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Shear Stress Induced mTOR Signaling in Cultured A7r5 Aortic Smooth Muscle Cell Line

Sriram Prasad Mupparaju
msriramprasad@yahoo.com

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By
Sriram Prasad Mupparaju

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Committee:

_____ Dr. Eric R Blough, Thesis adviser

_____ Dr. David Mallory, Committee member

_____ Dr. Simon collier, Committee member

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Abstract

Vascular intervention procedures such as balloon angioplasty and stent implantation lead to denudation of endothelium exposing the underlying smooth muscle layer to effects of blood shear stress. The effect of shear stress on smooth muscle cell signaling has not been studied in depth. In this study, we examined time dependent changes in activation of Akt, mTOR and p70S6k -related signaling in response to orbital shear stress on cultured A7r5 cells. A7r5 cells cultured to sub-confluence on 150mm culture dishes were subjected to orbital shear of 9.8dynes/cm^2 for zero, 5 min, 15 min, 30 min, 1h, 4h and 24h. The cells were found to be reoriented with shear. Immunoblotting demonstrated increased phosphorylation of mTOR/p70s6k- related signaling proteins, suggesting an association between mTOR/p70s6k related signaling and shear induced reorientation of vascular smooth muscle cells. These data may provide insight into possible pharmacological interventions to prevent or retard the development of restenosis. (149 words)

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LIST OF SYMBOLS

AAALAC	Association for assessment and accreditation of laboratory animal care
ANOVA	One-way analysis of variance on ranks
BSA	Bovine serum albumin
CHD	Coronary heart disease
CVD	Cardio vascular disease
DMEM	Dulbecco's modified Eagle medium
EC	Endothelial cells
ECL	Enhanced chemiluminiscence
ECM	Extra cellular matrix
KRB	Krebs-Ringers Buffer Solution
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.5% tween
PI3K	Phosphoinositide 3-kinases
PTCA	Percutaneous transluminal coronary angioplasty
PTEN	Phosphotase and tensin homologue deleted on chromosome ten
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.5% tween
VSMC	Vascular smooth muscle cells

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Chapter 1

INTRODUCTION

Coronary heart disease (CHD) is the major cause of morbidity and mortality in the United States causing 1 in every 5 deaths in the year 2000 [1]. The health care costs associated with CHD are enormous as it is estimated that 151.6 billion will be spent on the treatment of disorder in 2007 alone [2]. The factors which regulate the progression of vascular disease are not well understood however blood flow disturbances such as nonuniform laminar flow and altered gradients in wall shear stress have been postulated to be involved [3]. Indeed, recent data has suggested that—hemodynamic forces may act as important modulators of vascular cell morphology and function. One such condition that may be associated with alterations in vascular flow is intimal hyperplasia, an important clinical problem associated with most vascular interventions. Intimal hyperplasia is characterized by increased vascular smooth muscle cell (VSMC) proliferation, migration of VSMC to the intima, and the synthesis of extra cellular matrix (ECM) after injury [4] which if allowed to proceed unchecked can lead to restenosis, a condition seen in approximately 40% of coronary balloon angioplasties and arterial bypass grafts [5].

The initiating event(s) causing intimal hyperplasia are not well understood however alterations in vascular shear stress have been postulated to be a contributory factor. Shear stress, has been shown to alter the function of endothelial cells (EC) and induce the secretion of various bioactive molecules [6-8], cell proliferation [9], and the rearrangement of the cell cytoskeleton [10, 11]. How VSMC respond to shear stress is not well studied.

It is well established that the different functions VSMC can perform translate into a diversity of VSMC phenotypes, ranging from the contractile to the synthetic type. Although part

of the variation in VSMC populations can be explained by the diverse embryological origins of VSMC, it is thought that phenotypic transitions between cell types is governed by the activation of intracellular signaling pathways [12]. One protein that may be involved in regulating VSMC phenotype is the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription [13, 14]. The p70s6k, a member of ribosomal s6 kinase family of proteins, is believed to function downstream of mTOR and is postulated to have a role in controlling cell growth through regulating ribosome biogenesis [15-17] and the expression of several cell cycle proteins [18]. Similar to mTOR and p70S6k, Akt or protein kinase B is another important molecule in mammalian cellular signaling that has regulatory functions in cell survival, metabolism and angiogenesis [19, 20]. Recent data suggest that Akt lies upstream of both mTOR and p70s6k and that the sequential activation of these proteins regulates the VSMC phenotypic modulation that is associated with proliferative pathologies like restenosis [4, 18, 21-28]. How shear stress may influence the regulation of Akt / mTOR / p70S6k signaling in VSMC has not been investigated.

Purpose:

The purpose of this study was to determine whether shear stress can alter the orientation and morphology of VSMC and examine the mTOR-mediated p70S6k regulation. Given the role that mTOR and p70S6k may play in regulating VSMC growth and proliferation, and on the basis of previous shear studies demonstrating the ability of shear stress to influence VSMC phenotype, we hypothesized that shear stress would be a powerful activator of mTOR-p70s6k signaling. To test this hypothesis, we examined the effects of shear stress on the phosphorylation of p70S6k and several of its potential upstream and parallel activators in VSMC subjected to various durations of shear stress.

Specific Aim #1:

To determine how shear stress alters vascular smooth muscle cell morphology and orientation.

Hypothesis:

Shear stress will alter vascular smooth muscle cell orientation.

Specific Aim#2:

To determine how shear stress alters mTOR / p70S6k signaling in vascular smooth muscle cells.

Hypothesis:

Shear stress will be a potent stimulus for the activation of mTOR and p70s6k related signal transduction cascades in the vascular smooth muscle cells.

Chapter 2

REVIEW OF LITERATURE

Introduction

A review of the pertinent literature concerning the present study will be presented in the following chapter. The following areas will be addressed: 1) Coronary heart disease 2) Vascular pathophysiology and 3) p70s6k pathway related proteins and p70S6k signaling as a regulator of vascular smooth muscle cell phenotype.

Coronary Heart Disease

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in the United States causing 1 in every 5 deaths in the year 2000 [1]. The monetary costs associated with CVD are staggering with an estimated $151.6 billion in direct and indirect costs in 2007 alone [2]. Intimal lipid accumulation, hyperplasia, and scarring are stigmata of atherosclerotic vascular disease, whose major complications—myocardial ischemia and infarction—continue to be major health problems in developed nations [29]. These lesions of vascular disease typically show nonrandom distribution within the arterial vasculature and are associated with blood flow disturbances such as nonuniform laminar flow and altered gradients in wall shear stress [3]. As a result, hemodynamic forces are recognized as important modulators of vascular cell morphology and function. Imbalance of these forces may lead to intimal hyperplasia which is an important clinical problem associated with most vascular interventions. Intimal hyperplasia is characterized by increased vascular smooth muscle cell (VSMC) proliferation, migration to the intima, and

synthesis of extra cellular matrix (ECM) [4]. Hyperplasia refers to the proliferation of cells within an organ or tissue beyond that which is ordinarily seen. Intimal hyperplasia is thought to be a major contributing factor in the development of restenosis (narrowing of the vascular lumen) a post-surgical complication observed in up to 40% of coronary balloon angioplasties and arterial bypass graft procedures [5]. The factors causing intimal hyperplasia are not known but alterations in vessel flow patterns have been implicated. One such stimulus may be the development of alterations in vessel shear stress.

Shear stress is a function of blood flow, vessel geometry and fluid viscosity which can be estimated using fluid dynamics [30]. It is thought that understanding the physiological and biochemical signaling associated with vascular shear stress may provide insight into the potential role that shear stress may play in pathophysiological alterations. How alterations in shear stress may influence vascular signaling is not well understood.

Vascular Pathophysiology

Vascular endothelial cells and vascular smooth muscle cells (VSMC) are the major cellular components of the vessel wall. Vascular smooth muscle cells refer to the particular type of smooth muscle cells found within, and composing the majority of the wall of blood vessels. The interactions between these cells play a significant role in defining the structure and function of the blood vessel. There are two different phenotypes of VSMC [31]. It has been suggested that a phenotypic modulation of VSMC occurs before VSMC division [32, 33]. During most of the fetal and early postnatal periods, the medial layer of the artery is made up of VSMC of the synthetic phenotype, characterized by extensive rough endoplasmic reticulum, a prominent golgi apparatus, and only a few myofilaments. These cells proliferate and secrete extra cellular matrix.

During development, the arterial VSMC shift from a synthetic to contractile phenotype. The cell in the contractile phenotype is quiescent with numerous myofilaments, and it provides both vasomotion and structural support to the vessel. Studies in animal models have shown that the VSMC are able to shift back to the synthetic phenotype during the process of atherogenesis [34, 35] and intimal thickening after endothelial injury [36]. The factor(s) which govern this de-differentiation are not well understood however it has been shown that shear stress is capable of altering the phenotype of some vascular cell types. For example, in areas of uniform laminar flow, endothelial cells exhibit an ellipsoidal cell shape and alignment in the direction of flow [37], whereas in regions of disturbed flow, this orderly pattern is disrupted [38]. It has been shown that cyclic strain induces endothelial cells to proliferate, elongate, and align perpendicularly to the vector force [39]. Cell reorientation may, therefore, be an early event in the phenotypic modulation. Whether VSMC exhibit a similar response to mechanical forces, such as when they are directly exposed to alterations in shear stress following endothelial injury is not known.

The control of shear stress levels is critical to maintaining normal physiologic vascular function as it plays a key role regulating vasculature pressure and flow. In endothelium-preserved lesions, shear stress has been shown to exert an inhibitory effect on intimal thickening [40] and to suppress VSMC proliferation via endothelial cell–derived inhibitory factors [41]. In contrast, nonlaminar shear stress has been shown to stimulate VSMC proliferation and migration *in vivo*, especially after endothelial denuding injury [42]. With the endothelium denuded, the endothelial derived inhibitory factors no longer exist and this could contribute to the VSMC proliferation and migration. These data are important as vascular restenosis after percutaneous balloon angioplasty of atherosclerotic arteries is primarily mediated by intimal hyperplasia due to

proliferation of VSMC [43]. Therefore, it is of interest to know if direct blood flow mechanics could stimulate intimal hyperplasia in the exposed VSMC at sites of endothelial damage.

Although the exact mechanisms that lead to an increased number of intimal VSMC in atherosclerotic lesions and restenosis remain largely unknown, the contribution of early migration and proliferation of medial VSMC has been suggested [44-46]. Work by numerous laboratories has demonstrated a variety of changes in the metabolic and synthetic activities of endothelial cells in response to defined flow stimulation, including the production of prostacyclin, nitric oxide, cytokines, growth factors, ECM components and vasoactive mediators [47-50]. These changes are critically discussed in the perspective of vascular geometry and associated flow alterations that modify the endothelial function and subsequent VSMC response. For the most part, these studies examined VSMC responses that were dependent on a functional endothelium. The mechanisms underlying VSMC migration, proliferation, and mechanotransduction in response to hemodynamic stress in the absence of the endothelial layer are not well understood.

Summary

CDC statistics indicate that heart diseases and particularly coronary heart disease is the leading cause of deaths in the United States. Interactions of the cellular components of the vessel wall influenced by the blood flow mechanics and shear stress play a major role in the pathophysiology of vascular diseases. Pathophysiological conditions such as hypertension, atherosclerosis, restenosis following vascular interventional procedures like percutaneous transluminal coronary angioplasty (PTCA) and vein grafts induce undesirable vascular smooth muscle cell growth, leading to further complications of the disease. The molecular mechanisms

underlying these changes are not fully understood. Intimal hyperplasia with proliferation of either EC or migration and proliferation of VSMC is thought to play a critical role in these lesions.

p70s6k pathway related proteins and regulation of Vascular Smooth Muscle Cells

The entry of vascular cells into the cell cycle plays an important role in the pathogenesis of proliferative vascular diseases, restenosis, transplant vasculopathy, vein graft disease, and primary atherosclerosis [51, 52]. Cellular proliferation involves changes not only in the level of gene transcription but also in the rate of protein synthesis. The p70s6k, is a serine/threonine kinase that is believed to function in the regulation of protein translation [15, 16]. The p70s6k along with Akt was found to upregulate the expression of several cell cycle proteins in coronary smooth muscle cells after both growth factor stimulation and balloon injury, consistent with a role in restenosis [18]. The primary structure of p70s6k consists of four functional domains or modules. Module 1 extends from the N-terminus to the beginning of the catalytic domain, and confers rapamycin sensitivity to p70s6k [53, 54]. Module II contains the conserved catalytic domain, while the module III links the catalytic domain with the carboxyl tail [55]. Module III also contains two additional sites of acute phosphorylation and is conserved in many members of the second messenger family of Ser/Thr kinases [56]. The module IV contains the putative auto-inhibitory domain, which has significant homology with substrate region of s6, and four closely clustered phosphorylation sites [57]. The activation of p70s6k occurs in a hierarchical fashion through the sequential phosphorylation of each module. It is thought that p70s6k accelerate translation of mRNAs containing a terminal oligopolypyrimidine (TOP) track at the 5' end. This

is important as the regulation of TOP containing proteins has been postulated to be a rate-limiting step in protein synthesis [58].

