SPHINGOSINE-1-PHOSPHATE INHIBITS VASCULAR SMOOTH MUSCLE CELL MIGRATION BY ACTIVATING $G_{\alpha 12}$ AND RhoA

A Senior Honors Thesis

by

NICHOLAS J. ANTHIS

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2004

Major: Biochemistry

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Approved as to style and content by:

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George E. Davis	Edward A. Funkhouser
(Fellows Advisor)	(Executive Director)

April 2004

Major: Biochemistry

ABSTRACT

Sphingosine-1-Phosphate Inhibits Vascular Smooth Muscle

Cell Migration by Activating $G_{\alpha 12}$ and RhoA. (April 2004)

Nicholas J. Anthis
Department of Biochemistry
Texas A&M University

Fellows Advisor: Dr. George E. Davis
Department of Pathology and Laboratory Medicine

Angiogenesis, the formation of new blood vessels, is important in growth, healing, and disease. Sphingosine-1-phosphate (S1P) is a lysophospholipid with potent angiogenic effects. One of these effects is its regulation of cellular migration, an early step in angiogenesis. S1P exerts contradictory effects on different types of vascular cells, strongly inducing endothelial cell (EC) migration while strongly inhibiting vascular smooth muscle cells (VSMC) migration. Here, we demonstrate that S1P inhibits the migration of VSMCs by activating $G_{\alpha 12}$ and RhoA. S1P abolished the migration of VSMCs in response to platelet-derived growth factor (PDGF), a potent stimulator of VSMC migration. PDGF strongly induced the formation of lamellipodia, which are membrane ruffles associated with cell migration. S1P eliminated these and induced the formation of stress fibers, which are actin cytoskeletal structures that stabilize the cell. The members of the Rho family of small GTPases regulate cytoskeletal morphology and function. Rac1 induces lamellipodia, RhoA induces stress fibers, and Cdc42 induces filopodia, which are small finger-like membrane spikes. We

introduced dominant-negative (DN) and constitutively active (CA) mutants of the Rho GTPases into VSMCs using adenoviral vectors. The results indicated that S1P inhibits VSMC migration by activating RhoA. RhoA activation was also demonstrated directly using a RhoA pull-down assay. S1P exerts its cellular effects by activating the endothelial differentiation gene G-protein-coupled receptors, which initiate signaling pathways by activating different G_{α} subunits of heterotrimeric G proteins. To determine which G_{α} subunit S1P activates in order to activate RhoA, we introduced DN and CA mutants of $G_{\alpha 12}$ and $G_{\alpha 13}$ into VSMCs through adenoviral vectors. The results indicated that S1P activates RhoA by activating $G_{\alpha 12}$. These results are important because they further elucidate some of the key signaling pathways regulating angiogenesis. When angiogenesis begins, ECs migrate in response to S1P and other factors. Interestingly, our new data suggest that S1P may prevent VSMCs from interfering with early EC tube assembly, by inhibiting VSMC migration through selective activation of $G_{\alpha 12}$ and RhoA. Later, after the ECs have established a network of capillary tubes, they secrete additional factors, such as PDGF, which attract VSMCs to them, allowing mature blood vessels to form.

ACKNOWLEDGMENTS

I would like to acknowledge my advisor, Dr. George E. Davis, for his guidance on this project. I would like to acknowledge Dr. Kayla J. Bayless for her contributions to this work, including constructing adenoviruses carrying mutants of the Rho GTPases and providing helpful insight. I would like to acknowledge Dr. W. Brian Saunders for his assistance as well.

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INTRODUCTION

Angiogenesis, the formation of new blood vessels, is necessary for growth, development, and healing. Abnormal angiogenesis, though, is also implicated in many pathological conditions. In diabetic retinopathy, uncontrolled angiogenesis occurs in the eye, creating leaky vessels that lead to blindness[1;2]. Angiogenesis is also implicated in atherosclerosis, obesity, psoriasis, as well as ischemic heart and limb disease. Most notably, tumors stimulate angiogenesis, which is necessary for their growth[3]. Although angiogenesis is a highly studied topic, the basic molecular mechanisms controlling this process are still not well understood. Before abnormal angiogenic conditions can be effectively addressed, a clear understanding of factors that both stimulate and inhibit angiogenesis is necessary. Sphingosine-1-Phosphate (S1P) has recently emerged as a potent regulator of angiogenesis[4-10]. A detailed understanding of the signaling pathways it initiates will lead to a much greater understanding of angiogenesis and may offer several targets for controlling pathological angiogenesis.

The basic cellular stages of angiogenesis have been previously determined[11-15]. Blood vessels, regardless of type or size, all have a similar structure. The inner layer consists of endothelial cells, surrounded on the outside by a basement membrane. A layer of smooth muscle cells lies on the outside of this basement membrane. In larger vessels, this layer of smooth muscle cells is surrounded by an additional layer of

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This thesis follows the style and format of *Circulation Research*.

connective tissue. Angiogenesis begins when, in response to a stimulus, a portion of the vessel wall breaks down, and endothelial cells migrate away from it. These endothelial cells undergo morphogenesis to form capillary tubes. Smooth muscle cells then migrate to the new vessels, allowing them to stabilize and mature.

Many molecules have been characterized that are involved in angiogenesis. One of these, which is used in this study is platelet-derived growth factor (PDGF). PDGF is a well-characterized protein growth factor, which has a strong chemotactic (i.e. attractive) effect on smooth muscle cells[16;17]. Interestingly, endothelial cells also produce PDGF[18-20]. In fact, the production of PDGF is upregulated as ECs undergo morphogenesis into capillary tubes[21], indicating that it is a key molecule in the later stages of angiogenesis, when endothelial and smooth muscle cells communicate to achieve vessel maturation. The lipid growth factor S1P has emerged more recently as an angiogenic molecule. Its angiogenic effects have been well documented, and the molecular means by which it exerts these effects are being elucidated[4;5;7;9]. S1P stimulates endothelial cell migration[5;7] and induces their morphogenesis into capillary tube networks[6;10]. In contrast, S1P also inhibits vascular smooth muscle cell migration[8], emphasizing the importance of S1P in the earlier steps of angiogenesis, where S1P allows endothelial cells to migrate and develop while preventing smooth muscle cells from interfering with this process. There is still much to learn about the detailed molecular signaling pathways that S1P initiates in cells, especially in how they differ between cell types.

