Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Signature:

_____	_____
Mithu Maheswaranathan	4/15/2010

Platelet-derived Growth Factor-Mediated Regulation of Slingshot Phosphatase Activity in Vascular Smooth Muscle Cells

by

Mithu Maheswaranathan

Advisers

Dr. Kathy K. Griendling & Dr. Alejandra San Martín

Department of Biology

_____
Dr. Kathy K. Griendling
Adviser

_____
Dr. Alejandra San Martín Almeyda
Adviser

_____
Dr. Darrell Stokes
Committee Member

_____
Dr. Kathleen Campbell
Committee Member

_____
4/15/2010

Platelet-derived Growth Factor-Mediated Regulation of Slingshot Phosphatase Activity in Vascular Smooth Muscle Cells

By

Mithu Maheswaranathan

Advisers

Dr. Kathy K. Griendling & Dr. Alejandra San Martín

An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2010

Abstract

Platelet-derived Growth Factor-Mediated Regulation of Slingshot Phosphatase Activity in Vascular Smooth Muscle Cells

By Mithu Maheswaranathan

Vascular smooth muscle cell (VSMC) migration contributes to the development of cardiovascular diseases, including atherosclerosis and postangioplasty restenosis. Platelet-derived growth factor (PDGF) mediated VSMC migration requires the NADPH oxidase-1 (Nox1) dependent activation of Slingshot (SSH1L) phosphatase, which dephosphorylates and activates cofilin, a key protein in the regulation of actin dynamics. The overall goal of this project was to investigate the specific mechanism of PDGF-induced SSH1L activation in VSMCs, which is mostly unknown. Because SSH1L has been shown to interact with 14-3-3 regulatory proteins in other cell systems, we hypothesized that 14-3-3 plays a role in SSH1L activation in VSMCs. We demonstrated that the 14-3-3 gamma and beta isoforms form a complex with SSH1L in VSMCs in basal conditions, and that these complexes are disrupted by PDGF treatment, shown by co-immunoprecipitation followed by western blotting. We observed that PDGF induced serine dephosphorylation of SSH1L in a consensus 14-3-3 binding motif in VSMCs derived from wild type, but not Nox1 knockout, mice. We therefore sought to characterize potential phosphorylation sites within the Slingshot protein using site-directed mutagenesis to generate phosphomimetic mutations (serine to aspartic acid) and phosphodeficient mutations (serine to alanine) at position 834. We expressed the SSH1L S834A mutant in VSMC and confirmed that it acts as a constitutively active phosphatase and prevents the ability of PDGF to further regulate the system. Our results demonstrate that the dephosphorylation of SSH1L in a 14-3-3 binding motif site via Nox1-mediated

signaling is part of the mechanism of PDGF-induced activation of SSH1L in VSMC, and suggests that disruption of a SSH1L/14-3-3 inhibitory complex is involved. The phosphorylation mutants we generated will be a key tool to further study the specific regulation of SSH1L. These results help provide insight into mechanisms of regulation of VSMC migration, an important event in the development of cardiovascular pathology.

Platelet-derived Growth Factor-Mediated Regulation of Slingshot Phosphatase Activity in Vascular Smooth Muscle Cells

By

Mithu Maheswaranathan

Advisers

Dr. Kathy K. Griendling & Dr. Alejandra San Martín

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2010

Acknowledgements

I would like to thank my advisers, Dr. Kathy K. Griendling and Dr. Alejandra San Martín, for their encouragement, support, and guidance throughout my research. This work would not have been possible without their mentorship. Also, I would like to thank the Summer Undergraduate Research Program at Emory (SURE) for enabling me to have the opportunity to pursue research in the summer of 2009. Additionally, I would like to thank the members of the Griendling laboratory for their assistance and guidance, including Holly C. Williams, Lily Pounkova, and Dr. Bernard Lassègue. I am also grateful for the help of my committee members, Dr. Darrell Stokes and Dr. Kathleen Campbell, for their suggestions and advice. And I make a final, special thank you to my parents, my brother, and my friends who have supported me in this endeavor.

Table of Contents

Introduction

According to the American Heart Association, an estimated 79,400,000 Americans (or 1 in 3 adults) have one or more types of cardiovascular disease (Rosamond et al. 2008), such as atherosclerosis, post-angioplasty restenosis, stroke, and coronary heart disease. Atherosclerosis, the process of the thickening of artery walls and narrowing of arteries due to fatty build-up of plaques, is implicated in many cases of cardiovascular disease (CVD), including coronary heart disease. Atherosclerosis accounts for almost three-fourths of all deaths from CVD, including deaths from heart attack and stroke (Rosamond et al. 2008). Coronary heart disease is responsible for 1 out of every five deaths in the United States in 2004, making it the leading causing of death in America today (Rosamond et al. 2008). Considering the widespread prevalence of CVD, understanding the molecular mechanisms that lead to the pathogenesis of atherosclerosis is of critical importance.

The blood vessel wall consists of three concentric, distinct layers that surround the lumen, the central blood-containing space. The tunica intima (or tunica interna) is the innermost layer, surrounding the lumen. The intima is composed of endothelial cells which line the lumen of vessels, creating a smooth lining. The tunica media, or middle layer, consists of smooth muscle cells and elastin sheets, in which muscle cells contract and relax while elastin allows for the vessels to stretch and recoil. The tunica adventitia (or tunica externa) is the outermost layer, composed of collagen fibers that protect the blood vessel and anchor it to adjacent structures (Marieb and Hoehn 2006).

One of the processes that contributes to development of cardiovascular diseases, including atherosclerosis and postangioplasty restenosis, is vascular smooth muscle cell

migration (Schwartz 1997). Vascular smooth muscle cells (VSMCs) comprise the majority of cells in blood vessel walls, and are found in the tunica media layer. In physiological conditions, these cells are in a fully differentiated, contractile phenotype that allows them to regulate blood flow and pressure by contracting or relaxing. However, under pathological circumstances (such as vessel injury or atherosclerotic plaque development), the VSMC phenotype is altered, and VSMCs switch from a contractile, differentiated state to a proliferative, migratory state. In this proliferative state, VSMCs have the ability to migrate from the tunica media to the tunica intima (Orlandi and Bennett 2010) and then secrete matrix proteins that stabilize the plaque (Doran et al. 2008), thickening the intima and vessel wall, which results in atherosclerosis.

Migration

Migration is a dynamic process that requires signaling domains at the front and rear of the cell. A cell must sense a gradient and establish polarity, and the plasma membrane is then extended in the direction of movement. Actin filament reorganization is essential for change of cell shape, polarity formation, and migration (Lauffenberger 1996). In VSMCs, after the cell senses a signal, lamellipodia formation or localized protrusion of the cell membrane in the direction of the chemotactic stimulus occurs by extension of F-actin rich fibers (San Martín and Griendling 2010). Lamellipodia are cytoskeletal projections at the edge of a mobile cell, containing arrays of actin filaments which orient their plus (barbed) ends toward the plasma membrane. Growth of actin filaments adjacent to the plasma membrane enables the forward extension of

lamellipodia, pulling the cell across a substrate (Alberts et al. 2002). Cycles of actin polymerization and depolymerization are required for protrusion of actin-rich lamellipodia in moving cells (San Martín and Griendling 2010).

Cofilin

Cofilin is an important protein that controls actin dynamics, and is a ubiquitous actin-binding factor required for actin filament reorganization in eukaryotes (Huang et al. 2006). Cofilin is capable of binding both globular (G)-actin and filamentous (F)-actin to increase the rate of depolymerization of actin filaments (Bamburg 1999, Bamburg et al. 1999). It stimulates the disassembly of actin filaments at the pointed or plus ends, thereby generating actin monomers continuously for polymerization and rapid turnover of actin filaments (Chen et al. 2000). Activation of cofilin is essential to maintain and protrude lamellipodia at the leading edge of migrating VSMCs (San Martín and Griendling 2010).

Cofilin is negatively regulated by phosphorylation at conserved serine (Ser) residues at position 3 by LIM kinases, preventing its ability to bind and depolymerize actin, and is reactivated by the Slingshot (SSH) family of phosphatases through dephosphorylation (San Martin et al. 2008). The phosphorylation and dephosphorylation of cofilin serves a simple regulatory mechanism for actin reorganization. Dephosphorylation of cofilin is important to enable the processes of actin severing, depolymerization, and directional cell motility (Huang et al. 2006).

Slingshot

The Slingshot protein family refers to a conserved family of cofilin phosphatases. Upon activation, Slingshot acts as a cofilin phosphatase, dephosphorylating and activating cofilin. Slingshot was originally identified in genetic studies in *Drosophila*, where dysfunction of the protein resulted in disorganized epidermal cell morphogenesis causing splintered hair bristles, explaining the origin of its name (Huang et al. 2006). In humans and mice, the Slingshot phosphatases are represented by three genes (SSH1, SSH2, and SSH3), each with long and short variants with distinct patterns of tissue expression. In mammalian cells, SSH1L, along with SSH2L and SSH3L, dephosphorylates phospho-cofilin at the critical Ser3 residue (Huang et al. 2006), leading to its activation, actin depolymerization and lamellipodial protrusion. In VSMCs, SSH1L is activated by pro-migratory factors such as platelet-derived growth factor (PDGF) (San Martin et al. 2008).

PDGF Signaling

VSMC migration can be stimulated by different factors, but in vivo it is primarily the consequence of stimulation of PDGF-β receptors at the cell surface by PDGF (Clowes 1999). PDGF is a serum growth factor for not only smooth muscle cells, but also fibroblasts and glial cells. Human PDGF was originally identified as a dimer of two different polypeptide chains, A and B, linked by a disulfide bond (Johnsson et al. 1982). Although PDGFs have important roles during development, there is limited evidence to suggest normal physiological functions in adults. Increased PDGF signaling, however, has been linked to several pathological disease states; for instance, there are increased

levels of PDGF in atherosclerotic lesions compared to the normal vessel wall (Raines 2004). Levels of expression of PDGF-β and PDGF-α receptors are also increased in atherosclerotic vessels. Several conditions related to cardiovascular disease affect PDGF or PDGF receptor gene expression. For example, increased blood pressure enhances the expression of vascular PDGF-α receptors (Majesky et al. 1990; Negoro et al. 1995), and hypercholesterolemia (high blood cholesterol) dramatically increases PDGF expression in circulating mononuclear cells (Mondy et al. 1997). PDGF released from platelet aggregates at locations of vascular injury plays a key role in migration of VSMC from the media into the intima and proliferation of VSMCs in the intima (Andrae et al. 2008).

The signal transduction mechanisms by which PDGF induces migration of VSMCs have been studied extensively. Stimulation of the PDGF-β receptors at the cell surface by PDGF results in transduction of the external signal and the coordination of various signaling events, which remodel cytoskeleton structure (Gerthoffer 2007). Specifically, autophosphorylation of PDGF-β receptors upon binding of PDGF creates a binding site for the cytosolic Src family tyrosine kinase (Gerthoffer 2007, Shimokado and Higaki 1997). Src phosphorylates tyrosine residues in the PDGF-β receptor, and different signaling molecules then bind to these phosphorylated tyrosine residues (Shimokado and Higaki 1997). For instance, phosphatidylinositol 3-kinase (PI3 kinase) binds to the tyrosine kinase, producing phosphatidylinositol bisphosphate (PIP_2), which then helps to activate a guanine nucleotide exchange factor, leading to Rac activation (Claesson-Welsh 1996). Activated small G proteins (Rho, Rac, Cdc42) regulate actin-binding proteins (WAVE, WASP, actin-related protein 2/3 [ARP2/3] complex) and activate downstream protein kinases, which phosphorylate target proteins to trigger actin

nucleation and actin filament extension at plus ends near the plasma membrane. As mentioned above, the depolymerization of F-actin at the minus end is promoted by cofilin, which is activated by SSH1L. PDGF stimulation induces SSH1L activation required for cell migration in VSMCs (San Martin et al. 2008) by a still unclear mechanism. Specifics of the signaling pathways that lead to actin polymerization and ultimately migration are currently being investigated.

