastatin, mevastatin and simvastatin can induce KLF2 expression and the expression of its downstream targets eNOS and TM (Sen-Banerjee et al. 2005; van Thienen et al. 2006). Owing to the observation that both laminar wall shear stress and statins can upregulate these atheroprotective genes we wondered whether there may be interactions between the two stimuli, which could result in either a synergistic or antangonistic effect when combined.

In the present study, our findings show that treatment of endothelial cells with LSS and simvastatin separately enhances KLF2, eNOS, and TM mRNA, which is supported with numerous other studies as previously discussed. The combination of both LSS and simvastatin produces the greatest induction of these genes. Combining LSS and simvastatin resulted in a response that was in general not significantly different from the sum of the mean individual effects (i.e. additive effect). Consequently, no significant interactions between LSS and simvastatin were detected at each concentration investigated (0.1, 1 and 10 μM) for KLF2 and eNOS. In addition, no interaction between LSS and simvastatin was seen at 1 and 10 μM simvastatin for TM. The only interaction observed was for TM at 0.1 μM simvastatin and 25 dynes/cm^2 whereby an antagonistic effect was detected. With the exception of TM at 0.1 μM simvastatin, our results suggest that under static and dynamic stimulation from LSS, the endothelial cells are equally responsive to statins. The additive nature of LSS and simvastatin on KLF2, eNOS, and TM expression provides further evidence that statin drugs may have non-low

density lipoprotein (LDL) lowering benefits in vascular biology through its effects on endothelial cells. We have shown that simvastatin can increase KLF2, eNOS, and TM gene expression in endothelial cells conditioned with shear stress which is more representative of *in vivo* conditions than static culture. Further studies are recommended to determine whether the modulation of other genes by statins observed in static culture hold when cells are under physiologically realistic biomechanical stimulation (shear stress, stretch and/or pressure). Interestingly, Ali et al. (2009) observed that cultured endothelial cells became more responsive to statin treatment when pre-conditioned with LSS (Ali et al. 2009). They reported that atorvastatin induction of HO-1 was enhanced when HUVEC were pre-conditioned for 12 h with LSS (12 dynes/cm^2). They showed that synergy was detected at low atorvastatin concentration (0.6 and 1.25 μM), whereas only an additive effect was observed at higher concentrations (2.5 μM). Aside from this work, to the best of our knowledge no other study has evaluated the synergy/antagonism of statins and LSS. In the same paper, Ali et al. (2009) showed that LSS alone and with atorvastatin enhanced KLF2 and eNOS mRNA in an Akt dependent pathway. Although they presented KLF2, eNOS, and TM mRNA levels, they did not compare the difference in expression between LSS alone and LSS with atorvastatin nor whether the increase was additive or synergistic.

Our data shows that mevalonate, a precursor to geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP) synthesis, eliminates the upregulation of KLF2, eNOS, and TM by simvastatin at each LSS investigated. These results suggest that the inhibition of HMG-CoA reductase is the mechanism for the statin-induced changes in gene expression in endothelial cells and that statin pleiotropy is conserved under low, medium, and high LSS. By blocking mevalonate synthesis, statins prevent the formation of isoprenoid intermediates FPP and GGPP. These molecules serve as lipid attachments for small GTP-binding proteins such as Rho, Rac and Ras. These intracellular signaling molecules require isoprenylation by FPP or GGPP for

proper membrane localization and activation and are involved in many cellular processes including the activation of proinflammatory pathways, the generation of reactive oxygen species, and regulation of cell proliferation, shape, and motility (Cernuda-Morollon and Ridley 2006; Jain and Ridker 2005; Loirand et al. 2006). Thus, by inhibiting isoprenylation, the GTPases remain inactive in the cytoplasm, and may be a mechanism for the cholesterol independent effects of statins. In agreement with our findings, several previous studies have attributed the modulation of KLF2, eNOS, and TM expression by statins as well as other genes to inhibition of GTPase activity by preventing mevalonate and isoprenoid intermediate synthesis (Laufs and Liao 1998; Masamura et al. 2003; Parmar et al. 2005; Sen-Banerjee et al. 2005).

6.8. Conclusions

We have shown that simvastatin increases KLF2, eNOS, and TM mRNA levels in cultured endothelial cells exposed to low, medium, and high LSS through inhibition of HMG-CoA reductase. Both LSS and simvastatin separately increase these genes and combining both stimuli produces an additive increase in mRNA levels. These results provide additional support that statin drugs may have potential non-lipid lowering effects.

6.9. Acknowledgments

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CHAPTER 7: DIFFERENTIAL RESPONSE OF ENDOTHELIAL CELLS TO SIMVASTATIN WHEN CONDITIONED WITH STEADY, NON-REVERSING PULSATILE OR OSCILLATING SHEAR STRESS

7.1. Preface

In chapter 6, we demonstrated that steady laminar wall shear stress and simvastatin separately upregulated KLF2, eNOS and TM in endothelial cells, and combining the two stimuli additively increased gene expression. In this study, we tested the hypothesis that the expression of these genes is sensitive to pulsatile flow. We evaluated the effect of simvastatin on KLF2, eNOS and TM expression in endothelial cells that have been exposed to temporal gradients in shear stress (non-reversing pulsatile and oscillating) and compared it to steady flow and static culture. We demonstrated that simvastatin can increase KLF2, eNOS and TM expression in endothelial cells exposed to static culture and all three flow conditions investigated. However, impairment in gene expression was observed under oscillating shear stress at lower concentrations. Synergy was detected at the highest simvastatin concentration evaluated under all flow conditions. These findings provide additional evidence that statin drugs may have non-lipid lowering benefits in vascular biology through its effects on endothelial cells. This paper will be published in Annals of Biomedical Engineering DOI: 10.1007/s10439-010-0145-9.

7.2. Article 4

Differential Response of Endothelial Cells to Simvastatin when Conditioned with Steady, Non-Reversing Pulsatile or Oscillating Shear Stress

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7.3. Abstract

Few studies have investigated whether fluid mechanics can impair or enhance endothelial cell response to pharmacological agents such as statin drugs. We evaluated and compared Kruppel-like factor 2 (KLF2), endothelial nitric oxide synthase (eNOS), and thrombomodulin (TM) expression in human abdominal aortic endothelial cells (HAAEC) treated with increasing simvastatin concentrations (0.1, 1 or 10 μM) under static culture and shear stress (steady, non-reversing pulsatile, and oscillating). Simvastatin, steady flow, and non-reversing pulsatile flow each separately upregulated KLF2, eNOS, and TM mRNA. At lower simvastatin concentrations (0.1 and 1 μM), the combination of statin and unidirectional steady or pulsatile flow produced an overall additive increase in mRNA levels. At higher simvastatin concentration (10 μM), a synergistic increase in eNOS and TM mRNA expression was observed. In contrast, oscillating flow impaired KLF2 and TM, but not eNOS expression by simvastatin at 1 μM. A higher simvastatin concentration of 10 μM overcame the inhibitory effect of oscillating flow. Our findings suggest that oscillating shear stress renders the endothelial cells less responsive to simvastatin than cells exposed to unidirectional steady or pulsatile flow. Consequently, the pleiotropic effects of statins *in vivo* may be less effective in endothelial cells exposed to atheroprone hemodynamics.

