EFFECTS OF SIMVASTATIN ON ENDOTHELIAL CELL CONTRACTILITY AND BARRIER INTEGRITY

A Thesis

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ABSTRACT

Cardiovascular diseases are the leading cause of death in the United States. Atherosclerosis, a cardiovascular disease, can lead to stroke and heart failure, affecting 50% of the U.S. population over the age of 65[1]. One of the first steps in the progression of atherosclerosis is increased endothelium permeability, ultimately resulting in the accumulation of lipids and formation of plaque. It has been shown that increased substrate stiffness, mimicking the stiffening that occurs naturally with age, results in increased cell contractility, cell-cell junction width, and endothelium permeability via Rho mediated contractility [2]. To date therapeutics to reverse increased vascular stiffness, and in turn prevent endothelial cell dysfunction leading to increased endothelium permeability, have been limited and unsuccessful. Previous findings suggest that statins may be beneficial beyond lowering cholesterol levels by having a positive effect on endothelium integrity through the inhibition of RhoA in the actomyosin contractility pathway [3–5]. As increased stiffness leads to increased permeability via Rho mediated contractility [2] and statins have been shown to decrease RhoA activity, this investigation examined the effect of simvastatin (Zocor®), a common statin, on endothelial cell morphology, cell traction forces, cell-cell junction widths, and endothelium permeability for endothelial cells seeded on 2.5, 5, and 10 kPa polyacrylamide substrates, mimicking the stiffness of healthy and aged vessels respectively.

Our data show that simvastatin results in an elongated cell morphology, reduced endothelial cell contractility, enhanced cell-cell junction integrity, and decreased substrate-stiffness-dependent and thrombin-dependent increased endothelium permeability on physiologically relevant substrate stiffnesses. Our data suggest that simvastatin may be beneficial

in preventing atherosclerosis beyond its traditional lipid lowering effects by decreasing monolayer permeability due to vascular stiffening that naturally occurs with aging.

BIOGRAPHICAL SKETCH

Courtney Faber was born in Goeppingen, Germany on April 10, 1988 and grew up in Columbia, SC with her mother Donna, father Gary, and two younger sisters, Chelsey and Carly. After graduating from Ridge View High School in Columbia, SC in May 2006, she attended Clemson University to pursue a Bachelors of Science degree in Bioengineering with a concentration in Materials Science. She graduated summa cum laude with a B.S. in Bioengineering and a minor in Chemistry from Clemson in May 2010. Courtney started her work at Cornell University in August 2010 and was awarded a Morgan Fellowship. In May 2011, she was awarded the National Science Foundation (NSF) Graduate Research Fellowship and received a NSF GK-12 Fellowship in May 2012. While at Cornell University, she was an active member of the Society of Women Engineers (SWE), co-founding a graduate student section during the Fall of 2010 and serving as the president through the Spring of 2012. She was recognized as the Diversity Programs in Engineering First Year Graduate Student of the Year in May 2011 for her contribution to SWE and for positively impacting and supporting her fellow peers. Courtney completed her Master's work under the supervision of Professor Cynthia A. Reinhart-King in December 2012 before joining Clemson University's Ph.D. program in Engineering and Science Education in January 2013. In her free time, she enjoys spending time with her family and friends.

For my family and friends, who have always encouraged and supported me in the pursuit of my dreams.

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CHAPTER 1

INTRODUCTION

1.1 Atherosclerosis

Cardiovascular diseases are the leading cause of death in the United States, claiming approximately 2,150 lives per day, according to the American Heart Association [1]. Atherosclerosis, which can lead to stroke and heart failure, is a progressive disease that begins with endothelial cell dysfunction followed by the accumulation of low-density lipoprotein (LDL) particles in the intima. This results in the formation of sub-endothelial accumulations of LDL and cholesterol-engorged macrophages, also known as foam cells [6,7]. Over time, as white blood cells, LDL, and other fatty materials permeate the endothelial cell layer an atherosclerotic plaque begins to form [8]. Eventually, as this plaque continues to grow it will begin to occlude the vessel and restrict blood flow [9]. These plaques can become unstable and rupture, leading to the formation of thrombi causing complications such as a myocardial infarction or stroke [9,10].

Atherosclerosis affects 50% of the population over the age of 65 [1]. This suggests a connection between atherosclerosis and aging; however, the exact mechanism by which aging leads to the progression of atherosclerosis remains unclear. Notably, during both aging and atherosclerosis, the blood vessel stiffens due to increased elastin fragmentation, collagen deposition, and collagen cross-linking [2,11]. Measurements of vessel stiffness, quantified through pulse wave velocities, are used clinically to determine a patient's risk of developing cardiovascular diseases [12,13]. While the connection between macro-scale artery stiffness and cardiovascular diseases is well characterized, the relationship between increased vessel stiffness and endothelial cell function is less clear. Recent work has shown, using *in vitro* and *ex vivo*

models of vessel stiffness and aging, that increasing substrate stiffness alone can promote endothelial monolayer disruption including increased endothelium permeability, the first step in the progression of atherosclerosis [2]. In addition, inhibition of Rho-dependent cell contractility restores monolayer integrity, reversing the increased endothelium permeability, destabilized cell-cell junctions, and increased cell contractility due to increased substrate stiffness. This work suggests targeting the Rho-dependent cell contractility pathway to prevent the cellular response to increasing extracellular matrix stiffness as a means to prevent the progression of atherosclerosis.

1.2 Importance of Matrix Mechanics

Mechanotransduction is the process by which cells convert forces to biochemical signals. When a mechanical force is applied to a cell, it activates numerous signaling pathways that mediate the cell's response [14–16]. Matrix mechanics have been shown to play a key role in endothelial cell behavior, and as such it is an important factor to account for when studying vascular diseases, such as atherosclerosis [2,17,18]. The vascular endothelium maintains its integrity by balancing cell-cell and cell-matrix forces [19]. Previous work shows that endothelial cell traction stresses are dependent on substrate stiffness, with increasing substrate stiffness leading to increased traction stress [18,20]. Traction forces have been shown to originate from actomyosin interactions within cells [2,18,21]. Myosin activity can be regulated through a number of mechanisms, one of which includes the phosphorylation of myosin light chain (MLC) by either myosin light chain kinase (MLCK) or Rho associated kinase (ROCK) [22,23].

As previously mentioned, it has been shown that vessel stiffness increases with both age and the progression of atherosclerosis. The exact stiffness of the intima of human arteries is not well characterized as it is difficult to obtain samples; as such, Peloquin et al. and Huynh et al. have have characterized the stiffness of bovine and mouse arteries respectively. The stiffness of 18 to 30 month old bovine carotid intima was reported to be 2.7 ± 0.6 kPa [24]. As the structure of bovine arteries is analogous to that of human arteries, this value is a good estimate of the stiffness of a young or healthy adult's artery.

Mechanotransduction has been shown to be regulated by the crosstalk between cadherins and integrins during disease progression [25,26]. A key cadherin in endothelial cells is vascular endothelial (VE) cadherin. VE-cadherin is essential for limiting endothelium permeability and maintaining vascular integrity as it is a major component of endothelial adherens junctions [27]. Adherens junctions are zipper-like structures that form along cell borders, linking one cell to another [28]. VE-cadherin interacts with the actin cytoskeleton through scaffolding proteins called catenins. This interaction suggests that actomyosin contraction regulates these cell-cell adhesions [28–30]. In addition, when VE-cadherin is phosphorylated, adherens junctions separate due to VE-cadherin degradation and internalization resulting in increased endothelium permeability [31].

In addition to matrix mechanics, soluble mediators, such as thrombin, have been shown to play a role in regulating endothelial cell monolayer integrity [20]. Upon thrombin stimulation, it has been shown that endothelial monolayers become more permeable through the activation of RhoA and subsequent phosphorylation of MLC [32,33]. Recently, thrombin has been shown to be an important contributor to endothelial cell dysfunction in atherosclerosis. Borissoff et al. published in vivo results showing that deletion of a thrombin inhibitor promotes an accelerated atherogenic state, while the administration of a thrombin inhibitor ameliorated atherosclerotic plaque

progression [34]. Through this work thrombin has been shown to play an important role in both the progression of atherosclerosis and increased endothelium permeability.

1.3 Statins to Reduce Endothelial Dysfunction

1.3.1 Isoprenoid Biosynthesis Pathway

The most common treatment for hypercholesterolemia today is a class of drugs called 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, more commonly known as statins. This class of drugs lowers blood cholesterol levels by inhibiting HMG-CoA reductase, the enzyme that converts HMG-CoA to mevalonate, the rate-limiting step in the isoprenoid biosynthesis pathway (Figure 1), the end product of which is cholesterol [35]. In addition to lowing cholesterol levels, statins have been found to reduce the risk of stroke and myocardial infarction beyond what can be explained by the drug's cholesterol lowering abilities [3–5,36]. It is now evident that inhibition of the isoprenoid biosynthesis pathway leads not only to a decrease in cholesterol levels, but also blocks the synthesis of farnesylpyrophosphate (Farnesyl-PP) and geranylgeranylpyrophosphate (Geranylgeranyl-PP) which serve as important lipid attachments for the posttranslational modification of small GTP-binding proteins, such as those belonging to the Rho family guanosine triphosphatases (GTPases) [37]. Farnesylastion and geranylgeranylation have been shown to be important for proper function and intracellular localization of Rho GTPases, such as RhoA, Rac1, and Cdc42 [37]. Without proper posttranslational modification, these small GTPases are not able to properly function, which has been suggested to lead to the pleiotropic effects of statins on the vascular wall.