S1P, a lysosphingolipid released primarily by activated platelets[22;23], acts on cells through the G-protein-coupled endothelial differentiation gene (Edg) receptors, most importantly Edg1, Edg3, and Edg5[4]. The varied effects of S1P on different cell types appear to depend on the specific receptors present. Endothelial cells, which express high levels of the receptor Edg1, are stimulated to migrate and to invade tissues in the presence of S1P. The movement of smooth muscle cells, which express high levels of Edg5 and almost no Edg1, is almost completely inhibited by S1P[4]. The roles of the various Edg receptors are well established, but only a few details are known about the signaling pathways they initiate. Our goal is to further elucidate the molecular means by which S1P inhibits smooth muscle cell migration.

The Rho GTPases—which include Rac1, Cdc42, and RhoA—maintain a central role in cytoskeletal function, which is involved in cell migration[24-26]. Rac1 induces the formation of lamellipodia, which are actin cytoskeletal structures associated with cell migration. Cdc42 induces the formation of filopodia, which are finger-like membrane spikes. RhoA induces the formation of stress fibers, stress fibers, which are actin cytoskeletal structures that stabilize the cell. These proteins cycle between an active, GTP-bound, state and an inactive, GDP-bound state[24-26]. Another research group has already connected Edg5 activation with subsequent Rac1 inactivation to that may lead to inhibition of smooth muscle cell migration by S1P[27]. Although it is likely that Rac1 inactivation may play a role in S1P-mediated inhibition of migration, evidence from our laboratory provides new evidence that RhoA activation may play a more central, causative role.

When a ligand, such as S1P, activates a G-protein-coupled receptor, the receptor activates a heterotrimeric G-protein. Heterotrimeric G-proteins have three subunits, with the G_α subunit causing most of the effects of interest[28]. The G_α subunit cycles between an active, GTP-bound, state and an inactive, GDP-bound, state. There are a few different families of G_α, but the newest family, $G_{\alpha 12/13}$, is most relevant here. The $G_{\alpha 12/13}$ proteins have been previously shown to activate RhoA [29-32]. Although the two members of this family, $G_{\alpha 12}$ and $G_{\alpha 13}$, are often discussed together as performing similar functions, we have evidence that they can exert very different effects, especially in pathways related to S1P. Much more research is necessary on these molecules and the other molecules that couple the Edg receptors to the Rho GTPases.

My goal is to elucidate the molecular signaling pathways that lead to inhibition of smooth muscle cell migration by S1P. Gaining a better understanding of this one stage of angiogenesis will lead to a more complete picture of angiogenesis. This is important, because angiogenesis is relevant to a variety of pathogenic conditions, including cancer, and a better molecular understanding of this process could lead to future drug treatments for these disorders.

MATERIALS AND METHODS

Cell Culture

Human coronary artery smooth muscle cells (Clonetics, San Diego, CA) were grown in culture in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, Rockville, MD) with 10% fetal bovine serum (Invitrogen Life Technologies). Human umbilical vein endothelial cells (Clonetics) were grown as previously described[33]. Cells were cultured at 37^{o}C in a 5% CO_2 atmosphere. Cells from passages 3 to 8 were used in experiments. In preparation for an experiment, cells were removed with trypsin-EDTA, neutralized with fetal bovine serum, centrifuged for 5 minutes at 350 g, and resuspended in DMEM.

Preparation and Propagation of Recombinant Adenoviruses

Recombinant adenoviruses were constructed to express either dominant-negative (DN) and constitutively active (CA) forms of RhoA, Rac1, Cdc42, $G_{\alpha12}$, and $G_{\alpha13}$ using the system previously described by Vogelstein and colleagues[34]. Construction of the DN Rho GTPase viruses (N17Rac1, N17Cdc42, and N19RhoA) and the CA Rho GTPase viruses (V12Rac1, V12Cdc42, and V14RhoA) has been described previously[35]. Full-length cDNA clones of dominant negative G_{α} subunits ($G_{\alpha12}$Q231L/D299N and $G_{\alpha13}$Q226L/D294N) and constitutively active G_{α} subunits ($G_{\alpha12}$Q231L and $G_{\alpha13}$Q226L) were obtained from Guthrie cDNA Resource Center (Sayre, PA), and prepared as the DN and CA Rho GTPase mutants[35], using different primers and restriction endonucleases. The following clones were amplified using the respective primer sets:

$G_{\alpha 12}$Q231L/D299N and $G_{\alpha 12}$Q231L: GAGATCTGCCACCATGTCCGGGGTGGTGCGGACC and AGTCTAGATCACTGCAGCATGATGTCCTTCAG; $G_{\alpha 13}$Q226L/D294N and $G_{\alpha 13}$Q226L: AGAGATCTGCCACCATGGCGGACTTCCTGCCGTCG and AGTCTAGATCACTGTAGCATAAGCTGCTTGAG (Genosys, The Woodlands, TX). Restriction digests of the PCR products of the mutant $G_{\alpha 12}$ and $G_{\alpha 13}$ genes as well as the pAdTrack-CMV vector were carried out with *Bgl*II and *Xba*I restriction endonucleases (Invitrogen Life Technologies) for 3 hours at 37°C. Digested vector and insert were purified, quantitated and ligated at an insert to vector ratio of 4.5:1 overnight at 14°C. Positive clones were confirmed by restriction digest and colony PCR. Recombination and virus production were performed as previously described[35]. When these viruses were used in experiments, cells were infected for six hours in serum-free conditions, and then infection was continued overnight.

Measuring Cell Migration

Cell migration assays were performed in a 48-well modified Boyden chamber (Neuroprobe, Gaithersburg, MD). Either S1P (Avanti Polar Lipids, Alabaster, AL), PDGF (Sigma-Aldrich), or neither was added to the lower wells, and cells at $1x10^6$ cells/mL were added to the upper wells. A polycarbonate membrane with 8 μm diameter pores (Neuroprobe), coated with 1 mg/mL gelatin and blocked with fatty acid-free 1 mg/mL BSA (Sigma-Aldrich) separated the wells. Experiments were performed in DMEM with 0.4% reduced serum II and 10 μg/mL fibronectin (Invitrogen Life

Technologies). Cells were allowed to migrate for four hours at 37°C in a 5% CO_2 atmosphere. The membrane was then removed, fixed in glutaraldehyde and stained with amido black (30% methanol, 10% acetic acid, 0.1% naphthol blue black). The cells on the upper side were then removed, leaving only the cells on the lower side, which had migrated through the membrane. The membrane was scanned with an HP ScanJet 5370C Scanner and then quantitated with Scion Imager. The average darkness of each well was measured, and the number of cells were quantitated using the equation:

$$\text{Migrated cells} = 1.1987\ (\text{darkness})^2 + 69.096\ (\text{darkness})$$

This equation had an R^2 value of 0.988 (data not shown).