7.4. Introduction

Atherosclerosis, a condition in which blood vessels harden and narrow, is the underlying cause of most heart attacks and strokes. Although diet, lifestyle, and genetic risk factors play an important role in the development of atherosclerotic plaques [1], the disease is focal in nature forming preferentially at specific sites in the arterial tree [2, 3]. Dysfunction of the endothelial cells that line the cardiovascular system due to disturbed hemodynamic forces has been hypothesized to cause atherogenesis [4-6]. An extensive amount of work has been done to understand the effect of hemodynamic forces (shear stress, hydrostatic pressure, and stretch) on vascular cell function [7-9]. It is now well established that these forces play an important role in vascular health and disease. Wall shear stress in particular has been shown to be an important regulator of endothelial cell function and morphology [10-12]. Many genes have been identified as shear responsive including the transcription factor Kruppel-like factor 2 (KLF2) [13-15] and some of its downstream target genes, including endothelial nitric oxide synthase (eNOS) [16] and thrombomodulin (TM) [16]. Both nitric oxide and TM are associated with vascular health as they have important anti-inflammatory and anti-thrombotic functions [17-19].

Pharmacological agents such as statins (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors), are designed to slow down atherosclerosis progression by inhibiting the biosynthesis of cholesterol [20], however statins are now recognized to also display non-lipid lowering benefits [21-23]. Statins have been shown in numerous studies to modulate endothelial cell gene expression including KLF2, eNOS, and TM [24-28]. Most studies evaluating the effect of statins on endothelial cell gene expression have been done in static culture and have neglected the effect of blood flow. Recent studies have shown that endothelial cell response to statin therapy may be altered by wall shear stress [29, 30]. Ali *et al.* found that preconditioning endothelial cells with steady laminar flow enhanced the induction of heme oxygenase-1 (HO-1) by atorvastatin, while

oscillating shear stress impaired it [29]. We have recently reported that laminar wall shear stress and simvastatin separately upregulated KLF2, eNOS, and TM in endothelial cells, and combining the two stimuli additively increased gene expression with a trend towards synergy observed at high simvastatin concentration [30]. In this study we sought to evaluate whether different types of shear stress, namely steady (18 dynes/cm^2), non-reversing pulsatile (1Hz, 18 ± 9 dynes/cm^2), and oscillating (1 Hz, 0.3 ± 3 dynes/cm^2) flow, can affect statin pleiotropy. Specifically, we evaluated the simvastatin induced increase in KLF2, eNOS, and TM in endothelial cells. Knowledge of possible shear stress/statin interactions will provide a better understanding of statin therapy.

7.5. Methods

7.5.1. Materials

Simvastatin (S6196, Sigma-Aldrich) was converted from an inactive lactone prodrug to the active acid form by alkaline hydrolysis. Simvastatin lactone was first dissolved in dimethyl sulfoxide (DMSO) to a concentration of 25 mM. Afterwards, 300 µL of 0.1 M NaOH was added to 100 µL of the 25 mM simvastatin solution and then incubated at 50°C for 2 h. The pH was adjusted to 7.4 with 0.1 M HCl and diluted to 0.5 mM in phosphate buffered saline (PBS). Mevalonic acid lactone (M4667) was obtained from Sigma-Aldrich, dissolved in DMSO to a concentration of 1 M and diluted in PBS to 50 mM. Both simvastatin and mevalonic acid solutions were filter sterilized in a laminar flow cabinet through a 0.22 µM polyvinylidene fluoride (PVDF) membrane and diluted to working concentrations in PromoCell endothelial cell growth medium (C-22010, PromoCell).

7.5.2. Cell Culture

Human abdominal aortic endothelial cells (HAAEC) derived from a 20 year old male were purchased from American Type Tissue Collection (ATCC, CRL-2472). HAAEC were cultured on 0.1% pig gelatin (Sigma-Aldrich) pre-coated vented tissue culture flasks in PromoCell growth medium (C-22010, PromoCell) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and PromoCell supplement mix (C-39215, PromoCell). The cells were grown in a 37°C humidified chamber with 5% CO_2. Cell culture medium was refreshed every other day. At passage 5, the cells were gathered with 0.25% trypsin/EDTA (Invitrogen), diluted in growth medium and counted with a cell counter (Z2^{TM} Coulter$^{®}$ Particle Count and Size Analyzer, Beckman Coulter Canada). Cell concentration was adjusted and recounted. The cells were seeded at a density of $1.25x10^6$ cells/mL into fibronectin pre-coated (F0895, Sigma-Aldrich) three dimensional tubular models (2 mm in diameter) made of Sylgard^{TM} silicone elastomer (Dow Corning). The models were rotated (Labquake Rotor, Series 1104, Barnstead/Thermolyne) overnight at 8 rpm in a 37°C incubator to ensure even coverage of the endothelial cells on the model lumen surface. The cells were cultured until confluent with growth medium changes every day. A more detailed description of the flow models is provided elsewhere [31-33].

7.5.3. Flow Experiment

The models containing confluent endothelial cells were connected to a closed perfusion loop consisting of a programmable 8 or 12 channel peristaltic pump (Ismatec Pump, Cole-Parmer), silicone tubing (Cole-Parmer), and vented reservoirs containing either unaltered growth medium, 0.06% v/v DMSO (vehicle control), simvastatin (0.1, 1, or 10 µM) or 10 µM simvastatin and 200 µM mevalonate in growth medium. Sinusoidal oscillating (1 Hz, 0.3 ± 3 dynes/cm^2)

and non-reversing pulsatile (1 Hz, 18 ± 9 dynes/cm^2) flow was achieved by controlling the peristaltic pump with a Labview version 8 (National Instruments) interface. Steady flow (18 dynes/cm^2) was obtained by passing the flow through dampeners before entering the models. Static models were run in parallel. The entire setup was assembled in a laminar safety cabinet and placed in a humidified incubator at 37°C with 5% CO_2. Simvastatin and flow (steady, non-reversing pulsatile and oscillating) were introduced to the cells simultaneously. The experiment was stopped after 24 h.

7.5.4. Cell Morphology

The models were disconnected from the perfusion loop and rinsed three times in phosphate buffered saline (PBS). The cells were then fixed with 1% w/v paraformaldehyde (Sigma-Aldrich) in PBS for 20 min. After fixation, the paraformaldehyde was removed, the cells were rinsed once with PBS and stored in a 1:1 v/v glycerol:PBS solution at 4°C. Cell morphology was assessed by adding crystal violet (BD Biosciences) to the cells. After staining for 5 min, the cells were rinsed with PBS. Light microscopy images were obtained at 100X magnification with a Leica DMIL microscope and Leica DC300 camera (Leica Microsystems).

7.5.5. Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The endothelial cells were extracted from the three dimensional tubular cell culture models with 0.25% Trypsin-EDTA and rinsed in PBS. Total RNA was extracted from cell pellets using RNeasy Mini Spin columns (Qiagen) with on-column DNASE1 digestion (Qiagen) following the manufacturer's instructions. The RNA was quantified with a UV-Visible spectrophotometer at 260 nm and reverse transcribed into complementary DNA (cDNA) with random

hexamers (Applied Biosystems) and MultiScribe^{TM} reverse transcriptase (Applied Biosystems) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Quantitative real-time PCR was carried out in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) using SYBR green (Applied Biosystems) and QuantiTect Primer Assays (β-Actin QT01680476, KLF2 QT00204729, eNOS QT00089033, TM QT00199920, Qiagen). The cycling conditions were: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Relative quantification of gene expression was achieved by normalizing the mRNA data to the housekeeping gene β-actin by the ΔΔCt method.

