EFFECTS OF SIMVASTATIN AND FLUVASTATIN ON

ENDOTHELIAL INVASION

A Senior Scholars Thesis

by

EVAN MICHAEL CHERRY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Chemical Engineering

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Effects of Simvastatin and Fluvastatin on Endothelial Invasion. (April 2009)

Evan Michael Cherry
Department of Chemical Engineering
Texas A&M University

Research Advisor: Dr. Kayla Bayless
Department of Molecular & Cellular Medicine

Endothelial invasion is a crucial step in angiogenic blood vessel formation and has

ramifications in wound healing, tumor growth, and may have implications in heart

disease. During invasion, quiescent endothelial cells (ECs) proteolyze their basement

membrane, proliferate, and sprout into the extracellular matrix. This process is partially

mediated by contact feedback from integrins and stimulated by angiogenic growth

factors Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor

(bFGF), as well as the lysosphingolipid Sphingosine-1-Phosphate (S1P). Invasion is

distinguishable by dramatic EC morphological changes from simple squamous cells to

sprouting structures that ultimately form new lumens.

Cholesterol synthesis is inhibited by a class of drugs called HMG-CoA Reductase

inhibitors, also known as statins. Statins are the first line of pharmacological treatment

for reducing cholesterol levels when diet and lifestyle changes are not sufficient. 3-

hydroxy-3-methylglutaryl-CoenzymeA (HMG-CoA) Reductase is the key rate-

determining enzyme of the mevalonate pathway, the biological process by which

dimethylallyl pyrophosphate and isopentyl pyrophosphate (sterol precursors) are

synthesized. Statins are effective in reducing the risk of atherosclerosis, a major cause of

cardiovascular disease and studies have shown that statins may have secondary

protective effects.

In our study, we tested the effects of statins on endothelial cell invasion. To investigate

the effects of statins on endothelial function, we studied the effects of two different

statins on regulating endothelial invasion of three-dimensional collagen matrices. The

natural product-derived Simvastatin (ZOCOR®) and the synthetic Fluvastatin

(LESCOL®) both lowered cellular cholesterol levels and inhibited invasion. Both were

tested at concentrations within the range of human plasma levels and in the presence of

S1P, VEGF, and FGF. Differences in the known signaling proteins Erk 1/2, Akt, and

Paxillin were analyzed by gel electrophoresis and western blotting.

ACKNOWLEDGMENTS

I would like to thank Dr. Bayless for the opportunity to work with her and especially for her guidance, expertise, and patience.

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NOMENCLATURE

BSA	Bovine Serum Albumin
DAPI	4',6-Diamidino-2-Phenylindole
EC	Endothelial Cell
ECL	Enhanced Chemiluminescence
FBS	Fetal Bovine Serum
G3PDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GF	Growth Factor
SB	Sample Buffer
HDL	High-Density Lipoprotein
HMG CoA Reductase	3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase
HRP	Horseradish Peroxidase
LDL	Low-Density Lipoprotein
ME	2-Mercapto-Ethanol
P-	Phospho-
PBS	Phosphate Buffered Saline
PVDF	Polyvinylidene difluoride
S1P	Sphingosine-1-Phosphate
SDS-PAGE	Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis
Statin	HMG-CoA Reductase Inhibitor

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CHAPTER I

INTRODUCTION

Atherosclerosis and statins

Cholesterol regulation has become one of the primary methods for preventing heart disease. HMG-CoA Reductase inhibitors (statins), the first line of treatment for inhibiting cholesterol synthesis, lowering blood cholesterol levels and significantly reduce both atherosclerosis [1] and the instance of heart disease by 60%. [2]

Atherosclerosis is the buildup of fatty plaques along the interior lining of blood vessels. Various stress factors oxidize the cholesterol transport molecule Low density Lipoproteins (LDL). Oxidized LDL adheres to the lining of blood vessels and encourages foam cell accumulation and plaque growth which reduces blood flow and can lead to a heart attack. [3] Plaque rupture can result in a stroke. The Agency for Healthcare Research and Quality found that in 2005, 29.7 million people purchased statins for an estimated total of 19.7 billion dollars. [4] The structures of the two statins used in this project are shown in (Fig. 1) and their plasma levels after initial dose are shown in (Fig. 2). [5-6]