Examining Cytoskeletal Morphology

In order to examine the cytoskeletal morphology of VSMCs, cells on glass coverslips were stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Cells were grown to 70% confluency, then fixed with 3% paraformaldehyde for 30 minutes. Cell membranes were permeabilized with Tx-100 and then stained with 5 units/mL rhodamine-phalloidin for one hour. Cells were washed with PBS then mounted on slides and visualized using a Nikon fluorescent microscope.

Measuring Activated RhoA

RhoA activation was measured using a RhoA pull-down assay based on a previously described assay[36]. Before some experiments, cells were infected with virus overnight. Serum-free media was added to VSMCs, and then they were either left untreated or treated with 1 μM S1P and incubated for various times at 37°C. Cells were

washed with TBS, placed in a lysis buffer (Upstate, Lake Placid, NY), and scraped off. Cell lysates were centrifuged at 14,000 g for 5 minutes. The supernatant was added to 70 μL of Rhotekin Rho Binding Domain-bound agarose beads (Upstate) and mixed for 45 minutes. Agarose beads were then centrifuged for 5 seconds at 10,000 g and washed with lysis buffer three times. After a fourth centrifugation, the pellet was resuspended in 80 μL of binding buffer containing DTT. These samples were then boiled for 10 minutes.

The results of the RhoA activation assay were visualized on a western blot. Activated GTP-RhoA samples were prepared as above. The total RhoA samples were prepared by taking part of the original supernatant and boiling it with binding buffer and β-mercapto ethanol. Samples were added to an SDS-PAGE gel, electophoresed, and transferred to a PVDF membrane. The membrane was then stained with mouse anti-RhoA (Cytoskeleton, Denver, CO) and then horseradish peroxidase-conjugated rabbit anti mouse (Dako Cytomation, Carpinteria, CA). Detection reagents (Amersham Resources, Piscataway, NJ) for chemiluminescence were added, and the membrane was visualized using film (Kodak).

RESULTS

S1P Has Contradictory Effects on Vascular Cell Migration

The effects of S1P on different vascular cell lines were assessed using Boyden chamber migration assays. ECs or VSMCs were allowed to migrate for four hours towards S1P from 1 nM to 1000 nM in half-log doses. The results of a representative migration assay are shown in Figure 1A. S1P strongly induced the migration of ECs. This effect was still increasing at the high dose of 1 μM.

In contrast, S1P strongly inhibited the migration of VSMCs, completely abolishing their background migration. In the experiment shown, S1P inhibited VSMC migration even with the low dose of 10 nM, suggesting that the inhibitory effect is very potent (Figure 1A). In some experiments, effects were observed at doses as low as 1 nM. Because S1P exerted such opposing effects on different vascular cells, we performed further experiments to determine the cause of these effects.

S1P Inhibits PDGF-Induced Migration of VSMCs

The effect of PDGF on VSMCs was also assessed using migration assays. VSMCs were allowed to migrate for four hours towards PDGF from 1 ng/mL to 1000 ng/mL in half-log doses (Figure 1B). PDGF strongly induced the migration of VSMCs. This effect disappeared at high concentrations, though, when the VSMCs became saturated. Therefore, this is a chemotactic response, dependent on the direction of a concentration gradient.

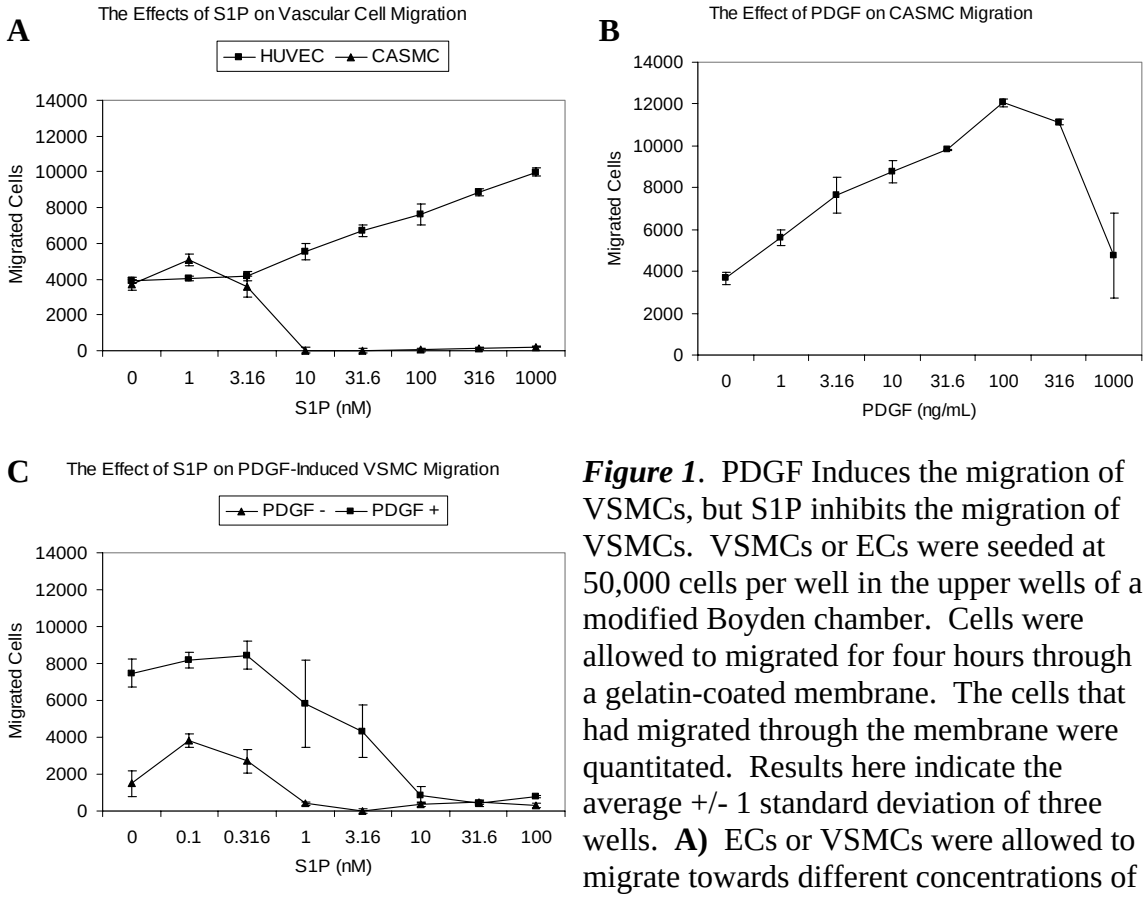

Figure 1. PDGF Induces the migration of VSMCs, but S1P inhibits the migration of VSMCs. VSMCs or ECs were seeded at 50,000 cells per well in the upper wells of a modified Boyden chamber. Cells were allowed to migrated for four hours through a gelatin-coated membrane. The cells that had migrated through the membrane were quantitated. Results here indicate the average +/- 1 standard deviation of three wells. **A)** ECs or VSMCs were allowed to migrate towards different concentrations of S1P. S1P had opposite effects on the two cell types, strongly inducing EC migration but strongly inhibiting VSMC migration. **B)** VSMCs were allowed to migrate towards various concentrations of PDGF. PDGF strongly induced the migration of VSMCs. **C)** VSMCs were allowed to migrate toward the indicated concentrations of S1P, with or with out additional 32 ng/mL PDGF. S1P eliminated the increase in migration of VSMCs induced by PDGF.