7.5.6. Statistical Analysis

The mRNA data is presented as the mean ± standard error (SEM) of 4 or 5 separate experiments of each flow condition on separate days. The data was analyzed either by t-tests or one-way analysis of variance (ANOVA) followed by Bonferroni post-tests. Differences were considered statistically significant with a p-value less than 0.05. All data was analyzed with GraphPad Prism 5.0 software (GraphPad).

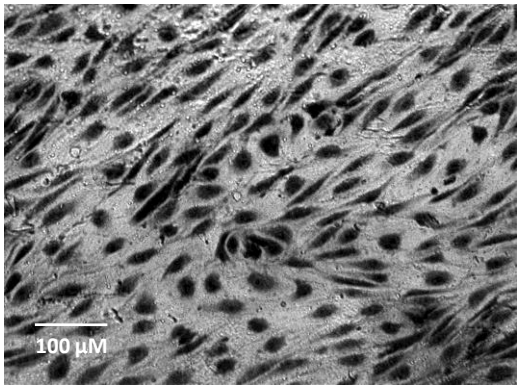
7.6. Results

7.6.1. Cell Morphology

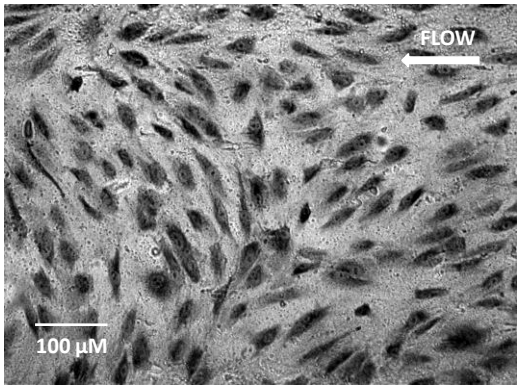
The influence of shear stress on endothelial cell morphology was examined by crystal violet staining using an inverted light microscope (Figure 7-1). As expected, unidirectional steady (18 dynes/cm^2) and pulsatile (1 Hz, 18 ± 9 dynes/cm^2) flow changed the morphology such that the cells were aligned and elongated in the flow direction (Figure 7-1C and D). The morphology of cells exposed to oscillating shear stress (1 Hz, 0.3 ± 3 dynes/cm^2) for 24 h was similar

to static culture in which the cells had a rounded and cobblestone shape with no obvious direction of alignment (Figure 7-1A and B).

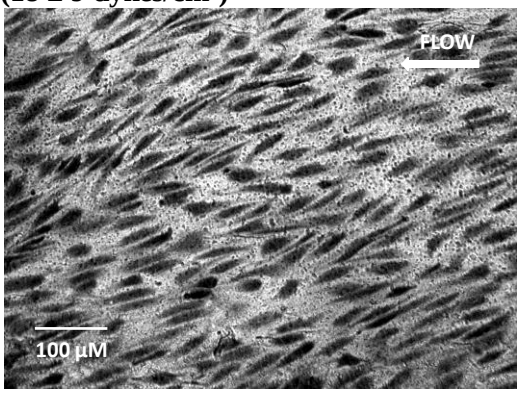
A: Static Culture



B: Oscillating Flow (0.3 ± 3 dynes/cm^2)



C: Non-reversing Pulsatile Flow (18 ± 9 dynes/cm^2)



D: Steady Flow (18 dynes/cm^2)

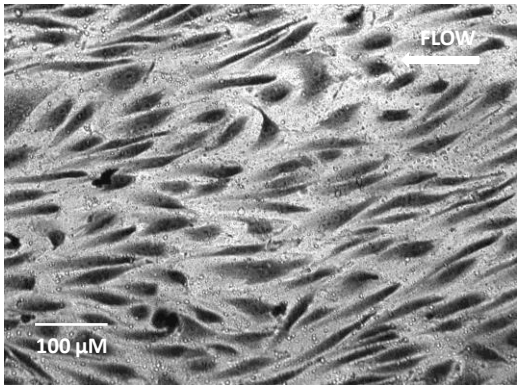
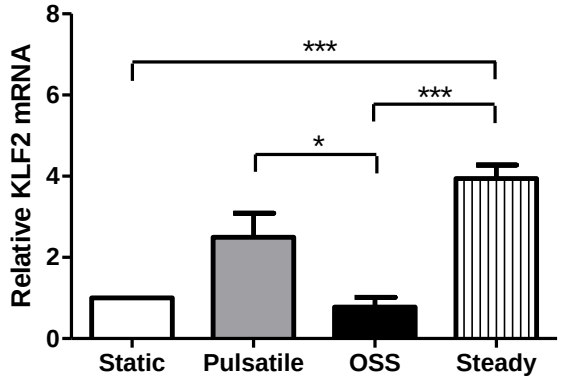


Figure 7-1: Effect of 24 h exposure to steady laminar (18 dynes/cm^2), non-reversing pulsatile (18 ± 9 dynes/cm^2) and oscillating shear stress (0.3 ± 3 dynes/cm^2) on endothelial cell morphology. The endothelial cells were stained with crystal violet and observed under an inverted light microscope (Magnification = 100X).

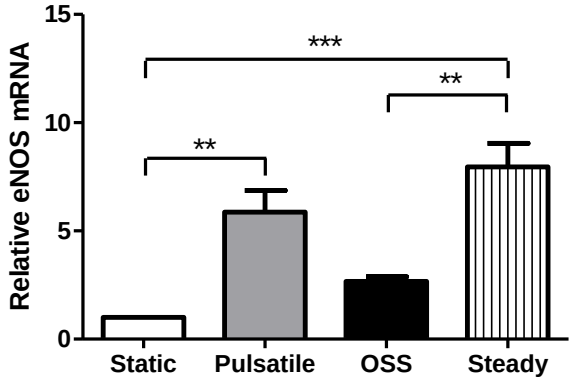
7.6.2. Effect of Steady, Pulsatile and Oscillating Flow on KLF2, eNOS, and TM Expression

The effect of steady, non-reversing pulsatile and oscillating shear stress on KLF2, eNOS and TM expression is shown in Figure 7-2. In all cases, the type of flow had a significant effect on the mRNA levels ($p<0.001$ for KLF2, eNOS and TM, one-way ANOVA). Endothelial cells exposed to steady laminar flow had significantly higher KLF2, eNOS, and TM mRNA levels at 24 h than cells under oscillating shear stress and static culture ($p<0.01$, Bonferroni's multiple comparison test), Figure 7-2. Specifically, KLF2, eNOS and TM mRNA levels were 3.9, 8.0 and 3.5-fold greater than static controls, respectively. Steady laminar flow had a greater effect on mRNA expression than unidirectional pulsatile shear stress. Statistically significant differences between steady and pulsatile flow was observed for TM only ($p<0.05$, Bonferroni's multiple comparison test). Unidirectional pulsatile shear stress significantly increased eNOS and TM expression to a value of 5.9 and 2.4-fold higher than static control cells, respectively ($p<0.01$, Bonferroni's multiple comparison test). A slight increase in KLF2 mRNA levels compared to static cells was found with non-reversing pulsatile flow although statistical significance was not observed. It was also detected that KLF2 and TM mRNA levels were significantly higher in cells treated with pulsatile flow than oscillating flow ($p<0.05$ for KLF2 and $p<0.001$ for TM, Bonferroni's multiple comparison test). Comparable KLF2, eNOS, and TM expression was observed in the static and oscillating flow treated cells.