Role of ROS in Migration and Cardiovascular Disease

An early clue that reactive oxygen species (ROS) might be important in migration, and cardiovascular disease states, came from a groundbreaking study which showed that hydrogen peroxide (H_2O_2) was required for PDGF-induced migration in VSMCs (Sundaresan et al. 1995). Evidence strongly suggests that oxidative stress, caused by excess amounts of ROS, plays an important role in pathophysiology of major cardiovascular diseases (Heistad 2006). The NADPH oxidase (Nox) proteins are a family of enzymes whose major function is to generate ROS, including superoxide ($O_2^{\cdot -}$) and H_2O_2. Although ROS derived from NADPH oxidases contribute to disease progression, they also play essential roles in the physiology of the vasculature, as well as the brain, immune system, digestive tract, and in hormone synthesis. Nox-derived ROS are involved in regulation of cytoskeletal remodeling, gene expression, proliferation, migration, and cell death (Selemidis et al. 2008). Under physiological conditions, ROS are produced in the blood vessel wall in a controlled fashion at low concentrations and act as signaling molecules to regulate contraction and relaxation of VSMCs as well as VSMC growth (Touyz and Schiffrin 2004). In various high-risk cardiovascular states

(including hypertension and hypercholesterolemia), the expression and activity of NADPH oxidases is upregulated in the blood vessel wall. ROS derived from these enzymes contribute to advancement of disease (Selemidis et al. 2008). In atherosclerosis and other disease states like hypertension, superoxide production is increased in blood vessels and endothelial vasomotor function is disrupted. Superoxide inactivates nitric oxide, an important signaling molecule which dilates smooth muscle of the blood vessels (Heistad 2006). Specifically, H_2O_2 is the ROS responsible for PDGF-induced migratory signaling (Brandes et al. 2001; Sundaresan et al. 1995; Weber et al. 2004), and can induce protein oxidation (such as thiol modifications) that alter the activation state of proteins (Ying et al. 2007). Consequences of increased H_2O_2 production include increased VSMC growth, increased contractility, monocyte migration, inflammation, and increased deposition of extracellular matrix proteins, all processes that are major contributors to vascular damage in cardiovascular disease states (Touyz and Schiffrin 2004).

NADPH Oxidases

The NADPH oxidases are multi-unit enzymes associated with the membrane, which catalyze reduction of oxygen using NADPH as an electron donor. NADPH oxidases produce $O_2^{\cdot -}$ through a single electron reduction (Brown and Griendling 2009). In VSMCs, there are two isoforms of the NADPH oxidase family which are expressed: Nox1 and Nox4 (Lassègue and Griendling 2009). Both Nox1 and Nox4 are catalytic subunits of NADPH oxidases that interact with another membrane spanning subunit, p22phox, but only Nox1 requires association with cytosolic subunits for activity. The

subunit composition of Nox1 varies by cell type, but in VSMCs, Nox1 associates with two cytosolic factors, p47phox and NoxA1, as well as the small-molecular weight G-protein Rac (Lyle and Griendling 2006). These cytosolic factors bind to the membrane subunits (Nox1 and p22phox) that form the central core of the enzyme, and are required for Nox1 enzymatic activation. Nox1 mRNA is highly expressed in colon epithelia, but is also expressed at lower levels in VSMCs, endothelial cells, osteoclasts, neurons, microglia, uterus, placenta, and prostate. Though the basal expression of Nox1 in VSMCs is low, it has been of interest since it is upregulated at the mRNA level and activated by vascular pathological stimuli including PDGF and Angiotensin II (Brown and Griendling 2009). Recent findings suggest that Nox1 is implicated in cell migration, since migration in response to PDGF or fibroblast growth factor (FGF) is impaired in VSMCs from Nox1 knockout mice (Lee et al. 2009).

PDGF-induced, Nox1-sensitive SSH1L activation

As discussed earlier, stimulation of the PDGF-β receptor by PDGF triggers signaling cascades leading to migration. PDGF activates PI3-kinase, which initiates downstream signaling pathways that ultimately activate Nox1 (Brandes and Kreuzer 2005). Nox1 then activates SSH1L phosphatase, which dephosphorylates and activates cofilin, leading to remodeling of actin cytoskeleton. However, very little is known about the mechanisms by which PDGF activates SSH1L in VSMCs. The focus of this research project was therefore to investigate the mechanism of PDGF-induced, Nox1-sensitive SSH1L activation.

14-3-3 Proteins and Role in SSH1L Activation

14-3-3 proteins are a family of regulatory proteins that self-assemble spontaneously as dimers, first detected due to their high abundance in brain extracts (Tzivion and Avruch 2002). The 14-3-3 proteins are ubiquitously expressed and found exclusively in eukaryotes, with a very large number of binding partners, allowing modulation of a variety of cellular processes (Bridges and Moorhead 2005). The proteins assemble as stable homodimers and heterodimers, and are a highly conserved group of proteins in amino acid sequences from yeast to mammals (Tzivion and Avruch 2002).

Although specific functions of 14-3-3 proteins are not fully understood currently (Yaffe 2002), this protein family includes seven isoforms that regulate important molecular and cellular processes. 14-3-3 proteins specifically bind phosphorylated motifs in their partner proteins, so that protein phosphorylation regulates protein-protein interactions with this family. 14-3-3s were the first proteins that were recognized to bind specifically to phosphoserine or phosphothreonine motifs of their partner proteins (Bridges and Moorhead 2005).

Research in other cell types indicates that SSH becomes activated by its release from its scaffold protein 14-3-3 in the cytoplasm, while association of 14-3-3 with SSH prevents cofilin dephosphorylation and SSH translocation to F-actin rich regions (Huang et al. 2006). The involvement of 14-3-3 in the PDGF-induced SSH1L activation is of particular interest, since PDGF has been shown to induce expression of the 14-3-3-gamma (γ) isoform in human VSMC (Autieri and Carbone 1999). Additionally, different isoforms of 14-3-3 have been shown to interact with SSH1L in various cell types,

including the 14-3-3 beta (β) isoform that is also expressed in VSMCs (Autieri et al. 1996).

Hypothesis and Objectives

We have demonstrated that SSH1L is activated in VSMC after PDGF stimulation and is required for VSMC migration. Activation of Slingshot results in several downstream consequences, including the dephosphorylation and activation of cofilin, actin depolymerization, lamellipodial protrusion, and ultimately migration. However, the mechanism of SSH1L activation is mostly unknown. In general, the mechanisms by which PDGF control VSMC migration are not yet clear, and are difficult to determine since PDGF is a proliferative growth factor which can initiate a wide diversity of signaling pathways. The purpose of this overall research is to investigate the mechanisms responsible for platelet-derived growth factor (PDGF)-mediated regulation and activation of Slingshot, which can lead to changes in actin dynamics required for VSMC migration. This project involves two major aims: (1) to determine whether PDGF-induced SSH1L activation is regulated through a mechanism that requires dephosphorylation of SSH1L at 14-3-3 binding motifs and consequent disruption of an inhibitory complex with 14-3-3, and (2) to identify and characterize PDGF-sensitive Ser phosphorylation sites within the SSH1L protein that mediate its binding with 14-3-3 and affect enzymatic activity. *We hypothesize that in VSMCs, PDGF-induced activation of SSH1L is mediated by Nox1-dependent disruption of a SSH1L/14-3-3 inhibitory complex through a mechanism that involves dephosphorylation of the phosphoserine residue in the 14-3-3 binding motif*

present in SSH1L (see Figure 1). These studies will help provide insights into mechanisms of regulation of VSMC migration, and will be useful in understanding potential therapeutic targets for pathologies like atherosclerosis and postangioplasty restenosis that involve dysregulated VSMC migration.

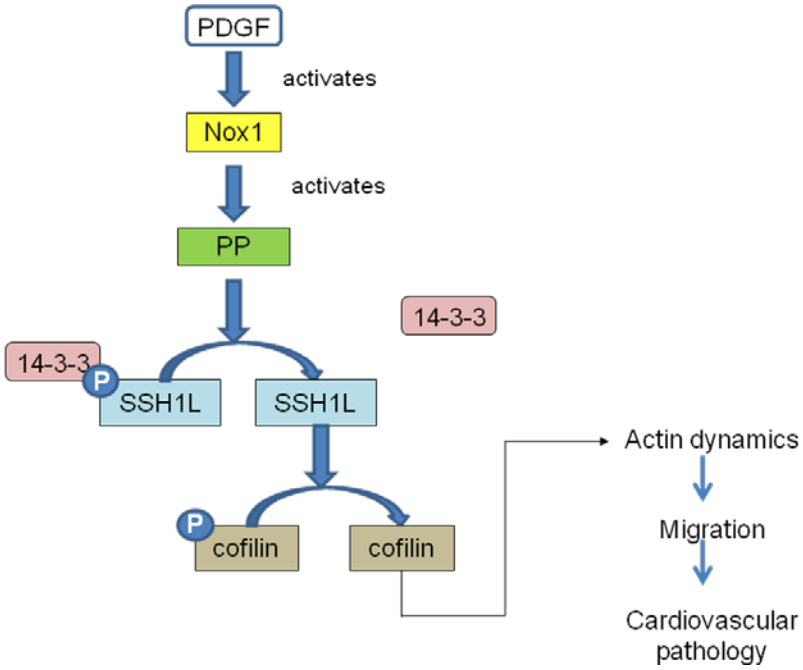


Figure 1. Working Model/Hypothesis. This figure illustrates our proposed hypothesis for the mechanism of SSH1L activation leading to VSMC migration. PDGF binds to PDGF-β receptors, activating Nox1 through a receptor tyrosine kinase pathway. Nox1 activates a protein phosphatase that dephosphorylates the SSH1L protein, disrupting the 14-3-3/SSH1L inhibitory complex. Activated SSH1L then dephosphorylates and activates cofilin.

Materials and Methods

Cell Culture

VSMCs were isolated from wild type (wt) and Nox1 knockout (KO) mouse aortas, by enzymatic disruption (Ohmi et al. 1997). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, and used between passages 6 and 12. Cultures were maintained at 37^{o}C in a 5% CO_2 atmosphere.

Total Cell Lysate Preparation

Media were aspirated from the cell culture dishes. Dishes were placed on ice and 5 mL of cold PBS was added. The dishes were rinsed again 2 more times with 5 mL of cold PBS. On the final rinse, the dishes were tilted for approximately 30 seconds and residual PBS removed. 100 µL of lysis buffer (containing aprotinin and leupeptin protease inhibitors and 1% Triton) was added to each plate. Lysed cells were scraped from dishes into tubes, and then shaken gently for 30 min at 4^{o}C. To pellet cellular debris and triton-insoluble fractions, we subjected cells to centrifugation at 25,000 x g for 10 minutes at 4^{o}C. The supernatant was transferred to a fresh microcentrifuge tube on ice.