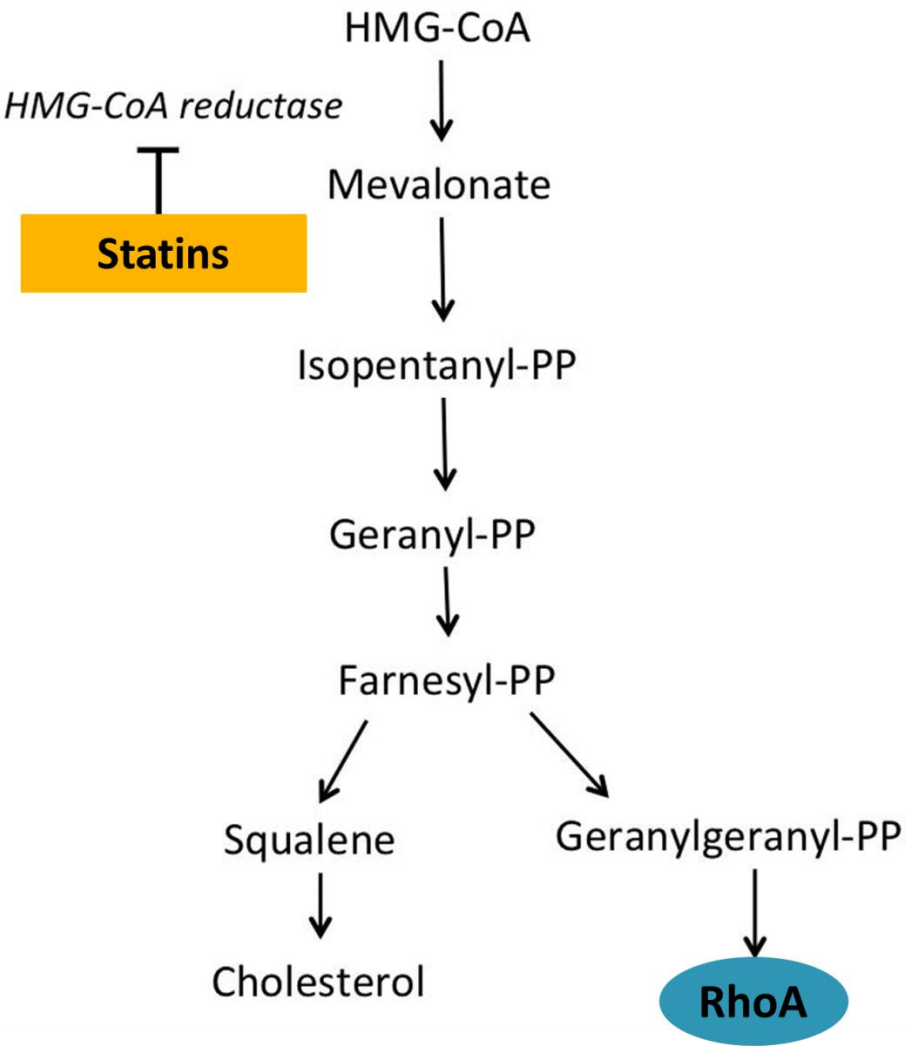

Figure 1: Pathway representing the connection between simvastatin and the isoprenoid biosynthesis pathway. This image was adapted from Rikitake et al. [37]

1.3.2 Actomyosin Contractility Pathway

The primary role of the Rho GTPases is to regulate the assembly and organization of the actin cytoskeleton [38], which has implications in the generation of contractile forces [39] and the maintenance of barrier integrity [40]. GTPases cycle between an inactive GDP-bound state and an active GTP-bound state, in doing so they act as molecular switches in a variety of signaling pathways [40]. Three classes of regulatory proteins control the activation state of Rho GTPases: 1) GTPase-activating proteins (GAPs) hydrolyzes GTP to GDP, inactivating the protein, 2) guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, activating the protein, and 3) guanine nucleotide dissociation inhibitors (GDIs) which bind to and stabilize the prenylated GDP-bound Rho GTPases [41] (Figure 2).

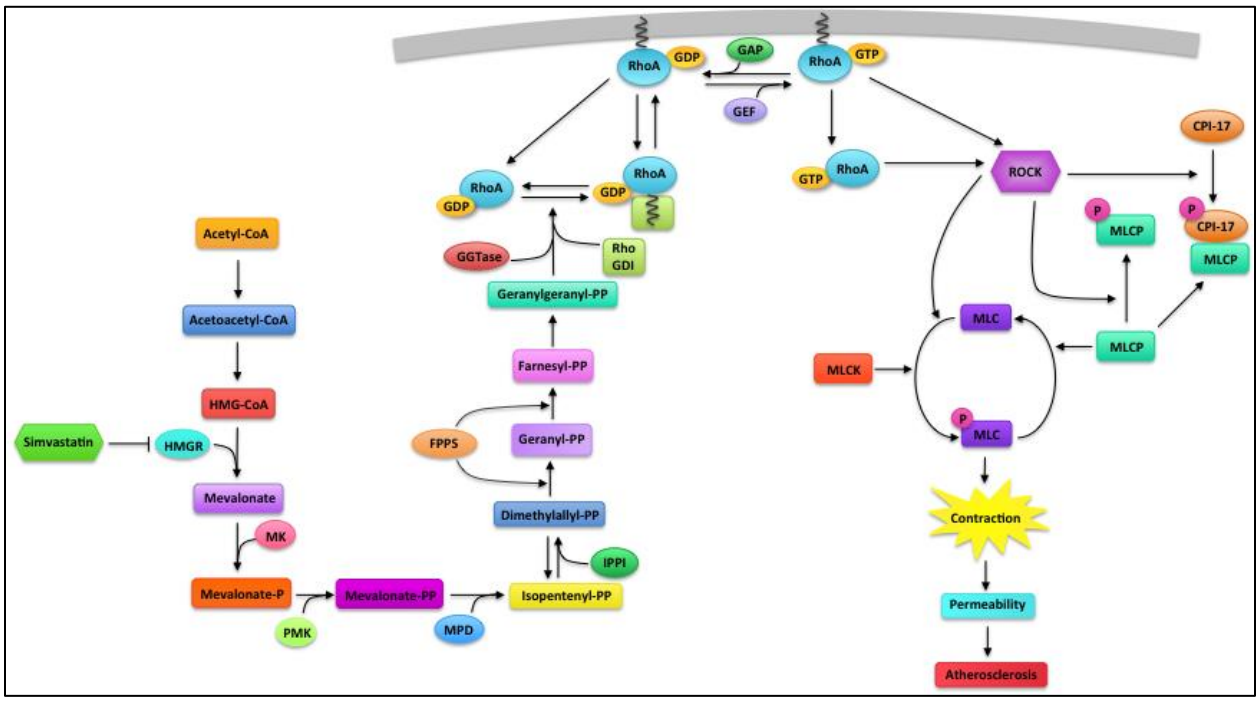

Figure 2: Biochemical pathway showing the interaction between simvastatin, the mevalonate pathway, and the actomyosin contractility pathway which ultimately leads to a change in endothelial contractility and permeability resulting in the progression of atheroscelerosis.

RhoA, Rac1, and Cdc42 are the best characterized Rho GTPases in terms of their role in the regulation of endothelial barrier integrity. RhoA has been shown to primarily have destabilizing effects on endothelial barrier integrity, while Rac1 and Cdc42 tend to stabilize junctional complexes [40,42]. RhoA acts to destabilize endothelial barrier integrity through the actomyosin contractility pathway by acting on its effector, Rho-associated kinase (ROCK) [22,43]. First, RhoA-GDP binds to the cellular membrane via its isprenoid group. RhoA-GDP can then be phosphorylated (activated) by a GEF converting it to membrane bound RhoA-GTP, which is the primary form that RhoA must be in to act on its effector ROCK. Once activated, ROCK can either directly or indirectly lead to increased levels of phosphorylated MLC [22,38,44]. ROCK inactivates myosin light chain phosphatase (MLCP), inhibiting the dephosphorylation of myosin light chain (MLC) indirectly leading to increased levels of phosphorylated MLC [22,44] (Figure 2). It has been suggested that as the ratio of phosphorylated MLC to MLC increases, endothelial cells become more contractile, in turn destabilizing cellular junctions [22,45] leading to increased endothelium permeability. It is through this pathway that statins are thought to modulate cellular contractility and ultimately decrease endothelium permeability.

1.3.3 Simvastatin

Statins, classically prescribed to lower serum cholesterol levels, work by inhibiting the hepatic enzyme HMG-CoA reductase, entering hepatocytes via the organic anion transporter. It is currently thought that statins enter nonhepatic cells via passive diffusion as specific transporters have yet to be identified [46]. There are a number of different statins currently on the market, each with varying levels of potency for inhibiting hepatic HMG-CoA reductase [3,36]. In addition, these statins differ in their tissue permeability and metabolism resulting in different potencies for

extrahepatic HMG-CoA reductase inhibition. The statins considered most likely to enter endothelial cells via passive diffusion are the lipophilic statins, such as simvastatin [46]. Simvastatin, originally marketed exclusively by Merck under the brand name Zocor®, was the number two best-selling pharmaceutical in 2002, 2003, and 2004, selling more than $3 billion each year [47]. Prior to losing U.S. patent protection, in 2006, Zocor was the second largest selling statin in the world behind Lipitor whose U.S. patent recently expired in 2011.

1.3.4 Previous Studies Conducted with Endothelial Cells

Thus far groups have primarily investigated the effect of simvastatin on endothelial cell monolayer integrity without accounting for biomechanical stimuli such as matrix stiffness [48–51]. These studies were primarily conducted using Transwell plates which serve as a nice model for permeability; however, Transwell plates have very high stiffnesses when compared to physiological stiffnesses. Using this model, it has been shown that pre-incubation with simvastatin reduces thrombin-induced endothelial barrier dysfunction [50–52]. In addition, simvastatin has been shown to downregulate key proteins in the actomyosin contractility pathway such as RhoA and phosphorylated MLC [50,51].

1.4 Scope of this Project

The primary goal of the work presented in this thesis was to investigate the potential for simvastatin to modulate increased endothelium permeability on substrates with physiologically relevant stiffnesses. The motivation for this research comes primarily from three areas of work.

First, numerous groups have shown that in addition to inhibiting the synthesis of cholesterol, simvastatin inhibits the activity of proteins in the actomyosin contractility pathway such as Rho [3,36,53–55]. Second, it has been demonstrated that increased substrate stiffness leads to increased cell contractility via the actomyosin contractility pathway ultimately resulting in increased endothelium permeability [2]. Third, a number of groups have shown that simvastatin treatment reduces endothelium permeability and prevents increased permeability induced by thrombin on tissue culture plastic [32,50,51,56]. As this work was conducted on tissue culture plastic, with stiffness ~10^6 kPa [57], it is unclear as to how these results will translate to substrates with physiologically relevant stiffnesses. Given that increased substrate stiffness leads to increased endothelial cell contractility and endothelium permeability via Rho mediated contractility [2] and statins have been shown to decrease RhoA activity, my goal was to examine the effect of simvastatin on endothelial cell morphology, cell contractility, cell-cell junction widths, and endothelium permeability for endothelial cells on 2.5, 5, and 10 kPa PA substrates, mimicking the stiffness of healthy and aged vessels respectively (Figure 3).