KLF2



eNOS



TM

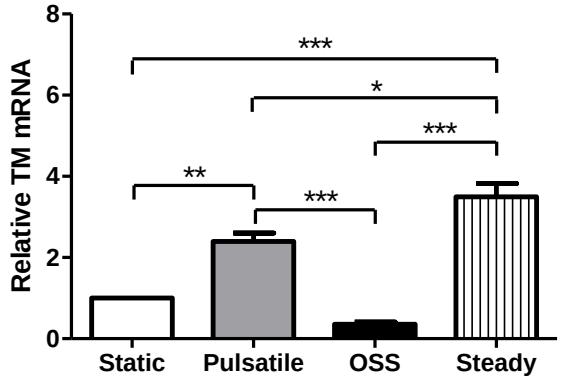


Figure 7-2: Effect of 24 h steady laminar (18 dynes/cm^2, Steady), non-reversing pulsatile (1Hz, 18 ± 9 dynes/cm^2, Pulsatile), and oscillating (1 Hz, 0.3 ± 3 dynes/cm^2, OSS) shear stress on KLF2, eNOS and TM mRNA levels in endothelial cells. The extracted RNA was reverse transcribed into cDNA and analyzed by quantitative real-time PCR. The data is presented as the mean ± SEM from 4 or 5 separate experiments. Statistically significant differences are labelled (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

7.6.3. Simvastatin Increases KLF2, eNOS, and TM mRNA Expression in HAAEC exposed to Steady, Pulsatile, and Oscillating Flow

The effect of increasing concentrations of simvastatin on KLF2, eNOS, and TM expression in endothelial cells under static and dynamic stimulation is presented in Figure 7-3. Flow and simvastatin with or without 200 μM mevalonate were introduced simultaneously. The RNA from cell lysates was extracted 24 h later. In static culture, simvastatin significantly enhanced the mRNA levels of all three genes in a concentration dependent manner at 1 and 10 μM ($p<0.001$, Bonferroni's multiple comparison test) in comparison to the control vehicle, Figure 7-3. As expected, the effect of simvastatin was completely abrogated when the endothelial cells were treated with 200 μM mevalonate. The increase in gene expression by simvastatin observed in static culture was also detected under pulsatile, steady, and oscillating shear stress at 10 μM only. Statistically significant differences between shear stress alone and combined 10 μM simvastatin and shear stress were found for all genes except KLF2 in endothelial cells exposed to pulsatile flow, however a trend towards greater mRNA levels with statin treatment was observed. Under each flow condition investigated, no significant increase in KLF2, eNOS, and TM mRNA above flowed controls was observed at lower simvastatin concentrations (0.1 or 1 μM). As with static culture, the effect of simvastatin was completely eliminated when the cells were treated with 200 μM mevalonate.

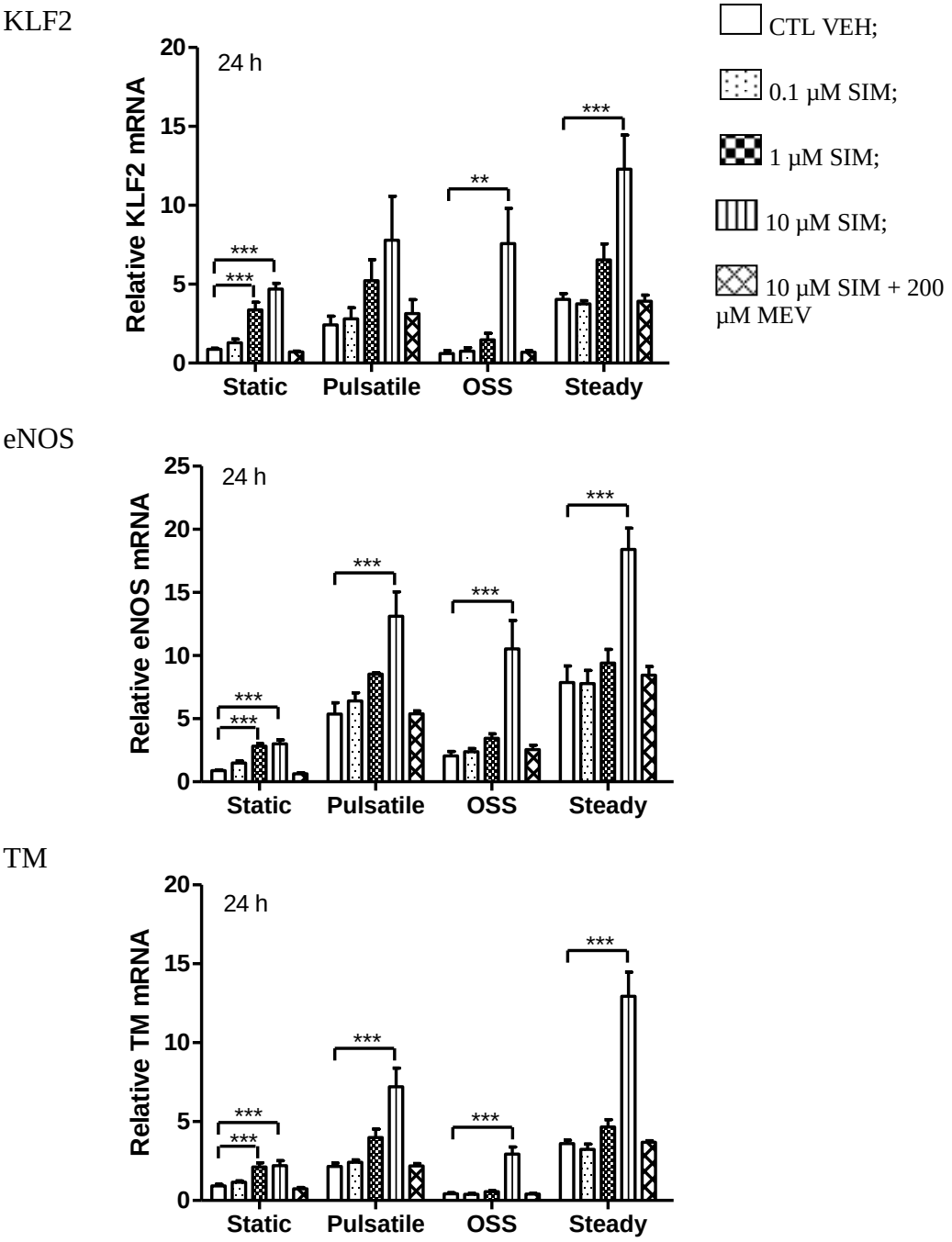
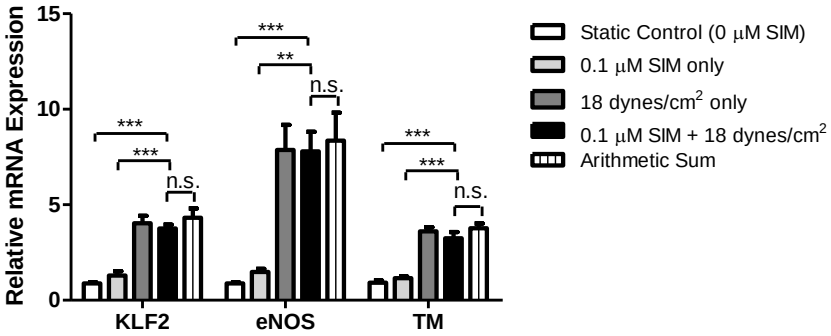