A Bradford based colorimetric assay was then performed to measure the concentration of protein in the samples using bovine serum albumin (BSA) as a standard. Samples were read using the µQuant spectrophotometer microplate reader.

Western Blot

Proteins of the cell lysate were separated by size through the use of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The proteins were electro-transferred to PVDF membranes, blocked with 5% milk for one hour and washed 3x with 0.1% TBS-Tween for 5 min/wash, and then incubated with a primary antibody (1:1000) against the specific protein of interest (SSH1L, 14-3-3, p-cofilin, or cofilin) in each experiment. Next, membranes were washed 3x with 0.1% TBS-Tween for 5 min/wash and a secondary antibody conjugated to horseradish peroxidase (HRP) was added (1:3000). Membranes were then washed 5x with 0.1% TBS-Tween for 5 min/wash. After incubation with the HRP-conjugated secondary antibody and the TBS-Tween wash, proteins were detected by ECL chemiluminescence using a light sensitive film. Western blots were quantified using ImageJ software.

Immunoprecipitation (IP)

VSMCs were lysed with lysis buffer containing 1% Triton. Cell lysates were normalized for the total amount of protein and SSH1L was immunoprecipitated in 1 mg of total protein with 1:1000 anti-SSH1L antibody or non-specific control IgG. After incubating for 2 hours at 4^{o}C, 20 μL of Protein A-Agarose was added and maintained at 4^{o}C on a rocker for 1 hour. Immunoprecipitates were collected by centrifugation at 3,000 rpm (approximately 1,000 x g) for 3 minutes at 4^{o}C. The supernatant was carefully aspirated and discarded.

Subsequently, phosphatase activity was measured in the immunoprecipitates using a commercial kit (see Phosphatase Activity Assay section) or immunoprecipitates

were separated by SDS-PAGE and blotted with the SSH1L antibody as described under Western Blot procedure.

Co-immunoprecipitation (Co-IP)

In these experiments, co-immunoprecipitation was performed to test for SSH1L/14-3-3 complex interactions. SSH1L was immunoprecipitated with the IP protocol described previously, using a Tris 7.5 buffer with 0.5% NP-40. After separating immunoprecipitates by SDS-PAGE as described under Western Blot procedure, the membranes were immunoblotted with antibodies for SSH1L and 14-3-3. Proteins were then visualized by chemiluminescence.

Site-Directed Mutagenesis

Primers were created to introduce mutations in the serine phosphorylation sites of SSH1L using the QuikChange XL site-directed mutagenesis kit from Stratagene. DNA was subjected to Sanger based automated DNA sequencing by Agencourt Bioscience.

Transfection

VSMCs were transfected with wild type (wt) or mutant SSH1L (5 µg of DNA) for the SSH1L SA/SD experiments and with wt or mutant 14-3-3 (3.5 µg of DNA) for the 14-3-3 wt/KE experiments by electroporation using a Nucleofector (Amaxa Biosystems).

Migration Assay

Prior to the migration assay, wt and Nox1 KO cells were transfected with the SA SSH1L mutant and serum-starved for 48 hours. A modified Boyden chamber assay was used to measure directed cell migration. VSMCs were added to the upper chamber of a Transwell dish, and exposed to PDGF in the lower chamber for 3 hours. Nonmigrated cells were removed from the upper chamber with a cotton swab. Then, cells remaining on the inserts were fluorescently stained with DAPI and visualized using a Zeiss Axioskop microscope. Migrated cells per membrane were quantified using ImageJ software.

Phosphatase Activity Assay

Cells from wt and Nox1 KO animals were stimulated with PDGF (10 ng/mL) or vehicle for 20 min. SSH1L was immunoprecipitated from VSMCs with a specific antibody using Tris 7.5 buffer. Phosphatase activity was measured in the immunoprecipitates using an in vitro phosphatase assay commercial kit (Promega) following the manufacturer's protocol, using a custom made phospho-cofilin peptide as a substrate for 30 min at 37^{o}C. The release of free phosphate was evaluated by its colorimetric reaction with malachite green, and the color intensity was measured in a 96-well plate using a μQuant spectrophotometer (microplate reader) at 600-630 nm. The amount of free phosphate was calculated using a standard curve and the enzymatic activity was expressed as pmol of phosphate per unit of protein per unit of time.

siRNA (small interfering RNA) Experiments, siSSH1L

Cells were transfected by electroporation using a Nucleofector (Amaxa Biosystems, U25 program) with 1.5 µg of annealed small interfering (si)RNA duplexes for SSH1L or nonsilencing control sequence no. 1 (Qiagen) per $1x10^6$ cells. siRNA duplexes were synthesized using a previously published sequence. The transfections were done three days before a 24-hour serum deprivation prior to experiments.

Statistical Analysis

Results are expressed as means ± SEM (standard error of the mean). Differences among groups were analyzed using 1-way ANOVA, with post hoc contrasts adjusted according to the Duncan correction using SPSS 14.0 for Windows. A value of $p<0.05$ was considered to be statistically significant.

Results

PDGF-induced SSH1L phosphatase activity requires Nox1

A phosphatase activity assay was performed by Dr. Alejandra San Martín to determine whether there is a difference in the activity of SSH1L in Nox1 knockout (KO) cells versus wt cells. VSMCs from wt and Nox1 KO mice were stimulated with PDGF (10 ng/mL) or vehicle for 20 minutes. An immunoprecipitation was performed for the SSH1L protein using a SSH1L antibody. Phosphatase activity was measured in the immunoprecipitates using a commercial kit (Promega). There was no significant difference in SSH1L phosphatase activity in wt versus Nox1 KO cells under basal conditions. However, a difference was noted in PDGF-stimulated conditions: SSH1L activity was induced by PDGF in wt cells, but not in cells derived from Nox1 KO mice (Figure 1). These results demonstrate the requirement of Nox1 for PDGF-induced SSH1L activation.

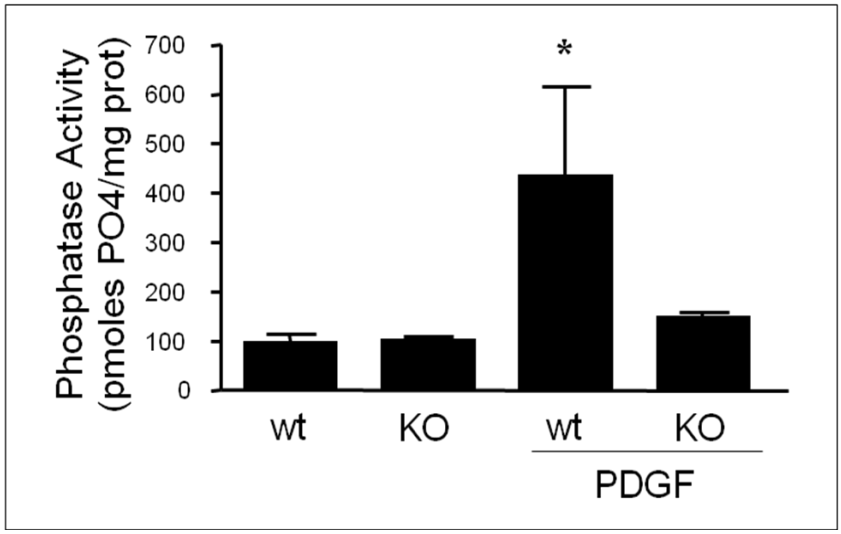
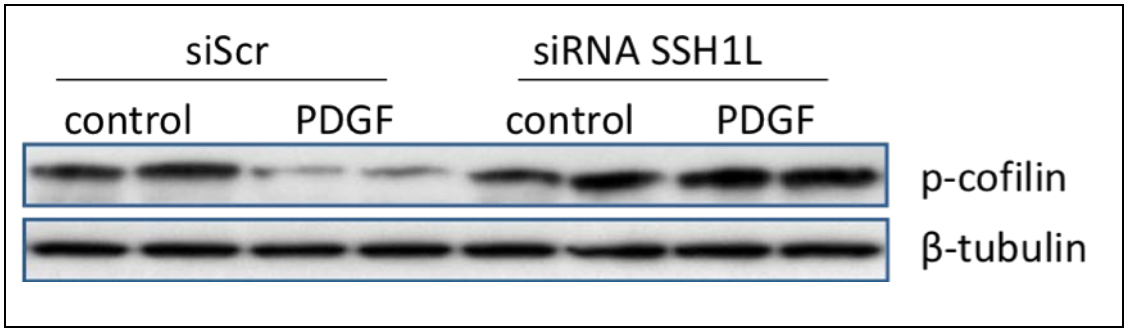


Figure 1. **SSH1L activity is induced by PDGF in wt VSMCs, but not in cells derived from Nox1 KO animals.** After PDGF stimulation of wt and Nox1 KO cells, SSH1L phosphatase activity was measured after immunoprecipitation using a commercial kit. Bars represent mean ± SEM average of four independent experiments. A value of $p<0.05$ was considered to be statistically significant. [Figure obtained courtesy of Alejandra San Martín].

SSH1L acts as a PDGF-sensitive cofilin phosphatase

An experiment using small interfering RNA (siRNA) was performed by Dr. Alejandra San Martín to determine if phospho-cofilin is a substrate for SSH1L enzymatic activity. Cells were transfected by electroporation using a Nucleofector (Amaxa Biosystems) with 1.5 µg of annealed small interfering (si) RNA duplexes for SSH1L or nonsilencing control sequence.

Results of the siRNA experiment are shown in Figure 2. siScr is a nonspecific double strand RNA that is used as a control for the siRNA against SSH1L. In control (siScr) lanes, PDGF stimulation dephosphorylates p-cofilin. We observed that with siRNA for SSH1L, the amount of p-cofilin remained the same in the control and PDGF-stimulated conditions. Downregulation of SSH1L prevented the ability of PDGF to dephosphorylate cofilin (Figure 2). These findings demonstrate that SSH1L acts as a PDGF-sensitive cofilin phosphatase in VSMCs.