The mammalian target of rapamycin (mTOR) functions as a growth factor and nutrient-sensing signaling molecule in mammalian cells. mTOR acts upstream of p70s6k and is thought to be responsible for regulating a number of proteins involved in controlling protein translation [59]. Indeed, p70S6k phosphorylation by mTOR activates p70s6k which in turn results in the phosphorylation of the 40S ribosomal protein S6 [60, 61]. Although the exact signaling pathways upstream of mTOR are incompletely resolved, insulin and activated Akt were shown to induce phosphorylation of mTOR in both *in vitro* and *in vivo* experiments [62-66]. How mTOR may function in the adaptation of VSMC to shear stress has not been investigated.

Akt or protein Kinase B is a serine/threonine kinase that plays an important role in executing multiple cellular metabolic pathways such as cell metabolism, cell survival and cell proliferation. Akt is activated in response to many different growth factors, including insulin and IGF-1 [67]. Akt is important for mediating the effects of these growth factors on the control of mammalian cell cycle progression and cell survival, as well as on the regulation of processes that influence growth, including protein synthesis and glucose metabolism. Akt is activated in a phosphoinositide 3-kinases (PI3K) dependent manner and is regulated by phosphorylation on Thr ^{308} and Ser ^{473}. The PI3K is a family of related enzymes that are capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol [68]. PI 3-kinases have been linked to an extraordinarily diverse group of cellular functions, including cell growth, proliferation, differentiation, cell motility, survival and intracellular trafficking. Many of these functions relate to the ability of PI 3-kinases to activate Akt. Previous work has shown that VSMC directly respond to oscillatory shear stress by increasing DNA synthesis, proliferation,

and activation of the PI3K/Akt signal transduction pathway indicating a mechanism of VSMC survival and proliferation. Whether PI3K/Akt signaling is involved in regulating the adaptation of VSMC to shear stress is not well understood.

Huang (2002) examining the role of PTEN (Phosphotase and tensin homologue deleted on chromosome ten) in VSMC proliferation and migration demonstrated that PTEN over-expression potently inhibits neointimal hyperplasia and restenosis [69]. PTEN functions as a phosphatidylinositol 3'-phosphatase to hydrolyze the lipid products of PI3K [70, 71], promote cell cycle arrest by down regulating PI3K/Akt signaling [72-74] and is inactive when phosphorylated [75, 76]. Therefore examining the shear induced regulation of PTEN in VSMC would aid in better understanding of the proliferative abnormalities of the vessel wall.

Summary

Intimal hyperplasia with proliferation of either endothelial cell or VSMC migration and proliferation is thought to play a critical role in restenotic lesions. Research to date has suggested that the Akt/mTOR/p70s6k signaling pathway may play a critical role in regulating VSMC cell growth, proliferation, and survival. If or how these molecules and pathways may regulated the phenotypic adaptation of VSMC to shear stress is not well understood. These findings of such a connection, if present, will provide new insight into possible pharmacological interventions to treat vascular diseases in general and restenotic lesions in particular.

Chapter 3

Shear Stress Induced mTOR Signaling in Cultured A7r5 Aortic Smooth Muscle Cell Line

INTRODUCTION

Vascular interventions such as coronary angioplasty and stent-implantation cause damage to the vascular endothelium, exposing the intima directly to the mechanical effects of blood flow. This alterations in blood flow mechanisms can lead to intimal hyperplasia, which results in restenosis in a vast majority of individuals undergoing such interventions. Restenotic lesions are generally more severe and difficult to excise than atherosclerotic plaques, and may lead to death or morbidity in the absence of timely re-intervention. Intimal hyperplasia is thought to be a response to mechanical injury and exposure to alterations in blood flow such as shear stress, circulating agonists and/or growth factors.

Vascular smooth muscle cells (VSMC) are not exposed to such stimuli under normal conditions and they maintain a differentiated and contractile phenotype. It is well known that vessel injury due to vessel stretch or excessive denudation of the vessel endothelium induces the quiescent VSMC to dedifferentiate and adapt a synthetic, migratory and proliferative phenotype, which if allowed to proceed unchecked can lead to VSMC hyperplasia and reocclusion of the vessel. A better understanding of the stimuli and signaling mechanisms involved in the phenotypic modification of VSMC may help identify pharmacological targets and aid in preventing or slowing down the progression on restenosis.

Shear stress has been postulated to play an important role in regulating VSMC proliferation, growth and function [77, 78]. Laminar shear stress inhibits VSMC proliferation [79] while conversely, shear stress in response to turbulent flow is associated with VSMC proliferation [42]. Fluid shear stress *in vitro* has also been found to induce VSMC reorientation [80]. In pathophysiological settings, shear stress is thought to play a role in the VSMC migration that contribute to vessel occlusion [81, 82]. However, the precise signaling pathways that

mediate vascular remodeling in response to shear stress remain largely unclear. Recent studies have suggested that VSMC growth and differentiation may be partially regulated by mammalian target of rapamycin (mTOR) and 70kDa ribosomal S6-kinase (p70S6k) [4, 83-85]. It is thought that these proteins play a critical role in the translation of proteins involved in the regulation of cell cycle progression and protein translation [60, 86].

The factors regulating mTOR and p70S6k phosphorylation have not been fully defined however, previous work has shown that mechanical factors alter their expression and activation [87-90]. Rapamycin inhibition of mTOR leads to differentiation of cultured synthetic VSMC from multiple species along with cell cycle withdrawal, altered gene expression and decreases in protein synthesis [4]. The decrease in protein synthesis was found to take place via inactivation of p70s6k. We and others have shown activation of p70s6k in aortic VSMC following application of mechanical stretch [25]. Fluid shear stress has also been found to regulate p70s6k activity in primary cultures of human umbilical vein endothelial cells [91], while Hornberger and colleagues reported that mechanical stimuli can elicit increased translational efficiency via mTOR dependent activation of p70s6k [90] in skeletal muscle. To our knowledge, it is not known whether shear stress exhibits a similar ability to activate mTOR and p70S6k signaling in VSMC.

The purpose of this study was to determine whether shear stress induced alterations in VSMC orientation are associated with changes in the mTOR mediated p70S6k regulation. Given the role that mTOR and p70S6k may play in regulating VSMC growth and proliferation, and on the basis of previous shear studies demonstrating the ability of shear stress to influence VSMC phenotype, we hypothesized that shear stress would be a powerful activator of mTOR-p70s6k signaling. To test this hypothesis, we examined the effects of shear stress on the phosphorylation

of mTOR and several of its potential upstream and parallel activators in VSMC subjected to various durations of shear stress. Our data suggest that shear stress results in VSMC realignment and this is associated with mTOR mediated activation of p70s6k.

MATERIALS AND METHODS

Materials

Antibodies against non-phosphorylated and phosphorylated forms of Phospho kinase B (Akt), PTEN (phosphatase and tensin homolog), mTOR and p70S6k were purchased from Cell Signaling Technology (Beverly, MA). Precast 10% SDS-PAGE gels were procured from Cambrex Biosciences (Baltimore, MD). Enhanced chemiluminescence (ECL) western blotting detection reagent was from Amersham Biosciences (Piscataway, NJ). Restore western blot stripping buffer was obtained from Pierce (Rockford, IL) and NIH 3T3 cell lysates were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Orbital Shear Stress

The A7r5 vascular smooth muscle cell line was obtained from American Type Culture Collection (Manassas, VA). Cultures were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum at 37°C in an incubator perfused with 5% CO2 and 95% O2. Shear stress was applied to serum-starved cells (about 60% confluent) by securing the culture dishes (150mm) to an orbital shaker that had been placed in the CO_2 incubator and rotated for 0 (Control), 5, 15, 30 min, 1h, 4h or 24h. Although the orbital shaker does not produce uniform laminar shear stress on the seeded cells, most of the cells are exposed to near-maximal shear stress (t max), as described previously [92, 93].

$$t\ max = a\sqrt{\rho\eta\ [2\pi f]^3}$$

where: a is the orbital radius of rotation of the shaker, ρ is the density of the culture medium, η is the viscosity of the medium, and f is the frequency of rotation. At 210 rpm, the frequency of

rotation used in these experiments, t max was calculated at 9.8 dyne/cm^2 which is similar to the magnitude of shear stress observed during arterial flow *in vivo*. The orbital shear stress model has been shown to yield results similar to other shear models when employed under the conditions used in our experiments [42] . Each experiment was repeated at least three times with a minimum of 8 dishes per time-point in each experiment.

Cell Orientation

Serum starved VSMC monolayers (about 60% confluent) on 150 mm culture dishes were exposed to 9.8 dyn/cm^2 fluid shear stress for 24hr and stained with H & E. The angle between the long axis of the cell and the vector force of shear was measured for each cell using ImageJ (Fig1). A minimum of three different fields from three different plates were analyzed for orientation.

Immunoblotting

At the end of each experiment cells were scraped and stored at -80^{0}C until use. Cells were washed three times with ice cold PBS and lysed on ice for 15 min in T-PER (2mL/1g tissue weight) (Pierce, Rockford, IL) supplemented with 100mM NaF, 1 mM Na_3VO_4, 2mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepsatin. After lysis, homogenates were centrifuged 10 min at 800g and the supernatant was collected. This procedure was repeated twice. Protein concentrations of homogenates were determined in triplicate via the Bradford method (Pierce, Rockford, IL) using bovine serum albumin as a standard. Samples were diluted to a concentration of 3 mg/ml in SDS loading buffer, boiled for 5 min, and 30µg of protein was separated using 10% SDS PAGE gels. Transfer of protein onto nitrocellulose membranes was

performed using standard conditions [94]. To verify transfer of proteins and equal loading of lanes the membranes were stained with Ponceau S. For immunodetection, membranes were blocked with a solution of 5% nonfat dry milk prepared in 1% Tris-buffered saline containing Tween 20 (TBST, pH 8.0) for 1 h at room temperature and then incubated with the appropriate primary antibody overnight. After washing in TBST, the membranes were exposed to horseradish peroxidase labeled IgG secondary antibody for 1 h at room temperature. Protein bands were visualized with ECL (Amersham Biosciences). Exposure time was adjusted to keep the integrated optical densities within a linear and nonsaturated range, and band signal intensity was quantified by densitometry using a flatbed scanner (Epson Perfection 3200 PHOTO) and Imaging software (AlphaEaseFC). Molecular weight markers (Cell Signaling) were used as molecular mass standards and NIH 3T3 cell lysates were included as positive controls. To allow direct comparisons to be made between the concentration levels of different signaling molecules, immunoblots were stripped and reprobed with restore western blot stripping buffer according to the manufacturer's instructions.

Data Analysis

Results are presented as mean ± SEM. Data were analyzed by using the SigmaStat 3.0. A one-way analysis of variance on ranks (ANOVA) was performed for overall comparisons with the Student-Newman-Keuls post hoc test used to determine differences between groups. The level of significance accepted *a priori* was $P \leq 0.05$.

RESULTS

Effect of Orbital Shear Stress on A7r5 cell morphology

Shear stress for 24 h in endothelial cells (EC) show marked elongation and reorientation towards the direction of shear flow [95]. We observed similar results using the A7r5 VSMC cell line. In the absence of flow, the angle of cell orientation was random in nature with no significant difference in the average angle of cell orientation (Fig 2). Conversely, with shear stress the cells tended to orientate themselves 45° relative to the axis of the shear stress (Control vs. Shear; 0^0-17^0: 17.7 ± 6.97 vs. 11.7 ± 2.54 ($p \leq 0.05$); 18^0-35^0: 19.1 ± 6.00 vs. 23.3 ±1.33; 36^0-53^0: 22.5 ± 3.46 vs. 35.7 ± 5.80 ($p \leq 0.05$); 54^0-71^0: 21.3 ± 6.27 vs. 17.8 ± 0.59 ($p \leq 0.05$), and 72^0-90^0: 19.4 ± 1.95 vs. 11.2 ± 7.86 ($p \leq 0.05$).

Effect of Orbital Shear Stress on mTOR/p70S6k activation in the A7r5 cell line

Previous reports have suggested that mTOR and p70S6k are regulated by Akt and that the phosphorylation of these molecules can be regulated by mechanical stimuli such as stretch [90, 96]. Phosphorylation of mTOR was found to be increased initially at 5 and 15 min to 118.88 ± 7.48 % and 117.16 ± 4.45 % of control, respectively ($p \leq 0.05$), before decreasing to 78.67 ± 2.86 %, 85.60 ± 1.29 %, and 79.04 ± 4.08 % of control at 30 min, 1h and 4h, respectively ($p \leq 0.05$) (Fig 3). The phosphorylation response of p70S6k increased to 169.33 ± 5 % at 5 min ($p \leq 0.05$), 188.42 ± 2.85 % at 15 min ($p \leq 0.05$), 187.04 ± 3.82 % at 30 min ($p \leq 0.05$), 270.80 ± 5.18 % at 1h ($p \leq 0.05$), 216.02 ± 7.03 % at 4 h ($p \leq 0.05$), and 170.58 ± 7.95 % relative to control ($p \leq 0.05$) at 24 h (Fig 4).