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This thesis follows the style of Biochemical and Biophysical Research Communications.
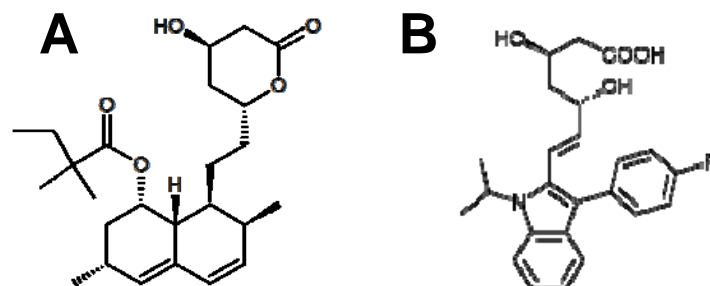
Fig.1. Structures of (A) Simvastatin and (B) Fluvastatin. Simvastatin is derived from lovastatin, a naturally-occurring compound in the fungus Aspergillus terreus. Fluvastatin, a synthetic statin, has a visibly different structure.
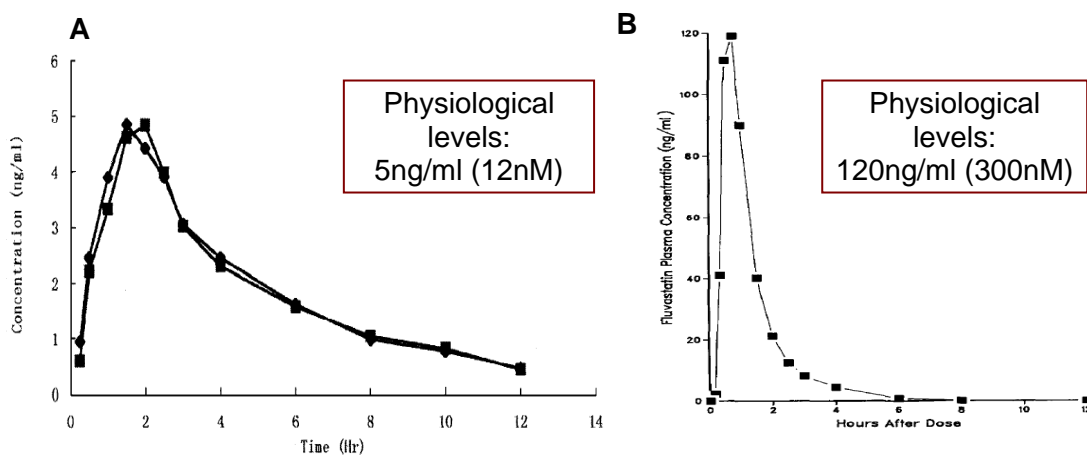

Fig 2. Plasma concentrations of (A) Simvastatin, and (B) Fluvastatin. Simvastatin reaches a peak of approximately 12 nM within the first two hours. Fluvastatin levels in plasma peak at approximately 300 nM within the first hour.

Angiogenesis

Patients treated with statins exhibit other cardiovascular protective effects, including a lower incidence of cancer. [7-8] New blood vessel growth, or angiogenesis, is required for tumor growth and wound healing. These data have helped form the basis for our hypothesis that statins may affect angiogenesis.