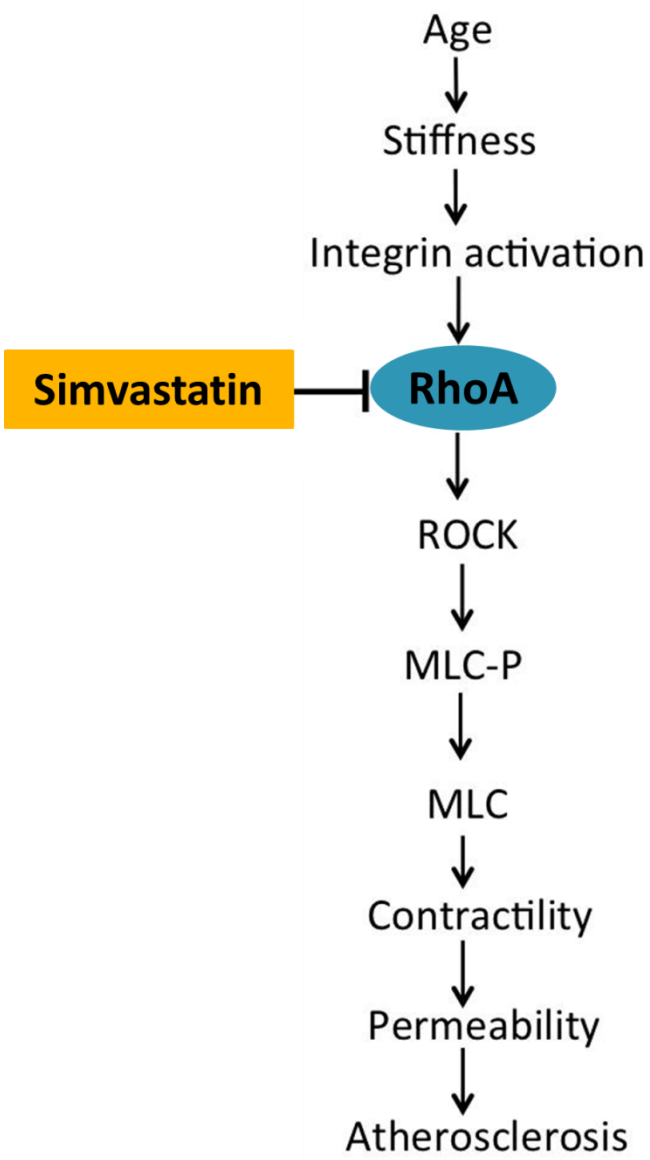

Figure 3: Pathway representing the connection between aging, simvastatin, and the progression of atherosclerosis. Aging leads to increased matrix stiffness which activates the actomyosin contractility pathway. Simvastatin inhibits a key protein, RhoA, in the actomyosin contractility pathway ultimately leading to a decrease in contractility and permeability preventing the progression of atherosclerosis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Overview

An in vitro atherosclerosis model that accounts for blood vessel stiffening that occurs with aging was used for this work. This model employs polyacrylamide gels of 2.5, 5, and 10 kPa seeded with bovine aortic endothelial cells (Figure 4). Peloquin et al. and Huynh et al. measured the stiffness of bovine and mouse arteries respectively. Bovine and mouse arteries were selected given their similarities to human arteries and availability at a variety of ages. The stiffness of 18 to 30 month old bovine carotid intima was reported to be 2.7 ± 0.6 kPa [24]. This is a good estimate of the stiffness of a young or healthy human adult artery because the structure of bovine arteries is analogous to that of human arteries. To get an idea of how vessel stiffness changes with age, Huynh et al. characterized the intimal stiffness of young (10 to 11 weeks) and old (21 to 25 months) mice. The measured modulus for old mice was found to be higher than the value for young mice confirming that there is a relative increase in blood vessel stiffness with increasing age [2]. In our in vitro model, 2.5 kPa polyacrylamide gels were selected to represent young vessels and two higher stiffness polyacrylamide gels, 5 and 10 kPa, were selected to represent aged vessels.

This in vitro atherosclerosis model was employed look at the effect of various treatment conditions on a series of readouts including, LIVE/DEAD assay, shape factor, cell traction force, cell-cell junction, endothelium permeability, and Rho activity (Figure 5). The treatment conditions included no simvastatin, simvastatin, thrombin, and simvastatin followed by thrombin. The treatment conditions were selected based on the literature and then optimized for our in vitro model.

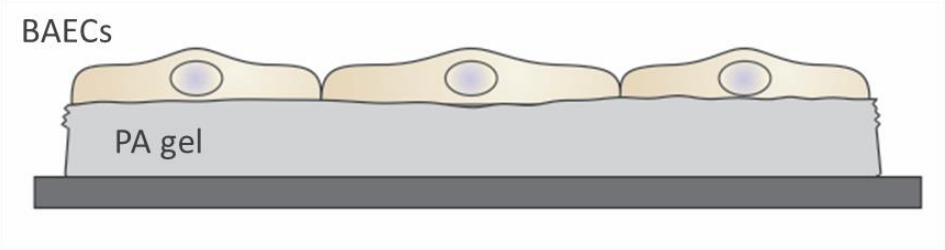

Figure 4: In vitro atherosclerosis model. Polyacrylamide (PA) gel on glass coverslip seeded with bovine aortic endothelial cells (BAECs).

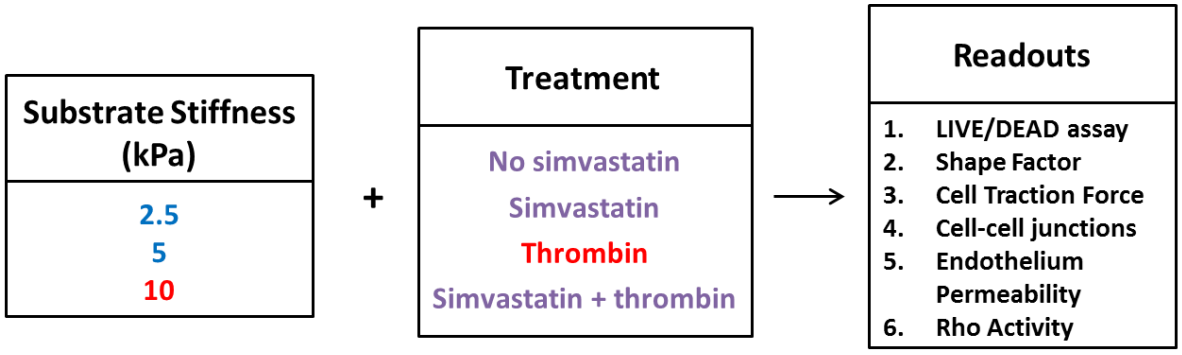

Figure 5: Schematic of research plan. Blue represents known atheroprotective conditions, red represents known atheroprone conditions, and purple represents conditions with no known effects on the progression of atherosclerosis.

2.2 Cell Culture

All experiments were conducted using bovine aortic endothelial cells (BAECs) purchased from VEC Technologies (Rensselaer, NY). Cells were maintained at 37ºC and 5% CO_2 in Medium 199 (Invitrogen, Carlsbad, CA) with 10% Fetal Clone III (HyClone, Logan, UT), 1% MEM amino acids (Invitrogen), 1% MEM vitamins (Medtech, Manassas, VA), and 1% penicillin-streptomycin (Invitrogen), this will be referred to as complete M199. BAECs were used from passages 7-10.

2.3 Preparation of Glass Coverslips

PA substrates are prepared by allowing polyacrylamide solutions to polymerize between two glass coverslips. One of the coverslips must be activated to allow the polymerized PA to adhere for the duration of the experiment. Glass coverslips (No. 2, 22 x 22mm, No. 1, 43 x 50mm, and No. 1, 48 x 65mm, VWR, West Chester, PA) were plasma cleaned for 2 minutes. Plasma cleaned samples were placed in a 1% PEI (polyethylenimine) solution in Milli-Q water for 10 minutes at room temperature. Samples were rinsed with Milli-Q water and inverted onto a 0.1% glutaraldehyde solution in Milli-Q water for 30 minutes at room temperature. Samples were thoroughly rinsed 3 times in Milli-Q water for 5 minutes each. Samples were allowed to completely dry prior to using. Activated coverslips were used within one month of activating.

The second coverslip must be removed after polymerization of the substrate. Glass coverslips (18mm diameter, 48 x 65mm, VWR) were coated with Rain-X and allowed to dry for approximately 5 minutes. Excess Rain-X was removed from the coverslip using a Kimwipe. Particulates were removed by gently spraying the coverslip with a stream of air.

2.4 Polyacrylamide substrate synthesis

PA gels were synthesized as previously described (Califano and Reinhart-King, 2008). 2.5, 5, and 10 kPa gels were polymerized onto activated glass coverslips. The stiffness of the gel is determined by the ratio of acrylamide to bis-acrylamide. For the 2.5, 5, and 10 kPa PA gels used throughout the experiments described the ratios (% acrylamide: % bis-acrylamide) were, 5:0.1, 7.5:0.175, 7.5:0.35, respectively. After polymerization, the gels were functionalized with 0.1 mg/ml rat tail type I collagen (BD Biosciences, San Jose, CA).

2.5 Drug Treatments

Simvastatin (Sigma-Aldrich) prodrug was activated prior to use as previously described [58–60]. Briefly, 5 mg of simvastatin prodrug was dissolved in 125 µl of 200 proof ethanol. This solution was incubated with 187.5 µl 0.1 N NaOH for 2 hours at 50ºC. Then, 600 µl MilliQ water was added and the pH was brought to 7.0 using 0.1 N HCl. The solution was diluted to yield a final concentration of 10 mM and was stored at 4ºC. For treatments, the 10 mM stock was diluted to concentration of 1, 5, 50, and 200µM in M199 or L15 (for permeability study only) media. Bovine thrombin (Calbiochem, San Diego, CA) was used at a concentration of 4 U/ml. 40-kDa FITC conjugated dextran (Sigma-Aldrich) was used at a concentration of 10 µM in L15 for permeability studies.

2.6 LIVE/DEAD® Cell Viability Assay

BAECs were seeded on 2.5 and 10 kPa PA gels at a density of 15,000 cells/cm^2 and allowed to grow to confluence. Two days post confluence cells were treated with 0, 0.1, 0.5, 1, 5, or 10 µM simvastatin or an ethanol control. The ethanol control contained an equivalent amount of ethanol as the 1 µM simvastatin treatment condition. This control was included to ensure that the ethanol used to dissolve the simvastatin prodrug was not toxic to the cells. Following treatment, cell viability was assessed using a LIVE/DEAD® cell viability kit (Invitrogen) according to the manufacturer's protocol. Ethidium homodimer-1 was used at a concentration of 4mM, and calcein was used at a concentration of 2mM. After a 30 minute incubation with the dye solution the solution was removed and replaced with complete M199. Samples were imaged within one hour to ensure no additional cell death. Fluorescent images were acquired on a Zeiss Axio Observer.Z1m microscope equipped with a Hamamatsu ORCA-ER camera using a 10x objective.