Effect of Orbital Shear Stress on Akt activation in A7r5 cell line

Oscillatory shear stress has been shown to increase bovine aortic smooth muscle cell proliferation via activation of the PI3K-Akt pathway [26]. We and others have shown the phosphorylation of Akt in response to mechanical stimuli such as stretch in vascular tissue [87, 97-99]. Upon application of orbital shear stress, we observed a time dependant increase in Akt phorphorylation in A7r5 cells (Fig 5). There was a gradual increase in phosphorylation which was 142.36 ± 0.53 % , 167.16 ± 1.25 %, 172.57 ± 3.39 %, 156.31 ± 2.22 %, 183.76 ± 6.30 % of control ($p \leq 0.05$), respectively, at 5, 15, 30 min, 1 h and 4 h before slight reduction to 124.05 ± 15.22 % of control at 24 h.

To confirm these shear dependent increase in Akt phosphorylation we examined the phosphorylation status of PTEN, upstream regulator of the PI3K-Akt pathway. As expected, the trend for PTEN phosphorylation had shown gradual increase to 125.4 ± 3.07 %, 133.93 ± 1.56 % and 135.45 ± 7.58 % of control ($p \leq 0.05$) at 5, 30 min and 4h respectively, before slightly reducing to 115.22 ± 2.88 % of control ($p \leq 0.05$), at 24h (Fig 6). PTEN functions as a phosphatidylinositol 3'-phosphatase to hydrolyze the lipid products of PI3K [70, 71], promote cell cycle arrest by down regulating PI3K/Akt signaling [72-74] and is inactive when phosphorylated [75, 76]. Therefore increased PTEN phosphorylation (inactivity) act to confirm up regulation of PI3K/Akt signaling. Similar to previous reports using other cell types, these data suggest the involvement of the PI3K-Akt pathway in shear stress signaling in VSMC [26, 27, 99].

DISCUSSION

In this study, we present a time course study of shear stress induced signal transduction in cultured VSMC. VSMC in normal, mature vessels are quiescent, contractile, and differentiated but assume a proliferative, migratory, and protein synthetic phenotype in response to injury [100]. VSMC proliferation, migration to the intima, and synthesis of extracellular matrix after injury is an important component of the intimal hyperplasia [101] that is thought to be involved in restenosis. Similar to previous reports using EC, exposure of VSMC to shear stress altered cell alignment (Fig. 2) and this effect was associated with increased phosphorylation of Akt, PTEN, p70s6k and a biphasic response of mTOR. Taken together these data suggest that mTOR- p70s6k signaling may be an important mediator of shear induced VSMC adaptation.

Effect of Shear Stress on mTOR Phosphorylation

mTOR is suggested to be mechanically regulated [90] and the activity of mTOR may be particularly important to the regulation of VSMC phenotype as recent studies have demonstrated that mTOR inhibition by rapamycin causes VSMC differentiation [4, 24]. Therefore, phosphorylation of mTOR may be crucial for VSMC dedifferentiation. We examined the phosphorylation of mTOR that showed a biphasic response with early activation followed by decrease in phosphorylation at 30 min and a recovery to near control levels by 24h. mTOR is suggested to be key regulator of p70s6k mediated proliferation of in-stent restenosis as the mTOR inhibitor, rapamycin, has been shown to inhibit p70s6k phosphorylation and proliferation of human VSMC following vascular injury [4, 22, 23]. In the present study we observed that SS increased mTOR phosphorylation only at 5 and 15 min of exposure. These data suggest that SS may be a strong stimulus for mTOR regulation and that only transient activation of mTOR is

necessary for initiation of downstream signaling such as activation of p70S6k, which in turn activates cell growth and translational processes.

Although the exact signaling pathways upstream of mTOR are incompletely resolved, substantial evidence indicates that mTOR activation is dependent on PI3K, with insulin and activated Akt being able to induce phosphorylation of mTOR both *in vitro* and *in vivo* experiments [62-66]. Therefore we examined the phosphorylation of Akt. Akt is mechanically regulated in the vascular tissue and cultured cells and has been shown to be involved in the regulation of endothelial and VSMC proliferation in response to shear stress [25-27, 99]. Here, we found a distinct up regulation of Akt (Fig 5) in response to shear stress in the A7r5 smooth muscle cell line. PTEN is a phosphatase that has been proposed to inhibit the PI3K/Akt signaling cascade and function in the control of cell cycle arrest [102, 103]. Recent study by Mourani et al (2004) had shown that unique, highly proliferative growth phenotype expressed by embryonic and neointimal smooth muscle cells is driven by constitutive Akt, mTOR, and p70S6K signaling and is actively repressed by PTEN [28].The ability of PTEN to inhibit the PI3K/Akt signaling cascade is thought to be negatively regulated by PTEN phosphorylation. Our findings showing a sustained elevation of PTEN phosphorylation with shear stress (Fig 6) act to confirm the involvement of Akt activation in the response of VSMC to shear stress. Thus, mechanical activation of Akt and mTOR might be the biochemical events occurring in the VSMC that might lead to eventual proliferative pathology of VSMC when they are exposed to SS at times vessel damage with endothelial denudation. Akt has been shown to be involved in increasing the expression of matrix metalloproteinases (MMPs) [104], a family of extra cellular enzymes that regulate a wide variety of processes associated with vascular structure and remodeling [105], and this is associated with increased cell migration [106]. Although assessing the MMP expression is

beyond the scope of the present study whether the elevated Akt we observe in the present study is associated with increased MMP activity remains to be determined.

Effect of Shear Stress on p70s6k phosphorylation

Cellular proliferation involves changes not only in the level of gene transcription but also in the rate of protein translation [15]. The serine/threonine kinase, p70s6k, is believed to play a critical role in regulating protein synthesis [16] and activation of VSMC differentiation via the PI3-K/Akt dependent activation of mTOR pathway [21, 107, 108]. This notion is further supported from the finding that (in vivo) balloon injury of the rat carotid artery is found to be accompanied by increases in p70s6k activity and the rapamycin-sensitive induction of cell cycle protein expression [18]. In the present study, shear stress increased the phosphorylation of p70s6k consistent with the upstream phosphorylation of Akt and mTOR. These data suggest that shear stress mediates VSMC remodeling via sequential activation of Akt, mTOR and p70s6k. These findings are in good agreement with the study by Mourani et al who found that Akt, mTOR and p70s6k signaling is responsible for the proliferative VSMC phenotype and that this is actively repressed by PTEN. A similar pattern of signaling mechanism has recently been reported in VSMC following Angiotensin II stimulation [85, 109] suggesting that mechanisms mediating VSMC remodeling following mechanical and endocrine stimulation may be conserved.

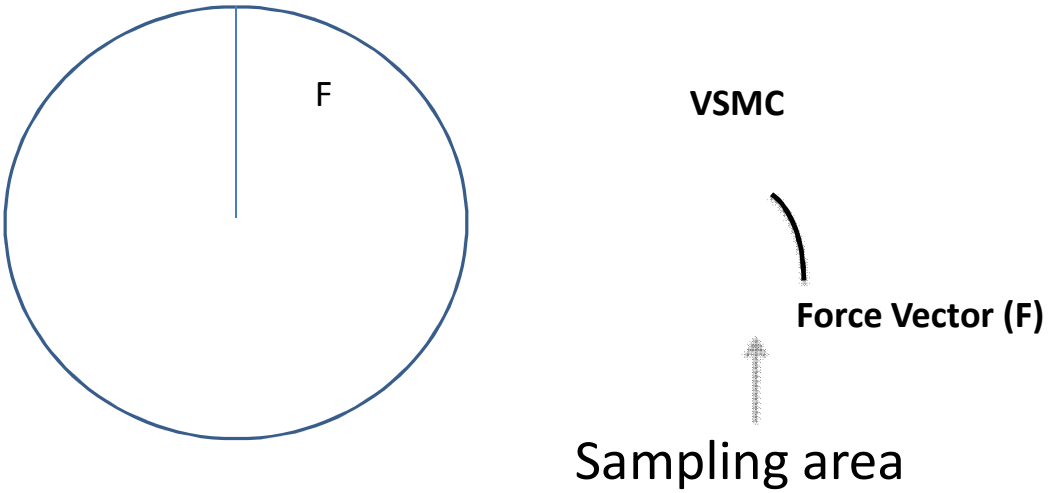
Effect of Shear Stress on PTEN:

PTEN, first discovered as a potent tumor suppressor, is a dual-specificity lipid and protein phosphatase [72-74]. As a direct negative regulator of PI3K/Akt- signaling, PTEN promotes cell cycle arrest, apoptosis, and decreased cell migration. Studies have shown PTEN inactivation results in early embryonic lethality and altered differentiation and organization of all 3 germ layers [110, 111]. Study by mourani et al demonstrated the capacity of VSMC to transiently reexpress the growth phenotype that is associated with PTEN inactivation [28]. The present data suggest that PTEN activity is downregulated in VSMC exposed to shear stress, thus indicating a dedifferentiated VSMC phenotype. Therefore, selectively targeting of PTEN and/or elucidation of upstream modulators of PTEN activity might hold promise for the development of antirestenotic and antiatherosclerotic therapies.

In summary, most studies have concentrated on flow induced alterations in EC and dependent changes in underlying VSMC [47-50] but the mechanotransduction pathways that regulate the VSMC function under direct influence of fluid mechanics have not been well understood. VSMC migration and proliferation is thought to be the hallmark of neointimal thickening and the consequent vascular occlusive diseases. In the present experiment, we demonstrate that shear stress elicits VSMC reorientation indicative of phenotypic modulation and that this has been found to be associated with Akt and mTOR mediated phosphorylation of p70s6k, a key enzyme in specialized translational control. Another important finding is inactivation of PTEN with shear stress which might hold promise to be targeted for the development of pharmacological interventions to restenosis.

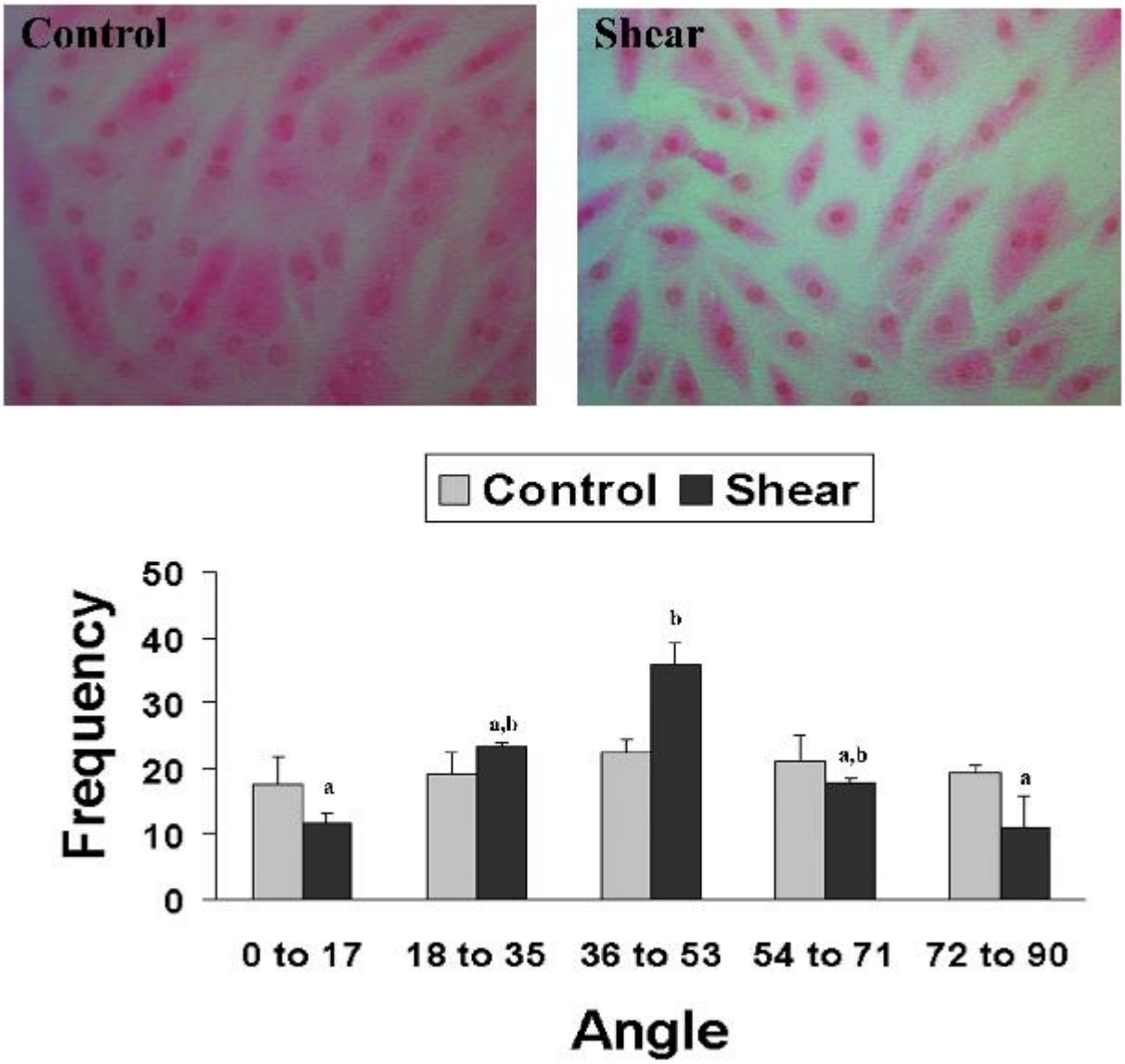
Figures

Fig 1. Determination of cell orientation



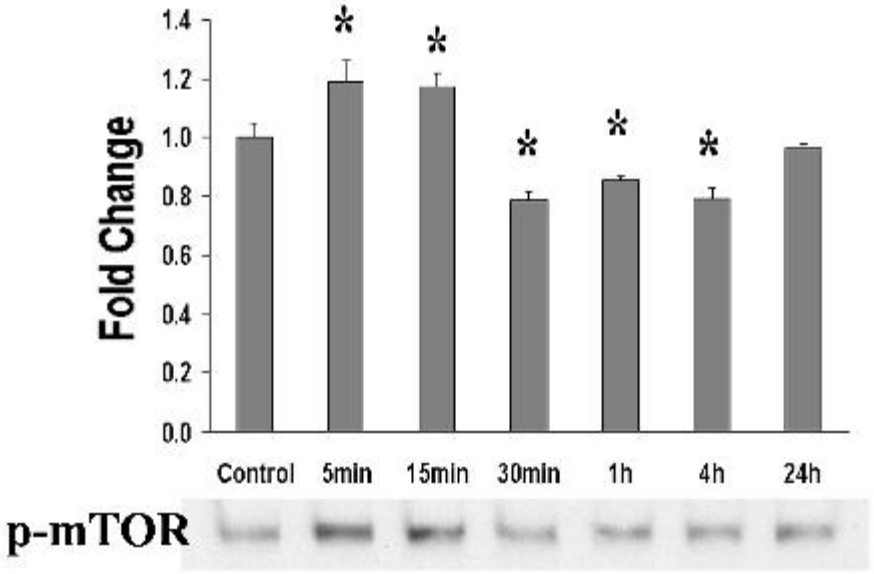
Cell orientation is determined by measuring the angle between the force vector and the long axis of the VSMC