The ability of S1P to affect the PDGF-induced migration of VSMCs was also determined. VSMCs were allowed to migrate for 4 hours towards S1P from 1 nM to 1000 nM in half-log doses, in the presence or absence of 32 ng/mL PDGF (Figure 1C). PDGF strongly induced the migration of VSMCs. At a dose as low as 10 nM, though, S1P had inhibited all migration, indicating that the inhibitory effect of S1P was dominant over the chemotactic effect PDGF.

A **B**

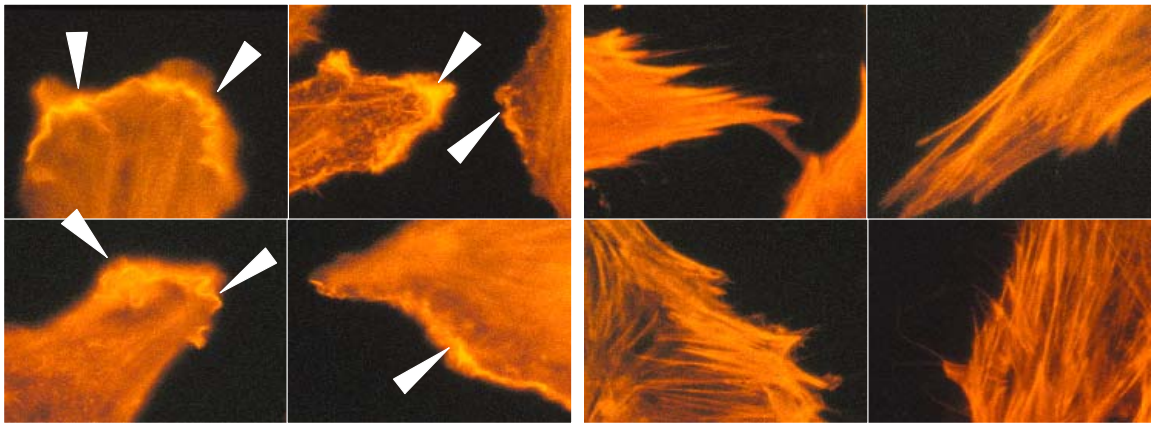

Figure 2. S1P eliminates lamellipodia and induces stress fibers in VSMCs. VSMCs on glass coverslips were treated for 30 minutes with 40 ng/mL PDGF with or without 1 μM S1P. Cells were then stained for cytoskeletal actin, and visualized under fluorescence microscopy **A)** VSMCs were exposed to PDGF. Arrows indicate lamellipodia, which were induced by PDGF. **B)** CASMCs were exposed to PDGF and S1P. No lamellipodia were observed, but prominent stress fibers were observed.

To further understand the effects of S1P on cell migration, the effects of S1P on cytoskeletal morphology were assessed. Changes in cytoskeletal morphology, including the formation of lamellipodia and the disappearance of stress fibers, accompany cell migration[25;37]. This was done by staining cells with rhodamine-phalloidin. Phalloidin binds to cytoskeletal filamentous actin (F-actin), and rhodamine fluoresces, allowing one to visualize the actin cytoskeleton. VSMCs were treated for thirty minutes with 40 ng/mL PDGF in the presence or absence of 1 μM S1P. Cells treated with PDGF displayed prominent lamellipodia (Figure 2A). PDGF increased the number of lamellipodia, although many lamellipodia were observed in untreated cells (data not shown). Cells treated with PDGF and S1P displayed no lamellipodia, but displayed

prominent stress fibers (Figure 2B). S1P eliminated lamellipodia, pro-migratory structures, and induced stress fibers, stabilizing structures, correlating with its effects on cell migration.

Inhibition of VSMC Migration by S1P Requires RhoA

Since the effects of S1P on cytoskeletal morphology correlated with its effects on cell migration, and since the Rho GTPases play a central role in regulating cytoskeletal morphology[24-26], the role of the Rho GTPases in the inhibition of VSMC migration was studied. The role of the Rho GTPases in inhibition of VSMC migration by S1P was assessed first by using dominant-negative (DN) mutants. In order to perform these experiments, recombinant adenoviruses carrying the gene for either a dominant-negative or constitutively active Rho GTPase were utilized. The preparation and characterization of these adenoviruses were previously described by Bayless and Davis[35]. After VSMCs were infected overnight, cells were then allowed to migrate towards 32 ng/mL PDGF, 100 nM S1P, both, or neither for four hours (Figure 3A). The control cells, infected with control adenoviruses carrying only green fluorescent protein (GFP), migrated in response to PDGF, but S1P eliminated this migration. The cells infected with DN Rac1 or DN Cdc42 behaved the same as the control cells. The cells infected with DN RhoA, though, migrated differently. They had little response to S1P. Although S1P had a slight inhibitory effect in the cells shown (Figure 3A), this experiment was repeated multiple times, and the inhibitory effect ranged from non-existent to the slight effect shown here. These results demonstrate that RhoA activation is necessary for the inhibition of VSMC migration by S1P.