Figure 7-3: Effect of shear stress (steady laminar, non-reversing pulsatile and oscillating) and increasing concentrations of simvastatin (0.1, 1 or 10 µM) on KLF2, eNOS and TM mRNA levels. The endothelial cells were exposed to simvastatin and shear stress simultaneously and the RNA was extracted 24 h later. The data is presented as the mean ± SEM from 4 or 5 separate experiments. Statistically significant differences are labelled (** $p<0.01$, *** $p<0.001$). Pulsatile: 18 ± 9 dynes/cm^2; OSS: 0.3 ± 3 dynes/cm^2; Steady: 18 dynes/cm^2; CTL VEH: control vehicle; SIM: simvastatin; MEV: mevalonate.

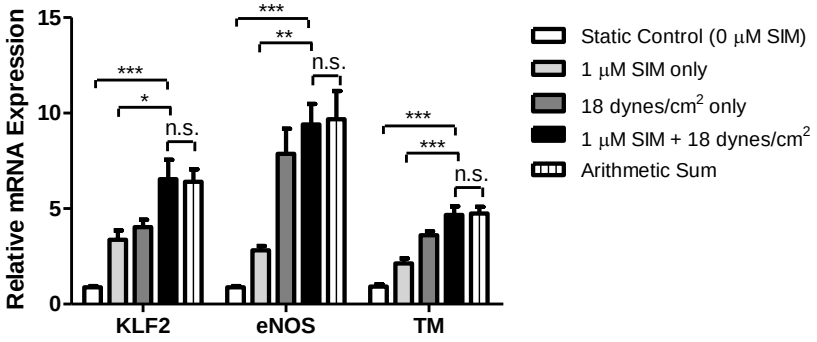
7.6.4. Combination of Steady Laminar Flow and Simvastatin Produces a Synergistic Increase in eNOS and TM mRNA at High Statin Concentration

To evaluate whether the combination of steady laminar shear stress (18 dynes/cm^2) and simvastatin produced an additive, synergistic or antagonistic induction of KLF2, eNOS and TM expression, the observed response was compared to the arithmetic sum of the mean individual effects (arithmetic sum = average relative mRNA expression from statin + average relative mRNA expression from shear stress – baseline (1)), Figure 7-4. As shown in Figure 7-4, combining steady flow and 0.1 or 1 µM simvastatin resulted in a response that was similar to the arithmetic sum. Interestingly, at 10 µM simvastatin + steady flow the mRNA levels of eNOS and TM were significantly greater than the arithmetic sum ($p=0.0080$ and $p=0.0025$ respectively, t-tests). Statistical significance between the observed and calculated responses was not detected for KLF2 at 10 µM simvastatin ($p=0.1123$, t-test). In general, at lower simvastatin concentrations (0.1 and 1 µM) the combination of steady laminar shear stress and simvastatin produced an overall additive increase in eNOS and TM mRNA levels, whereas at high statin levels a synergistic induction of eNOS and TM mRNA levels was observed. Synergy was not detected for KLF2 at any simvastatin concentration evaluated.

0.1 μM SIM



1 μM SIM



10 μM SIM

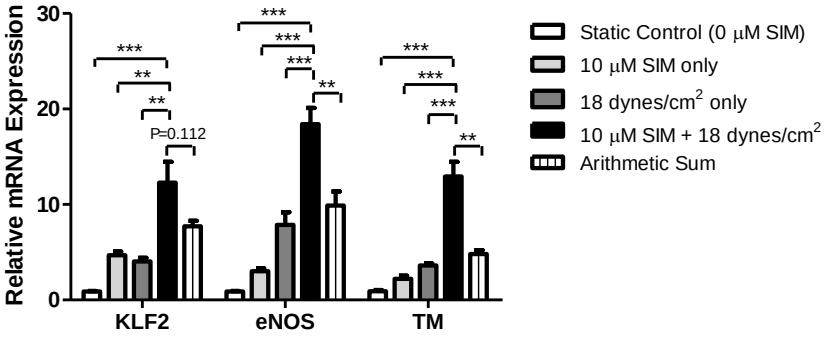
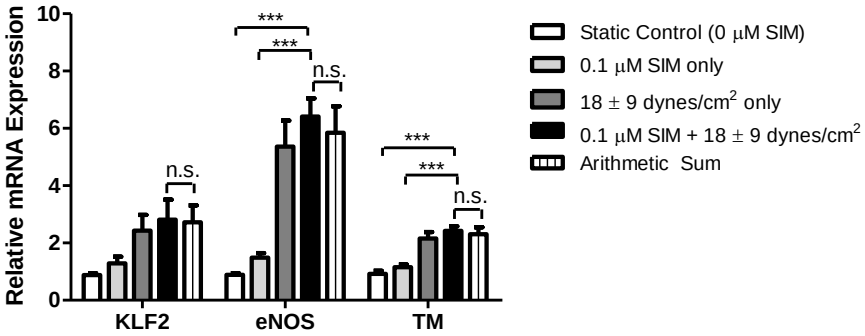


Figure 7-4: KLF2, eNOS and TM mRNA levels in endothelial cells exposed to steady laminar shear stress (18 dynes/cm^2) and increasing concentrations of simvastatin (0.1, 1 or 10 μM) for 24 h. The combined effect of shear stress and simvastatin was compared to the arithmetic sum of the mean individual responses with t-tests. The static control, SIM only, 18 dynes/cm^2 only and SIM + 18 dynes/cm^2 were compared with a one-way analysis of variance followed by Bonferroni's Multiple Comparison Tests. The data is presented as the mean ± SEM from 4 or 5 separate experiments. Statistically significant differences are labelled (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s.: not significant). Arithmetic sum = relative mRNA expression from statin + relative mRNA expression from shear stress – baseline (1). SIM: simvastatin.

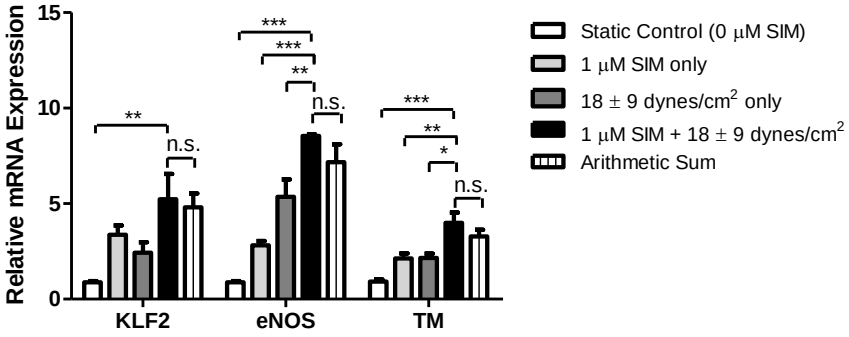
7.6.5. Combination of Non-reversing Pulsatile Flow and Simvastatin Produces a Synergistic Increase in eNOS and TM mRNA at High Statin Concentration

Similar to steady laminar shear stress, the combination of non-reversing pulsatile flow (1 Hz, 18 ± 9 dynes/cm^2) and simvastatin produced a response that was similar to the arithmetic sum of the mean individual effects for KLF2 at each statin concentration evaluated (0.1, 1 and 10 μM), Figure 7-5. Comparable observed (simvastatin + 18 ± 9 dynes/cm^2) and calculated mRNA levels were also detected for eNOS and TM at 0.1 and 1 μM. At 10 μM simvastatin + pulsatile flow, the mRNA levels of eNOS and TM were greater than the calculated sum ($p<0.05$, t-tests). Hence, the general observation was an additive increase in mRNA levels at lower simvastatin concentrations (0.1 and 1 μM) and a synergistic increase was detected at 10 μM for both eNOS and TM. Similar to steady flow, synergy was not observed for KLF2 at any simvastatin concentration evaluated.