2.7 In vitro quantification of cell shape factor

BAECs were plated in a 24-well plate at a density of 2,500 cells/cm^2 and allowed to reach 50% confluence prior to treatment with simvastatin. Simvastatin was diluted with M199 complete media to concentrations of 1 and 5µM. Cells were treated for 6, 16, 24, 36, and 48 hours. The media was then removed and cells were fixed with 3.7% formaldehyde (VWR International, West Chester, PA) and permeabilized with 1% Triton (VWR). Cells were incubated with a 1:200 dilution Alexa Fluor® 488 Phallodin (Life Technologies). Fluorescent images were captured on a Zeiss Axio Observer Z1m microscope equipped with a Hamamatsu ORCA-ER camera using a

20x objective. All cells in the field of view were hand traced in ImageJ. Data on the cell's area, perimeter, centroid, major and minor axis, and angle were collected and used to calculate each cell's shape factor [61]. Shape factor can take on values between zero and one, with zero representing a line and 1 representing a circle.

$$\boldsymbol{Shape\ Factor} = \frac{\boldsymbol{4\pi(Area)}}{\boldsymbol{Perimeter^2}}$$

2.8 Traction force microscopy

BAECs were seeded at a density of 300 cells/cm^2, to ensure the presence of isolated cells, on 2.5, 5, and 10 kPa PA gels embedded with 0.5 μm diameter fluorescent beads (Invitrogen). Cells were allowed to adhere for 16 hours after which the media was removed and replaced with complete M199 containing 1μM simvastatin for the treatment condition and complete M199 without simvastatin for the untreated condition. After 24 hours, phase contrast images of single cells were taken immediately followed by fluorescent images of the bead field beneath the cell (Figure 6). A second fluorescent image of the bead field in an unstressed state was taken after the cells were removed with 0.05% trypsin/EDTA (Invitrogen). Using the LIBTRC analysis library developed by M. Dembo (Dept. of Biomedical Engineering, Boston University), the bead displacements within the gel were calculated and used in combination with the gel's material properties to calculate the most likely cellular traction vectors, T, and the total magnitudes of force [62]. The overall force, | F |, exerted by the cell is the integral of the traction vector magnitudes, T, over the cell area, represented by the equation below, where $\boldsymbol{T}(\boldsymbol{x},\boldsymbol{y}) =$

$\left[\boldsymbol{T_x}(\boldsymbol{x},\boldsymbol{y}),\boldsymbol{T_y}(\boldsymbol{x},\boldsymbol{y})\right]$ is the continuous field of traction vectors defined at any spatial position $(\boldsymbol{x},\boldsymbol{y})$ over the cell area [63].

$$|\boldsymbol{F}| = \iint \sqrt{\left(\boldsymbol{T_x^2}(\boldsymbol{x},\boldsymbol{y}) + \boldsymbol{T_y^2}(\boldsymbol{x},\boldsymbol{y})\right)}\,\boldsymbol{dxdy}$$

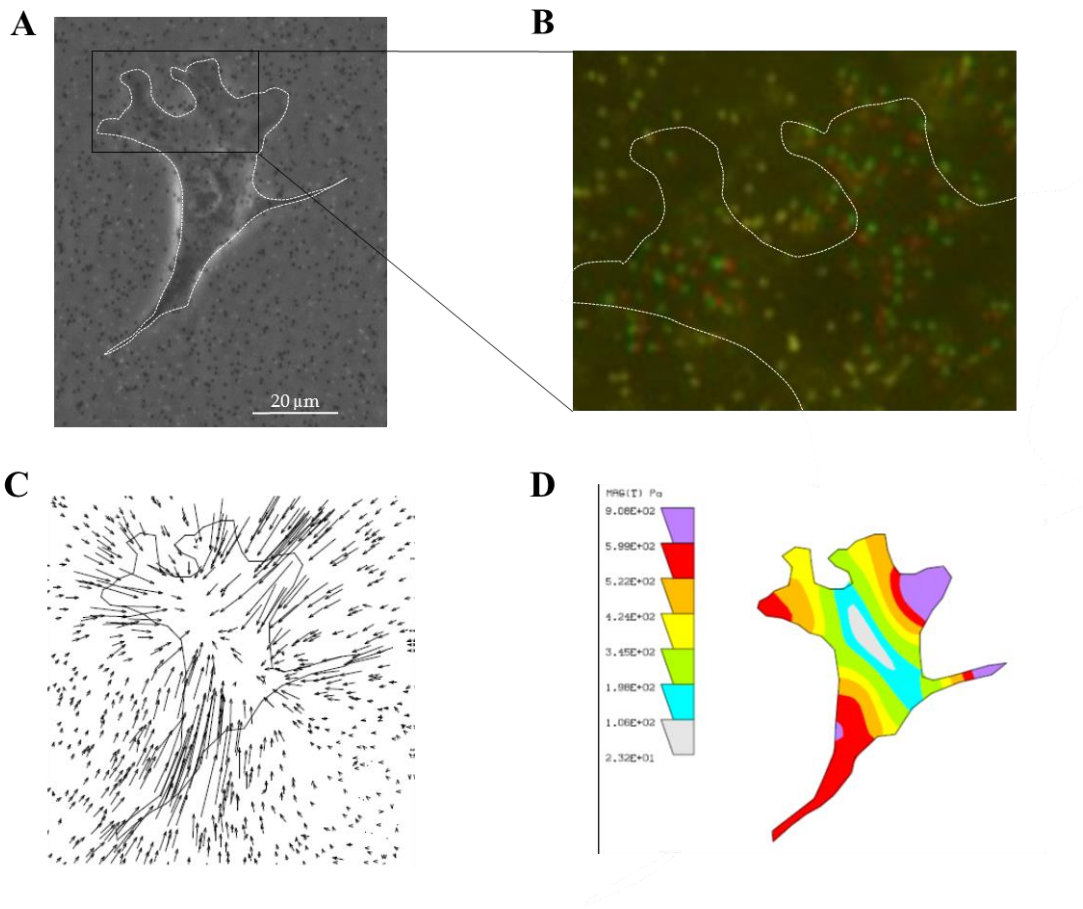

Figure 6: (A) Phase image of BAEC. (B) Zoomed in overlay of unstressed (green) and stressed (red) bead fields. (C) Image of force vectors calculated from bead trace. (C) Image of stress magnitudes, displaying magnitude of cellular force in Pascals.

2.9 In vitro quantification of VE-cadherin junction gap width

BAECs were seeded on 2.5, 5, and 10 kPa PA gels at a density of 15,000 cells/cm^2 and allowed to grow to confluence. Two days post confluence cells were treated with 1 μM simvastatin for 24 hours. Then cells were fixed and permeabilized with 3.7% formaldehyde (VWR) and 1% Triton (VWR), respectively. Cells were incubated with a 1:50 dilution of goat polyclonal VE-cadherin primary antibody (Santa Cruz Bio-technology, Santa Cruz, CA) and a 1:200 dilution of Alexa Fluor® 568 donkey anti-goat secondary antibody (Invitrogen). Fluorescent images were acquired on a Zeiss Axio Observer.Z1m microscope equipped with a Hamamatsu ORCA-ER camera using a 20x objective.

VE-cadherin junction width was quantified using ImageJ and a custom-written MATLAB algorithm previous described [2]. Briefly, a line was drawn perpendicular to the junction in ImageJ using the fluorescent VE-cadherin images giving an intensity profile for each junction. The intensity profile was fit with a two-Gaussian curve in MATLAB. Junction widths were defined as the width of the two-Gaussian fit.

2.10 In vitro measurement of endothelium permeability

BAECs were seeded onto PA gels with a diameter of 18mm at a density of 15,000 cells/cm^2 and allowed to grow to confluence. At two days post-confluence, untreated cells were given Leibovitz's L15 media containing 10% FetalClone III (HyClone) and 1% penicillin-streptomycin (Invitrogen) and treated cells were given this media with 10, 20, or 50 μM simvastatin for the thrombin permeability experiment or 200 μM simvastatin for the no thrombin permeability experiements and maintained at 37ºC. After 6 hours, the permeability was evaluated by adding a

10 μM solution of 40 kDa FITC-dextran to the cells for 10 minutes. Confocal z-slice images were then acquired on a Leica TCS SP2 system equipped with a 40x dipping lens. Three images were taken at three different spots on each gel. For the thrombin permeability experiment, 4 U/ml of thrombin was added to the 10 μM solution of 40 kDa FITC-dextran for 10 minutes and imaged in the same manner as previously described.

Endothelium permeability was quantified by measuring the fluorescent intensity above and below the cell layer using ImageJ as previously described [2]. Briefly, the pixel intensity inside a 50 by 400 pixel box above the cell layer was recorded. Next, the pixel intensity within the gel was determined and recorded by drawing a 400 wide box encompassing the entire height of the gel. This value represents the amount of FITC-dextran that permeated the cell monolayer into the gel over 10 minutes. Average permeability was calculated by dividing the average pixel intensity within the gel by the average pixel intensity above the gel. Since the permeability of the gels is dependent on their stiffness, the average permeabilities were divided by the average permeability calculated for gels without cells giving the permeability value that were reported.

2.11 Measurement of RhoA Activity

BAECs were seeded at a density of 15,000 cells/cm^2 on 48 x 65 mm PA gels with Young moduli of 2.5, 5, and 10 kPa. Cells were grown to confluence and treated with 1 μM simvastatin for 24 hours at two days post confluence. In this case, larger PA gels were used to ensure that there were enough cells for the assay. Immediately following treatment, cells were lysed at 4ºC and stored at -80ºC until the RhoA assay was run. BAEC intercellular RhoA activity was measured using a RhoA G-LISA kit (Cytoskeleton, Denver, CO) according to the manufacturer's protocol.

RhoA activity was normalized to total DNA measured using the Quant-iT™ PicoGreen® Assay (Invitrogen).

2.12 Statistical Analysis

Statistical analysis was conducted in JMP v. 10. All data is mean ± SEM (standard error of means). For the cell viability and shape factor analyses, a Tukey's Honestly Significant Difference (HSD) test was performed. For all other experiments, a standard least square regression with a post hoc Tukey's HSD was performed.

CHAPTER 3

RESULTS

3.1 BAEC Viability with Simvastatin Treatment

To ensure that simvastatin treatment was not significantly affecting endothelial cell viability, cell death was quantified using a LIVE/DEAD assay. Cell viability was assessed for endothelial cell monolayers treated with 0, 0.1, 0.5, 1, 5, and 10 μM simvastatin for 24 hours on 10 kPa PA gels (Figure 7A). As ethanol is required to dissolve the simvastatin prodrug prior to activation, an ethanol control sample was also included. For the ethanol control, an equivalent amount of ethanol as in the 1 μM simvastatin condition was used to ensure that the ethanol was not having a negative effect on cell viability. Percent cell death was not statistically different than untreated values for the 0.1, 0.5, and 1 μM simvastatin treatments (Figure 7B). Additionally, the ethanol control caused no significant decrease in cell viability compared to the untreated and 1 μM simvastatin treated conditions. This suggests that the ethanol used to dissolve the simvastatin prodrug had no negative effect on cell viability. Percent cell death was found to increase compared to the no drug control for 5 and 10 μM simvastatin treatments, increasing by 1.7 and 2.4% respectively. However, the percent cell death remained below 5% for both the 5 and 10 μM simvastatin treatment conditions. Similar results were found for cells seeded on tissue culture plastic and 2.5 kPa PA gels.