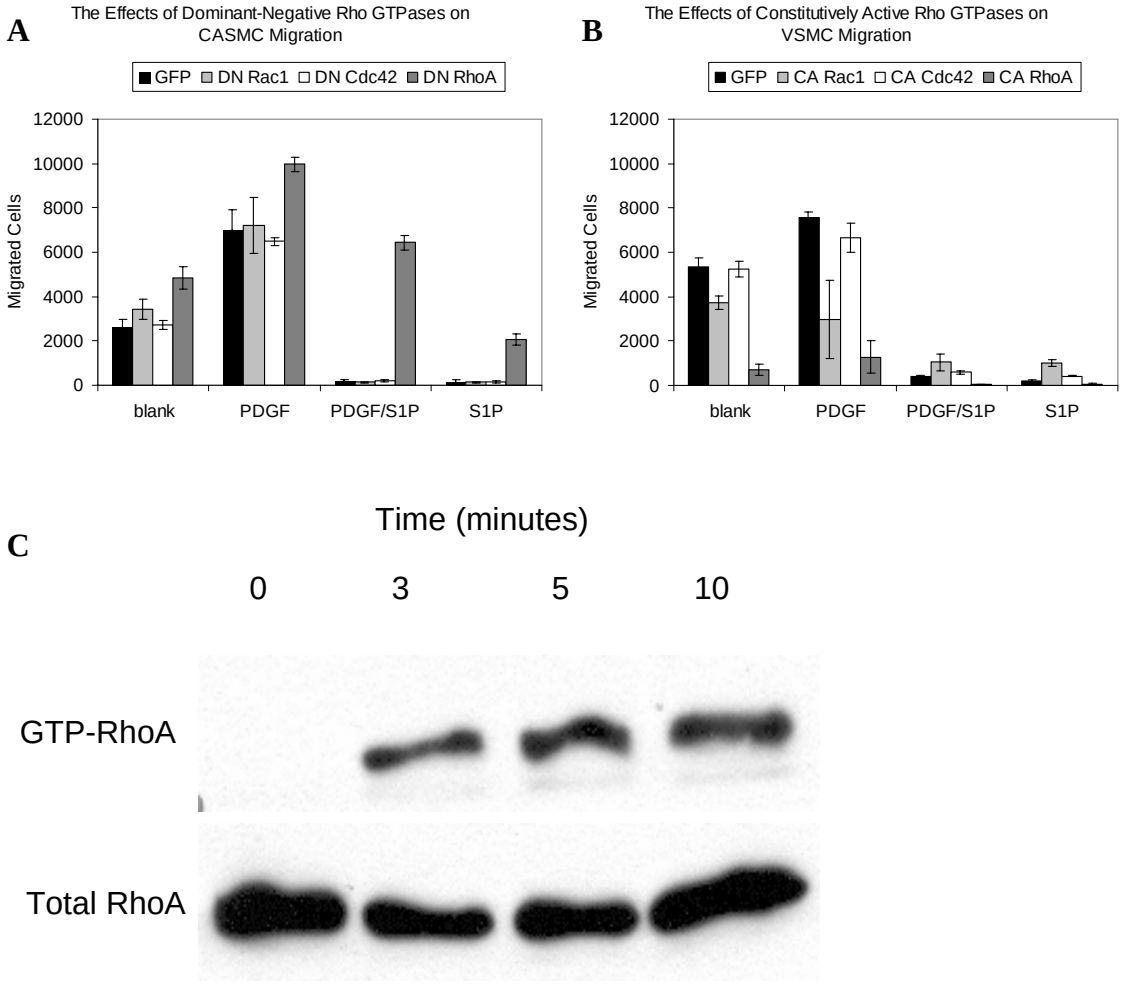

Figure 3. RhoA activation is required for the inhibition of VSMC migration by S1P. **A)** VSMCs were infected overnight with adenoviruses carrying either a dominant-negative (DN) mutant of one of the Rho GTPases or green fluorescent protein (GFP) as a control. VSMCs were then allowed to migrate towards 31.6 ng/mL PDGF, 100 nM S1P, both, or neither. DN RhoA prevented S1P from blocking migration. **B)** VSMCs were infected overnight with adenoviruses carrying either a constitutively active (CA) mutant of one of the Rho GTPases or GFP as a control. Cells infected with CA RhoA had greatly reduced migration overall. **C)** VSMCs were stimulated with S1P for the indicated amounts of time. Cells were lysed, and activated RhoA (GTP-RhoA) was collected with agarose beads bound to Rhotekin Rho Binding Domain. GTP-RhoA was then separated from the beads and observed using a western blot. A small amount of the cell lysate was kept and also observed on a western blot to demonstrate the level of total RhoA. Total RhoA remained fairly constant. There was almost no RhoA activation without exposure to S1P. After exposure to S1P, RhoA was quickly activated.

The role of the Rho GTPases in inhibition of VSMC migration by S1P was also examined using constitutively active (CA) mutants[35]. After VSMCs were infected overnight, cells were then allowed to migrate towards PDGF, S1P, both, or neither for four hours (Figure 3B). The cells infected with CA Cdc42 behaved the same as the control cells. The cells infected with CA RhoA had almost no migration, regardless of the presence of PDGF or S1P. The cells infected with CA Rac1 did not migrate as well as the control, with or without PDGF, but they were also not inhibited as strongly by S1P. These results demonstrate that RhoA activation inhibits VSMC cell migration.

RhoA activation in response to S1P was also demonstrated directly using a RhoA pull-down assay. VSMCs were activated with S1P for the indicated times (Figure 3C) or not at all. The active GTP-bound RhoA was collected and visualized on a western blot, along with the total RhoA. Untreated cells showed almost no activated RhoA. However, immediately after treatment with S1P, RhoA was activated, and this activation was sustained for at least 10 minutes. These results demonstrate that S1P strongly activates RhoA in VSMCs. This data coupled, with our previous results showing the direct involvement of RhoA in S1P-induced inhibition of migration, shows that RhoA appears to be necessary for the inhibition of migration by S1P.

Inhibition of VSMC Migration by S1P Requires $G_{\alpha 12}$

To determine which G-protein couples the Edg receptors to RhoA to regulate S1P-induced inhibition of VSMC migration, we studied the role of the $G_{\alpha 12/13}$ family of G-proteins, which have been shown to signal to RhoA[29-32]. The role of the $G_{\alpha 12/13}$ family