Figure 2: Shear stress alters the VSMC orientation



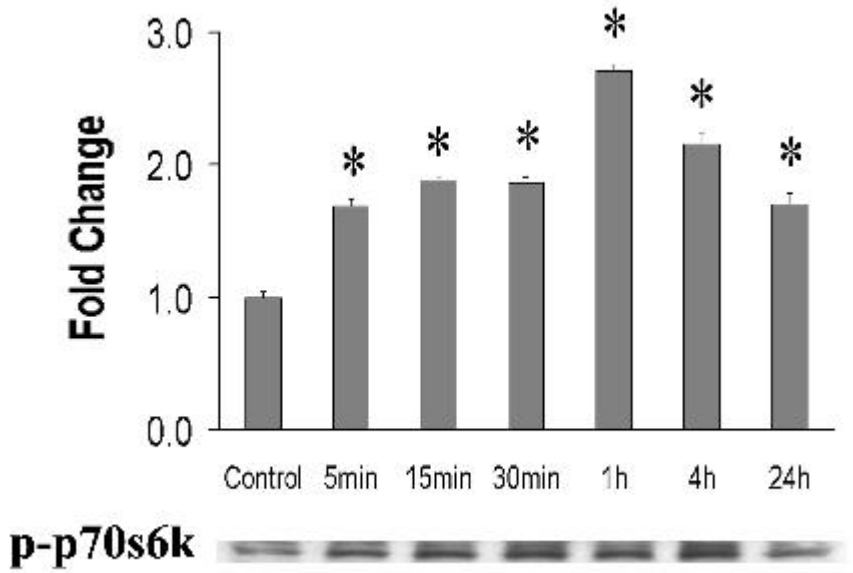
Histogram illustrating altered orientation of vascular smooth muscle cells under fluid shear stress. Sub confluent A7r5 rat aortic cell monolayers on 150mm culture dishes were exposed to 9.8 dyn/cm^2 fluid shear stress (orbital shaker) for 24 hr and stained with H&E. Results are representative of at least 3 experiments. The control cells were randomly oriented with no significant difference between the percentage of cells oriented at different angles. In the shear exposed cells the bars with different superscripts are significantly different ($p \leq 0.05$).

Figure 3: Shear induced alterations in mTOR phosphorylation



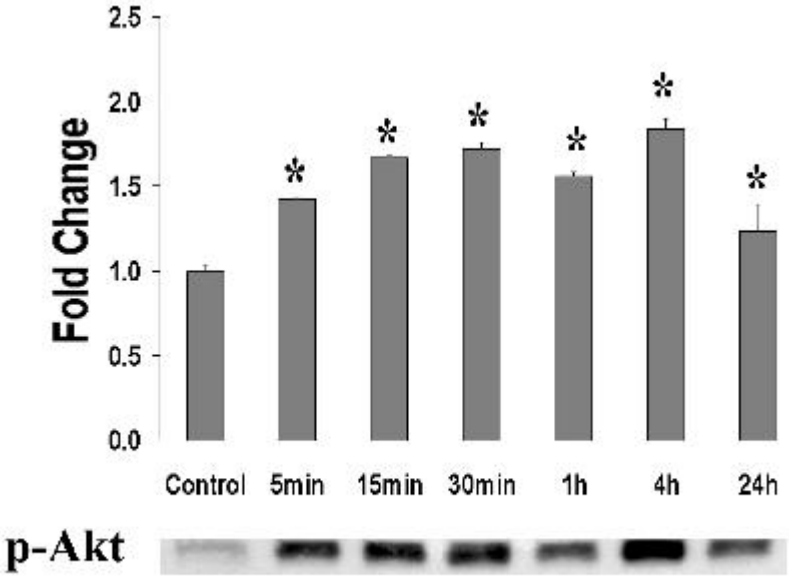
VSMC that were subjected to shear stress for 5 min, 15 min, 30 min, 1 h, 4 h or 24 hours were analysed for phospho-mTOR using immunoblotting. An asterisk (*) indicates significant differences ($P < 0.05$) from the control value.

Figure 4: Increased p70s6k phosphorylation with shear stress in VSMC



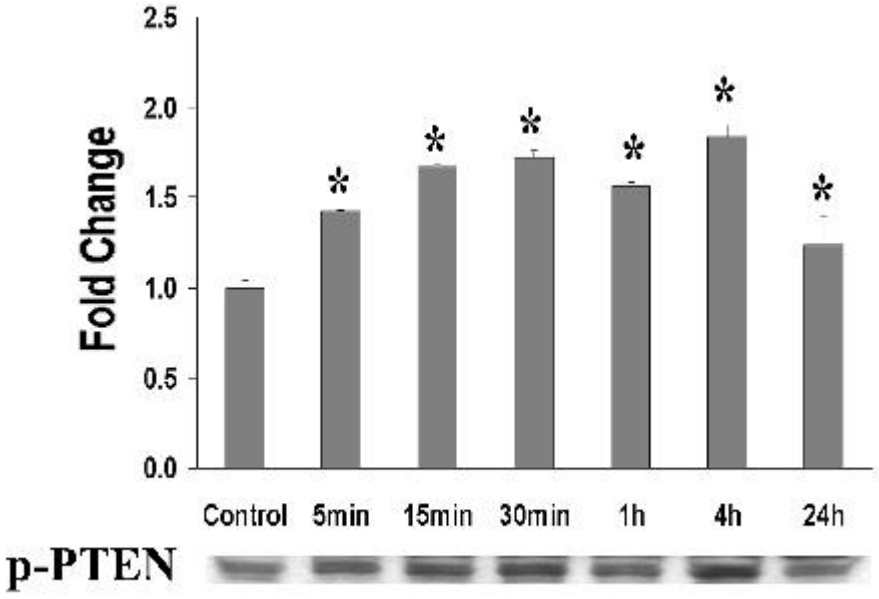
VSMC that were subjected to shear stress for 5 min, 15 min, 30 min, 1 h, 4 h or 24 hours were analysed for phospho-p70s6k using immunoblotting. An asterisk (*) indicates significant differences ($P < 0.05$) from the control value.

Figure 5: Akt is activated in VSMC when exposed to shear stress



VSMC that were subjected to shear stress for 5 min, 15 min, 30 min, 1 h, 4 h or 24 hours were analysed for phospho-Akt using immunoblotting. An asterisk (*) indicates significant differences ($P < 0.05$) from the control value.

Figure 6: PTEN phosphorylation increased with shear stress in VSMC



VSMC that were subjected to shear stress for 5 min, 15 min, 30 min, 1 h, 4 h or 24 hours were analysed for phospho-PTEN using immunoblotting. An asterisk (*) indicates significant differences ($P < 0.05$) from the control value.

Appendix

Raw data for the cell alignment in the control group

Class interval	**0 to 17**	**18 to 35**	**36 to 53**	**54 to 71**	**72 to 90**
% cells	18.34862	25.68807	18.80734	19.26606	17.88991
% cells	24.24242	17.57576	23.0303	16.36364	18.78788
% cells	10.36036	13.96396	25.67568	28.37838	21.62162
N	3	3	3	3	3
Mean	17.65047	19.07593	22.50444	21.33602	19.43314
Strandard Deviation	6.967316	6.004297	3.464233	6.269137	1.947739
Standard Error of the mean	4.022582	3.466582	2.000076	3.619488	1.124528
	0 to 17	18 to 35	36 to 53	54 to 71	72 to 90
Relative Expression Level	1	1.080761	1.275005	1.208808	1.100998
Standard error of the mean	0.227902	0.100965	-0.18613	-0.03917	-0.04313
	0 to 17	18 to 35	36 to 53	54 to 71	72 to 90
% RE	100	108.0761	127.5005	120.8808	110.0998
SE	22.79022	10.09649	-18.613	-3.91656	-4.31317

shear group

% cells	11.875	21.875	40.625	17.5	8.125
% cells	9.134615	23.55769	29.32692	17.78846	20.19231
% cells	14.21569	24.5098	37.2549	18.62745	5.392157
N	3	3	3	3	3
Mean	11.74177	23.31417	35.73561	17.97197	11.23649
Strandard Deviation	2.543154	1.334177	5.800244	0.585699	7.875413
Standard Error of the mean	1.468291	0.770287	3.348772	0.338153	4.546872
	0 to 17	18 to 35	36 to 53	54 to 71	72 to 90
Relative Expression Level	0.665238	1.320881	2.024627	1.018215	0.636611
Standard error of the mean	0.45981	-0.28784	-0.93092	0.000601	0.768041
	0 to 17	18 to 35	36 to 53	54 to 71	72 to 90
% RE	66.52382	132.0881	202.4627	101.8215	63.66113
SE	45.98103	-28.7841	-93.0917	0.060071	76.80411

Statistics

One Way Analysis of Variance for control data

Normality Test: Passed (P = 0.757)

Equal Variance Test: Passed (P = 0.392)

Group Name	N	Missing	Mean	Std Dev	SEM
0 to 17 S	3	0	17.650	6.967	4.023
18 to 35 S	3	0	19.076	6.004	3.467
36 to 53 S	3	0	22.504	3.464	2.000
54 to 71 S	3	0	21.336	6.269	3.619
72 to 90 S	3	0	19.433	1.948	1.125

Source of Variation	DF	SS	MS	F	P
Between Groups	4	44.258	11.065	0.396	0.807
Residual	10	279.383	27.938		
Total	14	323.642			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.807).