During angiogenesis, endothelial cells lining blood vessels respond to extracellular signals and change into invading structures (Fig. 3). In wound healing, the process relies on platelets, fibroblasts, and neighboring endothelial cells. The endothelial cells entering the surrounding matrix initiate new blood vessel growth. Endothelial cells propagate across the gap until they make contact other endothelial cells, cease multiplying, and migrate into the extracellular matrix in response to a sphingosine-1-phosphate (S1P) and angiogenic growth factor gradient. [9] The simultaneous combination of lipid inducers, contact feedback, and growth factors is required for endothelial invasion. [10-11]
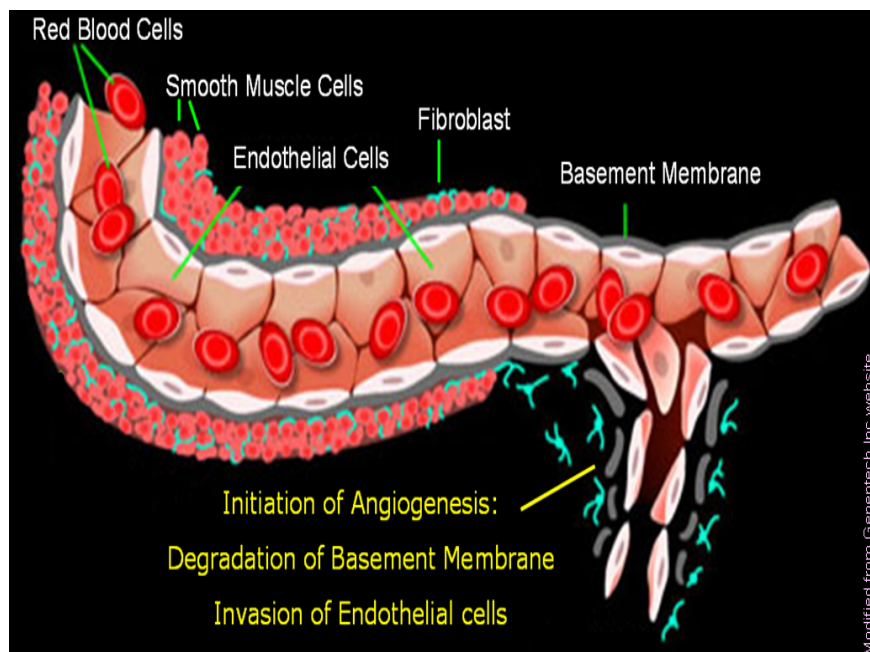
Fig 3. Summary of Angiogenesis. Endothelial cells lining blood vessels degrade the basement membrane and invade into the extracellular matrix in response to lipid and growth factor gradients.

CHAPTER II

METHODS

Reagents

D-Erythro-Sphingosine-1-Phosphate (S1P) was purchased from Avanti Polar Lipids. Simvastatin sodium salt and fluvastatin sodium salt were purchased from Calbiochem. Simvastatin sodium salt was reconstituted in DMSO and fluvastatin sodium salt was reconstituted in sterile water. Fetal Bovine Serum was obtained from Invitrogen. All other reagents were from Sigma-Aldrich unless otherwise stated.

Collagen extraction

Rat tail collagen type I was isolated by soaking frozen rat tails in ethanol, removing the epidermis from the rat tails, extracting tendons, and removing any muscle or fatty tissue. Tendons were then soaked in 150 ml of sterile 0.1% acetic acid and gently shaken for 48 h. Every 24 h the solution was centrifuged and 40 ml extracts were removed from the supernatant and added to separate tubes. Extracts were combined in a lyophylizer bottle and rotated while shell-freezing in dry ice until all liquid was frozen. Frozen solution was lyophilized until dry product was white and fibrous. Dry collagen product was weighed and dissolved in 0.1% acetic acid to a final solution of 7.1 mg collagen/ml.

Cell culture

Human umbilical vein endothelial cells (HUVECs) (Clonetics) were used between passages 2 and 6 and grown in 15 ml of Supermedia [Medium 199 (M199) containing 15% FBS, 0.1mg/ml Heparin, 0.4 mg/ml ECGM from bovine hypothalamus (Pel-Freeze Biologicals), 0.01 mg/ml Gentamicin Reagent Solution (Invitrogen), and 1X Antibiotic-Antimycotic (Invitrogen)] in 75 cm^2 flasks (Corning Incorporated). During collection, cells were washed twice with HEPES-buffered saline, removed with trypsin-EDTA and neutralized with FBS. Cells were washed with M199 to remove serum, centrifuged, re-suspended in M199, and counted using a hemocytometer. After calculating final dilution volume, the cells were centrifuged and re-suspended in equilibrated M199 (37°C and 5% CO_2) to $4x10^4$ cells/50μl.