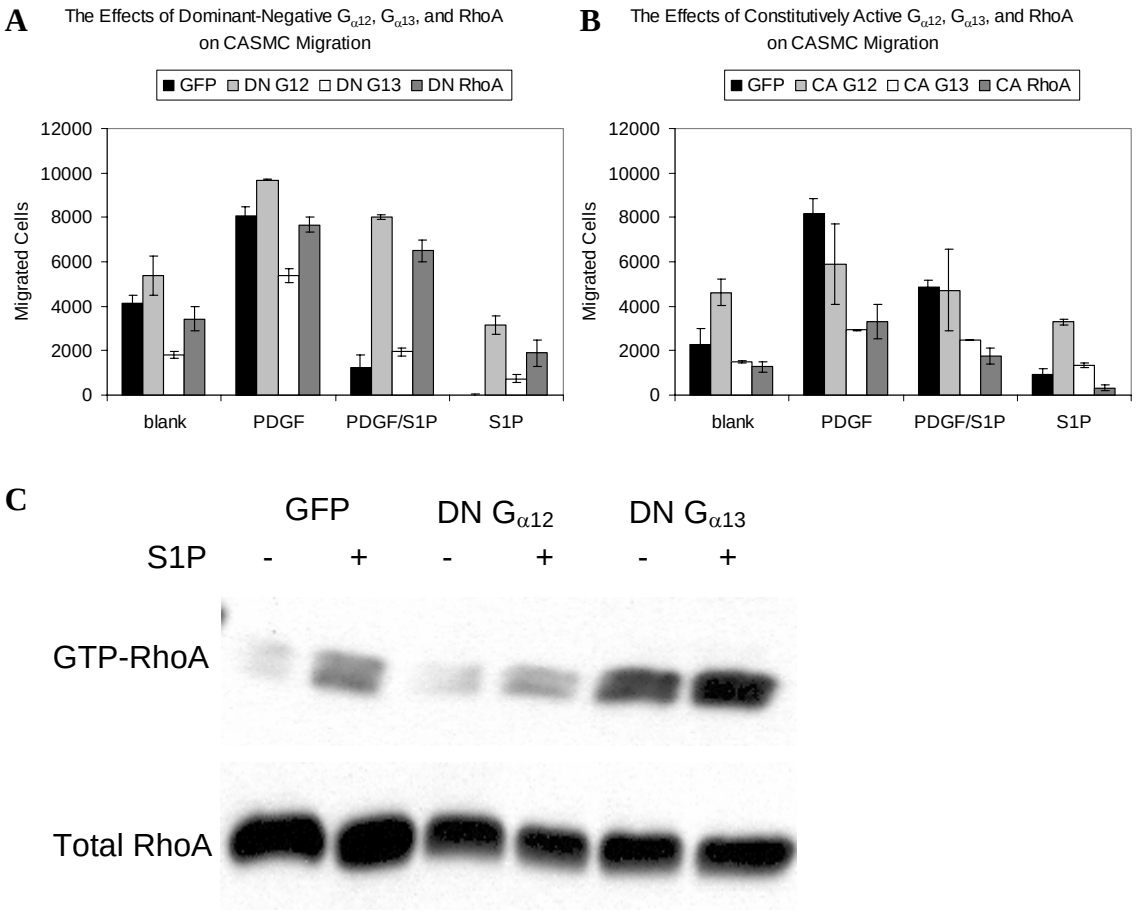

Figure 4. $G_{\alpha 12}$ activation is required for the inhibition of VSMC migration by S1P. **A)** VSMCs were infected overnight with adenoviruses carrying DN $G_{\alpha 12}$, DN $G_{\alpha 13}$, DN RhoA, or GFP. VSMCs were then allowed to migrate towards 32 ng/mL PDGF, 100 nM S1P, both, or neither. DN $G_{\alpha 12}$ had the same effect as DN RhoA, preventing S1P from blocking migration. **B)** VSMCs were infected overnight with adenoviruses carrying CA $G_{\alpha 12}$, CA $G_{\alpha 13}$, CA RhoA, or GFP. The same migration assay was performed as above. CA $G_{\alpha 13}$ had the same effect as CA RhoA, greatly reducing migration overall. CA $G_{\alpha 12}$ prevented migration from being induced or inhibited. **C)** After overnight infection with viruses carrying GFP, DN $G_{\alpha 12}$, or DN $G_{\alpha 13}$, VSMCs were stimulated with S1P for the indicated amounts of time. Cells were lysed, and activated RhoA (GTP-RhoA) was collected with agarose beads bound to Rhotekin Rho Binding Domain. GTP-RhoA was then separated from the beads and observed using a western blot. A small amount of the cell lysate was kept and also observed on a western blot to demonstrate the level of total RhoA. There was decreased RhoA activation in response to S1P in cells expressing DN $G_{\alpha 12}$. Cells infected with DN $G_{\alpha 13}$ displayed greatly increased RhoA activation, with or without S1P.

G-proteins in this effect was investigated first using DN mutants. These mutants were inserted into adenoviral vectors, then introduced to VSMCs. After VSMCs were infected overnight, cells were allowed to migrate towards PDGF, S1P, both, or neither for four hours (Figure 4A). Cells infected with DN $G_{\alpha 12}$ or DN RhoA were not inhibited as strongly by S1P. The cells infected with DN $G_{\alpha 13}$ had lower background migration. S1P did not inhibit their migration as much as that of the control, but S1P did inhibit their migration much more than the migration of cells infected with DN $G_{\alpha 12}$ or DN RhoA. These results indicate that activation of $G_{\alpha 12}$ appears to be necessary for the inhibition of VSMC migration by S1P.

The role of the $G_{\alpha 12/13}$ family G-proteins in inhibition of VSMC migration by S1P was also examined using CA mutants. After VSMCs were infected overnight with viruses carrying these mutants, cells were then allowed to migrate towards PDGF, S1P, both, or neither for four hours (Figure 4B). Cells infected with CA $G_{\alpha 13}$ or CA RhoA had low migration, regardless of the stimulus. The cells infected with CA $G_{\alpha 12}$ migrated well, but did not respond positively or negatively to any stimulus. These results indicate that both $G_{\alpha 12}$ and $G_{\alpha 13}$ can contribute to activation of RhoA in VSMCs and regulate their migratory responses.

The role of the $G_{\alpha 12/13}$ family G-proteins in activating RhoA in response to S1P was also examined using a RhoA pull-down assay. VSMCs were infected overnight with viruses containing GFP, DN $G_{\alpha 12}$, or DN $G_{\alpha 13}$. They were then either left untreated or activated with S1P for five minutes. The active GTP-bound RhoA was collected and

visualized on a western blot, along with the total RhoA. The two mutants had significant effects on RhoA activation in response to S1P (Figure 4C). In cells infected with the GFP control, there was very low RhoA activation before S1P treatment, but high activation after treatment. In cells infected with DN $G_{\alpha 12}$, the increase in RhoA activation due to S1P was much lower. In cells infected with DN $G_{\alpha 13}$, though, there was very strong RhoA activation observed before or after activation with S1P. These results indicate that $G_{\alpha 12}$ activation appears to be directly involved in the activation of RhoA by S1P in VSMCs.

DISCUSSION AND CONCLUSIONS

S1P is a Potent Regulator of Angiogenesis

The results of this study support the concept that S1P is a potent regulator of angiogenesis. Here we demonstrate that S1P strongly induces the migration of ECs while inhibiting the migration of VSMCs (Figure 1A). This is in contrast to the strong induction of VSMC migration caused by PDGF (Figure 1B), another important regulator of angiogenesis. From these results, a simple model of angiogenesis can be constructed (figure 5).

