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Effects of cPLA-2 on the Migration and Proliferation of Human Vascular Smooth Muscle Cells and the 2-D Migratory Patterns of Tropomyosin in Femoral and Abdominal Aorta Tissue

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EFFECTS OF cPLA-2 ON THE MIGRATION AND PROLIFERATION OF HUMAN VASCULAR SMOOTH MUSCLE CELLS AND THE 2-D MIGRATORY PATTERNS OF TROPOMYOSIN IN FEMORAL AND ABDOMINAL AORTA TISSUE

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ABSTRACT

Abstract 1: Platelet derived growth factor BB (PDGF BB) has an important influence on smooth muscle cell proliferation in restenosis and atherosclerosis. Our understanding of different signal transduction pathways involved in the response of smooth muscle cells to PDGF BB is potentially significant for understanding and manipulating these processes. Prior studies have demonstrated a crucial activation of cytosolic phospholipase A_2 ($cPLA_2$) in smooth muscle cells to PDGF BB with the production of arachidonic acid and prostaglandin E2. In these studies we investigated the role for another PLA_2, calcium-independent PLA_2 ($iPLA_2$) in comparison to $cPLA_2$ on smooth muscle cell migration and proliferation. Pharmacological inhibitors of $cPLA_2$ were found to substantially inhibit proliferation, but not migration. AACOCF3 ($cPLA_2$ and $iPLA_2$ inhibitor) and 1,2,4-trisubstituted pyrrolidine derivative ($cPLA_2$ inhibitor) both inhibited smooth muscle proliferation where Bromoenol lactone ($iPLA_2$ inhibitor) had no effect. None of these inhibitors prevented smooth muscle chemotaxis to PDGF BB in a modified Boyden chamber. In reconstitution experiments, arachidonic acid fully restored smooth muscle cell proliferation after treatment with 1,2,4-trisubstituted pyrrolidine derivative. These data demonstrate the distinct role of $cPLA_2$ on smooth muscle cell proliferation, which is a critical step in the pathogenesis of restenosis and atherosclerosis.

Abstract 2: There are major histological differences between normal murine aorta and femoral artery. There are also major differences in the effects of

atherosclerosis on these two arteries with aneurysm formation affecting the aorta and long complex atherosclerotic lesions affecting the femoral artery, which result in lumen narrowing. Our overall understanding of the different proteins expressed in these two arteries is not well understood. In these studies we investigated the difference in protein expression in normal murine abdominal aorta compared to femoral artery. We found that tropomyosin alpha 1 and beta from murine femoral artery migrates to different locations on 2D gel electrophoresis. Further investigation using western blot analysis shows that these two proteins migrate to the same location. We hypothesize that another protein(s) is bound to tropomyosin in femoral artery and not in abdominal aorta to form a complex that causes the protein to migrate differently on 2D analysis.

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LIST OF ABBREVIATIONS

AA……..……Arachidonic Acid

$AACOCF_3$ ……...…….Arachidonyltrifluoromethyl Ketone

ATP…….Adenosine Triphosphate

BEL……...…….Bromoenol Lactone

cPLA-2 ..... Calcium Dependent Cytosolic Phospholipase A-2

DMSO……...…….Dimethyl Sulfoxide

GTP…….Guanosine Triphosphate

HAVSMC .....……Human Arterial Vascular Smooth Muscle Cells

iPLA2……..……...…..Calcium Independent Cytosolic Phospholipase A-2

MALDI-TOF……..Matrix-Assisted Laser Desorption/Ionization – Time of Flight

MS……..……..…Mass Spectrometry/Spectrometer

PDGF……...……Platelet Derived Growth Factor

PTA……...…….Percutaneous Transluminal Angioplasty

siRNA……...……..small interfering Ribonucleic Acid

SMA……...…….Smooth Muscle Actin

SMC ..... Smooth Muscle Cells

TPM……..Tropomyosin

TPMI…….Tropomyosin

CHAPTER 1

EFFECTS OF CPLA-2 ON THE MIGRATION AND PROLIFERATION OF HUMAN VASCULAR SMOOTH MUSCLE CELLS

1.1 INTRODUCTION

Migration and proliferation of vascular smooth muscle cells (VSMCs) are important initial steps in the progression of atherosclerotic lesions, restenosis, and transplant vasculopathy. The common characteristics of vascular responses to balloon injury are proliferation and migration of VSMCs and neointima formation in the injured arteries.[1] Neointimal hyperplasia and restenosis are the major problems limiting the long-term efficacy of percutaneous transluminal angioplasty.[2] Although the mechanisms responsible for the proliferation and migration of VSMCs are not fully understood, several factors produced in response to vascular injury have been implicated in this process. One of the major growth factors includes platelet-derived growth factor (PDGF). PDGF is both mitogenic and chemotactic for medial VSMC.[3] Denudation of endothelial cells after balloon angioplasty results in release of PDGF and other growth factors which stimulates VSMC proliferation and migration into the intima resulting in intimal hyperplasia.[4]

PDGF is a growth factor that plays a role in embryonic development, cell proliferation, cell migration, angiogenesis, atherosclerosis and restenosis.[5,6] It is a dimeric glycoprotein composed of two A (-AA) or two B (-BB) chains or a combination of the two (-AB). Two receptors for PDGF are tyrosine kinase receptors classified as

alpha and beta type. Upon activation by PDGF, these receptors dimerize, auto-phosphorylate several sites on their cytosolic domains, which serve to mediate binding of cofactors and subsequently activate signal transduction through the PI3K and MAPK pathways.[7,8,9] Accumulating data suggest that although this molecule is generally part of growth signaling complex, it plays a more profound role in controlling cell migration. PDGF-BB is the highest-affinity ligand for the PDGFR-beta and is the most potent stimulator of VSMC migration.[10] Stimulation of VSMC with PDGF-BB produces a rapid release of arachidonic acid and prostaglandin E2 through the activation of $cPLA_2$.[11]

Phospholipase A_2 specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing fatty acid and lysophospholipid.[12] PLA_2 includes four unrelated protein families with common enzymatic activity composed of secretory PLA_2, $cPLA_2$ which is dependent on calcium, calcium independent PLA_2 or $iPLA_2$, and lipoprotein-associated PLA_2s (lp-PLA_2), also known as platelet activating factor acetylhydrolase (PAF-AH). Only $cPLA_2$ is selective for arachidonic acid at the sn-2 position which can be further metabolized into eicosinoids such as prostaglandins and leukotrienes which are categorized as inflammatory mediators.[13,14,15,16,17] The exact role these PLA_2s play in smooth muscle migration and proliferation is currently unknown, however it has been proposed that these eicosanoids activate GTPases, which in turn activate SMC proliferation and motility.[11]

This study investigates the role of cPLA2 and $iPLA_2$ in migration and proliferation of human smooth muscle cells to PDGF-BB in vitro.

1.2 METHODS AND MATERIALS

Tissue Culture and SMC Isolation

Human arterial vascular smooth muscle cell lines (HAVSMC) were purchased from ATCC (CLR-1999) which were obtained from a 11-month-old female aorta. The cells were cultured in HAVSMC Media (FI2K, $NaHCO_3$, Hepes, TEST, ECGS, ITS) containing 10% fetal bovine serum (FBS) at 37^{o}C with 5% CO_2. Cells were grown to >80% confluence and non-adherent cells were removed.

Protein Extraction for cell lines

Cells were treated in their six-well plates with lysis buffer containing NP-40 and incubated at room temperature for five minutes. Lysate was sonicated for fifteen seconds, three times. Cells and lysis buffer were transferred to tubes and spun at 13,000 rpm for 20 minutes at 4 ^{o}C. Nuclear pellet was discarded.

Immunofuorescence assay

For immunofluoresence, human aortic smooth muscle cells were grown in eight chamber slides with two wells stimulated with 25ng/ml of PDGF BB for 2hrs. Slides were fixed in HistoChoice (Ameresco, Solon, Ohio) until stained. Cells were permeablized with 0.01% triton X-100 and blocked with 1% bovine serum albumin. Primary antibodies were applied to recognize smooth muscle α-actin (mouse monoclonal antibody, clone 1A-4) and vimentin (rabbit polyclonal antibody) in the concentration of 1μg/mL. All antibodies were purchased from Neomarkers Inc, Fremount, Calif. After washing in PBS three times, fluorescein isothiocyanate-tagged secondary antibodies were applied (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Rabbit anti-mouse immunoglobulin G (IgG) was applied to chambers stained for smooth muscle actin

5μg/mL, and 5μg/mL of goat anti-rabbit IgG was used for vimentin staining. Negative control chambers were stained with secondary antibodies alone. This experiment was repeated in triplicate with representative micrographs. Slides were analyzed with Leica Qwin system (Leica, Wetzlan, Germany) by using Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, Md).

Pharmacological inhibitors

HAVSMC were washed once in PBS and resuspended in DMEM without serum. The cells were treated with the pharmacologic inhibitors and incubated for 1 h at 37°C with 10% CO_2 before performing the chemotaxis or proliferation assays. All inhibitors were reconstituted in DMSO. Varying concentrations of Bromoenol lactone (iPLA2 inhibitor), 1,2,4 trisubstituted pyrrolidine (cPLA2 inhibitor) or $AACOCF_3$ (cPLA2 and iPLA2 inhibitor) were added in less than 1/200 inhibitor volume to total volume of culture media. Unstimulated control cells were only stimulated with PBS in DMEM without serum.

Chemotaxis assay

HAVSMC migration was evaluated using a microchamber technique.[37] Human recombinant PDGF BB (25 ng/ml) in DMEM with 0.1% BSA was added to the lower compartment of the disposable 96-well chemotaxis chamber (NeuroProbe, Gaithersburg, MD) in a volume totaling 29 μl. The cell suspension (50 μl of 2 x 10^6 cells/ml; 1 x 10^5 cells/well) was added to the upper compartment of the chamber that had been precoated with collagen type 1 for 2 h. The two compartments were separated by a 5-μm pore size, polycarbonate, polyvinylpyrrolidone-free filter. The chamber was incubated at 37°C in air with 5% CO_2 for 90 min. At the end of the incubation, the filter facing the upper

compartment was scraped with a sponge and rinsed gently with PBS to remove all non-migrated cells. The side of the filter with the migrated cells was fixed and stained with Hema 3 Stain Set (Biochemical Science, distributed by Fisher Scientific, Pittsburgh, PA). Migrated SMC were counted in five high-power fields (x400) using a light microscope. All samples were tested in triplicate, and the data are expressed as the mean ± SD.

Proliferation assay

5x103 HAVSMC were cultured in DMEM without serum and in the presence or absence of various concentrations of AACOCF3, cPLA2 INH, and BEL for 1hr prior to stimulation with 25ng/ml of PDGF-BB or 10%FBS (Pos Con) for 48hrs at 37°C in air with 5% CO2. The number of cells were quantified by nonradioactive colorimetric WST-1 assay (Roche Applied Sciences, IN) according to manufactures protocol. Briefly, 10ul of Cell Proliferation Reagent WST-1 was added to each well and allowed to incubate for 4 hours at 37°C in air with 5% CO2. The plate was shaken for 1 minute and read at 440nm on a microplate reader (Bio-Rad, Hercules, CA).