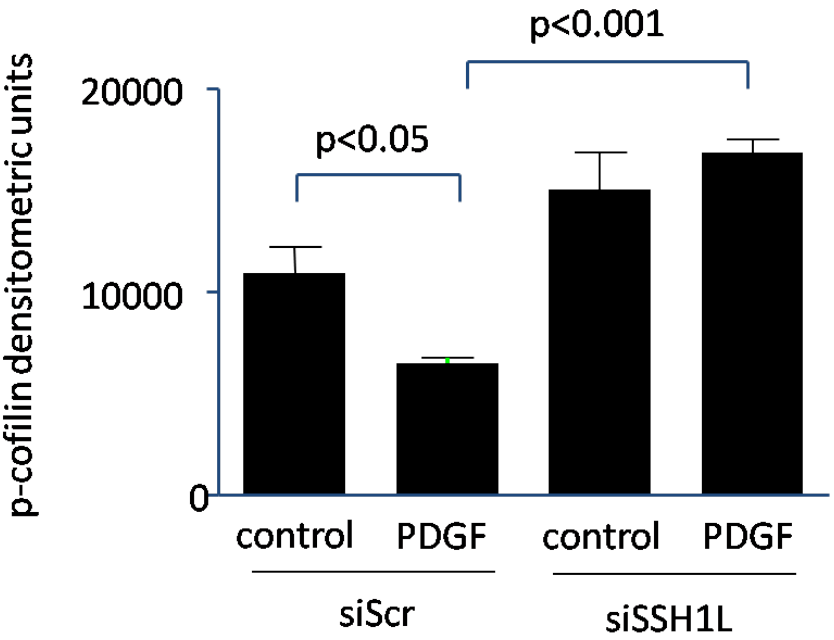


Figure 2. **PDGF-induced SSH1L activation and dephosphorylation of p-cofilin is lost in siSSH1L cells, implicating SSH1L as the phosphatase involved in PDGF-induced cofilin dephosphorylation.** Cells were transfected by electroporation using a Nucleofector with 1.5 µg of annealed small interfering (si) RNA duplexes for SSH1L or nonsilencing control sequence. Bars represent mean ± SEM average of five independent experiments. A value of $p<0.05$ was considered to be statistically significant. [Figure obtained courtesy of Alejandra San Martín and *Circulation Research*, San Martín et al. 2008]

PDGF induces SSH1L/14-3-3 complex disruption

Interestingly, recent reports indicate that in other cell types, 14-3-3 proteins can inhibit SSH1L activity by binding SSH1L in the cytoplasm through a complex formation dependent on the phosphorylation state of SSH1L (Nagata-Ohashi et al. 2004). This suggests that PDGF might activate SSH1L by inducing its release from 14-3-3. To test this possibility, we treated cells with PDGF and the interaction between the two isoforms of 14-3-3 expressed in VSMCs, 14-3-3γ and 14-3-3β (Autieri et al. 1996, Ellis et al. 2003), and SSH1L was examined.

VSMCs from mouse aorta were incubated with vehicle or PDGF (10 ng/mL for 15 min). An immunoprecipitation was performed using a SSH1L antibody followed by western blotting. The upper part of the membrane was immunoblotted using anti-SSH1L (130 kD) antibody and the lower part of the membrane was immunoblotted with an antibody for 14-3-3γ (30 kD). Non-related IgG was used as a negative control. We found that in basal (unstimulated) conditions, SSH1L coimmunoprecipitates with 14-3-3γ, suggesting an inhibitory SSH1L/14-3-3γ complex formation in VSMCs. This complex formation is disrupted after PDGF treatment, resulting in a decrease in the amount of 14-3-3γ which coimmunoprecipitated with SSH1L in PDGF-stimulated cells (Figure 3A).

In the second experiment, we incubated VSMCs from mouse aorta with vehicle or PDGF (10 ng/mL for 15 min) to test for the 14-3-3β isoform. In this experiment, the immunoprecipitation and immunoblotting steps were reversed. An immunoprecipitation was performed using a 14-3-3β antibody followed by western blotting. The upper part of the membrane was immunoblotted using anti-SSH1L (130 kD) antibody and the lower part of the membrane was immunoblotted with an antibody for 14-3-3β (28 kD). We

found that in basal (unstimulated) conditions, SSH1L coimmunoprecipitates with 14-3-3β, suggesting a SSH1L/14-3-3β complex formation in VSMCs. This complex formation is disrupted after PDGF treatment, resulting in a complete disappearance in SSH1L from 14-3-3β coimmunoprecipitates in PDGF-stimulated cells (Figure 3B).

These experiments demonstrated the expression of 14-3-3β and 14-3-3γ isoforms in VSMCs and also the ability for these isoforms to undergo complex formation with the SSH1L protein. Additionally, results from both experiments correspond with our hypothesized role of SSH1L/14-3-3 complex disruption in the mechanism of PDGF-induced SSH1L activation.

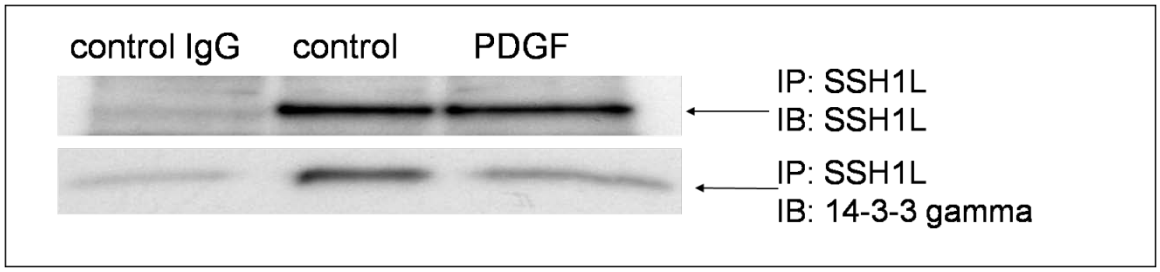


Figure 3A. **Under basal conditions 14-3-3γ forms a complex with SSH1L, which is disrupted after PDGF treatment.** Western blot results from a co-immunoprecipitation of the 14-3-3γ and SSH1L proteins. VSMCs from mouse aorta were incubated with vehicle or PDGF (10 ng/mL for 15 min). An immunoprecipitation (IP) was performed using a primary antibody against SSH1L. The upper part of the membrane was then immunoblotted (IB) using anti-SSH1L antibody, with the lower part of the membrane immunoblotted (IB) for 14-3-3γ. Representative of 3 independent experiments.

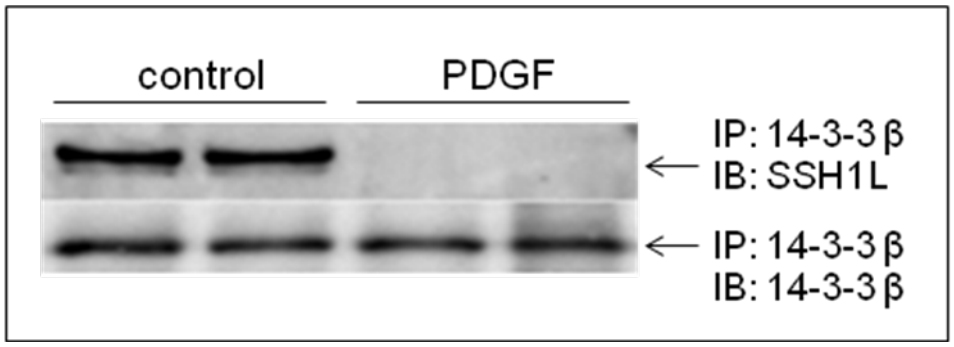


Figure 3B. **Under basal conditions 14-3-3β forms a complex with SSH1L, which is disrupted after PDGF treatment.** Western blot results from a co-immunoprecipitation of the 14-3-3β and SSH1L proteins. VSMCs from mouse aorta were incubated with vehicle or PDGF (10 ng/mL for 15 min). An immunoprecipitation (IP) was performed using a primary antibody against 14-3-3β. The upper part of the membrane was then immunoblotted (IB) using anti-SSH1L antibody, with the lower part of the membrane immunoblotted (IB) for 14-3-3β. 1 experiment.

PDGF-induced dephosphorylation of SSH1L is lost in Nox1 KO cells

Although 14-3-3 proteins have been reported to bind partner proteins independent of phosphorylation in some cases, they typically recognize and bind serine or threonine phosphorylation motifs within partner proteins (Bridges and Moorhead 2005). This suggests a mechanism by which Nox1 might mediate PDGF-induced disruption of the 14-3-3/SSH1L complex: Nox1-dependent, PDGF-induced dephosphorylation of SSH1L at 14-3-3 binding motifs. To test this possibility, we examined the effect of PDGF on dephosphorylation of serine residues within the SSH1L protein at 14-3-3 binding motifs in wt and Nox1 KO cells.

Wt and Nox1 KO VSMC from mouse aorta were incubated with vehicle or PDGF (10 ng/mL for 15 min). An immunoprecipitation was performed using the SSH1L antibody followed by western blotting. The membrane was immunoblotted using a phospho Ser 14-3-3 binding motif antibody, which recognizes the phosphorylated serine 14-3-3 binding sites within target proteins. The membrane was then stripped and reblotted with an antibody for SSH1L, to ensure the total amount of SSH1L was equal across all lanes.

Results of this experiment are shown in Figure 4. Under control conditions, bands in the wt cells show the presence of the phospho-Ser 14-3-3 binding motif of SSH1L, indicating the presence of phosphorylated SSH1L. PDGF induces dephosphorylation of SSH1L at these 14-3-3 binding motifs in wt cells, resulting in a decrease in the amount of phospho-SSH1L in PDGF-stimulated wt lanes. PDGF-induced dephosphorylation of phospho-SSH1L is lost in Nox1 KO cells (Figure 4). These results

suggest that (1) PDGF induces dephosphorylation of SSH1L in a 14-3-3 binding motif, and (2) Nox1 is required for PDGF-induced SSH1L dephosphorylation.

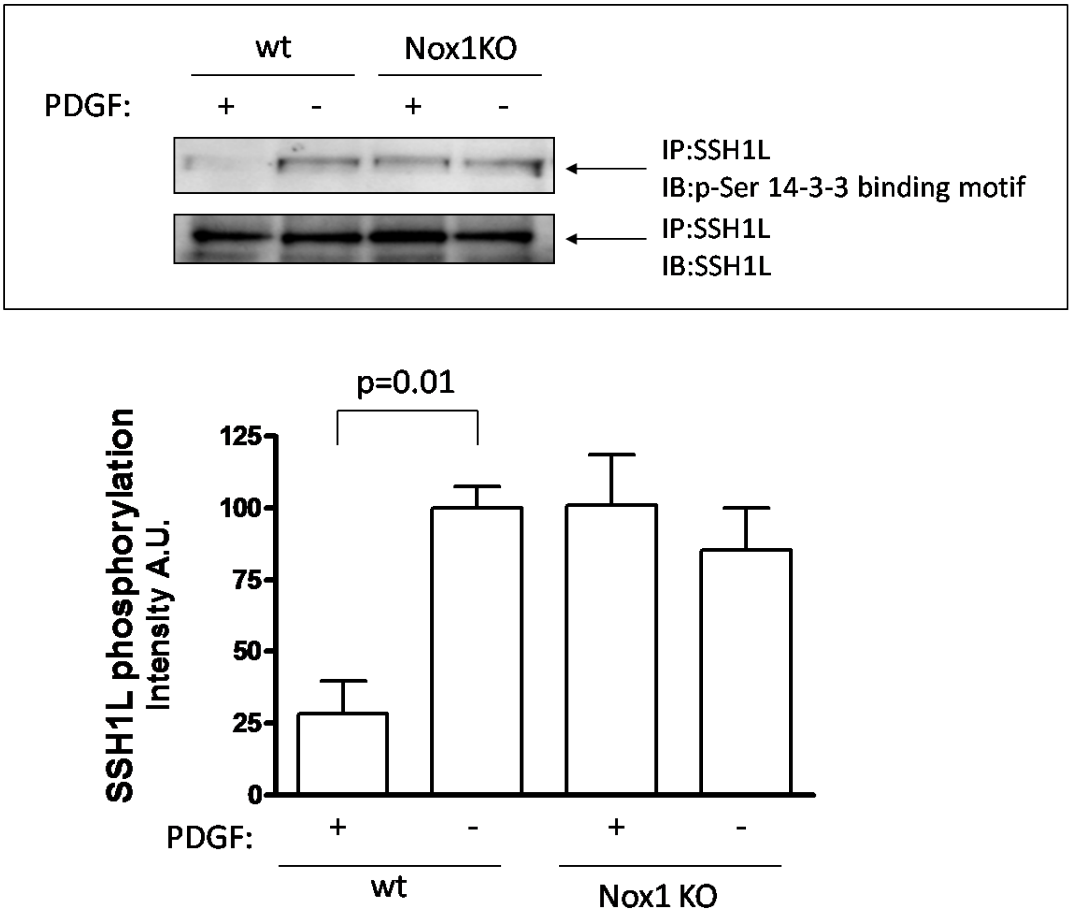


Figure 4. PDGF induces SSH1L Ser dephosphorylation at 14-3-3 binding motifs in wt but not in Nox1 KO cells. Immunoprecipitation and western blot for the p-Ser 14-3-3 binding motif of SSH1L. Both wt and Nox1 KO VSMCs were incubated with either vehicle or PDGF (10 ng/mL for 15 min). After immunoprecipitation of SSH1L, the membrane was immunoblotted for the phospho Ser 14-3-3 binding motif. Bars represent mean ± SEM average of three independent experiments. A value of $p<0.05$ was considered to be statistically significant.