S1P is most important in the earlier stages of angiogenesis. In the first stage of angiogenesis (figure 5B), the outer layer of an existing vessel break down, and ECs migrate away from the vessel. Due to its pro-migratory effects on ECs (figure 1A), S1P is probably involved in this stage. S1P would be especially relevant in a wound environment, where the S1P concentrations would be very high, since S1P is released from activated platelets[22;23]. As S1P is inducing the migration of ECs, it is inhibiting the migration of VSMCs. Even though PDGF, a molecule that induces the migration of VSMCs (Figure 1B), would also be present in this environment, our results indicated that the inhibitory effect of S1P overrides the pro-migratory effect of PDGF (figure 1C). This would presumably prevent VSMCs from interfering with the formation of capillary tubes by the ECs. S1P has also been demonstrated to be involved in the morphogenesis of ECs into capillary tubes[6;10]. Once these tube networks are established, VSMCs can migrate towards them, now without interfering with their morphogenesis (Figure 5C). The ECs secrete factors to draw VSMCs towards them, including PDGF[18-20], which

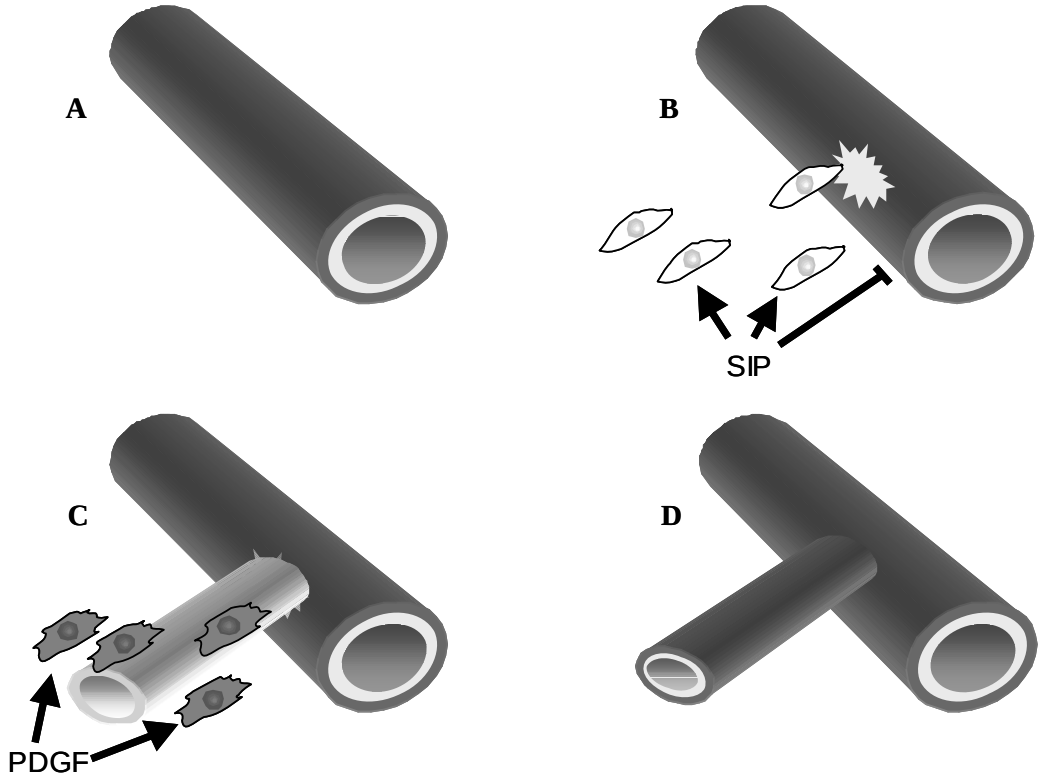

Figure 5. Model of angiogenesis based on the differential influence of PDGF and S1P on the migration of VSMCs and ECs. **A)** A short section of a blood vessel is shown here. The inner area corresponds to the vessel lumen, the light middle layer corresponds to ECs, and the dark outer layer corresponds to VSMCs. **B)** In response to S1P and/or other molecules, ECs break away from an established vessel and migrate toward the chemical stimulus. S1P also induces the morphogenesis of the ECs into a capillary tube. **C)** Once the capillary tube is established, ECs induce VSMCs to migrate toward the immature vessel by secreting PDGF. **D)** VSMCs are incorporated into the vessel structure, stabilizing it and allowing it to mature.

stimulates the directional migration of VSMCs (Figure 1B). PDGF is particularly relevant here because the expression of PDGF by ECs is upregulated during morphogenesis[21], so ECs will produce larger amounts of PDGF when they form capillary tubes. The VSMCs will form a stabilizing layer around the EC tubes (Figure 5D), allowing the new blood vessel to mature.

S1P Inhibits VSMC Migration by Activating $G_{\alpha 12}$ and RhoA

Because S1P has such drastic and opposing effects on different vascular cell types, it is important to understand the molecular basis for them. To determine which molecules play key roles in the inhibition of VSMC migration by S1P, we looked at cytoskeletal morphology in response to S1P and then molecular regulators in this pathway. Our results indicate that S1P inhibits VSMC migration by activating $G_{\alpha 12}$ and RhoA (Figure 6).

After our initial migration assays, we observed VSMC cytoskeletal morphology in order to gain more insight into how S1P inhibits VSMC migration. We found that S1P eliminates PDGF-induced lamellipodia and induces the formation of stabilizing stress fibers. These results correlated with our observations of cell migration, since lamellipodia are structures involved in migration and stress fibers are structures involved in cell stabilization[25;37]. From these results, it was clear that the Rho family of small GTPases may be involved in the inhibition of VSMC migration by S1P, since they are involved in regulating cytoskeleton morphology[24-26]. Also, RhoA is known to be primarily responsible for stress fiber formation[24-26], and our results clearly demonstrate its role in S1P-induced inhibition of VSMC migration.