Western Blot/SDS-Page

HAVSMC were washed three times with PBS to remove traces of DMEM and 10% BCS. The tubes were placed on ice, and the cells were lysed using 200 µl of lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCL, pH 7.4, 1 mM PMSF, and 10 µl of protease inhibitor mix (Sigma) per milliliter of lysis buffer). After 30 min, the lysate was centrifuged for 15 min at 9300 x *g*. The supernatant was collected, and the protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA) and loaded on a 7.5% SDS-PAGE gel (150 µg of total protein/well). The proteins were transferred to a polyvinylidene difluoride membrane (0.2 µm; Bio-Rad) using a TRANS-

BLOT SD electrophoretic transfer cell (Bio-Rad). The membrane was blocked in 5% nonfat milk in PBS and 1% Tween 20 overnight at 4°C and then probed with primary Ab. $cPLA_2$ protein was detected with a 1/1000 dilution of anti-mouse recombinant $cPLA_2$ mAb (Santa Cruz), followed by incubation with anti-mouse IgG HRP (1/1000 dilution; Transduction Laboratories, Lexington, KY).

siRNA Transfection

HAVSMC were plated in six well plates and cultured 37°C with 5% CO_2 until the cells were 70-90% confluent. siRNA transfection using a control A sequence, fluorescent conjugated control A, and $cPLA_2$ siRNA (h) (Santa Cruz Biotechnology, Inc, CA) was carried out according to manufacturer's protocol. Briefly, varying concentrations of siRNA tested from 60 to 120pM were each mixed with the transfection reagent for 45 minutes at room temperature, and then added to cells and allowed to incubate 7 hours at 37°C with 5% CO_2. One milliliter of normal growth medium with 2X FBS was added to each well and allowed to incubate for 18-24hours at 37°C with 5% CO_2. This was replaced with 1X normal growth media and allowed to incubate for 72 hours. Cells were prepared for either western blot analysis or fluorescent microscopy to determine protein inhibition or rate of transfection, respectively.

1.3 RESULTS

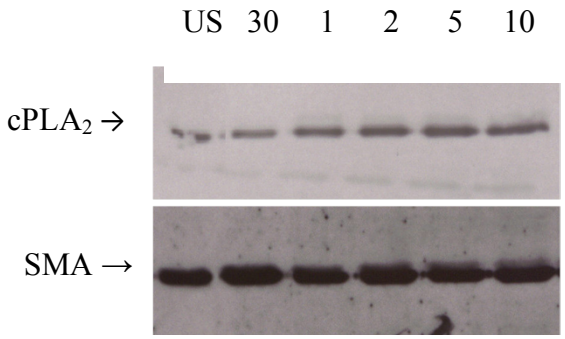

Figure 1.1. cPLA2 Protein Peaks at Two Hours in Human Aortic VSMCs After Stimulation with PDGF-BB. VSMCs were unstimulated (US) or incubated with 25ng/ml of PDGF-BB for 0.5, 1, 2, 5, and 10 hours. 100µg of total cytoplasmic protein was evaluated by Western Blot for cPLA2 protein (upper panel). The same blot was stripped and reprobed with an antibody to Smooth Muscle Actin (SMA – lower panel).

PDGF BB induces $cPLA_2$ protein expression, which appears to peak after 2 hours of stimulation in SMC (figure 1). $cPLA_2$ expression continues to 16 hours, and then attenuated to its PDGF BB un-stimulated levels at 24 hours (data not shown). SMC-Actin was used as a loading control. Next we investigated the effects on actin polymerization in SMC after stimulation with PDGF BB.

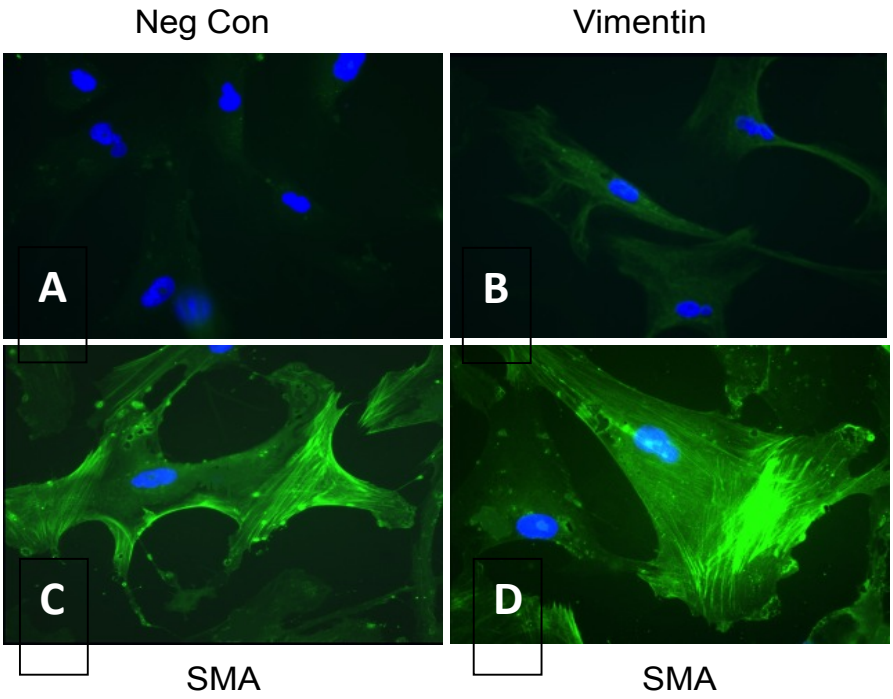

Figure 1.2. PDGF-BB Produces Actin Polymerization at the Leading Edge of Human Aortic VSMCs. Immunofluorescence of unstimulated VSMC stained with no primary antibody (A), vimentin (B), and smooth muscle actin (C). Stimulation of VSMC with 25ng/ml of PDGF-BB resulted in polymerization of smooth muscle actin fibers at the leading edge of cells (D).

Smooth muscle cells were observed in an immunofluorescence assay to reveal increased levels of smooth muscle actin polymerization in the PDGF-bb stimulated cells at the leading edge of cells. As a negative control un-stimulated human VSMC stained with no primary antibody exhibited no fluorescent staining (figure 2A). To rule out the presence of fibroblasts in the culture the cells were stained with vimentin (figure 2B). Normally, un-stimulated SMC stained with smooth muscle actin (SMA) show actin staining around areas of anchoring points (figure 2C). However, after stimulation with PDGF BB SMA filaments congregated at the leading edge of SMC (figure 2D). The cells became polarized to indicate movement with the SMA making up part of the lamellapodia of the polarized cell. This actin polymerization was observed in ~70% of

the cells within the culture colony. This demonstrates that PDGF BB plays a role in SMC movement. We next went to investigate if $cPLA_2$ played a role in SMC migration to PDGF-BB.

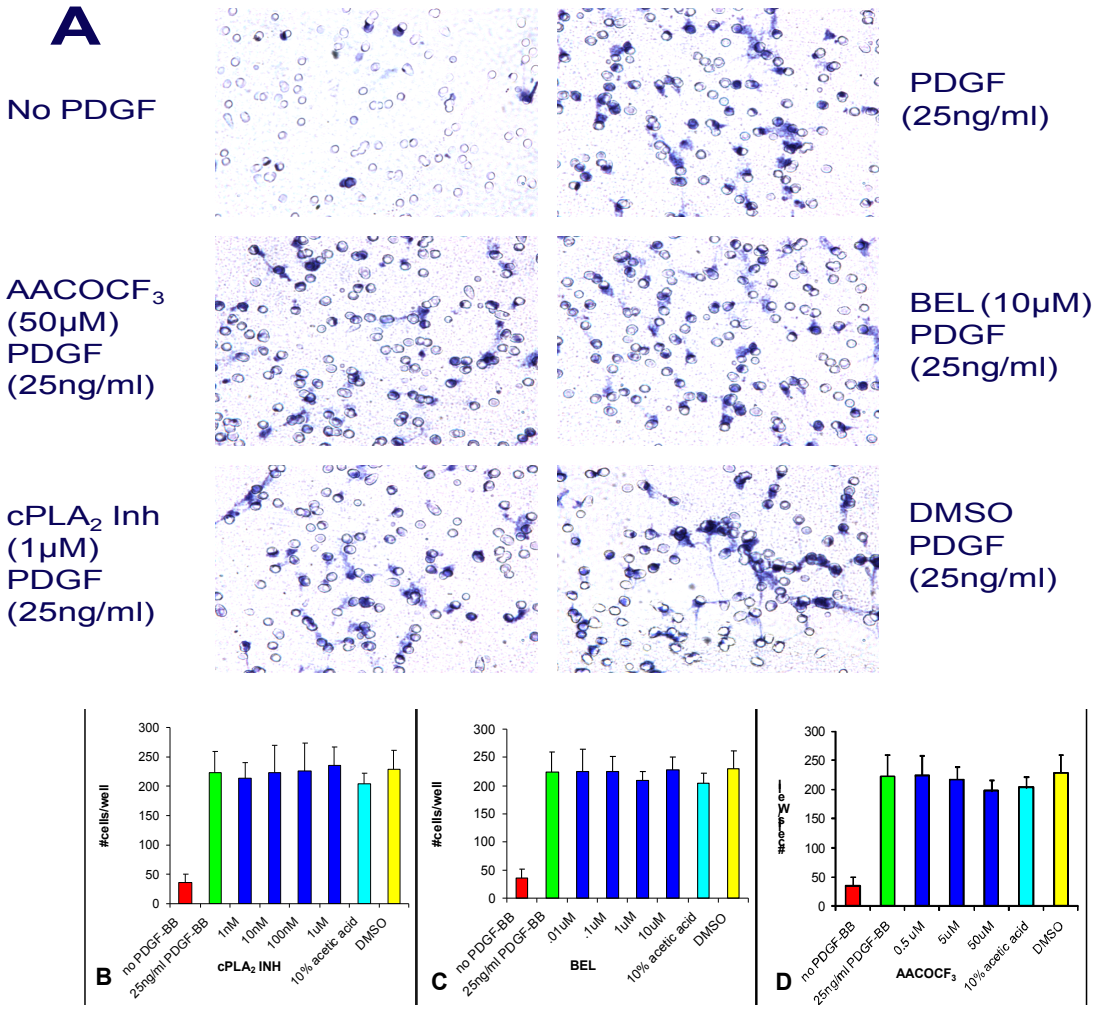

Figure 1.3. Pharmacological inhibitors of PLA_2 do not inhibit VSMC migration to PDGF-BB by in-vitro chemotaxis assay. Pictures of polycarbonate filter at 400X with the highest concentrations of all three pharmacological inhibitors (A). 10% acetic acid was used to dissolve the type 1 collagen and DMSO was the diluent for the pharmacological inhibitors. VSMCs were cultured in DMEM without serum and in the presence or absence of various concentrations of $cPLA_2$ INH (B), BEL, $iPLA_2$ inhibitor (C), $AACOCF_3$ $cPLA_2$ and $iPLA_2$ inhibitor(D) for 1hr. VSMCs chemotaxis across a type 1 collagen coated polycarbonate filter in response to 25ng/ml of PDGF-BB was then measured. All samples were performed in triplicate, and experiments were repeated three times. The results are expressed as the total number of migrated cells per well± SD and are from a representative experiment.

Inhibition of $cPLA_2$ or $iPLA_2$ with pharmacological inhibitors had no effect on smooth muscle cell chemotaxis to PDGF BB in a modified Boyden chamber (figure 3). 1,2,4-trisubstituted pyrrolidine derivative ($cPLA_2$ INH) is a specific inhibitor of $cPLA_2$ which had no significant effect on SMC chemotaxis to PDGF BB from doses of 1nM to 1μM (figure 3B). This was also seen for the $iPLA_2$ inhibitor, Bromoenol lactone (BEL), using concentrations from 0.01μM to 10μM (figure 3C). $AACOCF_3$ has been reported to inhibit both $cPLA_2$ and $iPLA_2$, but not $sPLA_2$.[18,19] $AACOCF_3$ also did not cause significant, dose-dependent inhibition of SMC migration to PDGF BB in up to 50μM concentration (Figure 3D).

Pictures of the filter at 400X with the highest concentration of inhibitors and the solvent used to dilute the inhibitors (DMSO) show an equal number of cells migrating to the other side of the filter (figure 3A). Therefore, the data shows that inhibition of $cPLA_2$ and $iPLA_2$ does not significantly affect SMC migration in a modified Boyden chamber migration assay.