0.1 μM SIM



1 μM SIM



10 μM SIM

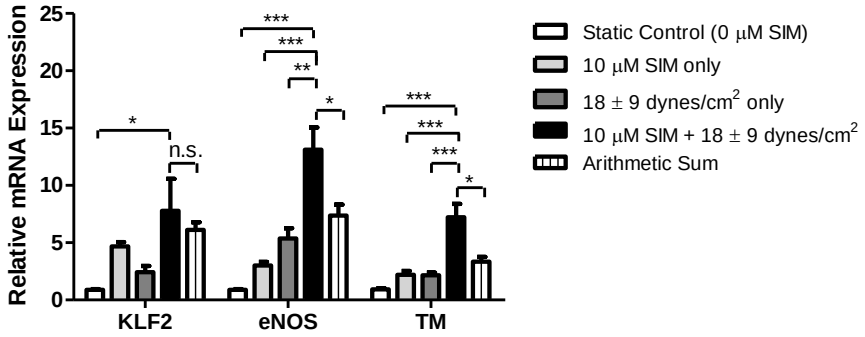
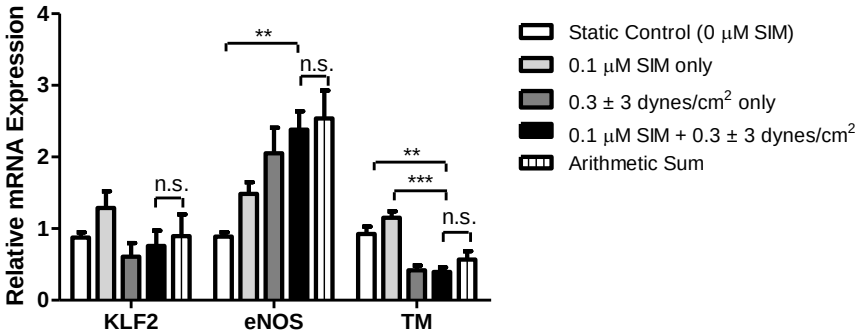


Figure 7-5: KLF2, eNOS and TM mRNA levels in endothelial cells exposed to non-reversing pulsatile shear stress (18 ± 9 dynes/cm^2) and increasing concentrations of simvastatin (0.1, 1 or 10 μM) for 24 h. The combined effect of shear stress and simvastatin was compared to the arithmetic sum of the mean individual responses with t-tests. The static control, SIM only, 18 ± 9 dynes/cm^2 only and SIM + 18 ± 9 dynes/cm^2 were compared with a one-way analysis of variance followed by Bonferroni's Multiple Comparison Tests. The data is presented as the mean ± SEM from 4 or 5 separate experiments. Statistically significant differences are labelled (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s.: not significant). Arithmetic sum = relative mRNA expression from statin + relative mRNA expression from shear stress – baseline (1).

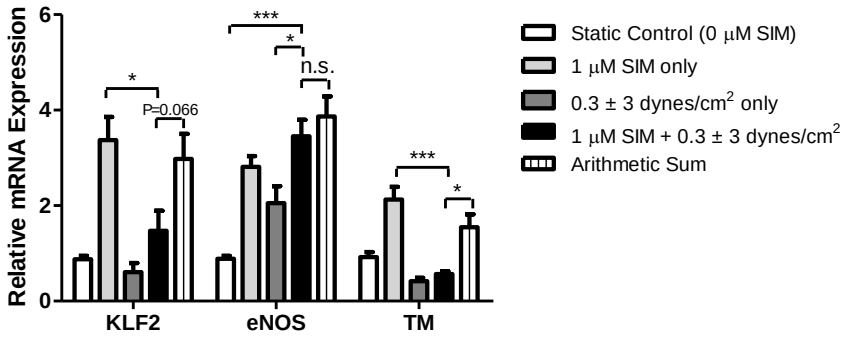
7.6.6. Oscillating Flow can impair the Simvastatin-induced Increase in KLF2 and TM mRNA Expression

The effect of simvastatin on KLF2, eNOS and TM expression in endothelial cells conditioned with oscillating shear stress (1 Hz, 0.3 ± 3 dynes/cm^2) is presented in Figure 7-6. Oscillating shear stress slightly reduced KLF2 and TM mRNA levels. As a result, the arithmetic sum is presented as being less than the effect of simvastatin alone for KLF2 and TM. For all three genes, the combination of oscillating shear stress and 0.1 μM simvastatin produced a response that was comparable to the arithmetic sum. At 1 μM simvastatin + oscillating shear stress, the mRNA levels of KLF2 and TM were less than the arithmetic sum ($p=0.0661$ for KLF2 and $p=0.0124$ for TM, t-tests) and less than 1 μM simvastatin alone ($p<0.05$ for KLF2 and $p<0.001$ for TM, Bonferroni's multiple comparison test). These findings suggest that oscillating flow impairs the induction of KLF2 and TM by simvastatin at 1 μM. Impairment was possibly not observed at 0.1 μM since no induction in gene expression was found under static conditions at this concentration, Figure 7-3. The impairment in KLF2 and TM expression was overcome by a higher simvastatin concentration of 10 μM. In fact, at 10 μM simvastatin the observed response was slightly greater than the expected value for all three genes investigated. However, statistical significance was observed for eNOS only ($p<0.05$, t-tests). No impairment in eNOS mRNA was detected at any simvastatin concentration evaluated.