In order to determine which Rho GTPases are involved in inhibition of VSMC migration, we infected VSMCs with viruses carrying a gene for a DN or CA mutant of Rac1, Cdc42, or RhoA. The dominant-negative mutants interfere and compete with

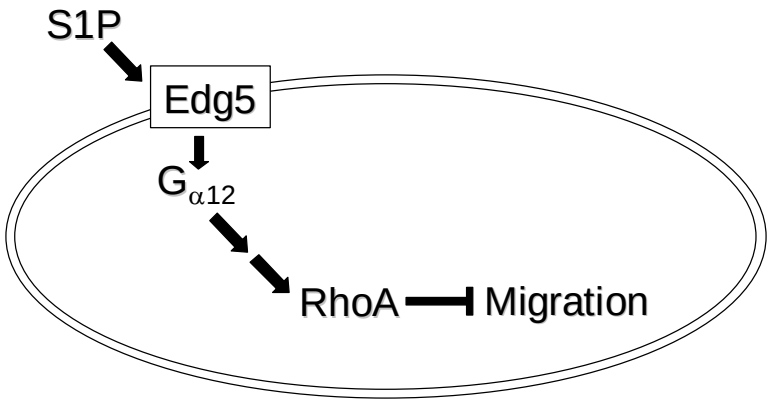

Figure 6. S1P inhibits the migration of VSMCs by activating $G_{\alpha 12}$ and RhoA. S1P activates Edg5 on the VSMC surface, which activates $G_{\alpha 12}$. This initiates a signaling pathway inside the cell, eventually activating RhoA and inhibiting VSMC migration.

endogenous Rho GTPases for binding to the guanosine nucleotide exchange factors (GEFs) that activate the GTPases by exchanging GTP for GDP. Constitutively active mutants prevent intrinsic GTPase-activating protein (GAP)-induced GTP hydrolysis[38]. Introducing a DN mutant is the functional equivalent of removing the protein from the cell, and introducing a CA mutant is the functional equivalent of exaggerating the effects of the protein in the cell. The combination of the data from experiments with these elucidates the role of the native protein.

Experiments with DN and CA mutants indicated that RhoA is necessary for inhibition of VSMC migration by S1P. CA RhoA inhibited VSMC migration, while S1P did not inhibit migration in the presence of DN RhoA. The mutants of Rac1 and Cdc42, on the other hand, did not have strong effects. In order to further confirm this conclusion, we performed an assay to see if RhoA is activated in response to S1P. We directly demonstrated RhoA activation in VSMCs in response to S1P (Figure 3C).

There was almost no RhoA activation before treatment with S1P, but there was strong activation after treatment.

Since S1P has been shown previously to activate Edg5[27], a G-protein-coupled receptor, we then performed experiments to determine which G-protein the Edg5 receptor activates. Since the $G_{\alpha 12/13}$ family of G-proteins has been shown to activate RhoA[29-32], we examined $G_{\alpha 12}$ and $G_{\alpha 13}$. In order to determine which G-protein is involved in inhibition of VSMC migration, we infected VSMCs with viruses carrying the gene for DN or CA mutants of $G_{\alpha 12}$ and $G_{\alpha 13}$[39]. The dominant-negative mutants interfere and compete with endogenous G-proteins for binding to the G-protein-coupled receptor responsible for activating them. Constitutively active mutants have reduced intrinsic GTP hydrolysis[40] and therefore have prolonged and sustained activity for activating their downstream effectors.

Experiments with DN and CA mutants indicated that, although $G_{\alpha 12}$ and $G_{\alpha 13}$ are both capable of activating RhoA, $G_{\alpha 12}$ is necessary for inhibition of VSMC migration by S1P. CA $G_{\alpha 13}$ inhibited VSMC migration, and CA $G_{\alpha 12}$ prevented up- or down-regulation of migration (Figure 4A). Most importantly, though, DN $G_{\alpha 12}$, had the same effect as DN RhoA, preventing VSMC migration from being inhibited by S1P, but DN $G_{\alpha 13}$ did not (Figure 4B). In order to further confirm this conclusion, a RhoA activation assay was performed to determine if either DN mutant affected the activation of RhoA by S1P (Figure 3C). Confirming the conclusion that S1P activates $G_{\alpha 12}$ in VSMCs, leading to RhoA activation, RhoA activation was markedly lowered in cells infected with DN $G_{\alpha 12}$. Interestingly, though, DN $G_{\alpha 13}$ markedly increased the activation of

RhoA, with or without S1P. This was unexpected, since DN $G_{\alpha 13}$ would be expected to either inhibit RhoA activation if $G_{\alpha 13}$ is involved in the pathway or it would be expected to have no effect if it was not involved in the pathway. These results indicate that this signaling pathway is more complicated than just one protein activating another. Properly functioning $G_{\alpha 12}$ and $G_{\alpha 13}$ are both required for normal behavior of VSMCs. The results do, however, indicate that S1P causes activation of $G_{\alpha 12}$ in order to activate RhoA, leading to the inhibition of VSMC migration.

Implications

In this study, we define some of the important molecules in one stage of angiogenesis (i.e. the inhibition of VSMC migration). This process is important, because it prevents VSMCs from interfering with ECs while they form capillary tube networks. An understanding of all stages of angiogenesis is necessary, because angiogenesis is important to many life processes. Angiogenesis is an important part of growth, development, and healing, and if angiogenesis cannot occur, these processes will be severely limited. In addition, angiogenesis is involved in many pathological conditions. In both diabetic retinopathy, which eventually leads to blindness, and tumor growth, which eventually leads to cancer, unwanted angiogenesis occurs[1-3]. When the molecular basis of angiogenesis is fully understood, more therapeutic agents can be developed to directly target angiogenesis in these cases, leading to novel therapies for these disorders.

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VITA

Nicholas J. Anthis

3571 Norfolk Road
Fort Worth, TX 76109
Phone: (512) 563-9386
Email: BiochemNick@tamu.edu

Education

2001-2005 **Texas A&M University, College Station, Texas**
Candidate for Bachelor of Science degree in Biochemistry in May 2005 with a 4.0 GPR. Received the Barry M. Goldwater Scholarship in 2003.

Research Experience

2002-2004 **University Undergraduate Research Fellow**
Texas A&M College of Medicine, Department of Pathology
Performed laboratory research in molecular biology under Dr. George E. Davis. Predominately used tissue culture and DNA methods. Presented a research in the College of Medicine Summer Research Symposium 2002.

2003 Summer **L.T. Jordan Fellow**
TGR BioSciences, Adelaide, South Australia
Performed laboratory research in assay development under Dr. Michael Crouch. Gave presentations on Australian culture after return to Texas A&M University.

2002 Spring **Student Researcher**
Texas A&M University, Department of Biochemistry and Biophysics
Performed laboratory research under Dr. Gary R. Kunkel. Predominately used methods in molecular genetics.

Leadership Positions

2002-2004 **President, Texas Aggie Democrats**
Focused on education, campaigning, and activism. Increased active membership from ten to seventy members. Led voter registration drives.

2001-2004 **Executive Vice President, Honors Student Council**
Sponsored a variety of programs to increase community and exchange of ideas within the Honors Program.

2002-2003 **Director of Administration, On To Medicine**
Was a founding member. Focused on educating members through guest physician speakers and on community service in the medical field.