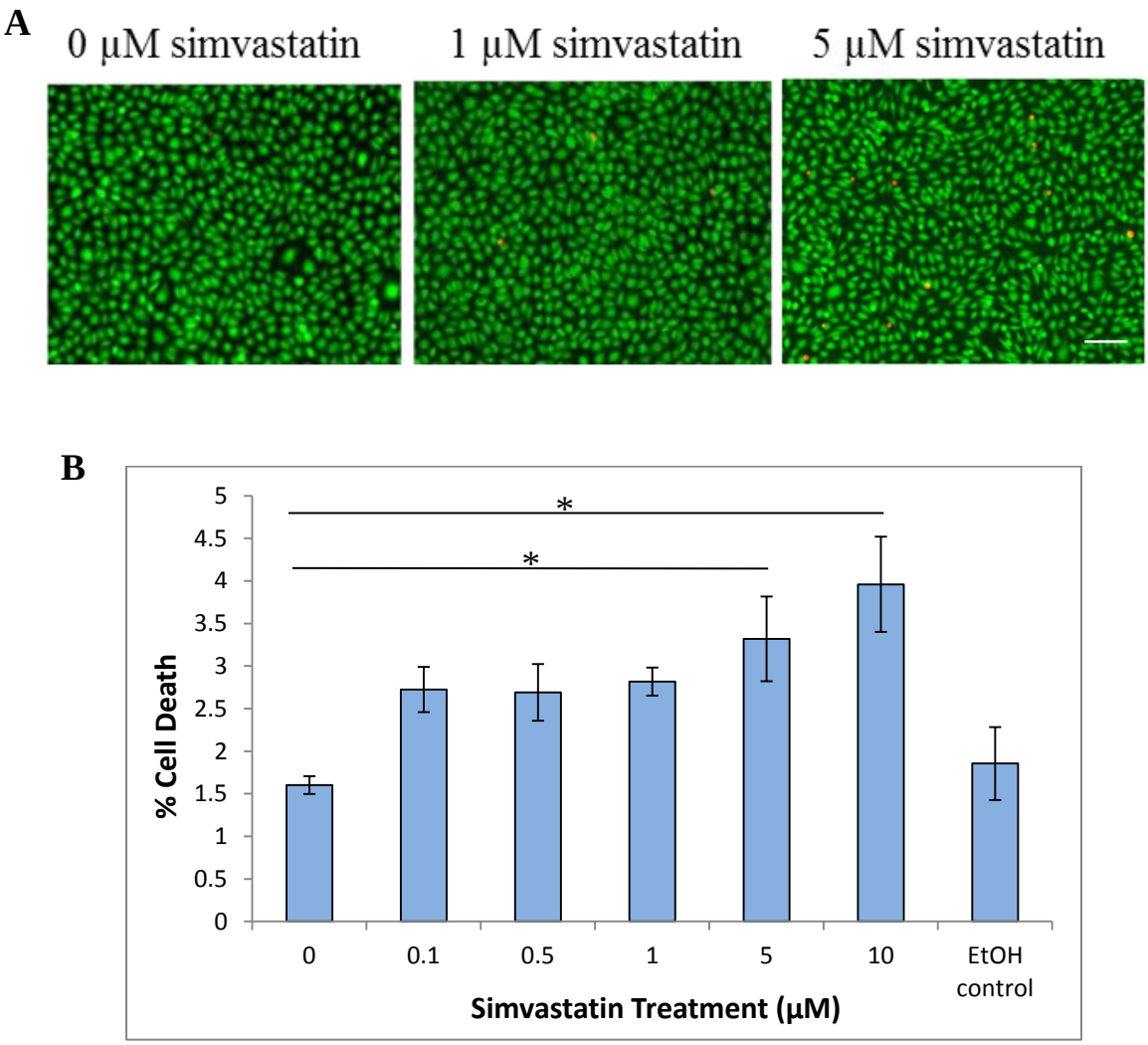

Figure 7: Quantification of cell viability. (A) Fluorescent images of endothelial cell monolayers on 10 kPa PA gels after a 24 hour 0, 1, or 5 µM simvastatin treatment, stained with green-fluorescent calcein-M to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Scale bar, 100 µm. (B) Quantification of LIVE/DEAD assay results for cells seeded on 10 kPa PA gels and treated with various simvastatin concentrations for 24 hours. Data collected in collaboration with Kate Gefell. Data ± SEM. N = 1 with 8-10 images and 2-3 independent gels per condition. *P < 0.005 (Tukey's HSD test).

3.2 Simvastatin Treatment Affects BAEC Morphology

Cell morphology has been shown to be associated with changes in actomyosin contractility, with inhibition of RhoA decreasing cellular contractility resulting in cells with a more elongated morphology [64]. A number of groups have shown that simvastatin treatment decreases RhoA activity [32,66,67]. Likewise, previous research has shown that endothelial cells treated with simvastatin have a more elongated morphology when compared to untreated cells [52]. As such, we sought to investigate the effect of simvastatin concentration and treatment time on cell morphology. Cell morphology was quantified by shape factor which distinguishes between elongated and round cells on a scale from 0 to 1, respectively (Figure 8A). Sub-confluent BAECs were treated with various concentrations of simvastatin (0, 1, and 5 μM) for up to 48 hours (Figure 8B). During this time period, the shape factor for untreated BAECs increased from 0.33 ± 0.009 to 0.53 ± 0.009. In contrast, the shape factor for cells treated with 1 μM simvastatin remained fairly consistent starting at 0.31 ± 0.008 and increasing slightly to 0.36 ± 0.007. After 6 hours of treatment with 5 μM simvastatin cells had a shape factor that was significantly lower than control and 1 μM simvastatin treated cells. For cells treated with 5 μM simvastatin, the shape factor was 0.29 ± 0.007 at 6 hours and decreased slightly to 0.24 ± 0.01 by 48 hours. As shown in Figure 8, the shape factor was found to be significantly lower compared to the untreated condition at all treatment times and concentrations except 1 μM for 6 and 16 hours. Our results show that simvastatin-treated endothelial cells were slightly elongated when compared with untreated cells. This result is consistent with previous work published on endothelial cell morphology in monolayers in response to simvastatin treatment [52]. Changes in cell morphology may have consequences for cell contractility and permeability [68] and suggests that simvastatin may be decreasing RhoA activity [21,64,65].

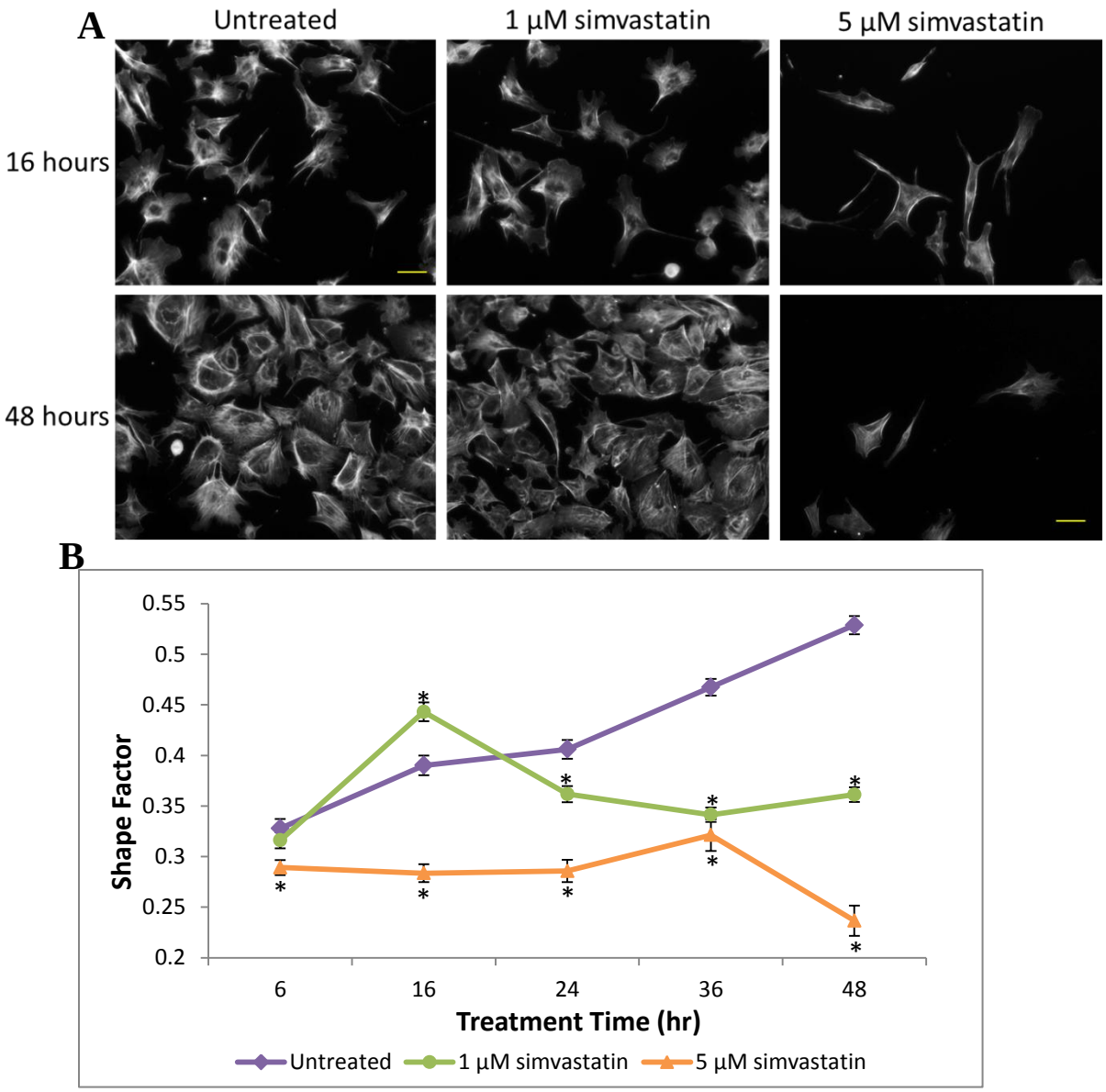

Figure 8: Shape factor as a function of simvastatin treatment time and concentration. (A) Fluorescent images of BAECs stained for actin after 16 and 48 hours of treatment with 0, 1, or 5µM simvastatin. Scale bar represents 40 µm. (B) Quantification of shape factor for BAECs treated with 0, 1, and 5 µM simvastatin for 6, 16, 24, 36, and 48 hours. Error bars represent SEM. N=1 with 170 to 230 cells per condition. Simvastatin treatments were compared to the untreated control for each time point using Tukey's HSD test. * $p<0.01$.

3.3 Simvastatin Treatment Decreases Substrate Stiffness-Dependent Increased Contractility

We previously showed that endothelial cells treated with simvastatin adapt an elongated cell morphology. To determine if this change in cell morphology translated to a change in cell contractility, we examined cell contractility using traction force microscopy. Additionally, it has been shown that endothelial cell contractility increases with increasing substrate stiffness and that this increase can be ameliorated with the addition of the ROCK inhibitor, Y-27632 [2]. From previous work, we hypothesized that simvastatin may inhibit substrate stiffness dependent increased contractility through Rho, as it has been shown to inhibit Rho activity [50]. To investigate the effect of substrate stiffness and simvastatin treatment on endothelial cell contractility, we quantified the forces exerted by the cells on their substrate using traction force microscopy (TFM) (Figure 9). A treatment of 1 μM simvastatin for 24 hours was selected for this experiment based on our cell viability results (Figure 7) and previously published data [52]. Our TFM results indicate that cellular force significantly ($p < 0.01$) increases for untreated cells seeded on 2.5, 5, and 10 kPa gels. These results support previously work showing that cellular force increases as a function of matrix stiffness [2]. With the addition of 1 μM simvastatin for 24 hours, we noted a decreasing trend for all stiffnesses tested when compared with the corresponding untreated condition. Most notably, there was a significant ($p < 0.01$) decrease in cell force upon simvastatin treatment on 10 kPa gels. These results demonstrate that simvastatin is able to reverse substrate stiffness-dependent increased cell contractility, with a significant decrease on stiffer (10 kPa) gels.