0.1 μM SIM



1 μM SIM



10 μM SIM

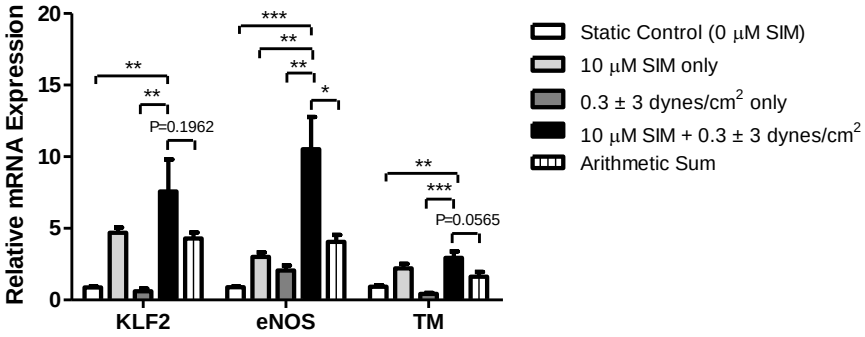


Figure 7-6: KLF2, eNOS and TM mRNA levels in endothelial cells exposed to oscillating shear stress (0.3 ± 3 dynes/cm^2) and increasing concentrations of simvastatin (0.1, 1 or 10 μM) for 24 h. The combined effect of shear stress and simvastatin was compared to the arithmetic sum of the mean individual responses with t-tests. The static control, SIM only, 0.3 ± 3 dynes/cm^2 only and SIM + 0.3 ± 3 dynes/cm^2 were compared with a one-way analysis of variance followed by Bonferroni's Multiple Comparison Tests. The data is presented as the mean ± SEM from 4 or 5 separate experiments. Statistically significant differences are labelled (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s.: not significant). Arithmetic sum = relative mRNA expression from statin + relative mRNA expression from shear stress – baseline (1).

7.7. Discussion

Few studies have investigated the interactions between shear stress and statins on endothelial cell gene expression [29, 30]. Knowledge of these possible interactions may improve our understanding of the non-lipid lowering effects of statins observed in the clinic [34-37]. In this study, we sought to determine whether wall shear stress can affect the statin-induced increase in KLF2, eNOS and TM observed in static culture [15, 24, 25, 27, 28, 38-42]. We evaluated and compared the response of endothelial cells to simvastatin under three different fluid mechanical environments: steady (18 dynes/cm^2), non-reversing pulsatile (1 Hz, 18 ± 9 dynes/cm^2) and oscillating (1 Hz, 0.3 ± 3 dynes/cm^2) shear stress. The magnitude of shear (18 dynes/cm^2) is representative of mean coronary shear stress and the pulsatility (reversing and non-reversing) was chosen to represent the temporal nature of arterial flow [43, 44]. The response of endothelial cells to simvastatin under all three flow environments was compared to static culture, where most effects of statins on cultured endothelial cells have been reported.

As expected, simvastatin (1 and 10 μM) and unidirectional shear stress (pulsatile and steady) each separately enhanced KLF2, eNOS and TM mRNA levels above static controls, Figure 7-2 and Figure 7-3. These results are consistent with the findings of many previous studies [13, 15, 24-26, 28, 39, 45-49]. Holding mean wall shear stress constant, we observed that KLF2, eNOS and TM mRNA levels were slightly greater under steady laminar flow (18 dynes/cm^2) than non-reversing pulsatile flow (18 ± 9 dynes/cm^2) at 24 h. Our results could suggest that wall shear stress magnitude has a larger effect on KLF2, eNOS and TM expression than temporal gradients in shear stress. In contrast with our findings, Dekker *et al.* reported that pulsatile flow (12 ± 7 dynes/cm^2) maintained greater KLF2 mRNA levels than steady flow (25 dynes/cm^2) over a 24 h time frame [46]. In another study, KLF2 mRNA expression was shown to be comparable between unidirectional steady and pulsatile shear stress after 6 h up to 15 dynes/cm^2, however at 30 dynes/cm^2 pulsatile flow produced higher levels of KLF2 mRNA

than steady flow [13]. Differences in amplitude, frequency and time points may account for the discrepancies between studies. In agreement with our results, Blackman *et al.* found that eNOS mRNA and protein expression in cultured endothelial cells was greater when treated with steady flow (7.5 dynes/cm^2) compared to arterial pulsatile flow (1 Hz, time-averaged shear stress of 7.5 dynes/cm^2, max 20 and min -3 dynes/cm^2) after 24 h [50]. However, it is difficult to compare the results since flow reversal was included in their waveform. To the best of our knowledge, comparison of unidirectional pulsatile versus steady shear stress on endothelial cell expression of TM has not been reported.

The expression of KLF2, eNOS and TM was further enhanced in our study when the endothelial cells were co-treated with a combination of steady laminar flow and 10 µM simvastatin or non-reversing pulsatile flow and 10 µM simvastatin (Figure 7-3). Upon comparing the arithmetic sum of the mean individual responses to the observed value, both steady flow and non-reversing pulsatile flow in conjunction with simvastatin produced an overall additive increase in eNOS and TM mRNA levels at lower statin concentrations (0.1 and 1 µM), Figure 7-4 and Figure 7-5. At 10 µM the combination of simvastatin and steady or pulsatile flow produced a synergistic increase in eNOS and TM mRNA levels. Synergy was not found for KLF2. We recently reported that an additive increase in KLF2, eNOS and TM mRNA was observed when simvastatin (0.1, 1 and 10 µM) was combined with 25 dynes/cm^2 laminar shear stress [51]. Although statistical significance was not observed, it was noted that the combination of 10 µM simvastatin and 25 dynes/cm^2 laminar shear stress generated greater mRNA levels than the calculated sum of the mean individual effects [51]. Ali *et al.* recently reported that unidirectional steady laminar flow (12 dynes/cm^2) enhanced endothelial cell responsiveness to atorvastatin [29]. A synergistic increase in heme oxygenase-1 (HO-1) gene expression was observed at lower statin concentrations (0.6 and 1.25 µM) and an additive effect was detected at a higher concentration (2.5 µM) [29]. The synergistic increase in HO-1 was associated in part to an upregulation in KLF2 and eNOS expression. Although, KLF2, eNOS and TM

mRNA levels were presented in their study it is not clearly stated whether combined statin and shear stress significantly increased KLF2, eNOS and TM mRNA levels above values achieved by shear stress alone [29].

Exposure of the cultured endothelial cells to oscillating flow (1 Hz, 0.3 ± 3 dynes/cm^2) did not significantly enhance or decrease KLF2, eNOS, or TM mRNA levels and the gene expression profile was comparable to static culture, Figure 2. Consistent with our results, Ziegler *et al.* showed that eNOS mRNA levels were similar between oscillating flow and static culture [52]. At 1 µM, the combination of simvastatin and oscillating flow resulted in KLF2 and TM mRNA levels that were less than the arithmetic sum and less than the effect of 1 µM simvastatin alone in static culture. A higher simvastatin concentration of 10 µM overcame this impairment and even increased gene expression above the calculated sum of the mean individual effects. Impairment was possibly not seen at 0.1 µM since this concentration was too low to increase mRNA levels under static conditions. In agreement with our study, Ali *et al.* reported that oscillating shear stress (1 Hz, ± 5 dynes/cm^2) impaired the atorvastatin-induced increase in HO-1 [29]. Our data suggests that endothelial cells are less responsive to statins under oscillating flow. This indicates that statin pleiotropy may be less effective in endothelial cells *in vivo* exposed to atheroprone hemodynamics.

The effect of simvastatin was completely abrogated when co-treated with 200 µM mevalonate suggesting that inhibition of HMG-CoA reductase is the mechanism in which statins enhance KLF2, eNOS and TM expression in endothelial cells. These results are consistent with other groups who have reported that the induction of KLF2, eNOS and TM expression by statins can be eliminated by co-incubation with mevalonate [24, 27, 28]. Mevalonate had no influence on the shear stress-induced endothelial cell expression of KLF2, eNOS and TM.