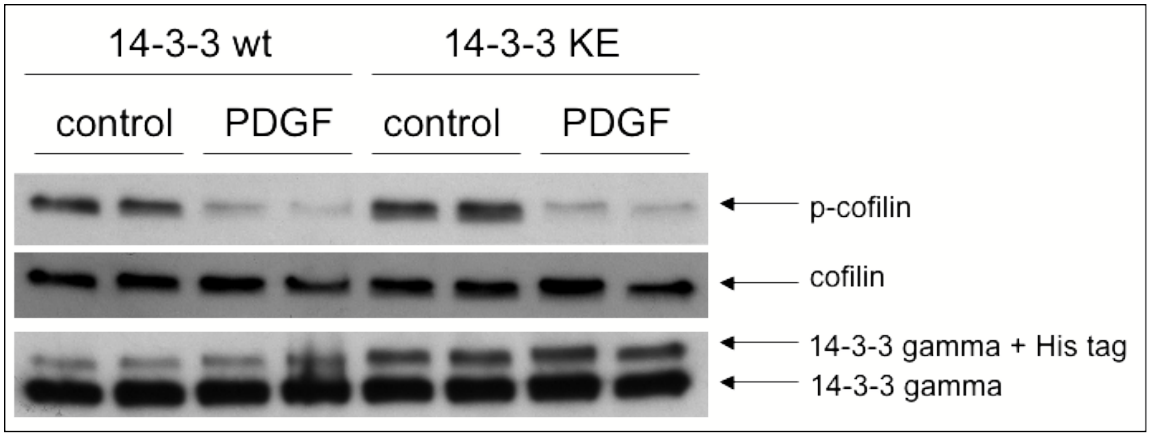
14-3-3 KE mutants may not have been able to displace endogenously bound SSH1L

The aim of this experiment was to investigate further the nature and regulation of SSH1L/14-3-3 binding and the mechanism of action of PDGF-induced SSH1L/14-3-3 complex disruption. 14-3-3 K49E (KE) mutants, which contain a mutation that disrupts the basic loop that recognizes and binds Ser/Thr-phosphorylated motifs in partner proteins such as Slingshot, were obtained from Dr. Haian Fu (Department of Pharmacology). These mutants are unable to recognize and bind the Ser phosphorylated motifs in their partner proteins. Additionally, the 14-3-3 KE mutant proteins bind to endogenous 14-3-3 proteins, forming inactive heterodimers. This creates a dominant negative effect, where the 14-3-3 KE mutants prevent endogenous 14-3-3 protein from carrying out its normal functions.

VSMCs from mouse aorta were transfected with wt 14-3-3 or the 14-3-3 K49E mutant (3.5 µg of DNA) by electroporation using a nucleofector (Amaxa Biosystems). In order to evaluate the ability of PDGF to dephosphorylate cofilin in transfected cells, we subjected samples to western blotting with a primary antibody against phospho-cofilin (p-cofilin). Since SSH1L is the major cofilin phosphatase activated by PDGF in VSMCs (San Martin et al. 2008), we used the PDGF-induced dephosphorylation of p-cofilin as a measure of SSH1L activity. Results of this experiment are shown in Figure 5.

Similar to previous results in untransfected cells (San Martin et al. 2008), in cells transfected with 14-3-3 wt, PDGF stimulated p-cofilin dephosphorylation. Cells transfected with 14-3-3 KE showed a similar response to PDGF (Figure 5). This result was unexpected based on our initial hypothesis, and repeated transfections provided similar results. We blotted with an antibody for 14-3-3γ to check for expression of 14-3-

3γ in the cells and to ensure transfection accuracy. The transfected proteins (14-3-3 wt or K49E) have a histidine tag, shifting the molecular weight of the protein and its band in the western blot. Presence of endogenous 14-3-3γ and transfected 14-3-3 (wt or KE) in all lanes indicated that the transfections were successful (Figure 5). Membranes were stripped with a commercial stripping solution and reblotted for total cofilin, to ensure that the amount of total cofilin was equivalent across all lanes (Figure 5). These findings led us to believe that the 14-3-3 KE mutants may not have been able to displace endogenously bound SSH1L.



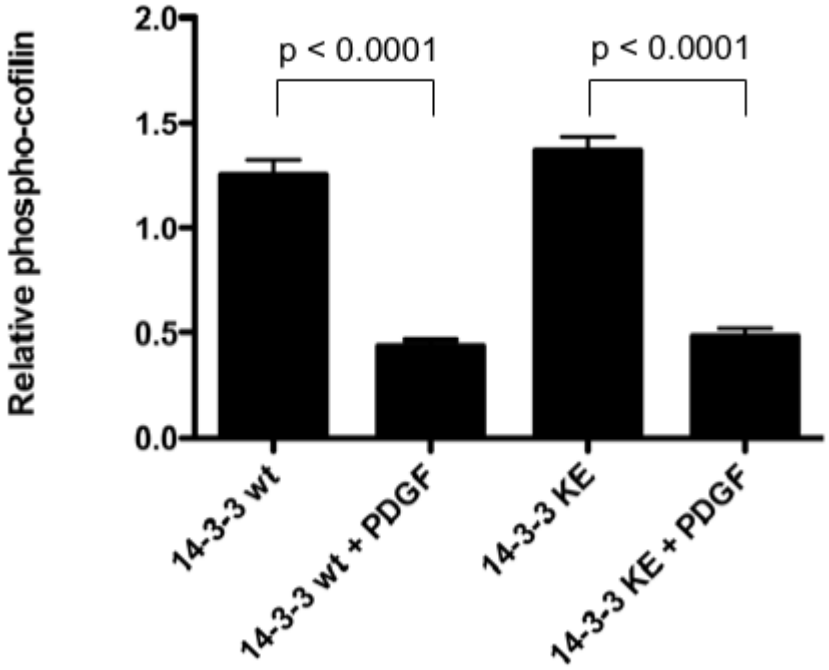


Figure 5. **VSMCs transfected with the 14-3-3 K49E mutant, in which the basic loop that recognizes Ser-phosphorylated motifs in partner proteins is altered.** Western blot using primary antibody against phospho-cofilin in cells transfected with 14-3-3 wt or 14-3-3 K49E mutant. Bars represent mean ± SEM average from three independent experiments. A value of $p<0.05$ was considered to be statistically significant.

Generation of SSH1L SA and SD Mutants

The above results confirm that SSH1L is phosphorylated at a 14-3-3 consensus sequence, but do not allow us to demonstrate the importance of this phosphorylation in 14-3-3/SSH1L interaction or SSH1L activation. We wanted to determine which serine residues on SSH1L are involved in this response and to use this information to answer the latter questions. With the help of Dr. Haian Fu, software analysis of SSH1L phosphorylation sites was performed to identify 14-3-3 binding motif sequences containing serines within the SSH1L protein. The Ser834 site (a phospho-Ser 14-3-3 binding motif) was selected as a primary site of investigation.

Site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) was then used to mutate SSH1L and generate a phosphomimetic mutation (Ser to Asp, SSH1L_S834D) and a phosphodeficient mutation (Ser to Ala, SSH1L_S834A) at position 834 (Figure 6). The mutations introduced created changes in amino acid side chain chemistry, and subsequently resulted in the generation of phosphorylation mimics (Figure 7). The serine to alanine mutation generates a phosphodeficient mutation, since the chemical structure of alanine resembles the dephosphorylated serine residue. The serine to aspartate mutation generates a phosphomimetic mutation, since the aspartate chemical structure resembles a phosphorylated serine residue.

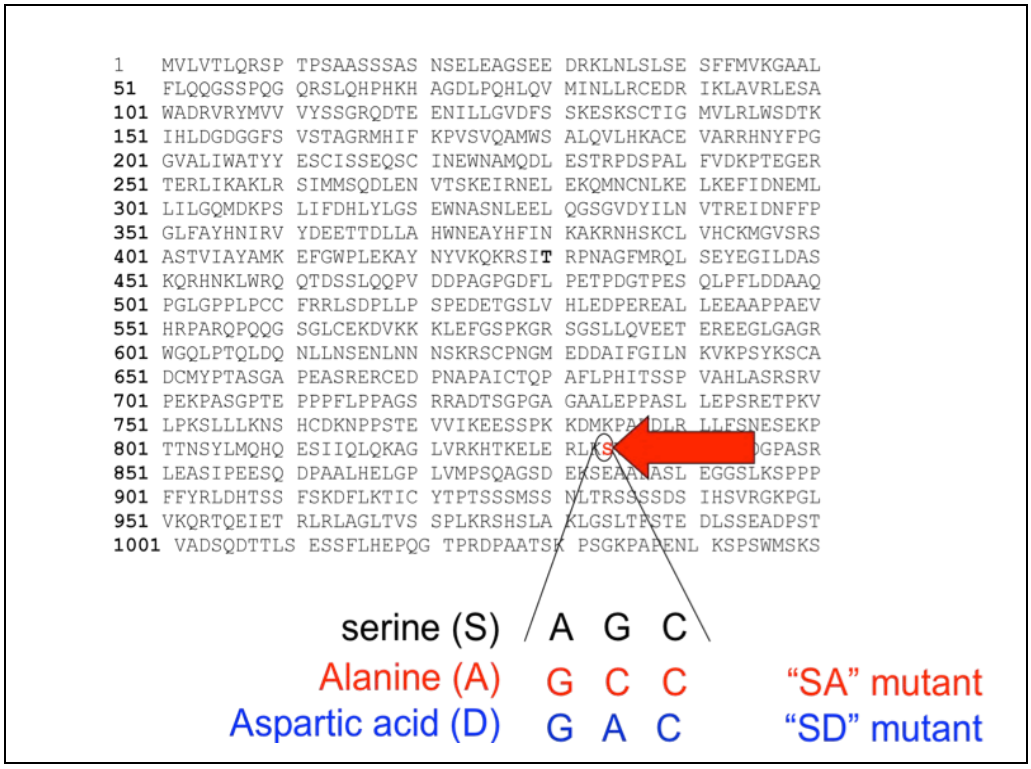


Figure 6. SSH1L protein. Ser834 (circled in red) is the Ser 14-3-3 binding motif within the SSH1L protein selected as the primary site of investigation. Mutations were introduced by site-directed mutagenesis.