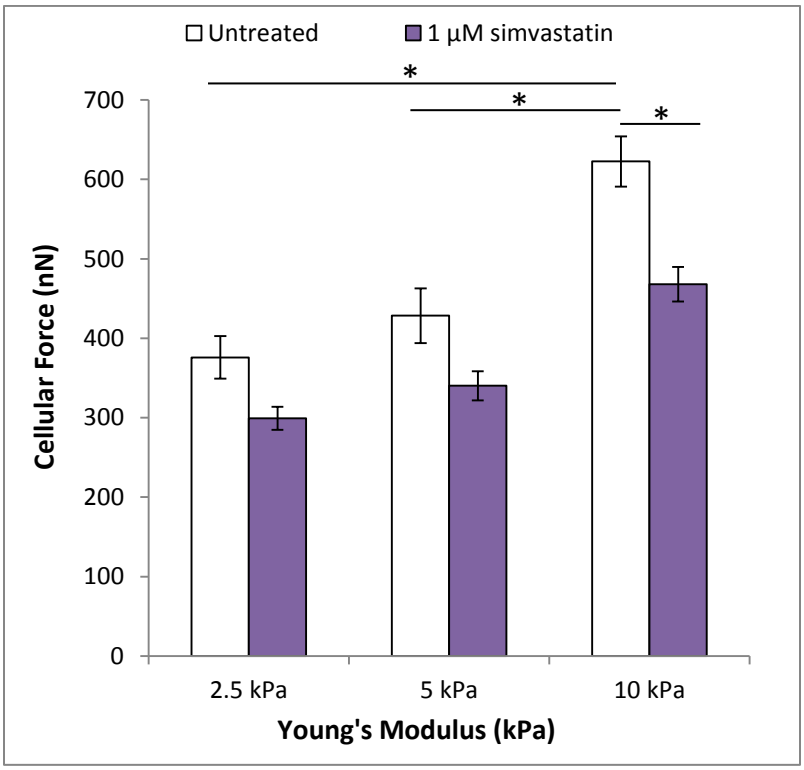

Figure 9: Quantification of cell contractility through TFM measurements. Effect of a 24 hour 1 µM simvastatin treatment and substrate stiffness on cellular traction force. Data are means ± SEM. *P < 0.01 (Standard Least Square Regression with post hoc Tukey's HSD). N=3 with 50 to 80 cells per condition.

3.4 Simvastatin Treatment Decreases Substrate Stiffness-Dependent Increased Cell-cell Junction Width

Cellular contractility and junction width play a key role in regulating endothelium permeability [42]. It has been previously shown that increased cellular contractility leads to destabilized cell-cell junctions causing increased endothelium permeability [2]. Our data show that simvastatin reduces cell contractility on 10 kPa PA gels, as such; we sought to investigate the effect of simvastatin on cell-cell junction width to determine whether the decrease in cell contractility translated to a decrease in cell-cell junction width. Increased cellular contractility has been shown to increase cell-cell junction width [2]. To investigate the effect of simvastatin on cell-cell junction width, fluorescent images of VE-cadherin junctions were analyzed to quantify the junction width (Figure 10A). A treatment of 1 μM simvastatin for 24 hours was used for this experiment because it significantly decreases cellular contractility on 10 kPa substrates (Figure 9). Overall cell-cell junctions on more compliant substrates (2.5 and 5 kPa) were tighter compared to the more destabilized, zipper-like junctions seen on less compliant (10 kPa) substrates (Figure 8A). Quantification of junction widths (Figure 10B) demonstrated that increasing matrix stiffness slightly widens intercellular junction widths on 5 kPa compared to 2.5 kPa substrates and significantly ($p < 0.05$) widens junction width on 10 kPa substrates. This observation is consistent with previous work showing a similar trend [2]. Our data suggests that simvastatin treatment slightly decreases cell-cell junction width on less compliant (2.5 and 5 kPa) substrates. In addition, our data demonstrates that simvastatin treatment results in a significant ($p < 0.05$) decrease in intercellular junction width on 10 kPa substrates.

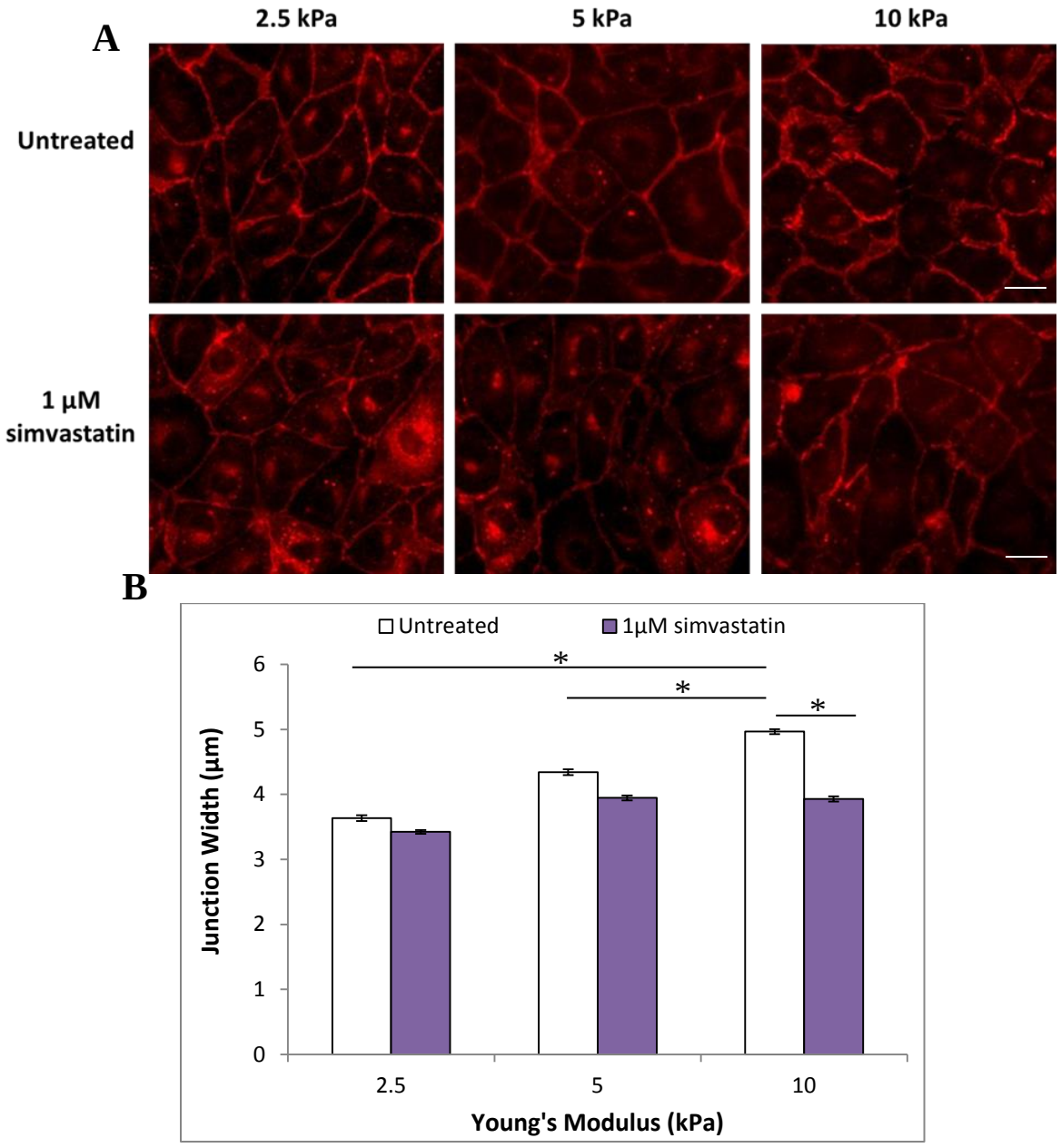

Figure 10: Quantification of cell-cell junction width. (A) Fluorescent images of VE-cadherin in endothelial cell monolayers on 2.5, 5, and 10 kPa gels with and without 1 µM simvastatin treatment. Scale bar, 25µm. (B) Quantification of cell-cell junctions as a represented as a function of substrate stiffness and simvastatin treatment. Data ± SEM. * $P < 0.05$ (Standard Least Squares Regression with Tukey's HSD post hoc.) N=2 with 100 to 200 lines drawn across cell-cell junctions at each condition.

3.5 Simvastatin Treatment Decreases Substrate Stiffness-Dependent Increased Permeability

It has been shown that cellular contractility and junction width play a key role in regulating endothelium permeability [2,42]. We previously showed that simvastatin decreases both cell contractility and cell-cell junction width. As such, we sought to determine if these changes translated to a change in endothelium permeability. To calculate the relative permeability of the endothelial cell monolayers, a solution of FITC-dextran was added to confluent monolayers and then imaged to obtain a z-slice (Figure 11B). These images were analyzed to compare the pixel intensities above and within the gel resulting in the relative permeability. Two sets of permeability experiments were conducted to compare the effect of a longer treatment time with a lower concentration (1 μM for 24 hours) to a shorter treatment time with a higher concentration (200 μM for 6 hours). Quantification of the endothelium permeability (Figure 11C and D) demonstrates that permeability significantly increases on 10 kPa substrates compared to 2.5 kPa substrates. This observation is consistent with previous work [2]. In addition, we note a decreasing trend for permeability upon simvastatin treatment at two different treatment conditions, 200 μM for 6 hours and 1 μM for 24 hours. Likewise, both treatment conditions (1 μM for 24 hours and 200 μM for 6 hours) resulted in the reduction of the permeability to a comparable value on 10 kPa substrates. While preliminary, this data suggests that simvastatin may be able to reverse substrate stiffness-dependent increased permeability under various treatment conditions.