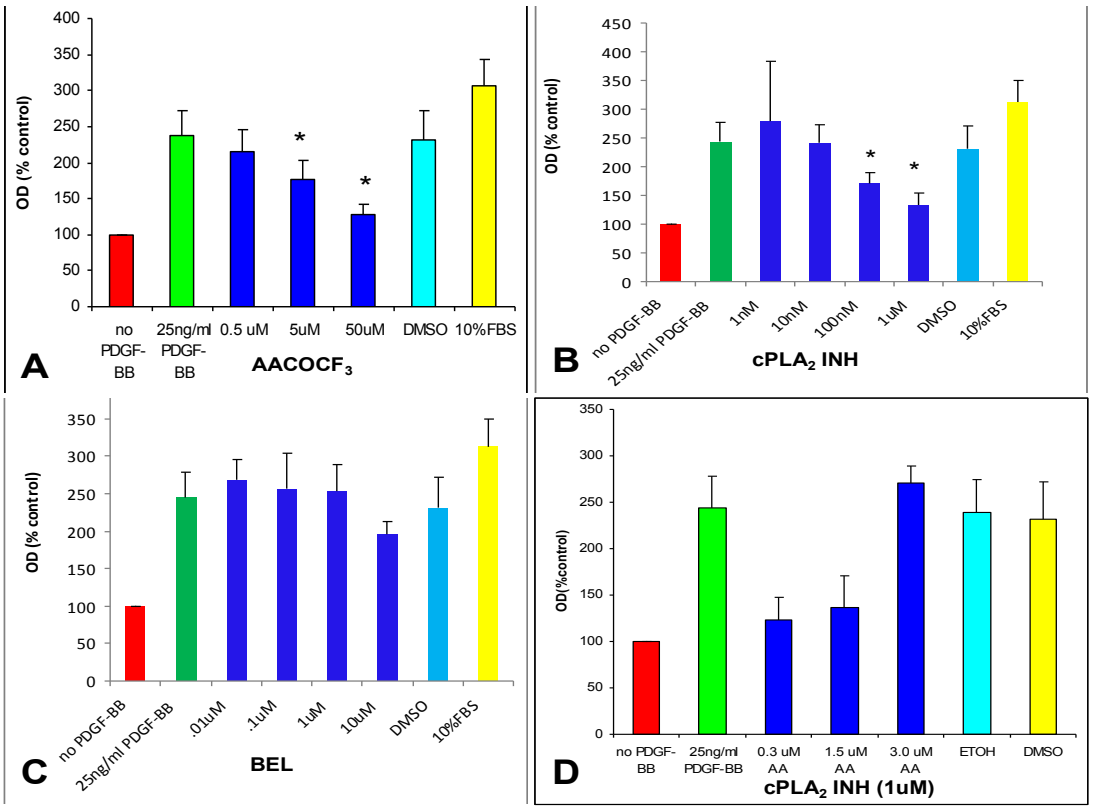

Figure 1.4. Pharmacological inhibitors of cPLA2 suppress PDGF-BB-induced proliferation of human aortic VSMCs, which is restored by AA. VSMCs were cultured in DMEM without serum and in the presence or absence of various concentrations of AACOCF3 (A), cPLA2 INH (B), and BEL (C) for 1hr prior to stimulation with 25ng/ml of PDGF-BB or 10%FBS (Pos Con) for 48hrs. The number of cells were quantified by nonradioactive colorimetric WST-1 assay (Roche Applied Sciences, IN). All samples were performed in triplicate, and experiments were repeated three times. The results are expressed as % control OD 450nm of wells not stimulated with PDGF-BB. PDGF-BB stimulated cell proliferation after inhibition by 1μM cPLA2 INH was restored by 3μM AA (D). *, $p < 0.05$

Inhibition of $cPLA_2$ with pharmacological inhibitors significantly reduces PDGF BB stimulated SMC proliferation (figure 4). Using the same inhibitors as in the migration assays, both $AACOCF_3$ and $cPLA_2$ INH significantly inhibited SMC proliferation in a dose dependent manner (figures 4A&4B). There was a 70 to 85% inhibition at their highest doses. Bromoenol lactone, the $iPLA_2$ inhibitor, did not inhibit SMC proliferation induced by PDGF BB even at its highest dose of 10μM (figure 4C). Arachidonic Acid (AA) a product of the enzymatic activity of $cPLA_2$ resulting from

cleavage at the sn-2 position of a phospholipid. We used AA in a dose dependent mannor to restore the inhibition of proliferation in SMC treated with $cPLA_2$ INH and stimulated PDGF BB. Inhibition of SMC proliferation was reconstituted in cells treated with $cPLA_2$ INH when the cells were stimulated with arachidonic acid, the principal downstream byproduct of cPLA2 (figure 4-D). It was found that a concentration of 3.0μM arachidonic acid best reconstituted SMC proliferation. This indicates that $cPLA_2$ and its pathway plays an important role in SMC proliferation.

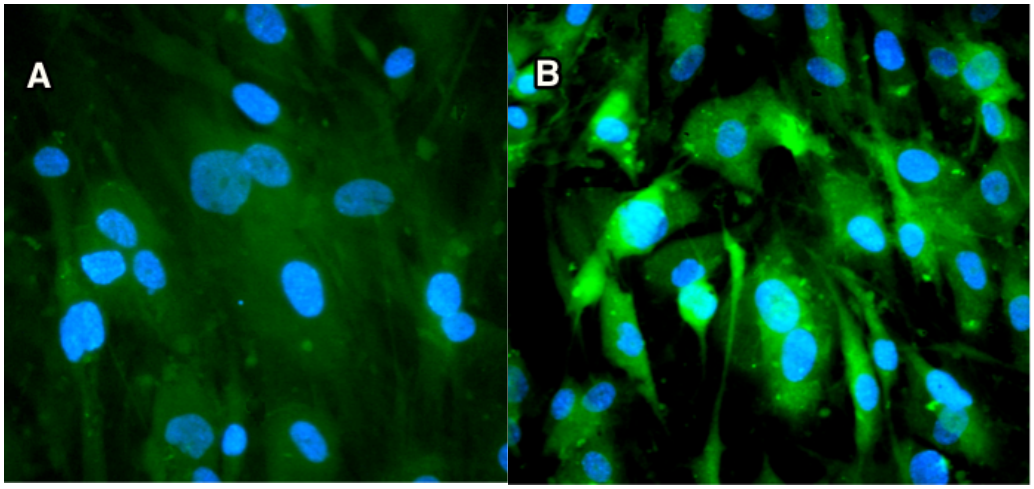

Figure 1.5 - Expression of fluoroscein conjugated siRNA after 72 hours of transfection in human aortic smooth muscle cells. VSMC's were not transfected and treated with PBS as a blank (left)**.** VSMC's were transfected with a control A scrambled siRNA at 120pM (right) and incubated for 72 hours at 37°C in 5% CO_2. Fluorescent microscopy was taken at 200x magnification
important role in SMC proliferation.

To further our studies of $cPLA_2$ inhibition, we sought to inhibit the expression of $cPLA_2$ by using targeted siRNA to $cPLA_2$ in SMC. First we characterized the number of cells and concentration of siRNA to transfect cells. We used a fluorescein-conjugated siRNA at different concentrations to observe the rate of transfection. Transfection of a fluorescein labeled siRNA at 120pM resulted in an 85-95% transfection rate after a 72-hour incubation period (figure 5C). This was confirmed in triplicate experiments.

Inhibition of cPLA2 in our siRNA model was confirmed via Western Blotting. Various concentrations of cPLA2 siRNA inhibitor revealed that 120pM best inhibited $cPLA_2$ protein expression (figure 6). Over several experiments we had a range from 70-90% of $cPLA_2$ protein inhibition after 72 hours of transfection with siRNA.

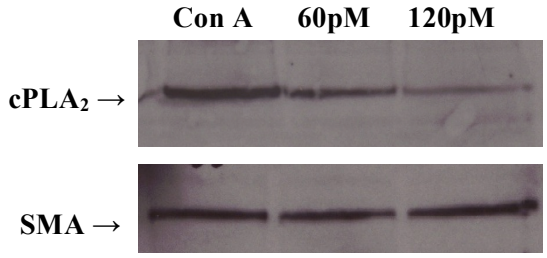

Figure 1.6 – $cPLA_2$ protein expression after 72 hours of transfection with $cPLA_2$ siRNA of human aortic smooth muscle cells. Western blot of VSMC's that were transfected with a 120pM control A scrambled siRNA, 60pM and 120pm of $cPLA_2$ siRNA (upper panel). The same blot was stripped and reprobed with an antibody to Smooth Muscle Actin (SMA – lower panel).

1.4 DISCUSSION

Our studies characterize the important relative contribution of $cPLA_2$ for regulating smooth muscle proliferation to PDGF BB. Since PDGF is believed to initiate a multitude of biological effects through signal transduction pathways such as MAP kinase cascade, phosphotidylinositol turnover, and calcium mobilization in different cells resulting in cell proliferation and directed migration.[10] PDGF stimulation results in edge ruffling, calcium mobilization, chemotaxis, and mitogenic effects in different cell lines.[6,7] It has been reported that smooth muscle cell growth and contraction require cAMP metabolism and calcium homeostasis to function.[20] $cPLA_2$ requires calcium mobilization from the endoplasmic reticulum initially to activate. Exactly how these key components interact is currently poorly understood. Because of our interest in the role of $cPLA_2$ and $iPLA_2$ in monocyte chemotaxis to MCP-1 during chronic inflammation[21,22], we have

turned to investigating the role of these PLA_2's in smooth muscle cell migration and proliferation which are essential components in the pathogenesis of atherosclerosis and restenosis.

Previous work has shown that the PDGF BB stimulated AA release from VSMC in a calcium dependent manner through the activation of $cPLA_2$.[23] Our studies confirm that $cPLA_2$ protein is increased after stimulation with PDGF BB (Figure 2) and remains elevated above 10 hours. This could lead to the overproduction of eicosanoids, AA metabolites, which play a key role as lipid mediators of inflammation. PGE_2 is released rapidly from PDGF BB stimulated VSMC.[24] COX-2 metabolizes AA to form PGE_2 which functions to control vascular smooth muscle constriction or dilation, regulate calcium movement, and mediate inflammation.[25] The exact role of eicosanoids on VSMC migration and proliferation is currently unknown.

Some of the first steps to cell movement are the formation of actin into long extending finger- like projections such as filopodia and other broad branching networks called lamellipodia in the front of the cell forming the leading edge.[26,27] The finger-like projections act to sense and explore the local environment where the lamellipodia provide a strong foundation over which the cell can move forward. Stimulation of smooth muscle cells with PDGF BB produced a broad branching network of smooth muscle actin at the leading edge of the cell forming a lamellipodia structure (Figure I-1D). This structure was seen in 60-70% of cells stimulated with PDGF BB in a culture made up primarily of smooth muscle cells without contamination of fibroblasts (Figure 1B- negative vimentin staining).

Eicosanoids have been implicated to play a role in migration of inflammatory cells and cancer cells.[28,29,30,31] AA can be converted into various metabolites, such as prostoglandins, lipoxins, LTB4, H(P)ETEs via COX-2, cytochrome P450, or lipoxygenase pathways.[32,33,34,35,36] LTB4 has been shown to play a role in the migration of different cells such as neutrophils and eosiophils.[37,38] Lipoxins induce RhoA and Rac –mediated actin reorganization in monocyte and macrophage, whereas protoglandin E2 increases migration of dendritic cells.[39,40] We have previously shown that $cPLA_2$ and $iPLA_2$ play distinct and separate roles in human monocyte migration to MCP-1.[21,22] However, pharmacological inhibition of both $cPLA_2$ and $iPLA_2$ had no effect on VSMC to PDGF BB (Figure 3). This may be due to differences in receptors and signaling between the two different cells, which would be subject to further investigation. Therefore, VSMC chemotaxis is not dependent on AA or its metabolites for its migration to PDGF BB.

PLA_2 plays a role in growth of several normal and cancerous cell types.[41,42,43,44] Insulin stimulates proliferation of VSMC which partially is dependent on $cPLA_2$.[45] Here we demonstrate that VSMC proliferation is dependent on $cPLA_2$ and not $iPLA_2$ (Figure 4 B&C). Since $AACOCF_3$ is an inhibitor of both $cPLA_2$ and $iPLA_2$ and significantly reduced PDGF BB stimulated proliferation of VSMC, but the specific $iPLA_2$ inhibitor BEL (Figure 4 A&C) did not, therefore $cPLA_2$ plays more of a role in proliferation. This was confirmed by the specific inhibitor of $cPLA_2$ significantly reducing PDGF BB stimulated proliferation of VSMC (Figure 4B). Since $cPLA_2$ is selective for cleavage of AA at the sn2 position of phospholipids, we used AA to see if it would restore $cPLA_2$-inhibited proliferation of VSMC stimulated with PDGF BB. Adding back AA fully

restored PDGF stimulated proliferation of VSMC after inhibition of $cPLA_2$ (Figure 4D). This leads us to conclude that $cPLA_2$ is involved in VSMC proliferation to PDGF BB in an AA dependent manor.