Vertical collagen invasion assay

Collagen matrices were prepared by combining 350 μl of collagen type I solution, 39 μl 10X M199, 2.1 μl 5N NaOH, and 609 μl M199 on ice and mixing thoroughly after adding each component. S1P was added at a final concentration of 1 μM. 28 μl of gel was added to wells in a 96-well plate (Corning Inc.) and allowed to polymerize and equilibrate at 37°C and 5%CO_2. HUVECs (40,000 cells/50 μl) were treated with statin at a physiological dose (160 nM fluvastatin or 10 nM simvastatin) (Fig 3) or nothing in a test tube for 20 min prior to being added to the wells and allowed to adhere. After seeding the cells, each well was given 50 μl of equilibrated media containing reduced-serum II supplement (Transferrin, BSA, oleic acid, and insulin), 40 ng/ml bFGF and

VEGF (Upstate Bio-technology), 50 μg/ml ascorbic acid, and the concentration of statin for pretreatment. Cultures were allowed to develop for 24 h before being fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with a 1:10000 dilution of DAPI in PBS, then counted via UV fluorescence using an eyepiece with an ocular grid. A field at the center of each well was selected and counted manually at 10x magnification using an Olympus CK3 Microscope. Data is presented as invading cells per field (±SD).

Cholesterol quantification

Cholesterol quantification was conducted using a commercial kit purchased from BioVision. HUVECs were grown in 75 cm^2 flasks for 3 d. Each day the media was changed and given a dose of fluvastatin, simvastatin, or nothing (control). On the fourth day, the cells were collected by trypsinization as described above. Cell numbers were and $3x10^6$ cells were extracted in 600 μl chloroform:isopropanol:Triton X-100 (7:11:0.1) and homogenized in a glass microhomogenizer. The extracts were then transferred to 1.7 ml eppendorf tubes and spun at 20,000 rpm for 10 min. Three drops of distilled water were added after centrifugation to aid in layer separation. 150 μl of the lower phase was removed and transferred to a fresh eppendorf tube. The solvent was evaporated under a steady nitrogen stream and the lipid residue was dissolved in 600 μl Cholesterol Reaction Buffer by vortexing for 5 min. Cholesterol standards and reaction mix were prepared as written in the instruction manual. 50 μl of sample or standard and 50 μl of reaction mix were added to wells in a 96-well plate, covered in foil, and incubated for 1

h at 37°C while protecting from light. The plate was then analyzed using a spectrophotometer at 570 nm in a microplate reader.

Protein analysis

Extracts were collected by dissolving 2 gels from a 96-well plate in 150 μl of RIPA Detergent (for P-Erk 1/2) or directly into sample buffer (SB) (for P-Paxillin and P-Akt). Extracts were collected at incubation of the invasion experiment, 1 h after incubation, and 2 h after incubation. Gels were heated to 100°C and spun at 14000 rpm. The supernatant was collected and loaded onto 10 or 12% agarose gels. Proteins were separated using electrophoresis at 400 mA and 175 V for 1 h. Afterwards, the proteins were transferred to PVDF paper at 450 mA and 175 V for 1.5 h. After transfer, the PVDF membrane was washed with Tween-20 Saline prior to blocking with 5% milk solution for 1 h. After rinsing for 15 min with Tween-20 Saline, membranes were probed with primary antibody specific for the target protein by adding primary antibody in 5 ml of 5% BSA blocking solution and refrigerated with gentle shaking overnight. After washing 15 min with Tween-20 Saline, the signal was amplified by probing with an HRP-bound secondary antibody specific to the primary antibody strain in 10 ml of 5% milk solution for 1 h. Membrane was developed using chemiluminescence (ECL) after rinsing 15 min with Tween-20 Saline prior to adding 2ml of a 1:1 solution of luminal substrate and peroxidase. The membrane and ECL substrate reacted for 5 min prior to developing. HRP reaction levels were analyzed by exposing the membrane paper with radiological film (Denville Scientific) prior to developing using a kodak film processor.