One Way Analysis of Variance for Shear data

Normality Test: Passed (P = 0.280)

Equal Variance Test: Passed (P = 0.314)

Group Name	N	Missing	Mean	Std Dev	SEM
0 to 17 S	3	0	11.742	2.543	1.468
18 to 35 S	3	0	23.314	1.334	0.770
36 to 53 S	3	0	35.736	5.800	3.349
54 to 71 S	3	0	17.972	0.586	0.338
72 to 90 S	3	0	11.236	7.875	4.547

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1223.111	305.778	14.665	<0.001
Residual	10	208.511	20.851		
Total	14	1431.622			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.999

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
36 to 53 S vs. 72 to 90 S	24.499	5	9.293	<0.001	Yes
36 to 53 S vs. 0 to 17 S	23.994	4	9.101	<0.001	Yes
36 to 53 S vs. 54 to 71 S	17.764	3	6.738	0.002	Yes
36 to 53 S vs. 18 to 35 S	12.421	2	4.712	0.008	Yes
18 to 35 S vs. 72 to 90 S	12.078	4	4.581	0.038	Yes
18 to 35 S vs. 0 to 17 S	11.572	3	4.390	0.028	Yes
18 to 35 S vs. 54 to 71 S	5.342	2	2.026	0.183	No
54 to 71 S vs. 72 to 90 S	6.735	3	2.555	0.217	No
54 to 71 S vs. 0 to 17 S	6.230	2	2.363	0.126	Do Not Test
0 to 17 S vs. 72 to 90 S	0.505	2	0.192	0.895	Do Not Test

Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

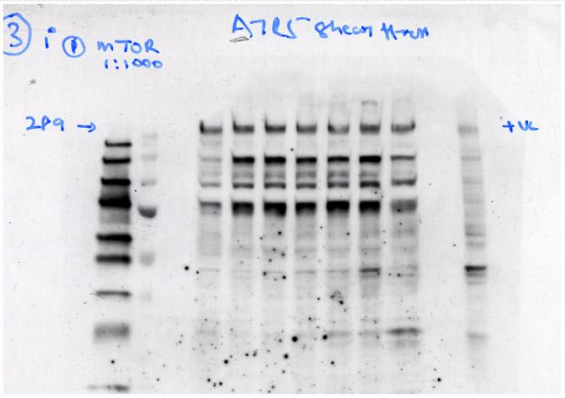
Primary Antibody

Type: phospho mTOR Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
Lane # 3	
Lane # 4	control
Lane # 5	5 min shear
Lane # 6	15 min shear
Lane # 7	30 min shear
Lane # 8	1 hr shear
Lane # 9	4 hr shear
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control



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Notes

email Report

Your Signature

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MURC billing address: 401 11th St., Suite 1400 Huntington, Wv 25701 304-696-6203 Fax 304-697-3861
University Billing: Marshall University, 1 John Marshall Drive, Huntington, WV, 25755

Laboratory of Molecular Physiology

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Project: A7r5 Shear Stress
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Incubation time: over night

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Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

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Lane # 2	mol wt marker
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Lane # 4	control
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Lane # 7	30 min shear
Lane # 8	1 hr shear
Lane # 9	4 hr shear
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control

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Media: 5% Milk in TBST
Incubation time: 1 hr

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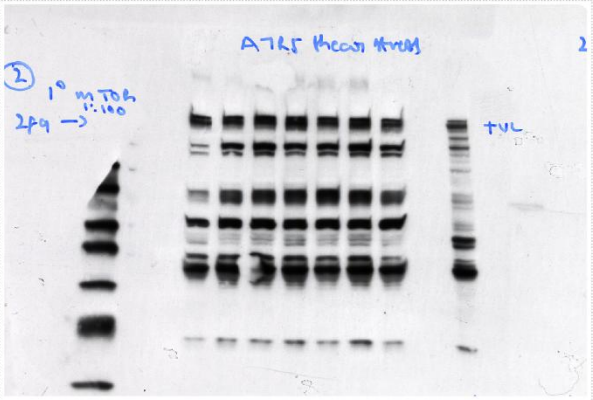
Lane # 9	4 hr shear
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control



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Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
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Transfer Voltage: 24 Duration: 45 min

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Incubation time: over night

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Media: 5% Milk in TBST
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Lane # 8	1 hr shear
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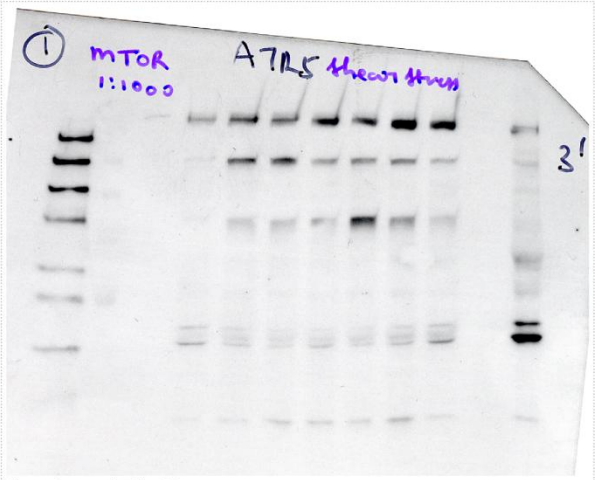
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control



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Raw data tables produced from spot densitometry of the immunoblot films probed for phosphor mTOR

	Control	5min	15min	30min	1h	4h	24h
%C	14.7	13.68663	13.8562	9.931555	10.53787	9.835592	12.08351
%C	11.7	18.71978	16.78235	8.359654	10.10599	7.664979	11.99127
%C	14.3	13.42173	13.94226	9.931555	10.96975	10.03909	11.89903
%C	11.7	18.89638	16.69629	8.359654	10.10599	7.936305	11.53007
%C	11.1	13.86323	14.88896	10.43171	10.71063	10.98873	11.80679
%C	12.9	12.98022	13.08163	10.71751	11.14251	10.85307	12.36023
%C	12.8	13.15682	12.99557	10.57461	11.22888	10.64957	12.63695
%C	10.7	14.03983	14.8029	10.28881	10.71063	10.98873	12.08351
%C							
N	8	8	8	8	8	8	8
Mean	12.4875	14.845588	14.630777	9.824383	10.689033	9.869508	12.048902
Strandard Deviation	1.454488	2.470818	1.470651	0.945581	0.427539	1.347396	0.337791
Standard Error of the mean	0.549745	0.933881	0.555854	0.357396	0.161595	0.509268	0.127673
Relative Expression Level	1	1.188835	1.171633	0.786737	0.855979	0.790351	0.964879
Standard error of the mean	0.044024	0.074785	0.044513	0.02862	0.012941	0.040782	0.010224
% RE	100	118.8835	117.1633	78.67372	85.59786	79.03511	96.48786
SE	4.402361	7.47853	4.451282	2.86203	1.294052	4.078221	1.022406

One Way Analysis of Variance

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.138)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	8	0	12.488	1.454	0.514
5min	8	0	14.846	2.471	0.874
15min	8	0	14.631	1.471	0.520
30min	8	0	9.824	0.946	0.334
1h	8	0	10.689	0.428	0.151
4h	8	0	9.870	1.347	0.476
24h	8	0	12.049	0.338	0.119

Source of Variation	DF	SS	MS	F	P
Between Groups	6	209.815	34.969	18.281	<0.001
Residual	49	93.728	1.913		
Total	55	303.544			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
5min vs. 30min	5.021	7	10.269	<0.001	Yes
5min vs. 4h	4.976	6	10.176	<0.001	Yes
5min vs. 1h	4.157	5	8.500	<0.001	Yes
5min vs. 24h	2.797	4	5.719	0.001	Yes
5min vs. Control	2.358	3	4.822	0.004	Yes
5min vs. 15min	0.215	2	0.439	0.758	No
15min vs. 30min	4.806	6	9.829	<0.001	Yes
15min vs. 4h	4.761	5	9.737	<0.001	Yes
15min vs. 1h	3.942	4	8.061	<0.001	Yes
15min vs. 24h	2.582	3	5.280	0.002	Yes
15min vs. Control	2.143	2	4.383	0.003	Yes
Control vs. 30min	2.663	5	5.446	0.003	Yes
Control vs. 4h	2.618	4	5.354	0.002	Yes
Control vs. 1h	1.798	3	3.678	0.032	Yes
Control vs. 24h	0.439	2	0.897	0.529	No
24h vs. 30min	2.225	4	4.549	0.012	Yes

24h vs. 4h	2.179	3	4.457	0.008	Yes
24h vs. 1h	1.360	2	2.781	0.055	No
1h vs. 30min	0.865	3	1.768	0.430	No
1h vs. 4h	0.820	2	1.676	0.242	Do Not Test
4h vs. 30min	0.0451	2	0.0923	0.948	Do Not Test

Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

Primary Antibody

Type: phospho Akt Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
Lane # 3	
Lane # 4	control
Lane # 5	5 min shear
Lane # 6	15 min shear
Lane # 7	30 min shear
Lane # 8	1 hr shear
Lane # 9	4 hr shear
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control

Place Scan of Film Here

Properties

Exposure time: 3 min Molecular Wieght: 60 kda ☑ Used For Analysis
Location of Scan: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Films\p-Akt 473
Location of Excell Data: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Excel

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Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

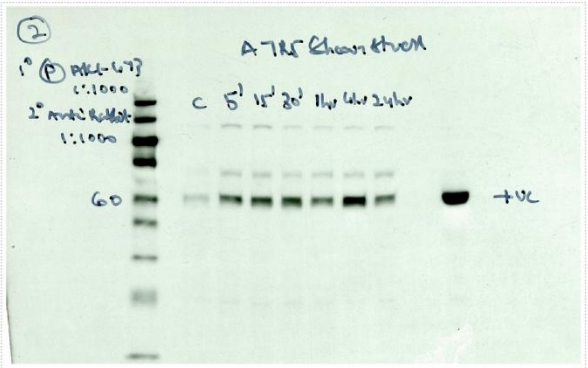
Primary Antibody

Type: phospho Akt Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
Lane # 3	
Lane # 4	control
Lane # 5	5 min shear
Lane # 6	15 min shear
Lane # 7	30 min shear
Lane # 8	1 hr shear
Lane # 9	4 hr shear
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control



Place Scan of Film Here

Properties

Exposure time: 3 min Molecular Wieght: 60 kda ☑ Used For Analysis
Location of Scan: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Films\p-Akt 473
Location of Excell Data: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Excel

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Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

Primary Antibody

Type: Akt Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
Lane # 3	
Lane # 4	control
Lane # 5	5 min shear
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Lane # 7	30 min shear
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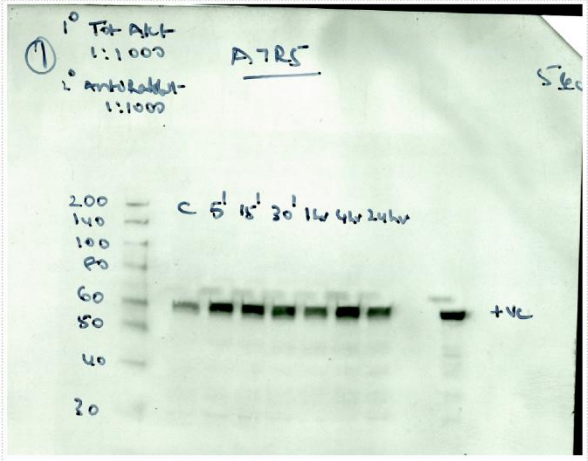
Lane # 9	4 hr shear
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control



Place Scan of Film Here

Properties

Exposure time: 5 sec Molecular Wieght: 60 kda ☑ Used For Analysis
Location of Scan: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Films\Akt 473
Location of Excell Data: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Excel

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Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

Primary Antibody

Type: Akt Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
Lane # 3	
Lane # 4	control
Lane # 5	5 min shear
Lane # 6	15 min shear
Lane # 7	30 min shear
Lane # 8	1 hr shear
Lane # 9	4 hr shear
Lane # 10	24 hr shear
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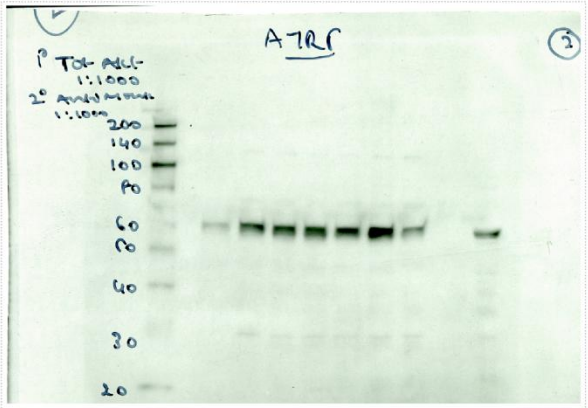
Lane # 12	possitive control



Place Scan of Film Here

Properties

Exposure time: 10 sec Molecular Wieght: 60 kda ☑ Used For Analysis
Location of Scan: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Films\Akt 473
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Raw data tables produced from spot densitometry of the immunoblot films probed for phospho Akt

	Control	5min	15min	30min	1h	4h	24h
%C	5.50	7.08	8.40	8.97	8.01	8.37	7.17
%C	5.30	6.98	8.50	8.97	8.01	8.49	3.17
%C	4.50	7.13	8.15	8.17	7.39	9.94	7.23
%C	4.60	7.13	8.20	8.22	7.70	9.77	7.11
%C	5.20	7.18	8.30	8.56	7.93	7.36	7.02
%C	5.18	7.21	8.10	8.75	7.81	7.40	7.27
%C	4.80	7.24	8.24	8.25	7.51	8.64	6.32
%C	4.70	7.27	8.20	8.08	7.62	8.68	6.19
N	8	8	8	8	8	8	8
Mean	4.98	7.08	8.32	8.59	7.78	9.14	6.17
Strandard Deviation	0.50	0.07	0.16	0.45	0.29	0.83	2.00
Standard Error of the mean	0.19	0.03	0.06	0.17	0.11	0.31	0.76

	Control	5min	15min	30min	1h	4h	24h
Relative Expression Level	1.00	1.42	1.67	1.73	1.56	1.84	1.24
Standard error of the mean	0.04	0.01	0.01	0.03	0.02	0.06	0.15

	Control	5min	15min	30min	1h	4h	24h
% RE	100.00	142.36	167.16	172.57	156.31	183.76	124.05
SE	3.79	0.53	1.25	3.39	2.22	6.30	15.22

One Way Analysis of Variance

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = <0.001)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	8	0	4.975	0.499	0.250
5min	8	0	7.082	0.0700	0.0350
15min	8	0	8.316	0.164	0.0821
30min	8	0	8.585	0.446	0.223
1h	8	0	7.776	0.293	0.146
4h	8	0	9.142	0.829	0.415
24h	8	0	7.173	0.0488	0.0244