Since pharmacological inhibitors can have other effect than specified, we have turned to siRNA as a more specific inhibitor of $cPLA_2$ and plan to repeat both proliferation and chemotaxis assay to further confirm our results. We are currently characterizing the doses of siRNA to inhibit $cPLA_2$ in human VSMC. In fluorescent-labeled siRNA a conconcentraton of 120pM results in about 90% of cellular transfection (Figure 5). At this concentration there is a about 80% inhibition of $cPLA_2$ protein (Figure 6). After characterizing our siRNA to $cPLA_2$ we plan to also do the AA restoration experiment to confirm that the proliferation of VSMC is dependent on this enzyme and it's substrate.

In summary, our studies provide novel insight into the role of $cPLA_2$ in VSMC migration and proliferation. $cPLA_2$ does not play a role in VSMC migration to PDGF BB, however, it is essential to PDGF BB stimulated proliferation. $cPLA_2$'s substrate AA is required for the proliferation and is currently being further investigated in addition to other metabolic products to determine its exact function. Since $cPLA_2$ is essential for monocyte migration[21,22] and VSMC proliferation, two essential components in the pathogenesis of atherosclerosis and restenosis, this could possibly lead to a valuable target for therapeutics to combat these crippling conditions.

CHAPTER 2

THE 2-D MIGRATORY PATTERNS OF TROPOMYOSIN IN FEMORAL AND ABDOMINAL AORTA TISSUE

2.1 INTRODUCTION

Arteries are classified into three types according to their size: large or elastic arteries; medium arteries; and small arteries or arterioles, which are less than 0.5 mm in diameter.[46] These types are all continuous with one another. A characteristic feature of arteries, regardless of size, is a well-defined lumen, rounded or oval, maintained by the muscularity of the vessel wall. An artery is an elastic blood vessel that transports blood away from the heart. The aorta is the main systemic artery and the largest artery of the body, which originates from the heart and branches out into smaller arteries and supplies oxygenated blood to the rest of the body.[47] The femoral artery is a large artery in the muscles of the thigh. The blood vessels are made of three layers, called from the luminal side outward, the tunica intima, the tunica media and the tunica adventitia.[48] The tunica intima consists of an endothelium and any subendothelial connective tissue that may be present. The tunica media is the layer of concentrically-arranged smooth muscle, the autonomic control of which can alter the diameter of the vessel and affect the blood pressure. Smooth muscle cells have secretory capabilities, and contain varying amounts of collagen fibers, elastic fibers, elastic lamellae, and proteoglycans.[49,50] The tunica adventitia is made chiefly of longitudinally arranged collagen fibers.

Vascular smooth muscle is an involuntary non-striated muscle, found within the tunica media layer of large and small arteries and veins. Smooth muscle may contract spontaneously (via ionic channel dynamic) or be induced by a number of physiochemical agents (hormones, drugs, neurotransmitters - particularly from the autonomic nervous system), and also mechanical stimulation (such as stretch).[51,52,53,54] In blood vessels smooth muscle contracts slowly and may maintain the contraction (tonically) for prolonged periods. The sliding of myosin and actin filaments, known as the sliding filament mechanism, causes smooth muscle contraction. The energy for this to happen is provided by the hydrolysis of ATP.[55] Myosin functions as an ATPase utilizing ATP to produce a molecular conformational change of part of the myosin and produces movement. Movement of the filaments over each other happens when the globular heads protruding from myosin filaments attach and interact with actin filaments to form crossbridges.[56,57]

Tropomyosin is an alpha helical coiled coil rod shaped protein dimer that binds end to end along F actin filaments in muscle.[58] Tropomyosin is an actin-binding protein that regulates actin mechanics. Tropomyosin, along with the troponin complex, associates with actin in muscle fibers and regulate muscle contraction by regulating the binding of myosin.[59] High molecular weight Tropomyosin can bind 7 actin monomers. In resting muscle, tropomyosin overlays the myosin binding sites on actin and is "locked" down in this position by troponin T (tropomyosin binding troponin) and troponin I (inhibitory troponin). Upon release of calcium from the sarcoplasmic reticulum calcium binds to troponin C (calcium binding troponin). This "unlocks" tropomyosin from actin, allowing it to move away from the binding groove. Myosin heads then access the

binding sites on actin. Once one myosin head binds, this fully displaces tropomyosin and allows additional myosin heads to bind, initiating muscle shortening and contraction.[59.60] Once calcium is pumped out of the cytoplasm and calcium levels return to normal, tropomyosin again binds to actin, preventing myosin from binding.

2.2 METHODS AND MATERIALS

Tissue Collection

Abdominal aorta and femoral artery tissue was excised from 8-week-old C57-Black mice. Mice were euthanized in CO_2 chamber according to protocol before tissue collection. Bilateral femoral arteries were collected in 1cm sections distal to the iliac crest. Abdominal Aorta tissue was collected in sections distal to the heart and proximal to the renal artery. The tissue was transported in DMEM and then snap frozen in liquid nitrogen and stored at minus 80°C.

Protein Extraction for Artery Tissue

Artery tissue extract was snap frozen in liquid nitrogen and dounce homogenized for 30 seconds. 200 µl of lysis buffer was added to the homogenizer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCL, pH 7.4, 1 mM PMSF, and 10 µl of protease inhibitor mix (Sigma) per milliliter of lysis buffer). Mixture was dounce homogenized again for 30 seconds. After 30 min, the lysate was sonicated three times for 15 seconds and then centrifuged for 15 min at 9300 x *g*. The supernatant was collected, and the protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA).

Histological Sections/Staining

Aortic tissue dissected from C57-Black mice and placed into 10 mL DMEM for paraffin embedding. Hematoxylin and eosin stained slides from abdominal aorta and femoral artery specimen were examined by light microscopy at 400X. Embedding and staining method details are given in the Armed Forces Institute of Pathology's 'Laboratory Methods in Histochemistry'(AFIP Cite).

Western Blot/SDS PAGE

100mg of protein extract were loaded on a 7.5% SDS-PAGE gel (100 μg of lysate/well). The proteins were transferred to a polyvinylidene difluoride membrane (0.2 μm; Bio-Rad) using a TRANS-BLOT SD electrophoretic transfer cell (Bio-Rad). The membrane was blocked in 5% nonfat milk in PBS and 1% Tween 20 overnight at 4°C and then probed with primary Ab. $cPLA_2$ protein was detected with a 1/1000 dilution of anti-mouse recombinant $cPLA_2$ mAb (Santa Cruz), followed by incubation with anti-mouse IgG HRP (1/1000 dilution; Transduction Laboratories, Lexington, KY). This Ab recognizes a distinct $cPLA_2$ protein band running at ~110 kDa and co-migrates with recombinant $cPLA_2$.

Two Dimensional Gel Electrophoresis

The protein extracted from the mice was quantified by the Bradford assay, with the loading quantity being 500mg per sample. 2-D was performed with a horizontal isoelectric focusing (IEF) system (Bio-Rad, America), using pre-cast pH 4–7 immobilized linear-gradient strips (170 mm) for the first dimension, and 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) for the second dimension. The SDS-PAGE was run in a Multi-gel casting chamber (Bio-Rad, America). Initial active

rehydration was set for 14 h at 50 V, IEF performed with the following voltage program: 250 V (linear over 1 h), 1000 V (rapid over 2 h), 10,000 V (linear over 5 h), 10,000 V (rapid for 60,000 V h), 500 V (rapid over 1 h). The limit electric current was at 50 μA/strip and the whole course was controlled at 20 °C. Prior to the 2-dimensional gel separation, the IPG strips were equilibrated for 2 × 15 min with gentle shaking in 6 ml of SDS equilibration buffer [Tris–Cl, pH 8.8 (50 mM), urea (6 M), glycerol (30%, v/v), SDS (2%)]. DTT (2%, w/v) was added in the first step and iodoacetamide (2.5%, w/v) in the second equilibration step. The second-dimensional SDS-PAGE with a 12% running gel was performed firstly at a constant current of 5 mA/gel for 2 h and then at 30 mA/gel till the bromophenol blue reached the bottom of the gel. After electrophoresis, the gels were stained with GelCode Coomassie Blue Stain (Pierce, America) overnight. After being de-stained, spots in the gels were excised.

MALDI TOF/TOF MS and MS/MS Analysis and Database Searching

The differential-expressed protein spots were excised from gels, rinsed by Milli-Q sterile water three times, and then de-stained with 25 mM NH_4HCO_3/50% ACN until the gel pieces were completely de-stained. The de-stained gel pieces were washed with 100% acetonitrile for 5 min and then dried. The dried gel pieces were rehydrated with 20μg/20μL trypsin in (Promega Corp., Madison, WI, USA) solution for 30 min. The excess liquid was removed and the pieces of the gel were immersed in 25 mM NH_4HCO_3 containing 10% acetonitrile at the 37 °C overnight. The digests were desalted with Ziptip (C-18, Millipore, Bedford, MA, USA) according to the manufacturer's instructions and subjected to analysis using MADLI TOF/TOF MS. Single MS and tandem mass spectrometer (MS/MS) experiments were performed by using a Bruker Ultraflex MALDI

TOF/TOF mass spectrometer (Bruker Daltonics, America). The MS and MS/MS spectra were searched within the MASCOT (MASCOT, Matrix Science, America) database system.

2.3 RESULTS

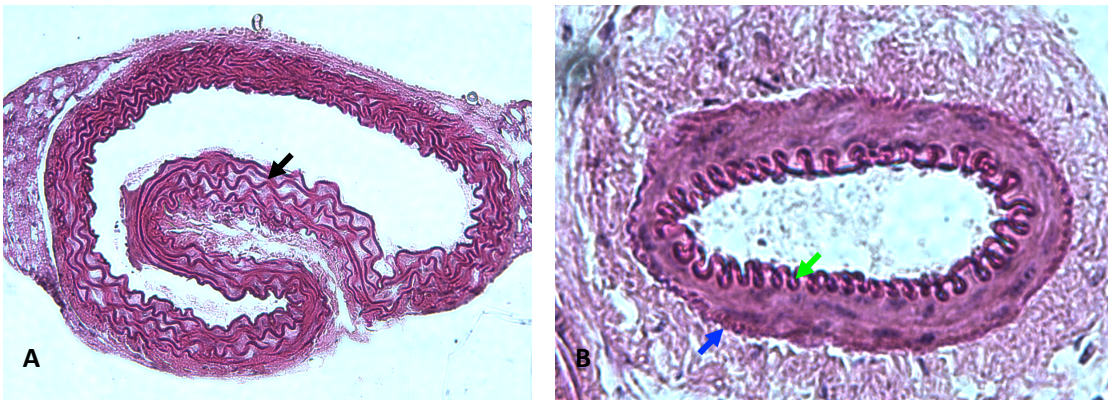

Figure 2.1. Hematoxylin and eosin stain of C57 black 6 mouse abdominal aorta (A) at 100x magnification and femoral artery (B) at 400x magnification. Note the multiple thick elastic bands throughout the entire wall of the aorta (black arrow) compared to the femoral artery which only has an inner elastic lamina (green arrow) and much smaller outer elastic lamina (blue arrow) separating the muscular layer from the serosa.

Hematoxylin and eosin stain of our C57-Black6 mice show obvious histological differences between the abdominal aorta and femoral artery. Most notably are the differences between elastic tissue expressions. External to the inner epithelial layer, the abdominal aorta exhibits multiple thick elastic bands spanning the entire arterial wall, whereas the femoral artery exhibited a more anatomical separation of internal and external elastica separated by a thick elastica free media separating the muscular layer from the serosa.