7.8. Conclusions

In this study, we observed that endothelial cells can respond differently to the combination of simvastatin and shear stress depending on the statin concentration and the type of flow imposed on the cells. At lower simvastatin concentrations (0.1 and 1 μM), the combined effect of unidirectional steady or pulsatile flow and statin produced an overall additive increase in KLF2, eNOS and TM mRNA levels, whereas impairment in KLF2 and TM expression was observed under oscillating shear stress. At high concentration, simvastatin overcame the inhibitory effect of oscillating shear stress and a synergistic increase in eNOS and TM expression was observed under steady and pulsatile flow. The highest concentration of simvastatin investigated in this study was 10 μM and far exceeds clinically realistic levels of 0.02 to 0.08 μM [53]. Consequently, the effect of shear stress type rather than high statin concentration on endothelial cells is more clinically relevant. The results of our study suggest that the pleiotropic effects of statins on endothelial cell gene expression may be reduced at sites in the vasculature exposed to low shear stress with reversing flow.

7.9. Acknowledgments

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7.10. References

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CHAPTER 8: NOVEL CONTRIBUTIONS

The novel contributions presented in this thesis are:

1. Documented the potential pitfalls of using dextran when investigating the dynamic response of endothelial cells to shear stress. Consequently, caution is advised when using dextran to increase the viscosity of the culture medium in *in vitro* flow experiments to avoid confounding results.
2. Characterized the effects of statins on endothelial cell gene expression under a wide range of flow conditions (steady, non-reversing pulsatile, and oscillating) and compared the response to static culture.
3. Demonstrated that significant statin-shear stress interactions are present for the cell adhesion molecules VCAM-1 and ICAM-1. Laminar shear stress reduced the TNFα-induced increase in VCAM-1 and ICAM-1, while surprisingly simvastatin potentiated the effect. The increase in VCAM-1 and ICAM-1 by simvastatin was eliminated in the presence of steady laminar shear stress.
4. Showed that the induction of KLF2, eNOS, and TM by simvastatin observed in endothelial cells in static culture is conserved under low, medium, and high steady laminar wall shear stress. In fact, the increase in KLF2, eNOS, and TM by steady laminar wall shear stress and simvastatin was, in general additive. A slightly greater than expected response was detected at the highest simvastatin concentration investigated, although statistical significance was not observed.
5. Showed that similar to steady flow, simvastatin was able to enhance the expression of KLF2, eNOS and TM in endothelial cells exposed to non-reversing pulsatile shear stress. In general, an additive increase was observed at lower simvastatin concentrations. A synergistic increase was observed at the highest concentration evaluated.

6. Demonstrated that oscillating shear stress impaired the increase in KLF2 and TM, but not eNOS by simvastatin. This impairment was overcome with a higher simvastatin concentration.
7. Showed that mevalonate eliminated the effect of simvastatin but had no effect on shear modulation of VCAM-1, ICAM-1, KLF2, eNOS and TM.

In summary, the findings presented in this thesis demonstrate that significant shear stress-statin interactions exist. Consequently, endothelial cells may respond differently to statins depending on concentration and the type of flow imposed. Our findings suggest that statins might have pleiotropic benefits in vascular biology by improving endothelial cell function.

CHAPTER 9: CONCLUSIONS

The benefits of statins in the clinic cannot be explained by cholesterol reduction alone, suggesting possible pleiotropy. Our understanding of the effects of statins on endothelial cell gene expression has been primarily explored in static culture. This model neglects the importance of shear stress in endothelial cell biology and may be limiting our understanding of the non-lipid lowering effects of statins. Investigations into statin-biomechanical interactions have only begun recently. Knowledge of these interactions are needed to provide a better understanding of statin therapy. Presented in this thesis is an investigation into the effects of statins on endothelial cells conditioned with shear stress (steady, non-reversing pulsatile and oscillating). In addition, the effect of dextran on endothelial cells was evaluated to determine whether it can be used to increase the viscosity of the perfusion medium.

The work presented in this thesis, demonstrates that significant interactions are present when shear stress and statins are combined. It was observed that simvastatin potentiated the TNFα-induced increase in VCAM-1 and ICAM-1 in statically cultured endothelial cells. This enhancement by simvastatin was prevented when endothelial cells were conditioned with laminar shear stress. Consequently, laminar wall shear stress had the dominating effect on cell adhesion molecule expression. These findings may help to explain why increased inflammation due to statin treatment is generally not observed in the clinic.

We also observed that simvastatin and unidirectional shear stress (steady and pulsatile) separately enhanced the expression of the atheroprotective genes KLF2, eNOS and TM. Combining both simvastatin and unidirectional shear stress resulted in an overall additive increase in KLF2, eNOS and TM at lower concentrations. At the highest concentration of simvastatin investigated, a synergistic increase in gene expression was observed. Under oscillating shear stress, a slight impairment in KLF2 and TM mRNA levels was observed at lower simvastatin concentrations. This impairment was overcome at higher

concentrations. These findings support the hypothesis that statins may have pleiotropic effects in vascular biology by improving endothelial cell function. In addition, our results suggest that endothelial cells in vivo may respond differently to statins depending on the hemodynamic environment.

We have also shown that dextran, which is commonly used to increase the viscosity of the perfusion medium in *in vitro* flow models, has a non-negligible effect on endothelial cell gene expression, attachment and neutrophile adhesion. In fact, dextran increased VCAM-1 and ICAM-1 expression in a concentration and time dependent manner and activated NF-κB. In terms of gene expression, different conclusions could be drawn from the effect of shear stress if time-matched dextran containing static controls were used or not. Consequently, caution is advised when using dextran to alter perfusion medium viscosity as confounding results may be possible.

The role of wall shear stress in endothelial cell biology and its potential interactions with pharmacological agents is a complex subject. Our work has shown that endothelial cells can respond differently to statins in static culture and under dynamic stimulation from fluid flow. Consequently, we believe that it is important to include biomechanical forces when modeling *in vitro* the effects of statins on endothelial cell function.

CHAPTER 10: RECOMMENDATIONS

In this study, we have investigated whether statin-induced changes in gene expression observed in static culture hold when endothelial cells are exposed to steady laminar flow and temporal gradients in fluid wall shear stress. It would also be of interest to evaluate endothelial cell response to statins under spatial gradients in shear stress, such as proximal and distal to an asymmetric stenosis or the inner and outer curvature of a 90° bend. Previous work by our group has shown that spatial gradients in shear stress in an asymmetric stenosis model, both acceleration and deceleration regions, can induce endothelial cell dysfunction. It is recommended to evaluate whether statins can prevent flow-induced dysfunction proximal and distal to the stenosis and/or restore function to these regions. This would provide valuable information that could be related clinically.

In addition to wall shear stress, other biomechanical forces exerted on endothelial cells such as pressure and stretch can also induce changes in endothelial cell gene expression. It would be of significant value to investigate the interactions between pressure and/or cyclic stretch and statins on endothelial cell gene expression. Specifically, evaluate whether stretch and pressure can alter endothelial cell response to statins.

In our studies, endothelial cells were exposed to simvastatin for a maximum duration of 24 h. As a result, high simvastatin concentrations were used in order to observe a response within 24 h. It would be valuable to develop a flow system that could perfuse endothelial cells for longer and determine whether lower, more clinical realistic, statin concentrations can alter gene expression.

Finally, simvastatin was used as a model statin in our studies. It would be interesting to compare statin-shear stress interactions with different types of statins to evaluate whether one is more effective than the other.

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