Equal sample amounts were confirmed by a loading control. 25 ml of stripping buffer with ME were added to membranes and heated in a water bath at 50°C for 20 min. Membranes were then washed with Tween-20 Saline until no ME was detectable. Membranes were blocked in 5% milk solution for 1 h prior to probing with G3PDH mouse antibody in 5% BSA for 1 h. The remainder of the protocol was repeated as described above.

CHAPTER III

RESULTS

**Statin therapy reduces cellular cholesterol levels**

When treated with simvastatin (10 nM), fluvastatin (160 nM), or vehicle (control) once a day for 3 days, the endothelial cells produced 12-17% less total cholesterol (free and esterified cholesterol) than the control cells, indicating statin treatment reduced cholesterol in ECs and demonstrating that treatment was successful. (Fig. 4) Fluvastatin was slightly more effective at reducing cholesterol levels than simvastatin.
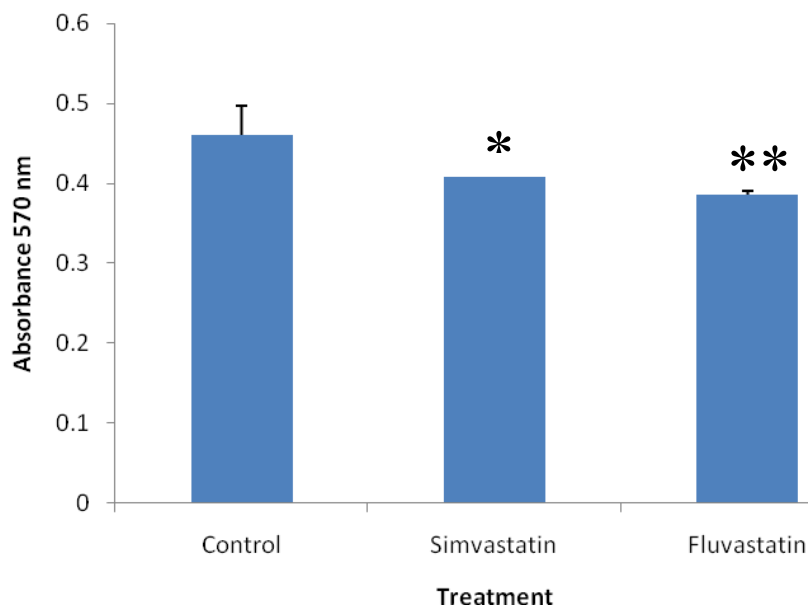
Fig. 4. Quantification of total cholesterol levels following statin treatment. 72 h statin treatment significantly reduced total cholesterol levels in endothelial cells (n=1). The average absorbance indicate cholesterol levels in the control was 2.28 μg/well, while the average cholesterol levels in simvastatin and fluvastatin were 1.95 and 1.79 μg/well, respectively, when extrapolated from a standard curve (not shown). Error bars indicate the standard deviation. P-values determined relative to control. (* indicates a p-value <0.05, ** indicates a p-value<0.02).