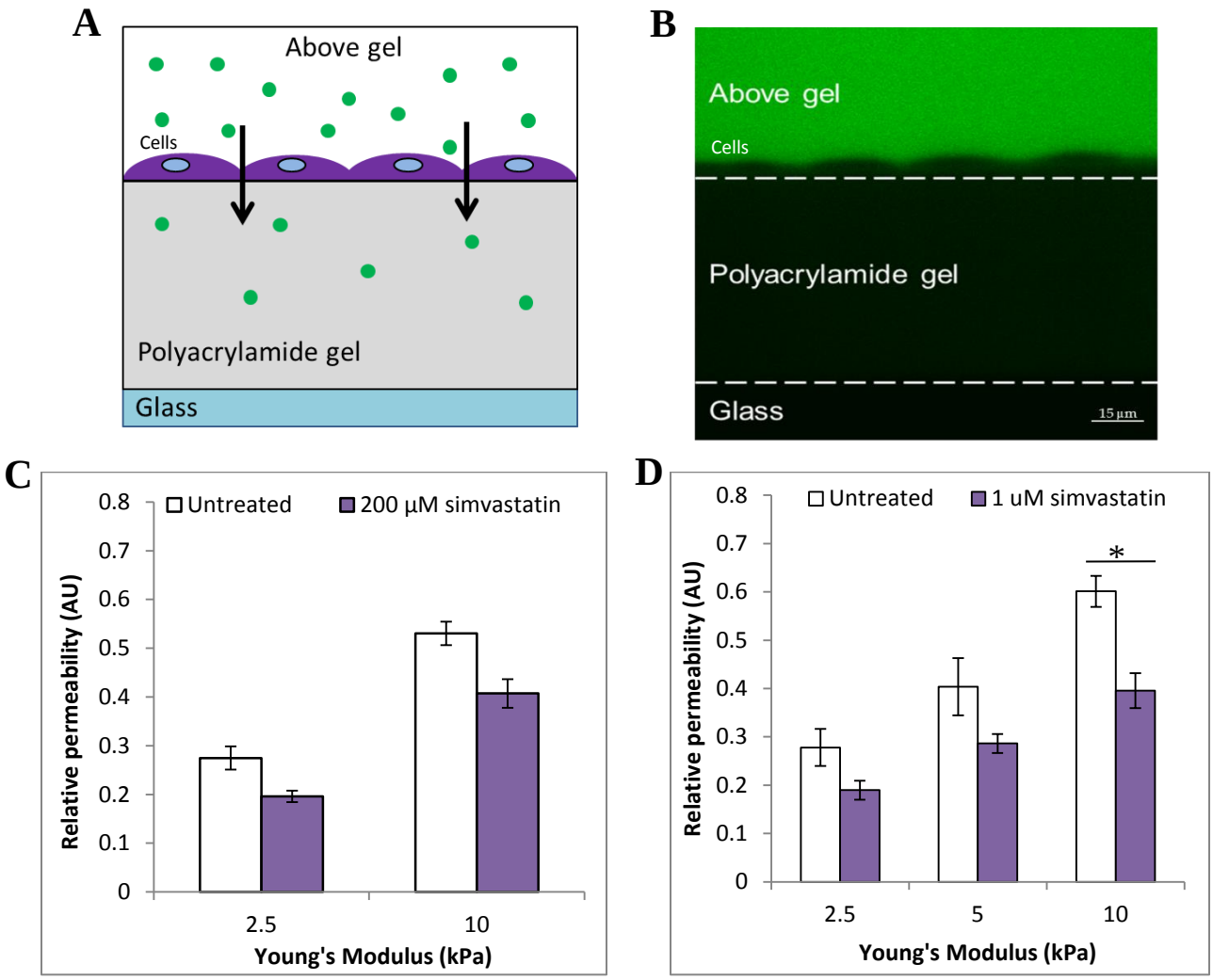

Figure 11: Quantification of endothelium permeability. (A) Schematic depicting dextran (●) accumulation within gels with cell monolayers. (B) Representative confocal cross sections of (A) after addition of FITC-dextran for 5 minutes. The dashed white lines represent the top and bottom edges of the gel. Permeability values were calculated by dividing the average pixel intensity within the gel by the average intensity above the gel. These values were then normalized to the permeability values calculated for no-cell gels to give the relative permeability. (C) Quantified endothelium permeability for a 6 hour 200 µM simvastatin treatment. N=1 with 4 to 8 gels per condition. (D) Quantified endothelium permeability for a 24 hour 1 µM simvastatin treatment. N=1 with 4 to 8 gels per condition. Data collected in collaboration with John Huynh, Ph.D. Data ± SEM. * $P < 0.01$ (Standard Least Squares Regression with Tukey's HSD post hoc).

3.6 Simvastatin Treatment Decreases Thrombin Induced Increased Permeability

Like substrate stiffness, thrombin has been shown to modulate endothelium permeability [69] and play a key role in the progression of atherosclerosis [34]. Previous studies have shown that simvastatin can reduce increased permeability caused by the transient activation of RhoA leading to altered cytoskeletal arrangement upon the addition of thrombin [32]. As such, we sought to investigate the effect of a 6 hour simvastatin pretreatment on attenuating the effect of thrombin on endothelium permeability. The concentrations (10, 20, and 50 μM) and time were chosen based on previously published data that investigated the ability of simvastatin to attenuate the increased permeability observed with thrombin treatment [50,70–72] Relative permeability values were calculated using the method as previously described (Figure 10A and B). The relative permeability of the monolayers (Figure 12) remained relatively consistent, approximately 0.48, at each simvastatin concentration for samples that did not receive thrombin after simvastatin pretreatment. Quantification of the endothelium permeability for 0, 10, and 20 μM simvastatin pretreatments demonstrate that permeability significantly ($P < 0.05$) increases with thrombin treatment. After a 50 μM simvastatin pre-treatment for 6 hours, thrombin no longer caused a significant increase in monolayer permeability. This data suggests that 50 μM simvastatin for 6 hours attenuates thrombin induced increased permeability.

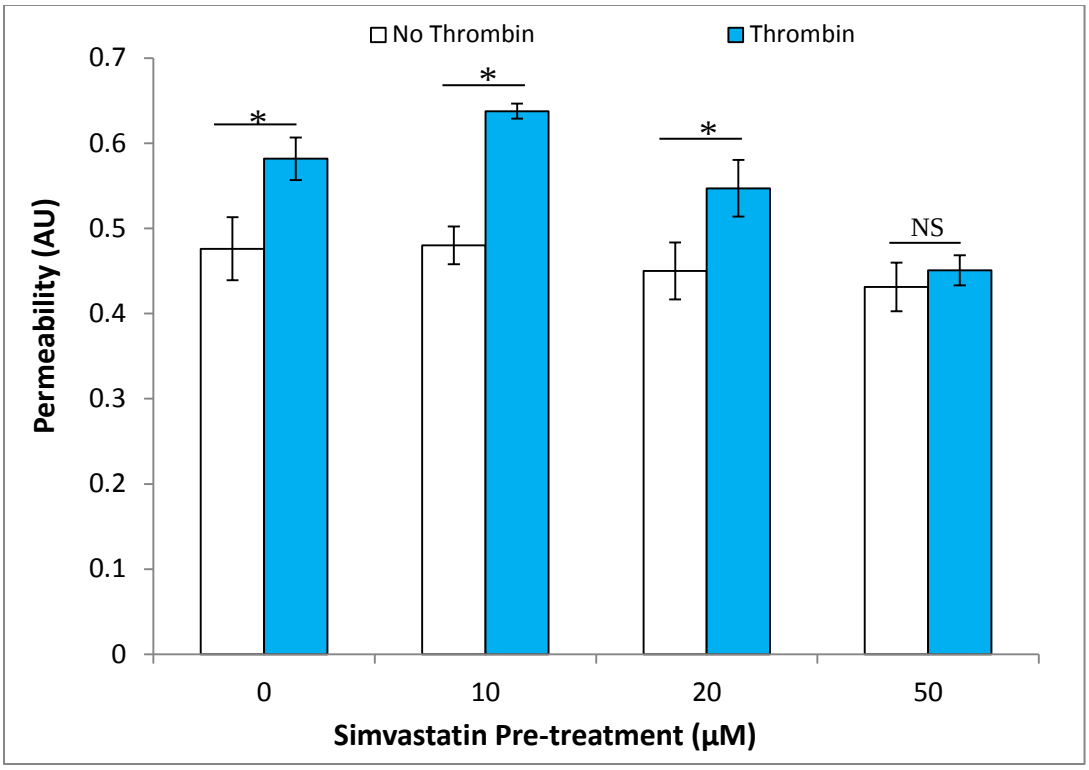

Figure 12: Quantification of endothelium permeability on 5 kPa PA gels with and without a 6 hour simvastatin pre-treatment prior to 10 minute treatment with 4 U/ml thrombin. Data ± SEM. N=2 with 8 to 12 gels per condition. * $P < 0.05$ and NS=not significant (Least Squares Linear Regression with Tukey's HSD post hoc test).

CHAPTER 4

DISCUSSION

One of the initial steps in the progression of atherosclerosis is endothelial cell dysfunction, resulting in increased endothelium permeability. This increased endothelium permeability ultimately leads to the accumulation of lipids and the formation of an atherosclerotic plaque. Traditionally, atherosclerosis is treated using statin therapy to reduce blood cholesterol levels. Interestingly, many studies have shown that the off-target effects of statins, such as simvastatin, may include the prevention of endothelial cell dysfunction and regulation of endothelium permeability. However, these studies were conducted on glass or plastic substrates with stiffness significantly higher than those found in the body, and it has recently been shown that increasing substrate stiffness has a significant effect on endothelial cell function resulting in increased cell contractility and endothelium permeability [2]. This increased contractility and permeability have been shown to be modulated by inhibiting ROCK, a key protein in the actomyosin contractility pathway downstream of RhoA, with Y-27632 [2]. As such, this study investigated the effect of simvastatin, which has been shown to inhibit RhoA, on endothelial cells in an in vitro atherosclerosis model that includes PA substrates with physiologically relevant stiffness. This work shows that simvastatin reduces endothelial cell contractility, enhances cell-cell junction integrity, and decreases substrate-stiffness dependent and thrombin-dependent increased endothelium permeability.

Simvastatin concentrations and treatment times for our in vitro atherosclerosis model were initially established by assessing cell viability and morphology. Cell viability results suggest that simvastatin concentrations up to 1 μM for 24 hours show no significant cell death compared to control and that concentrations up to 10 μM for 24 hours show minimal cell death.

In addition, the results presented show that simvastatin induces a more elongated morphology in endothelial cells treated for more than six hours. This finding is consistent with previous work showing that monolayers of endothelial cells treated with simvastatin have a more elongated morphology compared to untreated monolayers [52]. This work done with monolayers suggests that the morphology results obtained here for isolated cells may translate to monolayers. The time scale required to see this morphology change was also consistent with previously published work that suggests a simvastatin treatment of at least six hours is required to attenuate increased permeability caused by thrombin [50,51]. This longer time frame may be the result of a lag between the effects of simvastatin on Rho family proteins and translation to measurable changes in the actin cytoskeleton.

In this work, we showed that simvastatin reduces endothelial cell contractility, enhances cell-cell junction integrity, and decreases substrate stiffness-dependent permeability, all of which have implications in the initial stages in the progression of atherosclerosis. Simvastatin has been shown to inhibit the geranylgeranylation of RhoA leading to a decrease in membrane bound active RhoA which is important in the activation of ROCK and regulation of cell contractility. The decreases observed in this work for cellular contractility, cell-cell junction width, and endothelium permeability upon simvastatin treatment were not as pronounced as those reported after treatment with Y-27632 [2]. This could be explained by the fact that Y-27632 acts further downstream in the actomyosin contractility pathway, inhibiting ROCK, when compared to simvastatin, which inhibits the geranylgeranylation of RhoA. While ROCK is primarily activated by membrane bound active RhoA, a fraction is activated by cytosolic active RhoA which suggests that simvastatin treatment only partially inhibits ROCK in the actomyosin contractility pathway, while Y-27632 was used at a concentration to completely inhibit ROCK. Further work

needs to be conducted to gain a better understanding of the magnitude in which simvastatin is downregulating active RhoA, the activation of ROCK, and ultimately the phosphorylation of MLC.