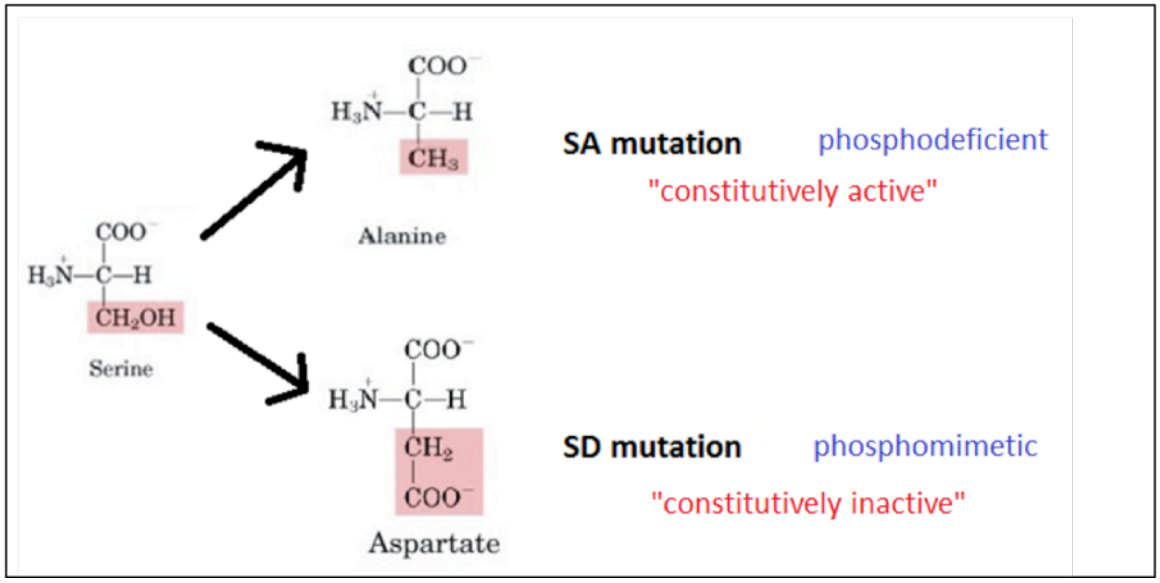


Figure 7. Generation of phosphodeficient (SA) and phosphomimetic (SD) mutations of the SSH1L protein, by changing amino acid side chain chemistry.
Amino acid figures obtained from: http://andromeda.rutgers.edu/~huskey/images/amino_acids.jpg

DNA from the SA and SD SSH1L mutant clones and wt SSH1L was digested with the Ear I restriction enzyme. This restriction enzyme recognizes and digests a site in the wt that is lost in the mutants (due to the nucleotide changes introduced), thus generating a specific band of 189 base pairs only in the wt. The pattern of digestion changed between wt and SA or SD mutants, with mutants lacking the 189 band present in the wild type. Successfully generated mutants were identified from the gels (Figure 8). For instance, the SA1, SA4, SA6, SA7, SA8, and SA9 lanes in the top gel contain successful mutants, with absence of the 189 band. Similarly for the SD gel, the SD1, SD2, SD3, SD5, SD8, and SD10 lanes contain successful mutants of interest.

wt SSH1L	S834A or S834D
877	877
210	210
189	
132	132
101	101
59	59

Expected size of Ear 1 digestion products

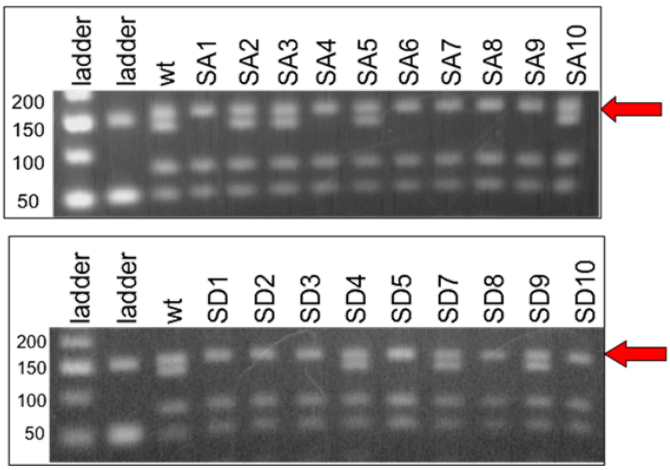


Figure 8. wt or SA and SD SSH1L mutants digested with Ear I restriction enzyme. In the top gel, the SA1, SA4, SA6, SA7, SA8, and SA9 lanes contain mutants of interest. In the bottom gel, the SD1, SD2, SD3, SD5, SD8, and SD10 lanes contain mutants of interest.

S834A-SSH1L acts as a Constitutively Active Enzyme

We next wanted to test the effects of these phosphomimetic and phosphodeficient SSH1L mutations in VSMCs, and decided to first test the phosphodeficient SA SSH1L mutants. We hypothesized that S834A-SSH1L would appear like its normal dephosphorylated, activated state, resulting in increased activity (increased p-cofilin dephosphorylation).

VSMC from mouse aorta were transfected with a GFP vector (control), wt SSH1L, and the S834A-SSH1L mutants (5 µg of DNA) by electroporation using a Nucleofector (Amaxa Biosystems). The cells were then incubated with either vehicle or PDGF (10 ng/mL for 15 min). A western blot was performed with a primary antibody against phospho-cofilin, again as a readout of SSH1L activity.

Results are shown in Figure 9. In the GFP vector control, PDGF stimulation resulted in a decrease in the amount of p-cofilin, indicating increased PDGF-induced SSH1L activity compared to non-stimulated cells. In the cells transfected with wt SSH1L, basal and PDGF-induced p-cofilin dephosphorylation was enhanced, as expected. Cells transfected with the S834A-SSH1L mutant (a phosphodeficient mutant that can no longer bind to the 14-3-3 regulatory protein) showed a partial dephosphorylation of cofilin even in the non-stimulated condition. Upon addition of PDGF, no further phospho-cofilin dephosphorylation is observed. These findings demonstrate that the S834A-SSH1L mutant acts as a constitutively active enzyme, and also implicates the phosphorylation of Serine 834 within SSH1L as one major component in the mechanism of regulation of SSH1L activity in VSMCs.

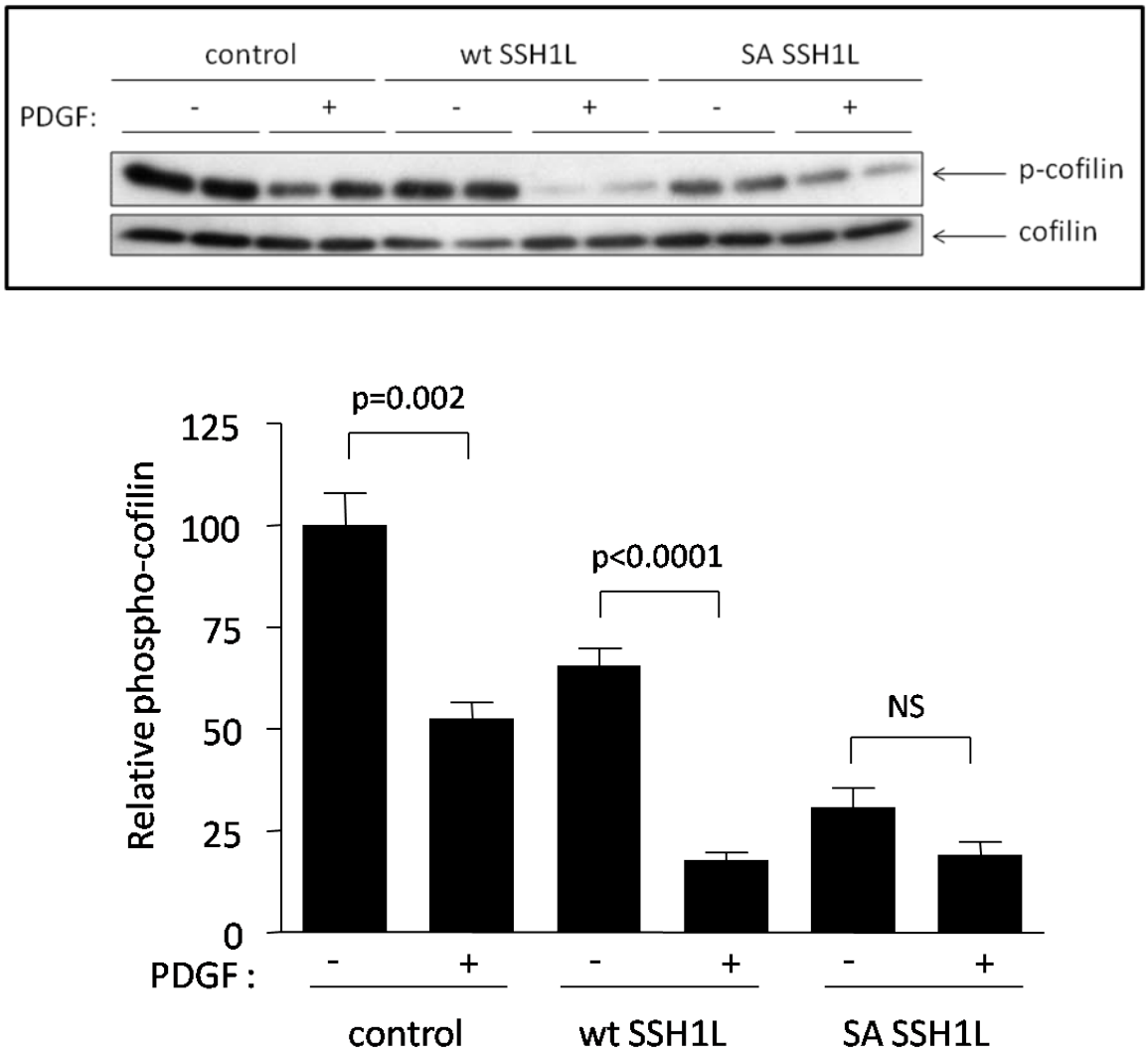


Figure 9. **SSH1L S834A mutant acts as a constitutively active enzyme.** Western blot using primary antibody against p-cofilin with transfected wt and SA SSH1L mutants. MASMC were transfected with GFP vector, wt SSH1L, or the SA SSH1L mutant by electroporation. Cells were then treated with vehicle or PDGF (10 ng/mL for 15 min). Bars represent mean ± SEM average from three independent experiments. A value of $p<0.05$ was considered to be statistically significant.

Migration Assay (Preliminary)

A migration assay was performed to test the physiological endpoint of the SSH1L S834A mutant and its ability to recover the Nox1 KO phenotype under PDGF-stimulated conditions. We have demonstrated that Nox1 KO cells migrate less than wt cells, since they cannot properly activate cofilin (Lee et al. 2009). We wanted to test whether the introduction of the constitutively active SSH1L S834A mutant by transfection into VSMCs could recover the Nox1 phenotype of impaired migration.