Statin treatment inhibits endothelial invasion

In the collagen matrix, statin treatment reduced endothelial invasion by approximately 20-30% (Fig. 5). Treating the cells with statins prior to invasion at lower doses did not differ significantly with treatment at higher doses. Earlier experiments involved pre-treating the cells with statin 24 or 72 h prior to invasion, but the same results were obtained with treating the cells 20 min prior to invasion (not shown).
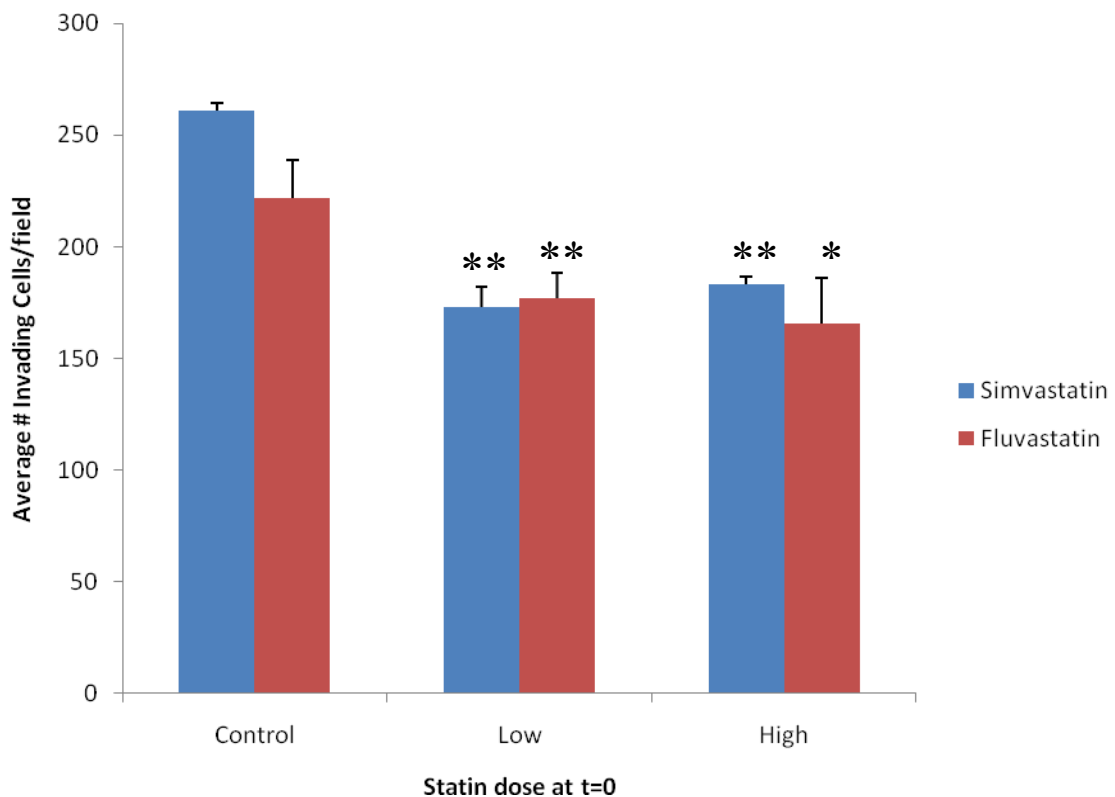
Fig 5. Inhibition of endothelial invasion with statin treatment. Treatment with statin significantly decreased the average number of invading endothelial cells (n=3). A lower dose of simvastatin (1 nM) and fluvastatin (50 nM) yielded the same general result as a higher dose (10 nM and 160 nM). Error bars indicate the standard deviation. P-values determined relative to control. (*indicates a p-value <0.05, ** indicates a p-value <0.02).
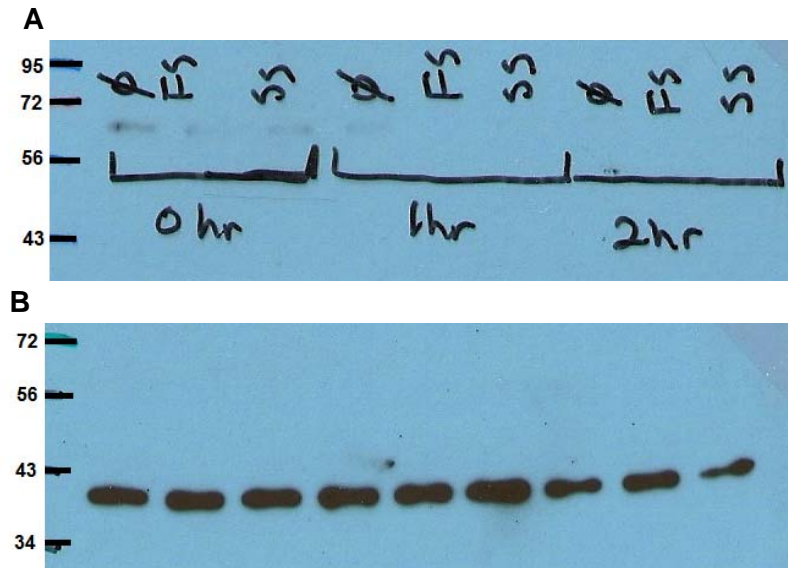
Protein analysis