Thrombin has been shown to induce an atherosclerotic phenotype leading to increased endothelium permeability through the activation of RhoA and play a role in the progression of the later stages of atherosclerosis[32,33]. Our data suggests that pre-incubation with simvastatin ameliorates thrombin-enhanced endothelial cell monolayer permeability in a dose-dependent manner. These results are consistent with results found in the literature, in which a simvastatin pre-treatment prevents thrombin-enhanced endothelium permeability on glass and tissue culture plastic substrates [50,51]. It has been suggested that simvastatin prevents this increased permeability through the down regulation of key proteins in the actomyosin contractility pathway such as RhoA and MLC-pp [50–52,56]. This mechanism is analogous to the mechanism by which we speculate simvastatin to decrease increased permeability with increasing substrate stiffness, as both thrombin and substrate stiffness activate RhoA. Further work needs to be conducted to explore the mechanism by which simvastatin ameliorates the dual effects of thrombin and increasing substrate stiffness on endothelium permeability.

The ability of simvastatin to enhance endothelium integrity on substrates with physiologically relevant stiffnesses suggests simvastatin as a potential treatment to prevent endothelial cell dysfunction and in turn the first step in the progression of atherosclerosis. The ability of simvastatin to further attenuate increased endothelium permeability caused by thrombin speaks to its ability to prevent endothelial cell dysfunction caused by thrombin which occurs during both the early and later stages of atherosclerosis. While further studies need to be conducted to understand the exact mechanism by which simvastatin induces these changes and

the effect of simvastatin on endothelial cells in a more robust atherosclerosis model that accounts for both substrate stiffness and fluid shear stress, these results suggest simvastatin as a potential option in the prevention of the initial stages of atherosclerosis.

CHAPTER 5

FUTURE WORK

The data presented in this thesis suggest that simvastatin reduces endothelial cell contractility, enhances cell-cell junction integrity, and decreases substrate-stiffness dependent and thrombin-dependent increased endothelium permeability. The exact mechanism by which simvastatin induces these changes remains unclear.

Several groups have shown that simvastatin decreases RhoA activity in endothelial cells [50–52,73]. Given its key role in the actomyosin contractility pathway and previous work suggesting that inhibiting RhoA with Y-27632 leads to a decrease in cellular contractility, cell-cell junction width, and endothelium permeability [2], preliminary experiments were conducted to look at RhoA activity as a function of substrate stiffness and 1 μM simvastatin treatment for 24 hours. RhoA activity was quantified using a RhoA G-LISA kit (Cytoskeleton). Values of RhoA activity were normalized to total DNA values, quantified with a PicoGreen Assay Kit, to account for the number of cells on each gel at confluence. RhoA activity was found to slightly increase with increasing substrate stiffness for 2.5, 5, and 10 kPa PA substrates (Figure 13). On glass Rho activity was much higher than on the PA substrates. While these results are not robust they support previous work showing that RhoA activity increases with increasing substrate stiffness [2]. Counter to our expectations, our results suggest that simvastatin does not decrease Rho activity on 2.5, 5, and 10 kPa PA substrates. In contrast, our results suggest that simvastatin decreases Rho activity on a glass substrate. As this is preliminary work, additional replications of this experiment are needed to determine the effect of simvastatin on RhoA activity for cells on 2.5, 5, and 10 kPa PA substrates.

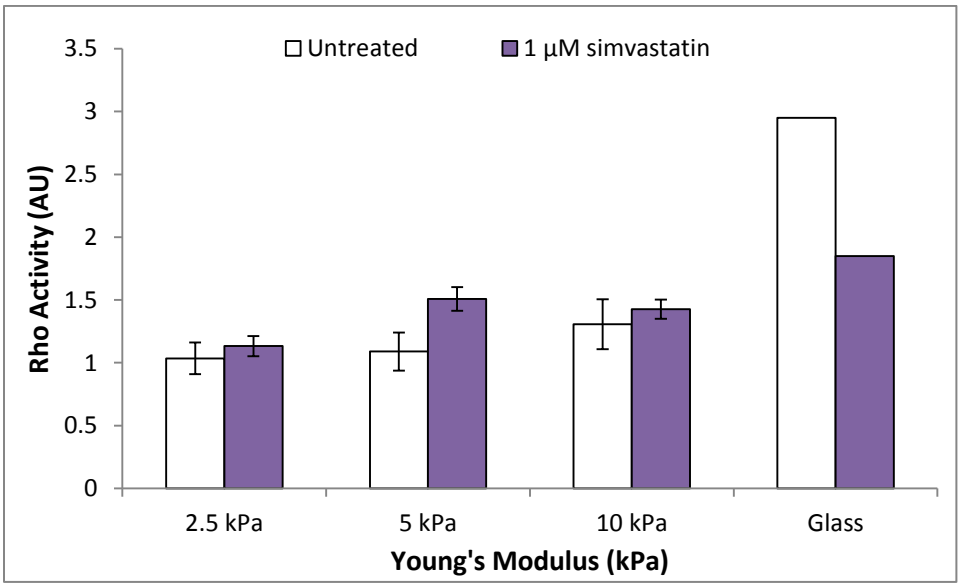

Figure 13: Effect of simvastatin on RhoA activity in cells plated on substrates of increasing stiffness. RhoA activity was quantified using a RhoA G-LISA with or without 1 µM simvastatin for 24 hours. RhoA activity was normalized to total DNA quantified using a PicoGreen assay to account for the number of cells on confluent samples. Data ± SEM. N=2 with 8 gels per condition for 2.5, 5, and 10 kPa PA gels and N=1for glass.

These preliminary results raise a number of questions about the mechanism by which simvastatin is altering cell morphology and reducing endothelial cell contractility, cell-cell junction width, and endothelium permeability on increasing substrate stiffnesses. Additional experiments need to be conducted to elucidate the role of RhoA and determine the mechanism by which simvastatin is acting on 2.5, 5, and 10 kPa PA substrates to alter cell morphology and decrease cell contractility, junction width and ultimately endothelium permeability.

As simvastatin is thought to reduce RhoA activity by preventing geranylgeranylation, it is possible that while overall levels of active RhoA are staying consistent or slightly increasing, the levels of membrane bound active RhoA are actually decreasing. To determine the role of cytosolic versus membrane bound active RhoA, separate lysing protocols can be employed to distinguish between these two forms of active RhoA. Based on the role of simvastatin, it would be expected that simvastatin reduces membrane bound active RhoA.

Additionally, it would be beneficial to determine the effect of simvastatin further downstream in the actomyosin contractility pathway by quantifying the amount of diphosphorylated myosin light chain, MLC-pp, using Western blot. As previously mentioned, cell contractility is related to the ratio of MLC-pp to MLC, with endothelial cells becoming more contractile as this ratio increases [22]. Based on this information we would expect to find that simvastatin treatment leads to a decrease in MLC-pp. This result has been previously reported in the literature under similar simvastatin treatment conditions for endothelial cells grown on glass [51].

Previous studies have suggested that both RhoA and Rac, another protein in the Rho GTPase family, act simultaneously to prevent endothelial cell dysfunction and junction

destabilization [50,65,74]. It has been suggested that simvastatin decreases membrane bound Rac by inhibiting geranylgeranylation through the isoprenoid biosynthesis pathway previously described [50,51]. In addition, cytosolic Rac has been shown to increase upon simvastatin treatment, leading to stabilization of junctional complexes [50]. Given its role in stabilizing intercellular junctions and the results of previous studies, looking at cytosolic and membrane Rac may help further elucidate the mechanism by which simvastatin prevents substrate-stiffness dependent and thrombin-dependent increased permeability.

Additionally, it would be interesting to investigate the effect of simvastatin on endothelial cells in a more robust in vitro atherosclerosis model that accounts for both substrate stiffness and fluid shear stress. As previously mentioned, substrate stiffness has been shown to affect endothelial cell contractility altering endothelium permeability. In addition to being affected by the mechanical properties of their matrix, endothelial cell phenotype is also affected by mechanical forces due to blood flow. Numerous studies have shown that fluid shear stress plays a role in atherosclerosis progression. Areas of low shear stress and disturbed flow patterns are more susceptible to plaque formation than areas of high shear stress and laminar flow [75,76]. A limited number of studies have been conducted to examine the effect of simvastatin on endothelial cell monolayers exposed to shear stress [48,77,78]. These studies were conducted with cells plated on polystyrene dishes ignoring the fact that in vivo, the intima is much more compliant. Because both shear stress[76,79–81] and matrix stiffness[2,17] have been shown to independently affect cell behavior through integrin-related mechanotransduction pathways and the involvement of simvastatin in the actomyosin contractility pathway[42,50,82,83], it is necessary to study these two mechanical stimuli in parallel along with simvastatin treatment to gain a more robust idea of the mechanism by which simvastatin modules increased endothelium permeability.

Completing the experiments presented above would lead to a better understanding of the mechanism by which simvastatin prevents endothelial cell dysfunction. This understanding could lead to better treatment and prevention strategies for patients presenting early signs of atherosclerosis.

The work presented in this thesis suggests that simvastatin may have beneficial effects in preventing the initial stages of atherosclerosis given its ability to prevent increased endothelium permeability caused by thrombin and increased substrate stiffness. It would be interesting to investigate the effect of simvastatin on the later stages of atherosclerosis and other aspects of the disease such as chemokine involvement and vessel calcification. There are numerous chemokines involved in the progression of atherosclerosis and formation of an atherosclerotic plaque. Chemokines such as tumor necrosis factor α and platelet-derived growth factor have also been shown to have an effect on endothelial cells leading to the activation of Rho family proteins[84–86]. The addition of these chemokines to the in vitro atherosclerosis model used in this work would allow for the effect of simvastatin on aspects of atherosclerosis beyond mechanical stimuli to be further investigated. Vascular calcification is found in most advanced atherosclerotic lesions and has been shown to contribute to overall morbidity by decreasing the elasticity of blood vessels [87]. It has been suggested that atherosclerotic calcification is the result of induced osteogenic differentiation in subpopulations of vascular cells, such as smooth muscle cells, by inflammatory factors [88]. The calcified plaque has been shown to have a higher stiffness than a healthy blood vessel, analogous to the relationship between vessel stiffness of young and old arteries [89]. Given this similarity it would be interesting to investigate the effect that the calcified plaque has on endothelial cell function and whether simvastatin is able to attenuate increased permeability that may result from the endothelial cells being exposed to a stiffer

matrix. Currently the ability of simvastatin to effectively treat the later stages of atherosclerosis remains unclear. This work would further our knowledge of simvastatin's ability to attenuate endothelial cell dysfunction and potential to treat the later stages of atherosclerosis.

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