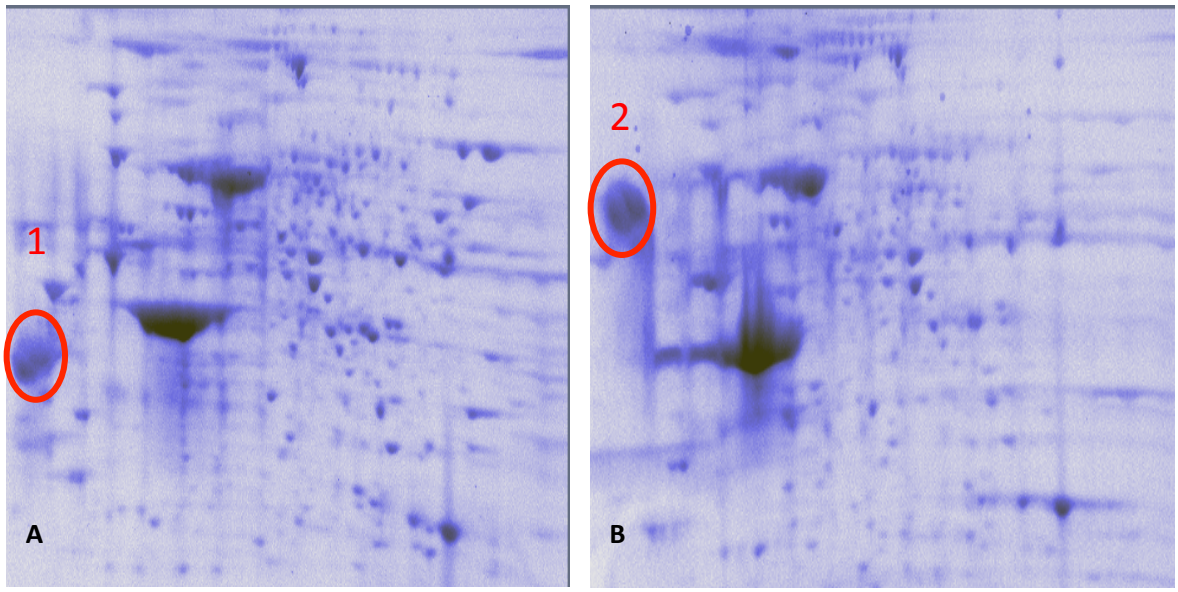

Figure 2.2. Tropomyosin migrates to different locations in murine abdominal aortic tissue compared to femoral artery. 2D gel of 150µg of whole C57 Black 6 abdominal aortic tissue (A) and femoral artery (B) extract run on 3-10 IPG strip and then a 4-20% SDS page gel and stained with Coomassie blue33. The abdominal aorta spot (spot 1) was at about 39kD where the femoral artery spot (spot 2) was located at the molecular weight of about 80kD.

When the histologically different abdominal aorta and femoral artery tissues were run through 2-D gel analysis, it was noted that a large protein (observed as spots 1 and 2 in figure 2) migrated to a significantly different location in the two gels. The abdominal aorta spot (figure 2 – 1) migrated to the same isoelectric focused spot, but migrated on the SDS gel to ~39kD. The same isoelectric spot on the femoral artery migrated to on the gel to ~80kD. This suggested that a protein within the two arteries was isoelectrically identical, however, was much larger in size in the femoral artery

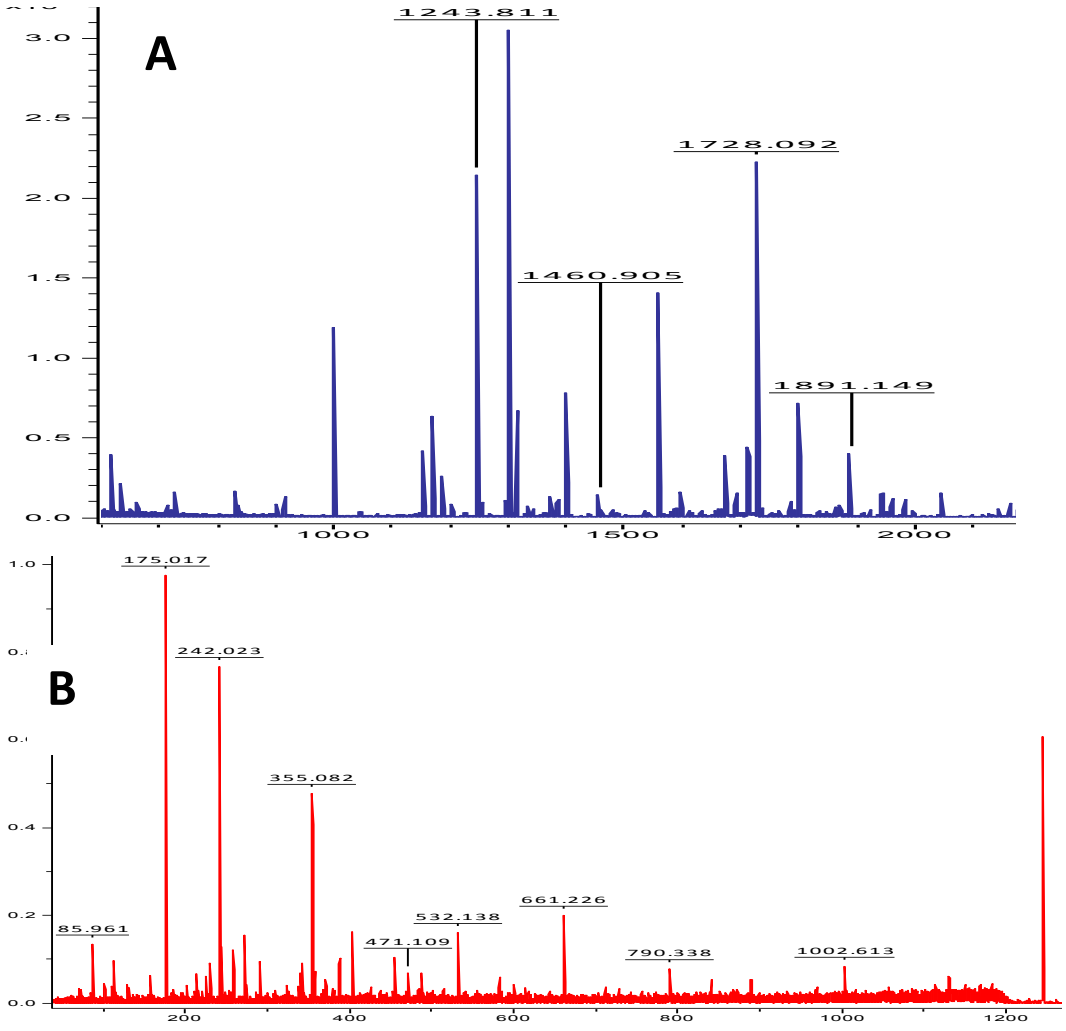

Figure 2.3. Analysis of the protein spot from a 2D gel (the red circle from figure 2-2B): MS spectrum (A) and MS-MS spectrum (B) showing peak 1243.81 corresponding to peptide IQLVEEELDR. Interpretations of both the MS and MS-MS spectra agreed that the protein was Tropomyosin alpha-1 and beta chain. The probability score (mowse) was 107 for MS and 90 for MS-MS. Scores >80 are statistically significant.

Initial mass spectrometry analysis of the two differing spots between the abdominal and aorta tissue revealed that this spot was indeed Tropomyosin. Probability scores from MASCOT search (Matrix Science) of 107 and 80 for MS and tandem MS/MS, respectively, indicate statistically significant results. Both Tropomyosin alpha-1 and beta chain isomers were discovered in the peptide analysis, which is a typical racemic mixture for natural state proteins.

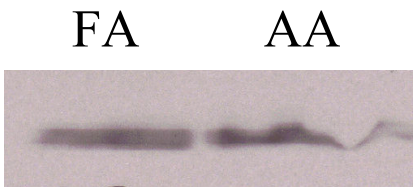

Figure 2.4. Murine Tropomyosin from femoral artery and abdominal aorta migrates to the same location on Western blot. 200ug of total protein from femoral artery (FA) and abdominal aorta (AA) were evaluated on an 8% SDS PAGE Gel and blotted for murine tropomyosin with a polyclonal antibody which detects both alpha and beta isoforms.

Western blotting analysis, which subjected the protein samples to denaturing SDS buffer prior to gel electrophoresis, exhibited a different Tropomyosin migratory pattern. In both the abdominal aorta and the femoral artery, tropomyosin migrates to its appropriate 36-38kD. This indicates a 40-50kD change in the migration of femoral artery Tropomyosin between western blotting and two-dimensional gel analysis, further suggesting the presence of a bound protein(s) in the femoral artery.

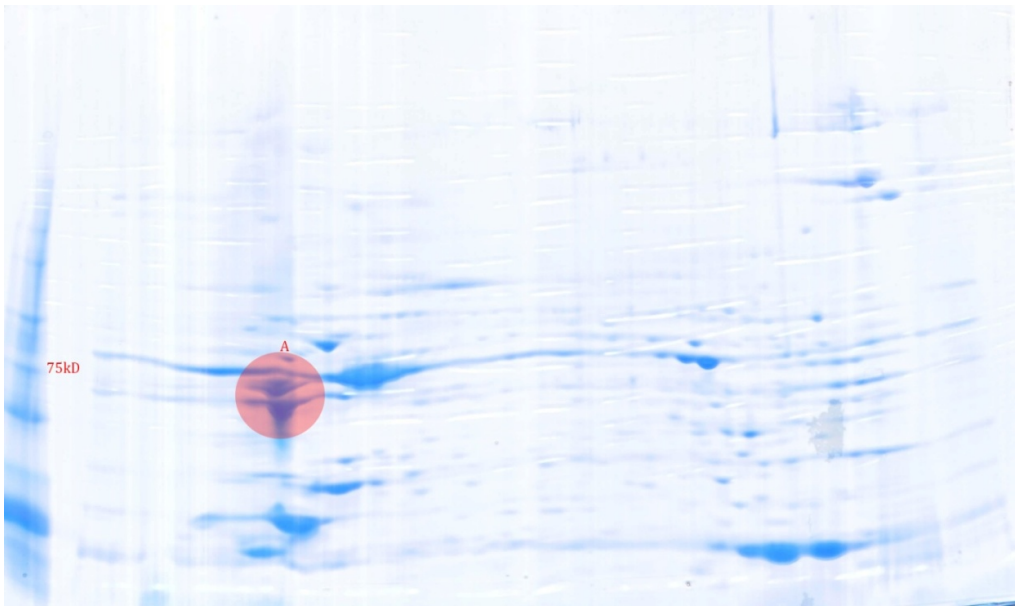

Figure 2.5 – Two Dimensional Gel Analysis of Tropomyosin in the Femoral Artery.
Migration of Tropomyosin was observed around 75kD

Two dimensional gel analysis was repeated with femoral artery tissue extracted from C57-Black6 mice. Tropomyosin again migrated to ~75kD, suggesting a protein is bound to the Tropomyosin isomer in the femoral artery. This spot was excised from the gel and analyzed via MS and tandem MS/MS analysis.