To explain why statins decrease invasion, we performed western blots with samples taken from invasion assays treated with vehicle (φ), fluvastatin (F), or simvastatin (S).The analysis of protein activation by SDS-PAGE indicated that there was no change in the phosphorylation or synthesis of Erk 1/2 (Fig. 6). Another term for Erk 1/2 are Mitogen-Activated Protein Kinase (MAPK) p42&p44. These numbers correspond to their molecular weight in kDa, which explains two bands on each side of the 43 kDa marker. A loading control was performed to verify the results; it is not known why there is only trace amounts of G3PDH in the 2 h fluvastatin sample.

We also performed experiments to determine whether altered levels of P-Akt and P-Paxillin might explain a decrease in invasion induced by simvastatin and fluvastatin. Conclusive data for Akt (Fig. 7) and Paxillin (Fig. 8) were note obtained. Light bands appeared corresponding to 60 and 68 kDa, respectively. A loading control performed on P-Akt verified that the lanes and extracts did contain cellular proteins and that the detection of protein on the blots was possible, therefore we must conclude that the antibodies used to prove for P-Akt and P-Paxillin bands were not functional in these experiments. P-Akt and P-Paxillin are known and crucial signaling proteins in the invasion process, usually highly expressed and maximized within the first 3 h. (unpublished data, Bayless laboratory)
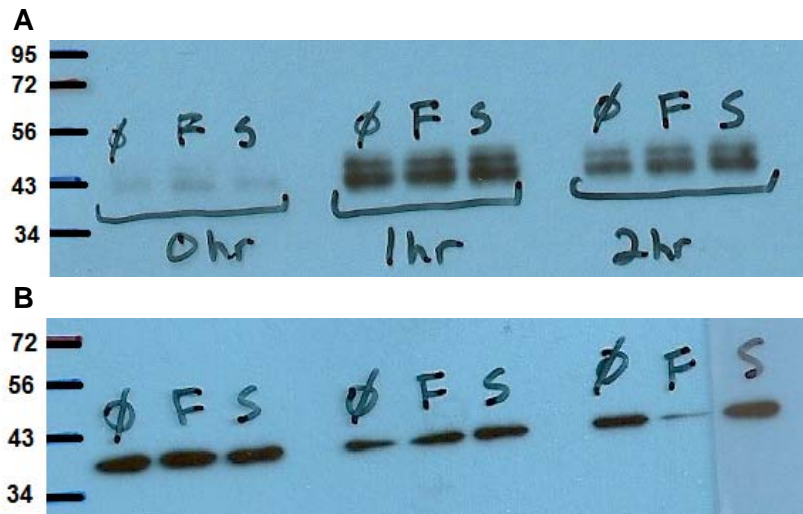
Fig 6. Protein analysis of (A) P-Erk 1/2 & (B) loading control (n=3). φ indicates the control, F indicates fluvastatin therapy, and S indicates simvastatin therapy. Extracts were removed at 0, 1, and 2 hours of invasion. Primary antibody was diluted 1:1000, secondary antibody was diluted 1:5000. MAP-K activation reaches a maximum around 1 h. The two bands correspond to two P-MAPK proteins with molecular weights 42 and 44 kDa.