Source of Variation	DF	SS	MS	F	P
Between Groups	6	44.921	7.487	41.727	<0.001
Residual	21	3.768	0.179		
Total	27	48.689			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
4h vs. Control	4.167	7	19.675	<0.001	Yes
4h vs. 5min	2.060	6	9.725	<0.001	Yes
4h vs. 24h	1.969	5	9.299	<0.001	Yes
4h vs. 1h	1.366	4	6.448	0.001	Yes
4h vs. 15min	0.826	3	3.900	0.031	Yes
4h vs. 30min	0.557	2	2.629	0.077	No
30min vs. Control	3.610	6	17.045	<0.001	Yes
30min vs. 5min	1.503	5	7.096	<0.001	Yes
30min vs. 24h	1.413	4	6.670	<0.001	Yes
30min vs. 1h	0.809	3	3.818	0.034	Yes
30min vs. 15min	0.269	2	1.270	0.379	No
15min vs. Control	3.341	5	15.775	<0.001	Yes
15min vs. 5min	1.234	4	5.826	0.003	Yes
15min vs. 24h	1.144	3	5.399	0.003	Yes
15min vs. 1h	0.540	2	2.548	0.086	No
1h vs. Control	2.801	4	13.227	<0.001	Yes
1h vs. 5min	0.694	3	3.278	0.075	No
1h vs. 24h	0.604	2	2.851	0.057	Do Not Test
24h vs. Control	2.198	3	10.376	<0.001	Yes
24h vs. 5min	0.0903	2	0.426	0.766	Do Not Test
5min vs. Control	2.107	2	9.949	<0.001	Yes

Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

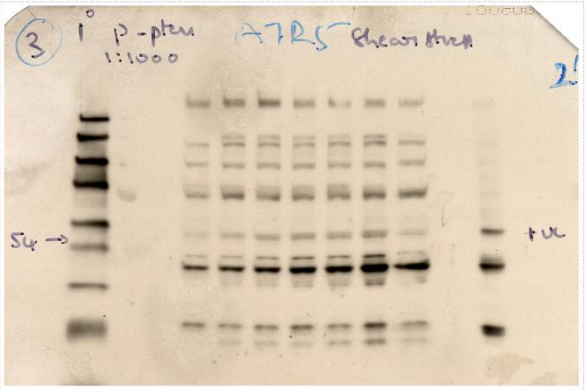
Primary Antibody

Type: phospho pten Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
Lane # 3	
Lane # 4	control
Lane # 5	5 min shear
Lane # 6	15 min shear
Lane # 7	30 min shear
Lane # 8	1 hr shear
Lane # 9	4 hr shear
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control



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Properties

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Location of Excell Data: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Excel

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Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

Primary Antibody

Type: phospho pten Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1: protein ladder
Lane # 2: mol wt marker
Lane # 3:
Lane # 4: control
Lane # 5: 5 min shear
Lane # 6: 15 min shear
Lane # 7: 30 min shear
Lane # 8: 1 hr shear
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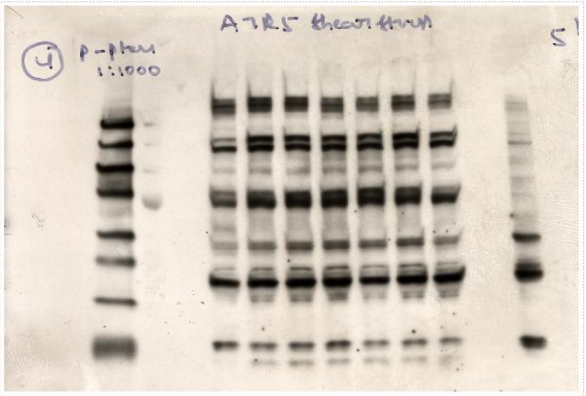
Lane # 10: 24 hr shear
Lane # 11:
Lane # 12: possitive control



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Properties

Exposure time: 5 min Molecular Wieght: 54 kda ☑ Used For Analysis
Location of Scan: \\Blough-raid\exp-data\Project folder\A7R5 shear stress\Films\p-p ten
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Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
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Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration:
Transfer Voltage: 24 Duration: 45 min

Primary Antibody

Type: pten Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
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Lane # 4	control
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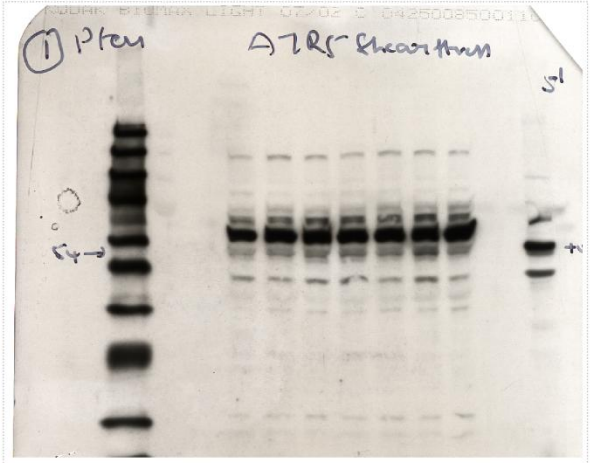
Lane # 10	24 hr shear
Lane # 11	
Lane # 12	possitive control



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Properties

Exposure time: 5 min Molecular Wieght: 54 kda ☑ Used For Analysis
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Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

Primary Antibody

Type: pten Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1	protein ladder
Lane # 2	mol wt marker
Lane # 3	
Lane # 4	control
Lane # 5	5 min shear
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Lane # 10	24 hr shear
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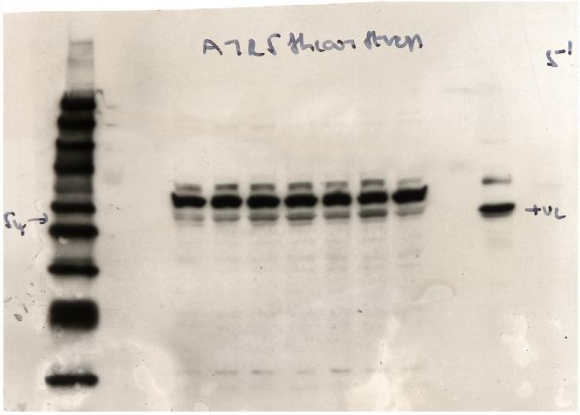
Lane # 12	possitive control



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Raw data tables produced from spot densitometry of the immunoblot films probed for phospho pten

	Control	5min	15min	30min	1h	4h	24h
%C	8.6	10.10884	10.86537	11.86475	12.84158	13.85247	9.606383
%C	8.5	11.12822	10.35146	11.43847	13.16875	13.65828	9.693714
%C	9.1	11.72286	11.81976	12.36208	11.94185	10.61591	11.00368
%C	9.5	11.80781	12.33366	12.14894	11.77826	10.22753	10.82901
%C	8.8	11.21637	10.96435	11.26581	12.61527	13.63924	10.28482
%C	9.0	11.32243	11.27638	11.51329	12.56979	13.77975	9.998713
%C	8.3	10.02843	9.739746	11.25272	11.24185	11.49236	9.164726
%C	8.1	9.894715	10.47912	11.59258	10.87635	10.53067	9.260893
N	8	8	8	8	8	8	8
Mean	8.925	11.19193	11.34256	11.95356	12.43261	12.08855	10.28325
Strandard Deviation	0.430116	0.724732	0.831518	0.369656	0.627507	1.789456	0.680876
Standard Error of the mean	0.162569	0.273923	0.314284	0.139717	0.237175	0.676351	0.257347

	Control	5min	15min	30min	1h	4h	24h
Relative Expression Level	1	1.253998	1.270875	1.339334	1.393009	1.354459	1.152179
Standard error of the mean	0.018215	0.030692	0.035214	0.015655	0.026574	0.075782	0.028834

	Control	5min	15min	30min	1h	4h	24h
% RE	100	125.3998	127.0875	133.9334	139.3009	135.4459	115.2179
SE	1.821498	3.069166	3.521393	1.565455	2.657425	7.578162	2.883439

One Way Analysis of Variance

Normality Test: Passed (P > 0.050)
Equal Variance Test: Failed (P = <0.001)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	8	0	4.975	0.499	0.250
5min	8	0	7.082	0.0700	0.0350
15min	8	0	8.316	0.164	0.0821
30min	8	0	8.585	0.446	0.223
1h	8	0	7.776	0.293	0.146
4h	8	0	9.142	0.829	0.415
24h	8	0	7.173	0.0488	0.0244

Source of Variation	DF	SS	MS	F	P
Between Groups	6	44.921	7.487	41.727	<0.001
Residual	21	3.768	0.179		
Total	27	48.689			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
4h vs. Control	4.167	7	19.675	<0.001	Yes
4h vs. 5min	2.060	6	9.725	<0.001	Yes
4h vs. 24h	1.969	5	9.299	<0.001	Yes
4h vs. 1h	1.366	4	6.448	0.001	Yes
4h vs. 15min	0.826	3	3.900	0.031	Yes
4h vs. 30min	0.557	2	2.629	0.077	No
30min vs. Control	3.610	6	17.045	<0.001	Yes
30min vs. 5min	1.503	5	7.096	<0.001	Yes
30min vs. 24h	1.413	4	6.670	<0.001	Yes
30min vs. 1h	0.809	3	3.818	0.034	Yes
30min vs. 15min	0.269	2	1.270	0.379	No
15min vs. Control	3.341	5	15.775	<0.001	Yes
15min vs. 5min	1.234	4	5.826	0.003	Yes
15min vs. 24h	1.144	3	5.399	0.003	Yes
15min vs. 1h	0.540	2	2.548	0.086	No
1h vs. Control	2.801	4	13.227	<0.001	Yes
1h vs. 5min	0.694	3	3.278	0.075	No

1h vs. 24h	0.604	2	2.851	0.057	Do Not Test
24h vs. Control	2.198	3	10.376	<0.001	Yes
24h vs. 5min	0.0903	2	0.426	0.766	Do Not Test
5min vs. Control	2.107	2	9.949	<0.001	Yes

Laboratory of Molecular Physiology

Film Properties Report

Experimenter: prasad
Date:
Project: A7r5 Shear Stress
Tissue/ cell line/ etc.: VSMC
Report Number:
Protien Concentration: 30 micrograms/ml
Gel type: 10% tris glycine

Electrophoresis Voltage: 125 V Duration: Transfer Voltage: 24 Duration: 45 min

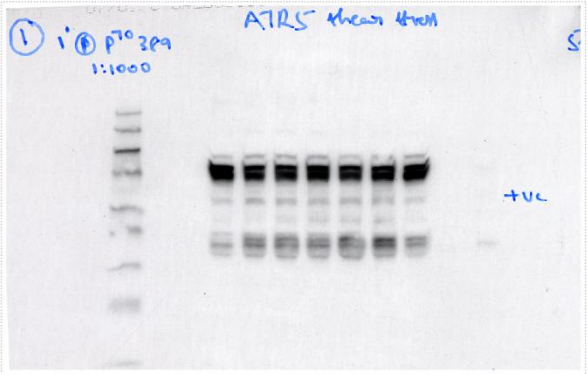
Primary Antibody

Type: phospho p70s6k Dilution: 1 : 1000
Media: 5% BSA in TBST
Incubation time: over night

Secondary Antibody

Type: Anti Rabbit Dilution: 1 : 1000
Media: 5% Milk in TBST
Incubation time: 1 hr

Lane # 1: protein ladder
Lane # 2: mol wt marker
Lane # 3:
Lane # 4: control
Lane # 5: 5 min shear
Lane # 6: 15 min shear
Lane # 7: 30 min shear
Lane # 8: 1 hr shear
Lane # 9: 4 hr shear
Lane # 10: 24 hr shear
Lane # 11:
Lane # 12: possitive control



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Incubation time: 1 hr

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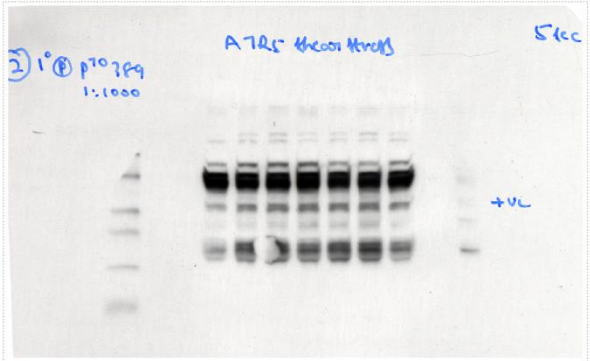
Lane # 12	possitive control



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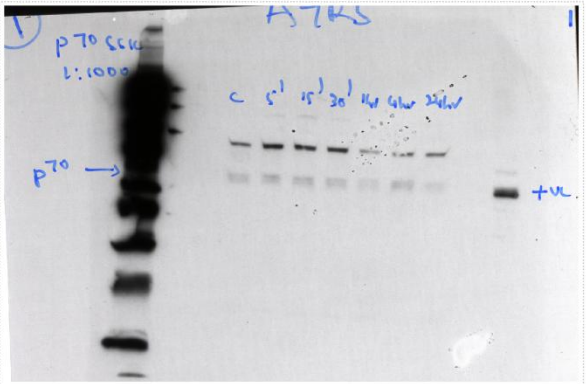
Lane # 12	possitive control