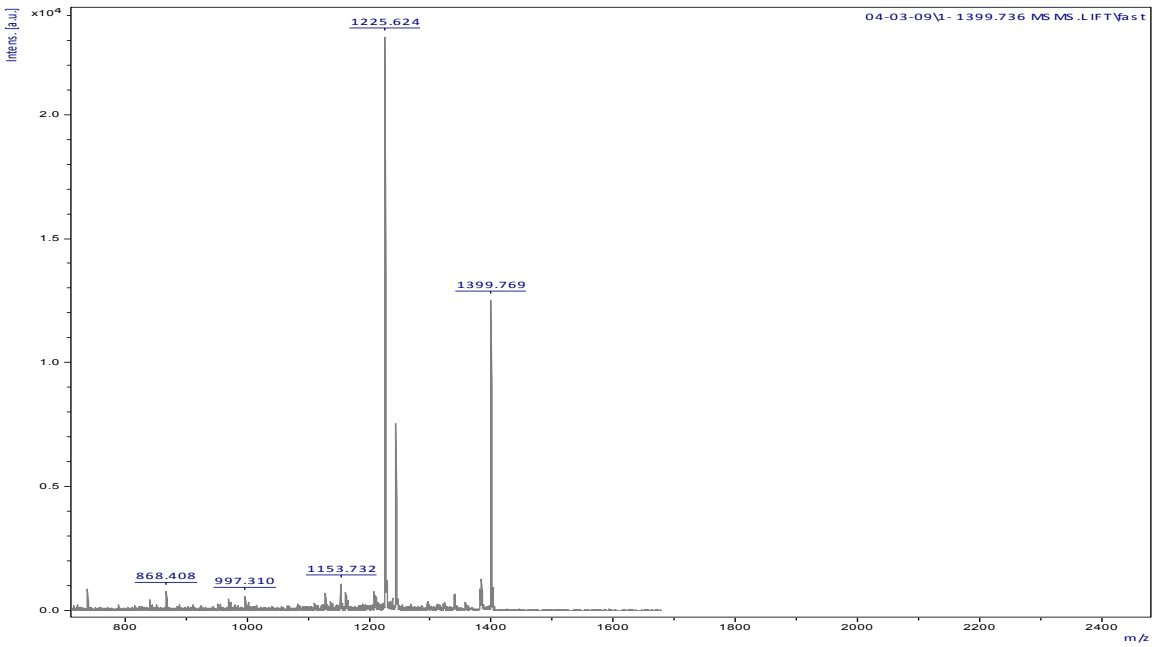

Figure 2.6 – MS Analysis of 75kD 2-D Gel spot (figure 5 – A) performed on Bruker MALDI TOF/TOF and analyzed with Matrix Science MASCOT Search Engine.

Excision of the 75kD spot was done in two sections because of the size and dimer nature of the spot. Figure 6 above shows the upper section of the spot on the femoral artery two-dimensional gel. This spot confirmed the identity of Tropomyosin I (TPMI). The probability score of this identification was 38 (scores >40 are considered statistically significant). The mass of this TPMI identification was 32kD still suggesting that a protein within the spot was hindering the proper migration of Tropomyosin to its 32-35kD location.

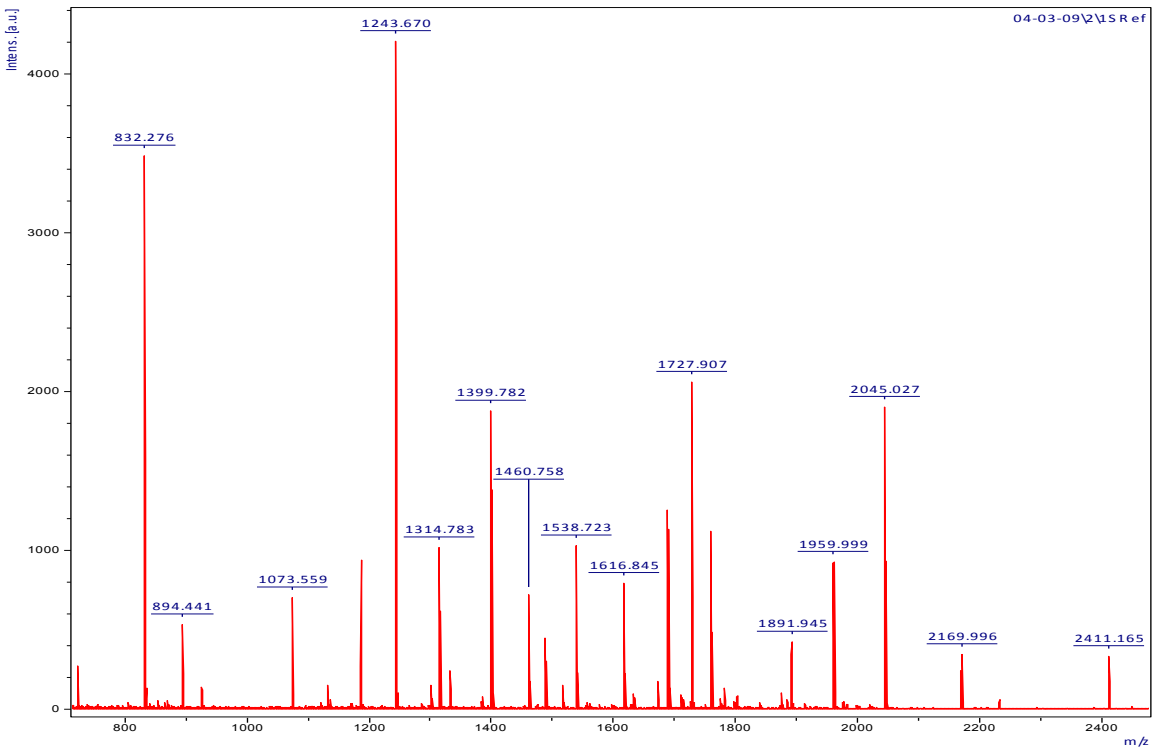

Figure 2.7 - MS Analysis of 75kD 2-D Gel spot (figure 5 – A) performed on Bruker MALDI TOF/TOF and analyzed with Matrix Science MASCOT Search Engine.

The second excisional section of the 75kD spot again revealed the identity of Tropomyosin I (TPMI). This spot however, also revealed the presence of Tropomyosin II (TPMII) or beta chain Tropomyosin. The MASCOT search score for TPMI was 128 (scores >40 are considered statistically significant) and 76 for TPMII. The mass of these proteins was still within the 33-36kD range. To further elucidate the phenomena of the 75kD migration, we sought to identify a specific peak within these MS spectra by tandem MS/MS analysis.

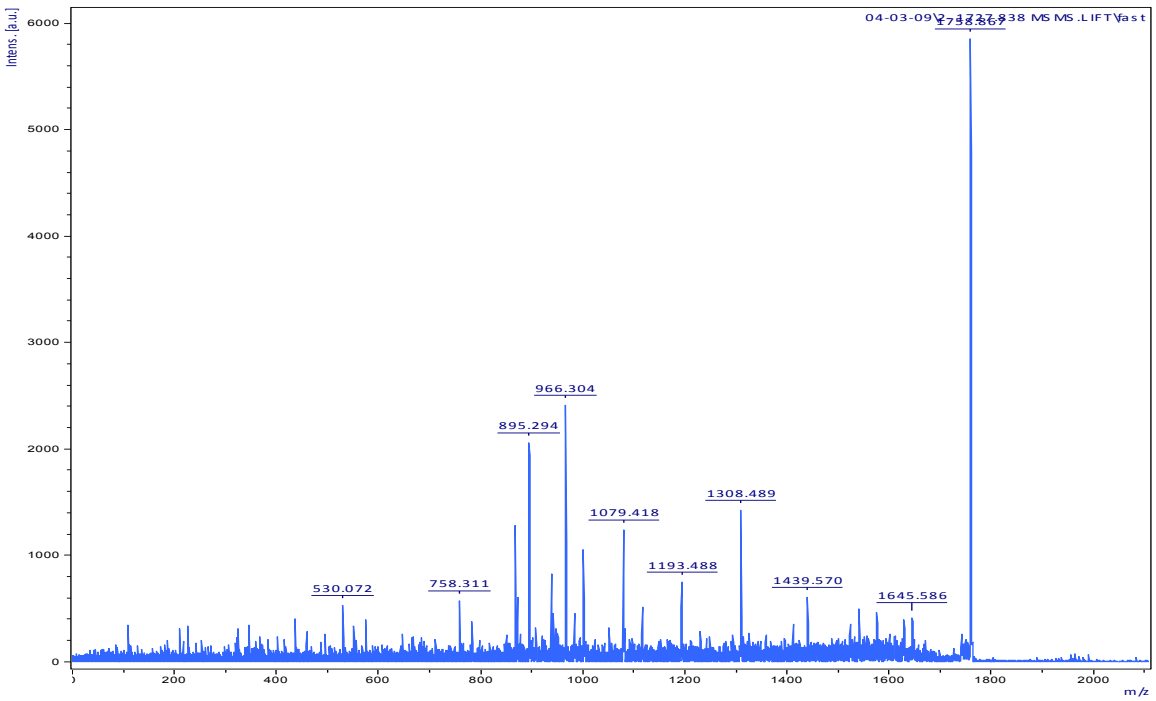

Figure 2.8 – MS/MS Analysis of 75kD 2-D Gel spot (figure 5 – A) on peak 1758.838 performed on Bruker MALDI TOF/TOF and analyzed with Matrix Science MASCOT Search Engine.

MS/MS analysis of all relevant peaks only confirmed one statistically significant protein, which is shown above in figure 8. Peak 1758.838 was successfully identified as Tropomyosin I (TPMI), with a MASCOT score of 104 (scores >40 are statistically significant). These data suggest that no other proteins within the 75kD were correctly identified as a source of the 40-50kD discrepancy between the 2-D gel and Western analysis of femoral artery tissue.

2.4 DISCUSSION

Since tropomyosin alpha 1(36kDa) and beta (38kDa) have a different migration pattern in 2D gel analysis between FA (~80kD) compared to AA (~39kD), we were interested in inquiring into the reasons for this difference. This led us to extract protein from FA and AA, strip and linearize the protein for analysis of tropomyosin by western

blot analysis. After stripping and linearizing the proteins, tropomyosin migrated to the same location between FA and AA with a molecular weight of 38kD(figure 4). This led us to conclude that tropomyosin may have had other protein attached to it in the FA that was not present in the AA. We are currently investigating the hypothesis that tropomyosin has other proteins bound to it in FA that are not present in AA.

In addition to calcium and actin, tropomyosin has also been shown to bind to a number of other proteins in both striated and smooth muscle fibers. The following table summarizes previous discoveries of Tropomyosin binding proteins. Of the possible proteins listed below:

Table 2.1 Proteins that have been shown to bind to Tropomyosin

Protein	**kDa**	**Function**	**Reference**
Calponin	34	Unknown	Childs & Watson, 1992
CEACAM1	44.6	Adhesion protein	Schumann *et al*, 2001
Endostatin	20	Unknown	MacDonald *et al*, 2001
Enigma	65	LIM	Guy *et al*, 1999
Gelsolin (sub-domain 2)	82	To regulate gelsolin severing function?	Koepf & Burtnick 1992, 1993; Maciver *et al*, 2000
S100A2	10	Unknown	Gimona*, et al*, 1997
Troponin	22.5	Calcium binding protein	
Smooth muscle actin (SMA)	42	Intermediate filament	

CEACAM1, SMA, and possibly calponin are of the proper molecular weight to be within the range of 35 to 45 kDA when added to the molecular weight of tropomyosin (36kDa alpha 1, 38kDa Beta) will give a total of about 70 to 85kDa where we find the tropomyosin spot on 2D analysis in FA. The largest, densest spot on FA and AA 2D gels

is about 45kDa on each blot (figure 2), and this turned out to be actin (data not shown), so we don't believe the change in tropomyosin molecular weight for FA is solely due to actin. Other proteins are being investigated.

Table 2.2 Secondary proteins found in Tropomyosin MS analysis

Protein	**Mass (Da)**
Retinol dehydrogenase 13	36411
Polycomb group RING finger protein 3	28028
Gamma-tubulin complex component 5	117903
Zinc finger protein 706	8492
Zinc finger protein GLI3	171549
DNA-directed RNA polymerase I SU RPA1	193987
Bone morphogenetic protein 3b	52457
Serine/threonine protein kinase SMG1	409840

First, we analyze the smaller peaks found around tropomyosin (peak 1243.81 in figure 3A) in the MS spectra of FA that were not found in the AA to point to clues around the identity of possible associated proteins. Table 2 show the proteins identified by of smaller peaks in the FA spectra that were not found in AA. None of these proteins match the known proteins that bind to tropomyosin in Table 1. Possible proteins from the list in Table 2 that would add up to 70-85kDa with the mass of tropomyosin would be retinol dehydrogenase 13, bone morphogenetic protein 3b, and serine/threonine protein kinase SMG1. Since this does not prove that tropomyosin is associated or bound to any of these proteins, we have moved to immunoprecipitation to pull down tropomyosin in both FA and AA and search of any bound proteins in FA that are not found in AA. (Reference table II-2)

Currently, we are performing immunoprecipitation assays using a polyclonal antibody that recognizes both tropomyosin beta and alpha 1 in both murine FA and AA. Our goal is to identify other bands in the FA that are not found in AA. We will excise

these bands and analyze them by MS and MS/MS analysis. This will hopefully allow us to identify the bound proteins in FA that are causing the higher migration of tropomyosin alpha 1 and beta on 2D electrophoresis. This may turn out to be a known protein or a possible protein that has been not associated with tropomyosin in the past. Other possibilities include a combination of heterodimers or homodimers made up in tropomyosin alpha 1 and beta that are conglomerated in FA and not in AA. This could lead to investigating different contractile properties of FA compared to AA in mice.