Results of the migration assay are shown in Figure 10. All VSMCs were stimulated with PDGF in the lower chamber of the modified Boyden chamber assay. The number of migrated cells per field decreased in wt cells transfected with SSH1L S834A compared to control wt cells. This result suggests that the wt cells stimulated with PDGF (control) are capable of activating SSH1L as required for migration. We believe the reduction in cell migration observed with addition of exogenous, constitutively active SSH1L S834A in the wt is due to an overactivity of SSH1L, which may be impairing cofilin and its ability to form lamellipodia, preventing proper migration.

As expected, Nox1 KO cells migrated less compared to wt cells stimulated with PDGF (Figure 10). Additionally, the number of migrated cells per field increased slightly in Nox1 KO cells transfected with SSH1L S834A compared to control Nox1 KO cells. This suggests the SSH1L S834A mutant is helpful in at least partially recovering the Nox1 KO phenotype of impaired migration. Refinement of conditions in the migration assay in this ongoing work in the future will hopefully reduce some of the variable results found in our initial experiments.

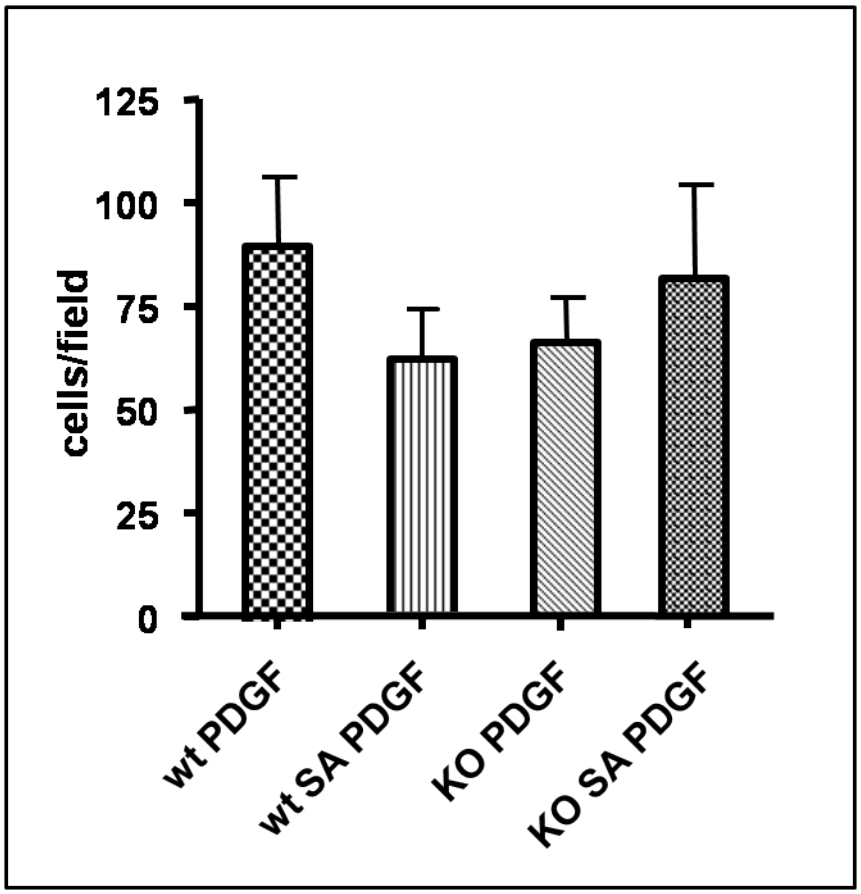


Figure 10. **The SSH1L S834A mutant is able to at least partially recover the Nox1 KO phenotype.** Wt and Nox1 KO VSMCs were transfected with the S834A SSH1L mutant and serum-starved for 48 hours. A modified Boyden chamber assay was used to measure directed cell migration. Cells were added to the upper chamber of the dish and exposed to PDGF in the lower chamber for 3 hours. Preliminary findings: 1 experiment with triplicate.

Discussion

The overall goal of this thesis was to investigate the specific mechanism of PDGF-induced SSH1L activation in vascular smooth muscle cells. Important experiments performed by Dr. Alejandra San Martín formed the basis for the development of experiments for this project. First, a SSH1L phosphatase activity assay was performed, demonstrating that SSH1L activity is increased in PDGF-stimulated wt VSMCs, and that this regulation is lost in Nox1 KO cells (Figure 1). Additionally, downregulation of SSH1L using directed siRNA showed that SSH1L is the cofilin phosphatase activated by PDGF in VSMCs (Figure 2). However, the mechanism by which PDGF activates SSH1L in these cells was unknown.

Because SSH1L has been shown to interact with 14-3-3 regulatory proteins in other cell systems, we hypothesized that 14-3-3 plays a role in SSH1L activation in vascular smooth muscle cells. The 14-3-3γ and β isoforms form a complex with SSH1L in VSMCs under basal conditions, and these complexes are disrupted by PDGF treatment as shown through co-immunoprecipitation followed by western blotting (Figures 3A and 3B). This supports our hypothesis that SSH1L forms an inhibitory complex with 14-3-3, which is disrupted by PDGF.

Next, we sought to examine the mechanism of activation of SSH1L through the disruption of the inhibitory SSH1L/14-3-3 complex. We found that PDGF stimulation dephosphorylates SSH1L at a phospho Ser within a 14-3-3 binding motif, in a process that requires Nox1 (Figure 4). This result confirmed our hypothesis that Nox1-mediated PDGF stimulation results in the dephosphorylation of SSH1L at a site that

binds 14-3-3. Next, we sought to use 14-3-3 K49E (KE) mutants to disrupt 14-3-3/SSH1L binding. Results did not follow what we predicted (Figure 5).

We hypothesized that 14-3-3 K49E mutants would bind to endogenous 14-3-3, blocking the SSH1L/14-3-3 complex formation. Since endogenous 14-3-3 would be unable to bind SSH1L (being bound to 14-3-3 K49E mutants), we expected the 14-3-3 K49E mutant would result in SSH1L activation. We anticipated an increase in p-cofilin dephosphorylation, and a corresponding decrease in the p-cofilin band, after transfection of 14-3-3 KE mutants alone. With 14-3-3 interaction with SSH1L blocked, we predicted that PDGF would not be able to further activate SSH1L, resulting in basal activity of SSH1L under control conditions to be equivalent to the one observed in PDGF-stimulated conditions, for the 14-3-3 KE transfected cells. These predictions did not match observed results, described earlier and shown in Figure 5.

After analyzing these results, we realized one plausible explanation for the lack of an effect in the 14-3-3 KE mutants. An equilibrium is presumably established in VSMC between the free endogenous 14-3-3 protein and the 14-3-3/SSH1L complex state (where 14-3-3 is bound in an inhibitory complex with SSH1L). We hypothesized the 14-3-3 KE transfections would cause 14-3-3 KE to bind and inactivate free endogenous 14-3-3, allowing for increased SSH1L activation. However, if the majority of endogenous 14-3-3 protein is already in complex formation with SSH1L and there is less free 14-3-3 available (in an unbalanced equilibrium), then the addition of 14-3-3 KE mutants may not affect system regulation anyway. We believe the PDGF-induced dephosphorylation of p-cofilin observed in the 14-3-3 KE transfected cells (Figure 5) matched the 14-3-3 wt transfected cell lanes because the 14-3-3 KE mutant was unable to compete with and

displace 14-3-3 wt (and consequently, unable to disrupt SSH1L/14-3-3 complex formation already present). We have designed an alternative experiment using the 14-3-3 KE mutant which will avoid the problem of endogenous 14-3-3 in the system. This experiment would involve co-transfection of 14-3-3 wt or KE with SSH1L, immunoprecipitating the transfected SSH1L protein (which has a tag), and then testing for presence of p-cofilin in the 14-3-3 wt versus 14-3-3 K49E samples. We predict that the 14-3-3 KE mutants co-transfected with SSH1L will be unable to bind SSH1L, allowing for increased p-cofilin dephosphorylation, compared to 14-3-3 wt co-transfected with SSH1L. Additionally, transfection experiments with the wt and 14-3-3 KE mutants will be performed with human embryonic kidney (HEK) cells, which have a high transfection efficiency rate, to test whether the effect of the KE is observed in these cells. Another option will be to investigate and utilize a 14-3-3γ knockout mouse model and transfect the wt or 14-3-3 KE mutant in KO cells derived from these mice, which also offers an avenue to avoid endogenous 14-3-3 protein (which can undergo complex formation with SSH1L prior to transfection). The next, future directions for this research project to be continued by Dr. San Martín will involve performing these co-transfection experiments.

Nevertheless, the dephosphorylation of SSH1L at 14-3-3 binding motifs by PDGF (Figure 4) supports our hypothesis that serine dephosphorylation of SSH1L (at 14-3-3 binding motifs) results in 14-3-3/SSH1L inhibitory complex disruption and SSH1L activation. To further characterize the activation of SSH1L, we generated and expressed phosphomimetic (SD) and phosphodeficient (SA) mutations in SSH1L at the 834 position. We found that S834A-SSH1L acted as a constitutively active form of the

enzyme. Cells transfected with this SA mutant (a phosphodeficient form) can no longer bind to 14-3-3 regulatory proteins, resulting in dephosphorylation of cofilin in the non-stimulated condition. This partial dephosphorylation in the non-stimulated condition with S834A-SSH1L explains why a further effect of PDGF on the amount of phospho-cofilin is not observed in the SA mutants (Figure 9).

To test the physiological relevance and effects of the SSH1L S834A mutants, we performed a migration assay in wt and Nox1 KO cells. We wanted to see whether the constitutively active SSH1L S834A mutant, introduced as an exogenous source by transfection, could recover the Nox1 KO phenotype under PDGF-stimulated conditions. We found that the SSH1L S834A mutant was able to partially recover the Nox1 KO phenotype (Figure 10), and possible explanations for why only partial recovery was observed are described in the limitations section below. Importantly, these results implicate a particular serine residue (Ser 834) in the 14-3-3 binding motif of the SSH1L protein which is likely involved in 14-3-3/SSH1L complex disruption, consequent SSH1L activation by PDGF in VSMC, and subsequent migration.

Limitations

One major limitation in this project was the fact that the 14-3-3 KE mutants transfected in the VSMCs appeared to have no major effect compared to the 14-3-3 wt transfected cells. As discussed above, this is perhaps due to a disequilibrium between the free endogenous 14-3-3 and bound 14-3-3 (in 14-3-3/SSH1L complex). Additional experiments to try to elucidate this unclear finding will be performed.

Additionally, there were several unexpected findings from the migration assay which require further investigation. The number of migrated cells per field decreased in the wt cells transfected with S834A SSH1L compared to wt control. As discussed earlier, perhaps the transfection of S834A SSH1L into wt cells resulted in an overactivity of SSH1L, which prevented its proper functioning. The migration assay will be repeated, with a decrease in the amount of plasmid DNA transfected, in an effort to prevent disrupted migration in S834A SSH1L transfected wt cells. We anticipate that the amount of migration in wt control cells versus wt S834A SSH1L transfected cells should be similar. Another limitation involves the only partial recovery of the Nox1 KO phenotype in KO cells transfected with S834A SSH1L compared to KO cells. This ongoing work will involve future repetitions of the migration assay using different conditions and also the use of less plasmid DNA vector in efforts to increase viability of the assay.