Fig 7. Protein analysis of P-Akt (A) & loading control (B) (n>3). φ indicates the control, FS indicates fluvastatin therapy, and SS indicates simvastatin therapy. Primary antibody was diluted 1:1000, secondary antibody was diluted 1:5000. Although the bands are light, P-Akt appears highest in the control after 1 h.
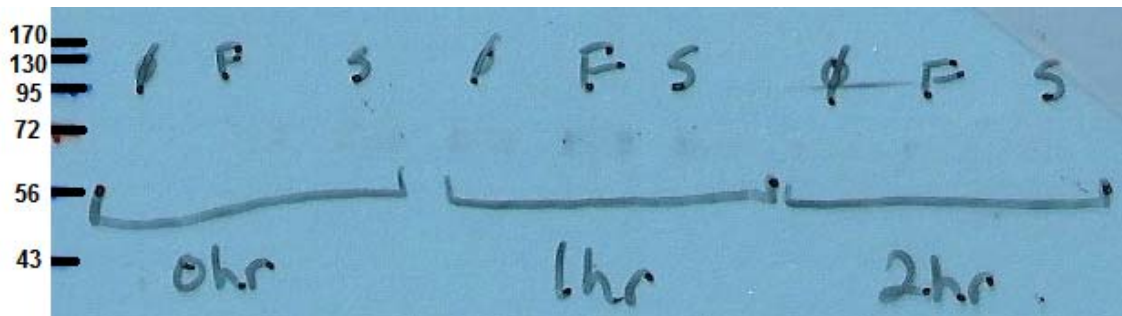
Fig 8. Protein analysis of P-Paxillin (n=1). φ indicates the control, F indicates fluvastatin therapy, and S indicates simvastatin therapy. Primary antibody was diluted 1:2000, secondary antibody was diluted 1:3500. The strongest bands appear at 1 h and there appears to be no noticeable difference in P-Paxillin expression.

CHAPTER IV

SUMMARY AND DISCUSSION

Summary

Statin therapy significantly reduced total cholesterol levels in endothelial cells and inhibited endothelial invasion in a three-dimensional collagen matrix. While convincing data was not obtained for Akt and Paxillin activation, we discerned that these changes are not due to signaling changes in phsphorylated levels of Erk 1/2 during invasion.

Discussion

Three signals can stimulate endothelial invasion: a lipid signal, growth factors, and contact feedback. Erk 1/2 is activated downstream in the signaling cascade from the dimerization of tyrosine kinase receptors VEGFR and FGFR. [12] Paxillin is activated downstream of integrins when cells come into contact with the extracellular matrix. [13-14] Akt is activated downstream of the S1P receptor EDG1. [15]

Integrins are transmembrane proteins with extra- and intra-cellular binding sites connected by a single membrane-spanning domain. [16] The $\alpha2\beta1$ integrin is responsible for connecting the cell cytoskeleton to the membrane to a type I collagen extracellular matrix, and allowing the cell to attach to collagen. [17] They are also mediators of signaling processes in angiogenesis. [18] Tyrosine kinase receptors such as the VEGR and FGFR families are pairs of monomers with one transmembrane domain each. [19]

Binding of the ligand to the monomers causes the two subunits to dimerize, leading to trans-phosphorylation and activating a signaling pathway that phosphorylates Erk 1/2. Excessive Erk 1/2 activation has been implicated in poor cancer prognosis. [20] EDG1 is part of the EDG receptor families, unique in that it has higher affinity for S1P than LPA.[21-22] The EDG1 receptor has seven transmembrane domains. [23-24]

We hypothesize that the lower cholesterol levels due to statin therapy cause less incorporation of cholesterol in the membrane, decreasing membrane fluidity and altering the conformation of receptors embedded in the membrane. We suggest that alterations in the membrane due to lower cholesterol would have the most profound effects on arrangement of the seven EDG1 domains, changing conformation of the receptor and decreasing its affinity for S1P. This would translate to a decrease in Akt phosphorylation and inhibition of endothelial invasion. Further work will be necessary to demonstrate this convincingly.

Other studies have shown that integrins mediate tumor growth [25] and inhibiting angiogenic growth factors retards tumor growth in vivo. [26] Our method provides evidence that cholesterol-lowering statin therapy inhibits endothelial invasion, a crucial step in angiogenesis. The link between angiogenesis and tumor growth may help explain the cause of secondary protective effects witnessed with statin therapy.

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CONTACT INFORMATION

Name:	Evan Michael Cherry
Professional Address:	c/o Dr. Kayla Bayless Department of Molecular & Cellular Medicine Reynolds Building Rm 442 Texas A&M University College Station, TX 77843-1114
Email Address:	evianc1@neo.tamu.edu
Education:	B.S., Chemical Engineering, Texas A&M University, May 2010 Summa Cum Laude Undergraduate Research Scholar Phi Kappa Phi Tau Beta Pi