Subcellular differences in major arteries are not well characterized, but may lead to major differences in function between different arteries. We have confirmed that microscopically there is a greater amount of elastic tissue in the AA compared to the FA (figure 1). Additionally, there are clear differences at the subcellular level in the contractile protein tropomyosin alpha 1 and beta between FA and AA on 2D analysis. The exact nature of these differences is under investigation. We hypothesize that tropomyosin alpha 1 and beta have different protein complexes bound to tropomyosin alpha 1 and beta in FA that are not present in AA. This may lead to distinctive functional characteristics that are found in FA and not present in AA.

WORKS CITED

Ackermann, E. J., K. Conde-Frieboes, E. A. Dennis. 1995. Inhibition of macrophage Ca2+-independent phospholipase A2 by bromoenol lactone and trifluoromethyl ketones. J. Biol. Chem. 270:445-50.

Ajtai K, Halstead MF, Nyitrai M, Penheiter AR, Zheng Y, Burghardt TP. The myosin C-loop is an allosteric actin contact sensor in actomyosin. Biochemistry. 2009 Jun 16;48(23):5263-75.

Ali M, Madjid M. Lipoprotein-associated phospholipase A2: a cardiovascular risk predictor and a potential therapeutic target. Future Cardiol. 2009 Mar;5(2):159-73.

Alvarez RH, Kantarjian HM, Cortes JE, Biology of platelet-derived growth factor and its involvement in disease. Mayo Clin Proc. 2006 Sep;81(9):1241-57.

Anderson JE, Grant's Atlas of Anatomy, Eighth Edition. Williams and Wilkins, 1983; pp 2-100, 4-11.

Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. 2008 May 15;22(10):1276-312.

Bai XM, Zhang W, Liu NB, Jiang H, Lou KX, Peng T, Ma J, Zhang L, Zhang H, Leng J. Focal adhesion kinase: important to prostaglandin E2-mediated adhesion, migration and invasion in hepatocellular carcinoma cells. Oncol Rep. 2009 Jan;21(1):129-36.

Balsinde J, Balboa MA, Dennis EA, Antisense inhibition of group VI Ca2+-independent phospholipase A2 blocks phospholipid fatty acid remodeling in murine P388D1 macrophages. J Biol Chem. 1997 Nov 14;272(46):29317-21.

Balsinde J, Balboa MA. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A2 in activated cells. Cell Signal. 2005 Sep;17(9):1052-62.

Balsinde J, Dennis EA, Function and inhibition of intracellular calcium-independent phospholipase A2. J Biol Chem. 1997 Jun 27;272(26):16069-72.

Bornfeldt KE, Campbell JS, Koyama H, Argast GM, Leslie CC, Raines EW, Krebs EG, Ross R, The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. J Clin Invest. 1997 Aug 15;100(4):875-85.

Bornfeldt KE, Raines EW, Nakano T, Graves LM, Krebs EG, Ross R, Insulin-like growth factor-I and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. J Clin Invest. 1994 Mar;93(3):1266-74.

Boullart AC, Aarntzen EH, Verdijk P, Jacobs JF, Schuurhuis DH, Benitez-Ribas D, Schreibelt G, van de Rakt MW, Scharenborg NM, de Boer A, Kramer M, Figdor CG, Punt CJ, Adema GJ, de Vries IJ. Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration. Cancer Immunol Immunother. 2008 Nov;57(11):1589-97.

Burgstaller G, Gimona M. Podosome-mediated matrix resorption and cell motility in vascular smooth muscle cells. Am J Physiol Heart Circ Physiol. 2005 Jun;288(6):H3001-5.

Carnevale KA, Cathcart MK. Calcium-independent phospholipase A(2) is required for human monocyte chemotaxis to monocyte chemoattractant protein 1. J Immunol. 2001 Sep 15;167(6):3414-21.

Chawengsub Y, Gauthier KM, Campbell WB. Role of arachidonic acid lipoxygenase metabolites in the regulation of vascular tone. Am J Physiol Heart Circ Physiol. 2009 Aug;297(2):H495-507.

Crosslin DR, Shah SH, Nelson SC, Haynes CS, Connelly JJ, Gadson S, Goldschmidt-Clermont PJ, Vance JM, Rose J, Granger CB, Seo D, Gregory SG, Kraus WE, Hauser ER. Genetic effects in the leukotriene biosynthesis pathway and association with atherosclerosis. Hum Genet. 2009 Mar;125(2):217-29.

Devin JK, Young PP. The effects of growth hormone and insulin-like growth factor-1 on the aging cardiovascular system and its progenitor cells. Curr Opin Investig Drugs. 2008 Sep;9(9):983-92.

Englesbe MJ, Deou J, Bourns BD, Clowes AW, Daum G. Interleukin-1beta inhibits PDGF-BB-induced migration by cooperating with PDGF-BB to induce cyclooxygenase-2 expression in baboon aortic smooth muscle cells. J Vasc Surg. 2004 May;39(5):1091-6.

Faury G. Function-structure relationship of elastic arteries in evolution: from microfibrils to elastin and elastic fibres. Pathol Biol. 2001 May;49(4):310-25.

French WJ, Creemers EE, Tallquist MD. Platelet-derived growth factor receptors direct vascular development independent of vascular smooth muscle cell function. Mol Cell Biol. 2008 Sep;28(18):5646-57.

Ger R, Abrahams P, Essentials of Clinical Anatomy. Pitman Publishing Limited, 1986; pp44.

Gomes A, Fernandes E, Silva AM, Pinto DC, Santos CM, Cavaleiro JA, Lima JL. Anti-inflammatory potential of 2-styrylchromones regarding their interference with arachidonic acid metabolic pathways. Biochem Pharmacol. 2009 Jul 15;78(2):171-7.

Graf K, Xi XP, Yang D, Fleck E, Hsueh WA, Law RE, Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. Hypertension. 1997 Jan;29(1 Pt 2):334-9.

Graves LM, Bornfeldt KE, Sidhu JS, Argast GM, Raines EW, Ross R, Leslie CC, Krebs EG, Platelet-derived growth factor stimulates protein kinase A through a mitogen-activated protein kinase-dependent pathway in human arterial smooth muscle cells. J Biol Chem. 1996 Jan 5;271(1):505-11.

Gunning P. Emerging issues for tropomyosin structure, regulation, function and pathology. Adv Exp Med Biol. 2008;644:293-8.

Guerrero AT, Verri WA Jr, Cunha TM, Silva TA, Schivo IR, Dal-Secco D, Canetti C, Rocha FA, Parada CA, Cunha FQ, Ferreira SH. Involvement of LTB4 in zymosan-induced joint nociception in mice: participation of neutrophils and PGE2. J Leukoc Biol. 2008 Jan;83(1):122-30.

Halka AT, Turner NJ, Carter A, Ghosh J, Murphy MO, Kirton JP, Kielty CM, Walker MG. The effects of stretch on vascular smooth muscle cell phenotype in vitro. Cardiovasc Pathol. 2008 Mar-Apr;17(2):98-102.

Henderson LM, Chappell JB, Jones OT, Superoxide generation is inhibited by phospholipase A2 inhibitors. Role for phospholipase A2 in the activation of the NADPH oxidase. Biochem J. 1989 Nov 15;264(1):249-55.

Hilgers RH, Webb RC. Molecular aspects of arterial smooth muscle contraction: focus on Rho. Exp Biol Med. 2005 Dec;230(11):829-35.

Inoue T, Node K. Molecular basis of restenosis and novel issues of drug-eluting stents. Circ J. 2009 Apr;73(4):615-21.

Kampmann A, Fernández B, Deindl E, Kubin T, Pipp F, Eitenmüller I, Hoefer IE, Schaper W, Zimmermann R. The proteoglycan osteoglycin/mimecan is correlated with arteriogenesis. Mol Cell Biochem. 2009 Feb;322(1-2):15-23.

Kanayasu-Toyoda T, Morita I, Murota S. Arachidonic acid pretreatment enhances smooth muscle cell migration via increased Ca2+ influx. Prostaglandins Leukot Essent Fatty Acids. 1998 Jan;58(1):25-31.

Kibos A, Campeanu A, Tintoiu I. Pathophysiology of coronary artery in-stent restenosis- Acute Card Care. 2007;9(2):111-9.

Levitzki A. PDGF receptor kinase inhibitors for the treatment of restenosis. Cardiovasc Res. 2005 Feb 15;65(3):581-6.

Maderna P, Cottell DC, Berlasconi G, Petasis NA, Brady HR, Godson C. Lipoxins induce actin reorganization in monocytes and macrophages but not in neutrophils: differential involvement of rho GTPases. Am J Pathol. 2002 Jun;160(6):2275-83.

Mantur M, Koper O. Platelet- derived growth factor--the construction, role and it's receptors. Pol Merkur Lekarski. 2008 Feb;24(140):173-6.

Masuda S, Yamamoto K, Hirabayashi T, Ishikawa Y, Ishii T, Kudo I, Murakami M. Human group III secreted phospholipase A2 promotes neuronal outgrowth and survival. Biochem J. 2008 Jan 15;409(2):429-38.

Meyer-Kirchrath J, Debey S, Glandorff C, Kirchrath L, Schrör K. Gene expression profile of the Gs-coupled prostacyclin receptor in human vascular smooth muscle cells. Biochem Pharmacol. 2004 Feb 15;67(4):757-65.

Mishra RS, Carnevale KA, Cathcart MK. iPLA2beta: front and center in human monocyte chemotaxis to MCP-1. J Exp Med. 2008 Feb 18;205(2):347-59.

Navarro-Tito N, Robledo T, Salazar EP. Arachidonic acid promotes FAK activation and migration in MDA-MB-231 breast cancer cells. Exp Cell Res. 2008 Nov 1;314(18):3340-55.

Qiao X, McConnell KR, Khalil RA. Sex steroids and vascular responses in hypertension and aging. Gend Med. 2008;5 Suppl A:S46-64.

Oliveira SH, Costa CH, Ferreira SH, Cunha FQ. Sephadex induces eosinophil migration to the rat and mouse peritoneal cavity: involvement of mast cells, LTB4, TNF-alpha, IL-8 and PAF. Inflamm Res. 2002 Mar;51(3):144-53.

Orr AW, Lee MY, Lemmon JA, Yurdagul A Jr, Gomez MF, Bortz PD, Wamhoff BR. Molecular mechanisms of collagen isotype-specific modulation of smooth muscle cell phenotype. Arterioscler Thromb Vasc Biol. 2009 Feb;29(2):225-31.

Park JL, Shu L, Shayman JA. Differential involvement of COX1 and COX2 in the vasculopathy associated with the alpha-galactosidase A-knockout mouse. Am J Physiol Heart Circ Physiol. 2009 Apr;296(4):H1133-40.

Patel MI, Singh J, Niknami M, Kurek C, Yao M, Lu S, Maclean F, King NJ, Gelb MH, Scott KF, Russell PJ, Boulas J, Dong Q. Cytosolic phospholipase A2-alpha: a potential therapeutic target for prostate cancer. Clin Cancer Res. 2008 Dec 15;14(24):8070-9.

Pfitzer G, Lubomirov LT, Reimann K, Gagov H, Schubert R. Regulation of the crossbridge cycle in vascular smooth muscle by cAMP signalling. J Muscle Res Cell Motil. 2006;27(5-7):445-54.

Pfister SL, Schmitz JM, Willerson JT, Campbell WB. Characterization of arachidonic acid metabolism in Watanabe heritable hyperlipidemic (WHHL) and New Zealand white (NZW) rabbit aortas. Prostaglandins. 1988 Oct;36(4):515-32.