Future Directions

Alternative experiments using the 14-3-3 KE mutant have been designed to avoid the problem of endogenous 14-3-3 effects in the system. Demonstrating an effect of the 14-3-3 KE mutant will provide further evidence that 14-3-3 binding to SSH1L regulates its activation, and that the partnership between SSH1L and 14-3-3 is based on the recognition of specific phosphorylated motifs. Additionally, experiments will be performed to repeat the migration assay under different experimental conditions (cell confluency, amount of plasmid DNA transfected, amount of collagen for the migration assay) in efforts to confirm or refute the ability of the SSH1L S834A mutant to recover

the Nox1 phenotype. Since SSH1L activation is implicated in migration which leads to cardiovascular pathologies such as atherosclerosis, experiments examining the role of the SSH1L mutants in migration are important to dissect the underlying mechanisms.

Future experiments will investigate PDGF-induced dephosphorylation of p-Ser 14-3-3 binding motifs in the SSH1L mutants and the effects of both SA and SD SSH1L mutants on 14-3-3/SSH1L association. Additionally, experiments will be performed to examine p-cofilin dephosphorylation in cells transfected with wt SSH1L versus the SD SSH1L mutants. There are some long-term research goals for the San Martín and Griendling laboratories involving this research as well, which include identifying the upstream protein phosphatase responsible for PDGF-mediated SSH1L dephosphorylation and elucidating whether the mutants alter SSH1L activity by affecting its cellular localization.

Conclusions

Our results establish that PDGF-induced SSH1L activation is regulated by Nox1-dependent disruption of the SSH1L/14-3-3 complex via dephosphorylation of SSH1L at particular serine residues. 14-3-3γ and 14-3-3β form a complex with SSH1L in VSMCs, which is disrupted by PDGF treatment. Stimulation by PDGF dephosphorylates SSH1L at a phospho Ser 14-3-3 binding motif, in a Nox1-dependent manner. Cells transfected with the SSH1L S834A phosphodeficient mutant had decreased amounts of p-cofilin (increased cofilin dephosphorylation) in non-stimulated conditions, and similar amounts in PDGF-stimulated conditions, demonstrating an inability for PDGF to further regulate

the system. To evaluate the biological relevance of these studies, we investigated the effects of S834A SSH1L mutants in vascular migration, with initial findings suggesting the S834A SSH1L mutant is able to partially recover the Nox1 phenotype. The ultimate goal will be to elucidate further the mechanism of SSH1L activation and determine if the identified pathway of SSH1L activation (involving SSH1L/14-3-3 complex disruption) results in VSMC migration dysregulation, and its consequent related pathologies including neointima formation and atherosclerosis.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D. Molecular Biology of the Cell. Fourth Edition. Garland Science, Taylor & Francis Group. New York, 2002: p. 908, 931, 973-975.

Andrae, J., Gallini, R., Betsholtz, C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. (2008). 22(10):1276-312.

Autieri, M.V., Carbone, C.J. 14-3-3 Gamma interacts with and is phosphorylated by multiple protein kinase C isoforms in PDGF-stimulated human vascular smooth muscle cells. DNA Cell Biol. (1999). 18(7): 555-64.

Autieri, M.V., Haines, D.S., Romanic, A.M., Ohlstein, E.H. Expression of 14-3-3 gamma in injured arteries and growth factor- and cytokine-stimulated human vascular smooth muscle cells. Cell Growth Differ. (1996). 7(11): 1453-60.

Bamburg, J.R., McGough, A., Ono, S. Putting a new twist on actin: ADF/cofilins modulate actin dynamics. Trends Cell Biol. (1999). 9(9): 364-70.

Bamburg, J.R. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Annu Rev Cell Dev Biol. (1999). 15: 185-230.

Brandes, R.P., Kreuzer, J. Vascular NADPH oxidases: molecular mechanisms of activation. Cardiovasc. Res. (2005). 65(1):16-27.

Brandes R.P., Viedt, C., Nguyen, K., Beer, S., Kreuzer, J., Busse, R., Gorlach, A. Thrombin-induced MCP-1 expression involves activation of the p22phox-containing NADPH oxidase in human vascular smooth muscle cells. Thromb Haemost. (2001). 85: 1104–10.

Bridges, D., Moorhead, G.B. 14-3-3 proteins: a number of functions for a numbered protein. Sci STKE. (2005). (296):re10.

Brown, D.I., Griendling, K.K. Nox proteins in signal transduction. Free Radic Biol Med. (2009). 47(9):1239-53.

Chen, H., Bernstein, B.W., Bamburg, J.R. Regulating actin-filament dynamics in vivo. Trends Biochem Sci. (2000). 25(1): 19-23.

Claesson-Welsh, L. Mechanism of action of platelet-derived growth factor. Int J Biochem Cell Biol. (1996). 28(4): 373-85.

Clowes, A.W. Regulation of smooth muscle cell proliferation and migration. Transplant Proc. (1999). 31: 810–11.

Doran, A.C., Meller, N., McNamara, C.A. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Atherioscler Thromb Vasc Biol. (2008). 28(5): 812-9.

Ellis, J.J., Valencia, T.G., Zeng, H., Roberts, L.D., Deaton, R.A., Grant, S.R. CaM kinase IIdeltaC phosphorylation of 14-3-3beta in vascular smooth muscle cells: activation of class II HDAC repression. Mol Cell Biochem. (2003). 242: 153-161.

Gerthoffer, W.T. Mechanisms of vascular smooth muscle cell migration. Circ. Res. (2007). 100(5): 607-21.

Heistad D.D. Oxidative stress and vascular disease: 2005 Duff lecture. Arterioscler Thromb Vasc Biol. (2006). 26(4):689-95.

Huang, T.Y., DerMardirossian, C., Bokoch, G.M. Cofilin phosphatases and regulation of actin dynamics. Curr Opin Cell Biol. (2006). 18(1):26-31.

Johnsson, A., Heldin, C.H., Westermark, B., Wasteson, A. Platelet-derived growth factor: Identification of constituent polypeptide chains. Biochem. Biophys. Res. Commun. (1982). 104: 66–74.

Lassègue, B., Griendling, K.K. NADPH Oxidases: Functions and Pathologies in the Vasculature. Arterioscler Thromb Vasc Biol. (2009). [Epub ahead of print].

Lauffenburger, D.A., Horwitz, A.F. Cell migration: a physically integrated molecular Process. Cell. (1996). 84(3): 359-69.

Lee, M.Y., San Martin, A., Mehta, P.K., Dikalova, A.E., Garrido, A.M., Datla, S.R., Lyons, E., Krause, K.H., Banfi, B., Lambeth, J.D., Lassègue, B., Griendling, K.K. Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury induced neointimal formation. Arterioscler Thromb Vasc Biol. (2009). 29(4):480-7.

Lyle, A.N., Griendling, K.K. Modulation of vascular smooth muscle signaling by reactive oxygen species. Physiology. (2006). 21: 269-80.

Majesky, M.W., Daemen, M.J., Schwartz, S.M. α 1-adrenergic stimulation of platelet-derived growth factor A-chain gene expression in rat aorta. J. Biol. Chem. (1990). 265: 1082–1088.

Marieb, E.N., Hoehn, K. Human Anatomy & Physiology. Seventh Edition. Pearson, Benjamin Cummings. New York, 2006.

Mondy, J.S., Lindner, V., Miyashiro, J.K., Berk, B.C., Dean, R.H., and Geary, R.L. Platelet-derived growth factor ligand and receptor expression in response to altered blood flow in vivo. Circ. Res. (1997). 81: 320–327.

Nagata-Ohashi, K., Ohta, Y., Goto, K., Chiba, S., Mori, R., Nishita, M., Ohashi, K., Kousaka, K., Iwamatsu, A., Niwa, R., Uemura, T., Mizuno, K. A pathway of neuregulin-induced activation of cofilin phosphatase Slingshot and cofilin in lamellipodia. J Cell Biol. (2004). 165(4): 465-71.

Negoro, N., Kanayama, Y., Haraguchi, M., Umetani, N., Nishimura, M., Konishi, Y., Iwai, J., Okamura, M., Inoue, T., Takeda, T. Blood pressure regulates platelet-derivedgrowth factor A-chain gene expression in vascular smooth muscle cells in vivo. An autocrine mechanism promoting hypertensive vascular hypertrophy. J. Clin. Invest. (1995). 95: 1140–1150.

Ohmi, K., Masuda, T., Yamaguchi, H., Sakurai, T., Kudo, Y., Katsuki, M., Nonomura, Y. A novel aortic smooth muscle cell line obtained from p53 knock out mice expresses several differentiation characteristics. Biochem Biophys Res Commun. (1997). 238: 154-8.

Orlandi, A., Bennett, M. In press. Progenitor cell-derived smooth muscle cells in vascular disease. (2010). Biochem Pharmacol, doi: 10.1016/j.bcp.2010.01.027

Raines, E.W. PDGF and cardiovascular disease. Cytokine Growth Factor Rev. (2004). 15: 237–254.

Rosamond, W., Flegal, K., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S.M., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C., Roger, V., Sorlie, P., Steinberger, J., Thom, T., Wilson, M., Hong, Y. (2008). Heart disease and stroke statistics – 2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circ. Res. 117: 25-146.

San Martin, A., Griendling, K.K. Redox control of vascular smooth muscle migration. Antioxid Redox Signal. (2010). 12(5): 625-40.

San Martin, A., Lee, M. Y., Williams, H. C., Mizuno, K., Lassegue, B., Griendling, K. K. Dual regulation of cofilin activity by LIM kinase and Slingshot-1L phosphatase controls platelet-derived growth factor-induced migration of human aortic smooth muscle cells. (2008). Circ. Res. 102: 432–8.

Schwartz, S.M. Perspectives series: cell adhesion in vascular biology. Smooth muscle migration in atherosclerosis and restenosis. (1997). J Clin Invest. 99(12): 2814-16.

Selemidis, S., Sobey, C.G., Wingler, K., Schmidt, H.H., Drummond, G.R. NADPH oxidases in the vasculature: molecular features, roles in disease and pharmacological inhibition. (2008). Pharmacol Ther. 120(3):254-91.

Shimokado, K., Higaki, M. Signal transduction for PDGF-induced chemotaxis of vascular smooth muscle cells. (1997). Ann N Y Acad Sci. 811:130-3.

Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K., Finkel, T. Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. Science. (1995). 270: 296–299.

Touyz, R.M., Schiffrin, E.L. Reactive oxygen species in vascular biology: implications in hypertension. Histochem Cell Biol. (2004). 122(4): 339-52.

Tzivion, G., Avruch, J. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem. (2002). 277(5): 3061-4.

Weber, D.S., Taniyama, Y., Rocic, P., Seshiah, P.N., Dechert, M.A., Gerthoffer, W.T., Griendling, K.K. Phosphoinositidedependent kinase 1 and p21-activated protein kinase mediate reactive oxygen species-dependent regulation of platelet-derived growth factor-induced smooth muscle cell migration. Circ Res. (2004). 94: 1219–26.

Yaffe, M.B. How do 14-3-3 proteins work? – Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett. (2002). 513(1): 53-7.

Ying, J., Clavreul, N., Sethuraman, M., Adachi, T., Cohen, R.A. Thiol oxidation in signaling and response to stress: detection and quantification of physiological and pathophysiological thiol modifications. Free Radic Biol Med. (2007). 43: 1099–1108.