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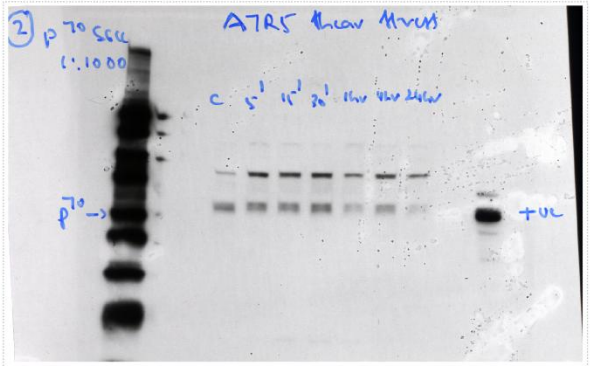

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Raw data tables produced from spot densitometry of the immunoblot films probed for phospho p70s6k

	Control	5min	15min	30min	1h	4h	24h
%C	11.6	20.02427	18.70229	17.53884	30.64721	20.08811	18.55011
%C	11.7	19.53883	18.82952	19.25593	27.22081	20.7489	18.71002
%C	8.5	16.62621	20.86514	19.62388	29.69543	22.20264	20.94883
%C	9.9	18.20388	20.35623	19.25593	27.22081	26.1674	14.71215
%C	9.2	16.14078	20.61069	19.62388	29.88579	22.46696	20.30917
%C	10.5	17.59709	19.84733	20.11447	28.36294	24.44934	15.1919
%C	11	16.86893	19.59288	20.97302	27.22081	23.25991	17.27079
%C	11.6	17.23301	19.46565	20.72772	27.22081	22.07048	17.59062
N	8	8	8	8	8	8	8
Mean	10.5	17.77913	19.78372	19.63921	28.43433	22.68172	17.91045
Strandard Deviation	1.204752	1.388262	0.793072	1.062047	1.438726	1.953774	2.209239
Standard Error of the mean	0.455354	0.524714	0.299753	0.401416	0.543787	0.738457	0.835014
Relative Expression Level	1	1.69325	1.884163	1.870401	2.708031	2.160164	1.705757
Standard error of the mean	0.043367	0.049973	0.028548	0.03823	0.051789	0.070329	0.079525
% RE	100	169.325	188.4163	187.0401	270.8031	216.0164	170.5757
SE	4.336701	4.997274	2.854793	3.82301	5.178927	7.032926	7.952512

One Way Analysis of Variance

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.258)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Control	8	0	10.500	1.205	0.426
5min	8	0	17.779	1.388	0.491
15min	8	0	19.784	0.793	0.280
30min	8	0	19.639	1.062	0.375
1h	8	0	28.434	1.439	0.509
4h	8	0	22.682	1.954	0.691
24h	8	0	17.910	2.209	0.781

Source of Variation	DF	SS	MS	F	P
Between Groups	6	1412.208	235.368	103.598	<0.001
Residual	49	111.325	2.272		
Total	55	1523.533			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
1h vs. Control	17.934	7	33.654	<0.001	Yes
1h vs. 5min	10.655	6	19.994	<0.001	Yes
1h vs. 24h	10.524	5	19.748	<0.001	Yes
1h vs. 30min	8.795	4	16.504	<0.001	Yes
1h vs. 15min	8.651	3	16.233	<0.001	Yes
1h vs. 4h	5.753	2	10.795	<0.001	Yes
4h vs. Control	12.182	6	22.859	<0.001	Yes
4h vs. 5min	4.903	5	9.200	<0.001	Yes
4h vs. 24h	4.771	4	8.953	<0.001	Yes
4h vs. 30min	3.043	3	5.709	<0.001	Yes
4h vs. 15min	2.898	2	5.438	<0.001	Yes
15min vs. Control	9.284	5	17.421	<0.001	Yes
15min vs. 5min	2.005	4	3.762	0.050	No
15min vs. 24h	1.873	3	3.515	0.043	Do Not Test
15min vs. 30min	0.145	2	0.271	0.849	Do Not Test
30min vs. Control	9.139	4	17.150	<0.001	Yes
30min vs. 5min	1.860	3	3.490	0.044	Do Not Test
30min vs. 24h	1.729	2	3.244	0.026	Do Not Test
24h vs. Control	7.410	3	13.906	<0.001	Yes
24h vs. 5min	0.131	2	0.246	0.862	Do Not Test
5min vs. Control	7.279	2	13.659	<0.001	Yes

Chapter 4

Conclusions

The research findings described herein can be summarized in the following major findings.

1. Shear stress has been found to reorient the VSMC. Under shear stress the cells were found to be aligned at an angle of 45^0 to the vector force of shear.
2. Shear stress was found to phosphorylate mTOR at 5 and 15 min but followed by decreased phosphorylation.
3. Akt was also found to phosphorylate with shear stress in VSMC.
4. In line with phosphorylation of Akt and mTOR the phosphorylation of p70s6k was also found to increase with shear stress.
5. The upregulation of Akt/mTOR/p70s6k activity was further confirmed with the increased phosphoryaltion of PTEN that is indicative of PTEN inactivity.

Taken together, our findings from the current study show that shear stress alters the VSMC orientation with sequential activation of Akt, mTOR and p70s6k and inactivation of PTEN, a pathway that is supposed to increase protein synthesis and result in restenosis.

Future Directions

Our data suggest that SS induces VSMC migration and proliferation that is associated with activation of mTOR, Akt and p70s6k related signaling. Additional studies perhaps employing strategies designed to directly inhibit or activate specific signaling proteins may prove to be useful in delineating the exact molecular mechanisms underlying SS induced VSMC phenotypic modulation.

Many studies have implicated MMPs in migration and proliferation of VSMC and consequent restenotic lesions [104-106, 112]. Therefore assessing MMP activity in the present study would be a valuable tool in further understanding the mechanisms underlying restenosis.

PTEN is demonstrated to be a key regulator of PI3K/Akt signaling being implicated in many proliferative pathologies [72-74, 110, 111]. Further studies to address issues related to upstream regulators associated with PTEN function would be helpful.

VSMC do not terminally differentiate but retain plasticity to dedifferentiate toward a proliferative, synthetic phenotype in response to injury. As the primary function of mature VSMC is contraction, the complement of contractile, structural, and regulatory proteins expressed in fully differentiated VSMC provides markers of differentiation status [113]. These include smooth muscle (SM) myosin heavy chain (SM-MHC), calponin, and SM α-actin. Estimation of expression of these markers of differentiation status in VSMC under shear stress would be another good direction to understand the pathophysiology of restenosis.

References

1. Ajani, U.A., E.S. Ford, and A.H. Mokdad, *Prevalence of high C-reactive protein in persons with serum lipid concentrations within recommended values.* Clin Chem, 2004. **50**(9): p. 1618-22.
2. *Prevalence of heart disease--United States, 2005.* MMWR Morb Mortal Wkly Rep, 2007. **56**(6): p. 113-8.
3. Gimbrone, M.A., Jr., *Vascular endothelium, hemodynamic forces, and atherogenesis.* Am J Pathol, 1999. **155**(1): p. 1-5.
4. Martin, K.A., et al., *The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation.* Am J Physiol Cell Physiol, 2004. **286**(3): p. C507-17.
5. McCaffrey, T.A., *TGF-betas and TGF-beta receptors in atherosclerosis.* Cytokine Growth Factor Rev, 2000. **11**(1-2): p. 103-14.
6. Diamond, S.L., S.G. Eskin, and L.V. McIntire, *Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells.* Science, 1989. **243**(4897): p. 1483-5.
7. Mitsumata, M., et al., *Fluid shear stress stimulates platelet-derived growth factor expression in endothelial cells.* Am J Physiol, 1993. **265**(1 Pt 2): p. H3-8.
8. Frangos, J.A., et al., *Flow effects on prostacyclin production by cultured human endothelial cells.* Science, 1985. **227**(4693): p. 1477-9.
9. Ando, J., H. Nomura, and A. Kamiya, *The effect of fluid shear stress on the migration and proliferation of cultured endothelial cells.* Microvasc Res, 1987. **33**(1): p. 62-70.
10. Dewey, C.F., Jr., et al., *The dynamic response of vascular endothelial cells to fluid shear stress.* J Biomech Eng, 1981. **103**(3): p. 177-85.
11. Wechezak, A.R., R.F. Viggers, and L.R. Sauvage, *Fibronectin and F-actin redistribution in cultured endothelial cells exposed to shear stress.* Lab Invest, 1985. **53**(6): p. 639-47.
12. Gittenberger-de Groot, A.C., et al., *Smooth muscle cell origin and its relation to heterogeneity in development and disease.* Arterioscler Thromb Vasc Biol, 1999. **19**(7): p. 1589-94.
13. Beevers, C.S., et al., *Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells.* Int J Cancer, 2006. **119**(4): p. 757-64.
14. Hay, N. and N. Sonenberg, *Upstream and downstream of mTOR.* Genes Dev, 2004. **18**(16): p. 1926-45.
15. Brooks, R.F., *Continuous protein synthesis is required to maintain the probability of entry into S phase.* Cell, 1977. **12**(1): p. 311-7.
16. Brown, E.J. and S.L. Schreiber, *A signaling pathway to translational control.* Cell, 1996. **86**(4): p. 517-20.
17. Dufner, A. and G. Thomas, *Ribosomal S6 kinase signaling and the control of translation.* Exp Cell Res, 1999. **253**(1): p. 100-9.
18. Braun-Dullaeus, R.C., et al., *Cell cycle protein expression in vascular smooth muscle cells in vitro and in vivo is regulated through phosphatidylinositol 3-kinase and mammalian target of rapamycin.* Arterioscler Thromb Vasc Biol, 2001. **21**(7): p. 1152-8.
19. Yang, Z.Z., et al., *Physiological functions of protein kinase B/Akt.* Biochem Soc Trans, 2004. **32**(Pt 2): p. 350-4.
20. Chen, J., et al., *Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo.* Nat Med, 2005. **11**(11): p. 1188-96.

21. Marx, S.O., et al., *Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells.* Circ Res, 1995. **76**(3): p. 412-7.
22. Sonenberg, N. and A.C. Gingras, *The mRNA 5' cap-binding protein eIF4E and control of cell growth.* Curr Opin Cell Biol, 1998. **10**(2): p. 268-75.
23. Rosner, D., N. McCarthy, and M. Bennett, *Rapamycin inhibits human in stent restenosis vascular smooth muscle cells independently of pRB phosphorylation and p53.* Cardiovasc Res, 2005. **66**(3): p. 601-10.
24. Martin, K.A., et al., *Rapamycin promotes vascular smooth muscle cell differentiation through IRS-1/PI 3-Kinase/Akt2 feedback signaling.* J Biol Chem, 2007.
25. Zhou, R., et al., *Stent implantation activates Akt in the vessel wall: role of mechanical stretch in vascuar smooth muscle cells.* Arterioscler Thromb Vasc Biol, 2003. **Nov 1;23(11)**: p. 2015-20.
26. Haga, M., et al., *Oscillatory shear stress increases smooth muscle cell proliferation and Akt phosphorylation.* J Vasc Surg, 2003. **37**(6): p. 1277-84.
27. Kudo, F.A., et al., *Differential responsiveness of early- and late-passage endothelial cells to shear stress.* Am J Surg, 2005. **190**(5): p. 763-9.
28. Mourani, P.M., et al., *Unique, highly proliferative growth phenotype expressed by embryonic and neointimal smooth muscle cells is driven by constitutive Akt, mTOR, and p70S6K signaling and is actively repressed by PTEN.* Circulation, 2004. **109**(10): p. 1299-306.
29. Ross, R., *Atherosclerosis--an inflammatory disease.* N Engl J Med, 1999. **340**(2): p. 115-26.
30. Resnick, N., et al., *Fluid shear stress and the vascular endothelium: for better and for worse.* Prog Biophys Mol Biol, 2003. **81**(3): p. 177-99.
31. Schwartz, S.M., G.R. Campbell, and J.H. Campbell, *Replication of smooth muscle cells in vascular disease.* Circ Res, 1986. **58**(4): p. 427-44.
32. Chamley-Campbell, J.H., G.R. Campbell, and R. Ross, *Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens.* J Cell Biol, 1981. **89**(2): p. 379-83.
33. Campbell, G.R. and J.H. Campbell, *Smooth muscle phenotypic changes in arterial wall homeostasis: implications for the pathogenesis of atherosclerosis.* Exp Mol Pathol, 1985. **42**(2): p. 139-62.
34. Campbell, G.R. and J.H. Chamley-Campbell, *Invited review: the cellular pathobiology of atherosclerosis.* Pathology, 1981. **13**(3): p. 423-40.
35. Chamley-Campbell, J., G.R. Campbell, and R. Ross, *The smooth muscle cell in culture.* Physiol Rev, 1979. **59**(1): p. 1-61.
36. Kocher, O., et al., *Phenotypic features of smooth muscle cells during the evolution of experimental carotid artery intimal thickening. Biochemical and morphologic studies.* Lab Invest, 1991. **65**(4): p. 459-70.
37. Levesque, M.J. and R.M. Nerem, *The elongation and orientation of cultured endothelial cells in response to shear stress.* J Biomech Eng, 1985. **107**(4): p. 341-7.
38. Levesque, M.J., et al., *Correlation of endothelial cell shape and wall shear stress in a stenosed dog aorta.* Arteriosclerosis, 1986. **6**(2): p. 220-9.
39. Yano, Y., J. Geibel, and B.E. Sumpio, *Tyrosine phosphorylation of pp125FAK and paxillin in aortic endothelial cells induced by mechanical strain.* Am J Physiol, 1996. **271**(2 Pt 1): p. C635-49.

40. Ku, D.N., et al., *Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress.* Arteriosclerosis, 1985. **5**(3): p. 293-302.
41. Kraiss, L.W., et al., *Shear stress regulates smooth muscle proliferation and neointimal thickening in porous polytetrafluoroethylene grafts.* Arterioscler Thromb, 1991. **11**(6): p. 1844-52.
42. Asada, H., et al., *Sustained orbital shear stress stimulates smooth muscle cell proliferation via the extracellular signal-regulated protein kinase 1/2 pathway.* J Vasc Surg, 2005. **42**(4): p. 772-80.
43. Libby, P., et al., *A cascade model for restenosis. A special case of atherosclerosis progression.* Circulation, 1992. **86**(6 Suppl): p. III47-52.
44. Schwartz, S.M., *Perspectives series: cell adhesion in vascular biology. Smooth muscle migration in atherosclerosis and restenosis.* J Clin Invest, 1997. **99**(12): p. 2814-6.
45. Abedi, H. and I. Zachary, *Signalling mechanisms in the regulation of vascular cell migration.* Cardiovasc Res, 1995. **30**(4): p. 544-56.
46. Ross, R., *The pathogenesis of atherosclerosis: a perspective for the 1990s.* Nature, 1993. **362**(6423): p. 801-9.
47. Topper, J.N. and M.A. Gimbrone, Jr., *Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype.* Mol Med Today, 1999. **5**(1): p. 40-6.
48. Gimbrone, M.A., Jr., T. Nagel, and J.N. Topper, *Biomechanical activation: an emerging paradigm in endothelial adhesion biology.* J Clin Invest, 1997. **99**(8): p. 1809-13.
49. Davies, P.F., *Flow-mediated endothelial mechanotransduction.* Physiol Rev, 1995. **75**(3): p. 519-60.
50. Resnick, N. and M.A. Gimbrone, Jr., *Hemodynamic forces are complex regulators of endothelial gene expression.* Faseb J, 1995. **9**(10): p. 874-82.
51. Ross, R., *Cell biology of atherosclerosis.* Annu Rev Physiol, 1995. **57**: p. 791-804.
52. Liu, M.W., G.S. Roubin, and S.B. King, 3rd, *Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia.* Circulation, 1989. **79**(6): p. 1374-87.
53. Chang, P.Y., et al., *Insulin stimulation of mitogen-activated protein kinase, p90rsk, and p70 S6 kinase in skeletal muscle of normal and insulin-resistant mice. Implications for the regulation of glycogen synthase.* J Biol Chem, 1995. **270**(50): p. 29928-35.
54. Dennis, P.B., et al., *The principal rapamycin-sensitive p70(s6k) phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases.* Mol Cell Biol, 1996. **16**(11): p. 6242-51.
55. Weng, Q.P., et al., *Multiple independent inputs are required for activation of the p70 S6 kinase.* Mol Cell Biol, 1995. **15**(5): p. 2333-40.
56. Pearson, R.B., et al., *The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain.* Embo J, 1995. **14**(21): p. 5279-87.
57. Ferrari, S., et al., *Activation of p70s6k is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs.* Proc Natl Acad Sci U S A, 1992. **89**(15): p. 7282-6.
58. Pause, A., et al., *Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function.* Nature, 1994. **371**(6500): p. 762-7.

59. Gingras, A.C., B. Raught, and N. Sonenberg, *Regulation of translation initiation by FRAP/mTOR.* Genes Dev, 2001. **15**(7): p. 807-26.
60. Jefferies, H.B., et al., *Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k.* Embo J, 1997. **16**(12): p. 3693-704.
61. Jefferies, H.B., et al., *Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family.* Proc Natl Acad Sci U S A, 1994. **91**(10): p. 4441-5.
62. Sekulic, A., et al., *A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells.* Cancer Res, 2000. **60**(13): p. 3504-13.
63. Reynolds, T.H.t., S.C. Bodine, and J.C. Lawrence, Jr., *Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load.* J Biol Chem, 2002. **277**(20): p. 17657-62.
64. Scott, P.H., et al., *Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7772-7.
65. Scott, P.H. and J.C. Lawrence, Jr., *Attenuation of mammalian target of rapamycin activity by increased cAMP in 3T3-L1 adipocytes.* J Biol Chem, 1998. **273**(51): p. 34496-501.
66. Thomas, G. and M.N. Hall, *TOR signalling and control of cell growth.* Curr Opin Cell Biol, 1997. **9**(6): p. 782-7.
67. Brazil, D.P. and B.A. Hemmings, *Ten years of protein kinase B signalling: a hard Akt to follow.* Trends Biochem Sci, 2001. **26**(11): p. 657-64.
68. Vanhaesebroeck, B., et al., *Synthesis and function of 3-phosphorylated inositol lipids.* Annu Rev Biochem, 2001. **70**: p. 535-602.
69. Huang, J. and C.D. Kontos, *Inhibition of vascular smooth muscle cell proliferation, migration, and survival by the tumor suppressor protein PTEN.* Arterioscler Thromb Vasc Biol, 2002. **22**(5): p. 745-51.
70. Cantley, L.C. and B.G. Neel, *New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4240-5.
71. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.* J Biol Chem, 1998. **273**(22): p. 13375-8.
72. Leslie, N.R. and C.P. Downes, *PTEN: The down side of PI 3-kinase signalling.* Cell Signal, 2002. **14**(4): p. 285-95.
73. Tsugawa, K., et al., *Biological role of phosphatase PTEN in cancer and tissue injury healing.* Front Biosci, 2002. **7**: p. e245-51.
74. Yamada, K.M. and M. Araki, *Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis.* J Cell Sci, 2001. **114**(Pt 13): p. 2375-82.
75. Vazquez, F., et al., *Phosphorylation of the PTEN tail regulates protein stability and function.* Mol Cell Biol, 2000. **20**(14): p. 5010-8.
76. Vazquez, F., et al., *Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex.* J Biol Chem, 2001. **276**(52): p. 48627-30.
77. Sterpetti, A.V., et al., *Shear stress modulates the proliferation rate, protein synthesis, and mitogenic activity of arterial smooth muscle cells.* Surgery, 1993. **113**(6): p. 691-9.

78. Ueba, H., M. Kawakami, and T. Yaginuma, *Shear stress as an inhibitor of vascular smooth muscle cell proliferation. Role of transforming growth factor-beta 1 and tissue-type plasminogen activator.* Arterioscler Thromb Vasc Biol, 1997. **17**(8): p. 1512-6.
79. Akimoto, S., et al., *Laminar shear stress inhibits vascular endothelial cell proliferation by inducing cyclin-dependent kinase inhibitor p21(Sdi1/Cip1/Waf1).* Circ Res, 2000. **86**(2): p. 185-90.
80. Nakazawa, T., et al., *Platelet-induced migration of smooth muscle cells under shear stress.* Microvasc Res, 1999. **58**(2): p. 177-82.
81. Schwartz, S.M. and M.A. Reidy, *Common mechanisms of proliferation of smooth muscle in atherosclerosis and hypertension.* Hum Pathol, 1987. **18**(3): p. 240-7.
82. Stamatas, G.N., C.W. Patrick, Jr., and L.V. McIntire, *Intracellular pH changes in human aortic smooth muscle cells in response to fluid shear stress.* Tissue Eng, 1997. **3**(4): p. 391-403.
83. Giasson, E. and S. Meloche, *Role of p70 S6 protein kinase in angiotensin II-induced protein synthesis in vascular smooth muscle cells.* J Biol Chem, 1995. **270**(10): p. 5225-31.
84. Sadoshima, J. and S. Izumo, *Rapamycin selectively inhibits angiotensin II-induced increase in protein synthesis in cardiac myocytes in vitro. Potential role of 70-kD S6 kinase in angiotensin II-induced cardiac hypertrophy.* Circ Res, 1995. **77**(6): p. 1040-52.
85. Li, N., et al., *Angiotensin II stimulates phosphorylation of 4E-binding protein 1 and p70 S6 kinase in cultured vascular smooth muscle cells.* Acta Pharmacol Sin, 2004. **25**(5): p. 593-6.
86. Chou, M.M. and J. Blenis, *The 70 kDa S6 kinase: regulation of a kinase with multiple roles in mitogenic signalling.* Curr Opin Cell Biol, 1995. **7**(6): p. 806-14.
87. Baar, K., et al., *Autocrine phosphorylation of p70(S6k) in response to acute stretch in myotubes.* Mol Cell Biol Res Commun, 2000. **4**(2): p. 76-80.
88. Bradley, J.M., et al., *Signaling pathways used in trabecular matrix metalloproteinase response to mechanical stretch.* Invest Ophthalmol Vis Sci, 2003. **44**(12): p. 5174-81.
89. Hornberger, T.A., et al., *Intracellular signaling specificity in response to uniaxial vs. multiaxial stretch: implications for mechanotransduction.* Am J Physiol Cell Physiol, 2005. **288**(1): p. C185-94.
90. Hornberger, T.A., et al., *Mechanical stimuli regulate rapamycin-sensitive signalling by a phosphoinositide 3-kinase-, protein kinase B- and growth factor-independent mechanism.* Biochem J, 2004. **380**(Pt 3): p. 795-804.
91. Kraiss, L.W., et al., *Fluid flow activates a regulator of translation, p70/p85 S6 kinase, in human endothelial cells.* Am J Physiol Heart Circ Physiol, 2000. **278**(5): p. H1537-44.
92. Ley, K., et al., *Shear-dependent inhibition of granulocyte adhesion to cultured endothelium by dextran sulfate.* Blood, 1989. **73**(5): p. 1324-30.
93. Tsao, P.S., et al., *Exposure to shear stress alters endothelial adhesiveness. Role of nitric oxide.* Circulation, 1995. **92**(12): p. 3513-9.
94. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979.* Biotechnology, 1992. **24**: p. 145-9.
95. Kataoka, N., S. Ujita, and M. Sato, *Effect of flow direction on the morphological responses of cultured bovine aortic endothelial cells.* Med Biol Eng Comput, 1998. **36**(1): p. 122-8.

96. Sedding, D.G., et al., *Mechanosensitive p27Kip1 regulation and cell cycle entry in vascular smooth muscle cells.* Circulation, 2003. **108**(5): p. 616-22.
97. Boppart, M.D., et al., *Static stretch increases c-Jun NH2-terminal kinase activity and p38 phosphorylation in rat skeletal muscle.* Am J Physiol Cell Physiol, 2001. **280**(2): p. C352-8.
98. Rice, K.M., et al., *Effects of aging on pressure-induced MAPK activation in the rat aorta.* Pflugers Arch, 2005.
99. Rice, K.M., et al., *Aging alters vascular mechanotransduction: Pressure-induced regulation of p70S6k in the rat aorta.* Mech Ageing Dev, 2005.
100. Campbell, G.R., et al., *Arterial smooth muscle. A multifunctional mesenchymal cell.* Arch Pathol Lab Med, 1988. **112**(10): p. 977-86.
101. Ferns, G.A., et al., *Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF.* Science, 1991. **253**(5024): p. 1129-32.
102. Furnari, F.B., H.J. Huang, and W.K. Cavenee, *The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells.* Cancer Res, 1998. **58**(22): p. 5002-8.
103. Tamura, M., et al., *Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN.* Science, 1998. **Jun 5;280(5369)**: p. 1614-7.
104. Risinger, G.M., Jr., et al., *Matrix metalloproteinase-2 expression by vascular smooth muscle cells is mediated by both stimulatory and inhibitory signals in response to growth factors.* J Biol Chem, 2006. **281**(36): p. 25915-25.
105. Vu, T.H. and Z. Werb, *Matrix metalloproteinases: effectors of development and normal physiology.* Genes Dev, 2000. **14**(17): p. 2123-33.
106. Uzui, H., et al., *The role of protein-tyrosine phosphorylation and gelatinase production in the migration and proliferation of smooth muscle cells.* Atherosclerosis, 2000. **149**(1): p. 51-9.
107. Poon, M., et al., *Rapamycin inhibits vascular smooth muscle cell migration.* J Clin Invest, 1996. **98**(10): p. 2277-83.
108. Reusch, H.P., et al., *Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells.* J Biol Chem, 2001. **276**(36): p. 33630-7.
109. Hafizi, S., et al., *ANG II activates effectors of mTOR via PI3-K signaling in human coronary smooth muscle cells.* Am J Physiol Heart Circ Physiol, 2004. **287**(3): p. H1232-8.
110. Suzuki, A., et al., *High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice.* Curr Biol, 1998. **8**(21): p. 1169-78.
111. Di Cristofano, A., et al., *Pten is essential for embryonic development and tumour suppression.* Nat Genet, 1998. **19**(4): p. 348-55.
112. Li, H., et al., *FoxO4 regulates tumor necrosis factor alpha-directed smooth muscle cell migration by activating matrix metalloproteinase 9 gene transcription.* Mol Cell Biol, 2007. **27**(7): p. 2676-86.
113. Sobue, K., K. Hayashi, and W. Nishida, *Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation.* Mol Cell Biochem, 1999. **190**(1-2): p. 105-18.