Pichon S, Bryckaert M, Berrou E. Control of actin dynamics by p38 MAP kinase - Hsp27 distribution in the lamellipodium of smooth muscle cells. J Cell Sci. 2004 May 15;117(Pt 12):2569-77.

Schulz R, Krueger C, Manickavel V, Steele JA, Cook DA. Production of 15-HETE by cultured smooth muscle cells from cerebral artery. Pharmacology. 1993 Apr;46(4):211-23.

Singh A, Hitchcock-Degregori SE. A peek into tropomyosin binding and unfolding on the actin filament. PLoS One. 2009 Jul 24;4(7):e6336.

Song Y, Wilkins P, Hu W, Murthy KS, Chen J, Lee Z, Oyesanya R, Wu J, Barbour SE, Fang X. Inhibition of calcium-independent phospholipase A2 suppresses proliferation and tumorigenicity of ovarian carcinoma cells. Biochem J. 2007 Sep 15;406(3):427-36.

Sternberg SS, Histology for Pathologists. Raven Press, 1992; pp195-198.

Street, I. P., H. K. Lin, F. Laliberte, F. Ghomashchi, Z. Wang, H. Perrier, N. M. Tremblay, Z. Huang, P. K. Weech, M. H. Gelb. 1993. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A2. Biochemistry 32:5935-40.

Watts SW, Priestley JR, Thompson JM. Serotonylation of vascular proteins important to contraction. PLoS One. 2009 May 25;4(5):e5682.

Weber AA, Zucker TP, Hasse A, Bönisch D, Wittpoth M, Schrör K. Antimitogenic effects of vasodilatory prostaglandins in coronary artery smooth muscle cells. Basic Res Cardiol. 1998;93 Suppl 3:54-7.

Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, Narumiya S. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nat Med. 2009 Jun;15(6):633-40.

Yang Z, Yamazaki M, Shen QW, Swartz DR. Differences between cardiac and skeletal troponin interaction with the thin filament probed by troponin exchange in skeletal myofibrils. Biophys J. 2009 Jul 8;97(1):183-94.

Zhou H, Das S, Murthy KS, Erk1/2- and p38 MAP kinase-dependent phosphorylation and activation of cPLA2 by m3 and m2 receptors. Am J Physiol Gastrointest Liver Physiol. 2003 Mar;284(3):G472-80.

ENDNOTES

[1] Inoue T, Node K. Molecular basis of restenosis and novel issues of drug-eluting stents. Circ J. 2009 Apr;73(4):615-21.

[2] Kibos A, Campeanu A, Tintoiu I. Pathophysiology of coronary artery in-stent restenosis-Acute Card Care. 2007;9(2):111-9.

[3] French WJ, Creemers EE, Tallquist MD. Platelet-derived growth factor receptors direct vascular development independent of vascular smooth muscle cell function. Mol Cell Biol. 2008 Sep;28(18):5646-57.

[4] Levitzki A. PDGF receptor kinase inhibitors for the treatment of restenosis. Cardiovasc Res. 2005 Feb 15;65(3):581-6.

[5] Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. 2008 May 15;22(10):1276-312.

[6] Alvarez RH, Kantarjian HM, Cortes JE, Biology of platelet-derived growth factor and its involvement in disease. Mayo Clin Proc. 2006 Sep;81(9):1241-57.

[7] Mantur M, Koper O. Platelet- derived growth factor--the construction, role and it's receptors. Pol Merkur Lekarski. 2008 Feb;24(140):173-6.

[8] Graf K, Xi XP, Yang D, Fleck E, Hsueh WA, Law RE, Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. Hypertension. 1997 Jan;29(1 Pt 2):334-9.

[9] Bornfeldt KE, Campbell JS, Koyama H, Argast GM, Leslie CC, Raines EW, Krebs EG, Ross R, The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. J Clin Invest. 1997 Aug 15;100(4):875-85.

[10] Bornfeldt KE, Raines EW, Nakano T, Graves LM, Krebs EG, Ross R, Insulin-like growth factor-I and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. J Clin Invest. 1994 Mar;93(3):1266-74.

[11] Graves LM, Bornfeldt KE, Sidhu JS, Argast GM, Raines EW, Ross R, Leslie CC, Krebs EG, Platelet-derived growth factor stimulates protein kinase A through a mitogen-

activated protein kinase-dependent pathway in human arterial smooth muscle cells. J Biol Chem. 1996 Jan 5;271(1):505-11.

[12] Henderson LM, Chappell JB, Jones OT, Superoxide generation is inhibited by phospholipase A2 inhibitors. Role for phospholipase A2 in the activation of the NADPH oxidase. Biochem J. 1989 Nov 15;264(1):249-55.

[13] Zhou H, Das S, Murthy KS, Erk1/2- and p38 MAP kinase-dependent phosphorylation and activation of cPLA2 by m3 and m2 receptors. Am J Physiol Gastrointest Liver Physiol. 2003 Mar;284(3):G472-80.

[14] Balsinde J, Balboa MA, Dennis EA, Antisense inhibition of group VI Ca2+-independent phospholipase A2 blocks phospholipid fatty acid remodeling in murine P388D1 macrophages. J Biol Chem. 1997 Nov 14;272(46):29317-21.

[15] Balsinde J, Dennis EA, Function and inhibition of intracellular calcium-independent phospholipase A2. J Biol Chem. 1997 Jun 27;272(26):16069-72.

[16] Balsinde J, Dennis EA, Function of calcium-independent phospholipase A2 in arachidonic acid metabolism in P388D1 macrophages. Adv Exp Med Biol. 1997;407:99-103.

[171717]Ali M, Madjid M. Lipoprotein-associated phospholipase A2: a cardiovascular risk predictor and a potential therapeutic target. Future Cardiol. 2009 Mar;5(2):159-73.

[18] Street, I. P., H. K. Lin, F. Laliberte, F. Ghomashchi, Z. Wang, H. Perrier, N. M. Tremblay, Z. Huang, P. K. Weech, M. H. Gelb. 1993. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A2. Biochemistry 32:5935-40.

[19] Ackermann, E. J., K. Conde-Frieboes, E. A. Dennis. 1995. Inhibition of macrophage Ca2+-independent phospholipase A2 by bromoenol lactone and trifluoromethyl ketones. J. Biol. Chem. 270:445-50.

[20] Meyer-Kirchrath J, Debey S, Glandorff C, Kirchrath L, Schrör K. Gene expression profile of the Gs-coupled prostacyclin receptor in human vascular smooth muscle cells. Biochem Pharmacol. 2004 Feb 15;67(4):757-65.

[21] Mishra RS, Carnevale KA, Cathcart MK. iPLA2beta: front and center in human monocyte chemotaxis to MCP-1. J Exp Med. 2008 Feb 18;205(2):347-59.

[22] Carnevale KA, Cathcart MK. Calcium-independent phospholipase A(2) is required for human monocyte chemotaxis to monocyte chemoattractant protein 1. J Immunol. 2001 Sep 15;167(6):3414-21.

[23] Kanayasu-Toyoda T, Morita I, Murota S. Arachidonic acid pretreatment enhances smooth muscle cell migration via increased Ca2+ influx. Prostaglandins Leukot Essent Fatty Acids. 1998 Jan;58(1):25-31.

[24] Weber AA, Zucker TP, Hasse A, Bönisch D, Wittpoth M, Schrör K. Antimitogenic effects of vasodilatory prostaglandins in coronary artery smooth muscle cells. Basic Res Cardiol. 1998;93 Suppl 3:54-7.

[25] Englesbe MJ, Deou J, Bourns BD, Clowes AW, Daum G. Interleukin-1beta inhibits PDGF-BB-induced migration by cooperating with PDGF-BB to induce cyclooxygenase-2 expression in baboon aortic smooth muscle cells. J Vasc Surg. 2004 May;39(5):1091-6.

[26] Burgstaller G, Gimona M. Podosome-mediated matrix resorption and cell motility in vascular smooth muscle cells. Am J Physiol Heart Circ Physiol. 2005 Jun;288(6):H3001-5.

[27] Pichon S, Bryckaert M, Berrou E. Control of actin dynamics by p38 MAP kinase - Hsp27 distribution in the lamellipodium of smooth muscle cells. J Cell Sci. 2004 May 15;117(Pt 12):2569-77.

[28] Gomes A, Fernandes E, Silva AM, Pinto DC, Santos CM, Cavaleiro JA, Lima JL. Anti-inflammatory potential of 2-styrylchromones regarding their interference with arachidonic acid metabolic pathways. Biochem Pharmacol. 2009 Jul 15;78(2):171-7.

[29] Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, Narumiya S. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nat Med. 2009 Jun;15(6):633-40.

[30] Bai XM, Zhang W, Liu NB, Jiang H, Lou KX, Peng T, Ma J, Zhang L, Zhang H, Leng J. Focal adhesion kinase: important to prostaglandin E2-mediated adhesion, migration and invasion in hepatocellular carcinoma cells. Oncol Rep. 2009 Jan;21(1):129-36.

[31] Navarro-Tito N, Robledo T, Salazar EP. Arachidonic acid promotes FAK activation and migration in MDA-MB-231 breast cancer cells. Exp Cell Res. 2008 Nov 1;314(18):3340-55.

[32] Chawengsub Y, Gauthier KM, Campbell WB. Role of arachidonic acid lipoxygenase metabolites in the regulation of vascular tone. Am J Physiol Heart Circ Physiol. 2009 Aug;297(2):H495-507.

[33] Park JL, Shu L, Shayman JA. Differential involvement of COX1 and COX2 in the vasculopathy associated with the alpha-galactosidase A-knockout mouse. Am J Physiol Heart Circ Physiol. 2009 Apr;296(4):H1133-40.

[34] Crosslin DR, Shah SH, Nelson SC, Haynes CS, Connelly JJ, Gadson S, Goldschmidt-Clermont PJ, Vance JM, Rose J, Granger CB, Seo D, Gregory SG, Kraus WE, Hauser ER. Genetic effects in the leukotriene biosynthesis pathway and association with atherosclerosis. Hum Genet. 2009 Mar;125(2):217-29.

[35] Schulz R, Krueger C, Manickavel V, Steele JA, Cook DA. Production of 15-HETE by cultured smooth muscle cells from cerebral artery. Pharmacology. 1993 Apr;46(4):211-23.

[36] Pfister SL, Schmitz JM, Willerson JT, Campbell WB. Characterization of arachidonic acid metabolism in Watanabe heritable hyperlipidemic (WHHL) and New Zealand white (NZW) rabbit aortas. Prostaglandins. 1988 Oct;36(4):515-32.

[37] Guerrero AT, Verri WA Jr, Cunha TM, Silva TA, Schivo IR, Dal-Secco D, Canetti C, Rocha FA, Parada CA, Cunha FQ, Ferreira SH. Involvement of LTB4 in zymosan-induced joint nociception in mice: participation of neutrophils and PGE2. J Leukoc Biol. 2008 Jan;83(1):122-30.

[38] Oliveira SH, Costa CH, Ferreira SH, Cunha FQ. Sephadex induces eosinophil migration to the rat and mouse peritoneal cavity: involvement of mast cells, LTB4, TNF-alpha, IL-8 and PAF. Inflamm Res. 2002 Mar;51(3):144-53.

[39] Maderna P, Cottell DC, Berlasconi G, Petasis NA, Brady HR, Godson C. Lipoxins induce actin reorganization in monocytes and macrophages but not in neutrophils: differential involvement of rho GTPases. Am J Pathol. 2002 Jun;160(6):2275-83.

[40] Boullart AC, Aarntzen EH, Verdijk P, Jacobs JF, Schuurhuis DH, Benitez-Ribas D, Schreibelt G, van de Rakt MW, Scharenborg NM, de Boer A, Kramer M, Figdor CG, Punt CJ, Adema GJ, de Vries IJ. Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E2 results in high interleukin-12 production and cell migration. Cancer Immunol Immunother. 2008 Nov;57(11):1589-97.

[41] Patel MI, Singh J, Niknami M, Kurek C, Yao M, Lu S, Maclean F, King NJ, Gelb MH, Scott KF, Russell PJ, Boulas J, Dong Q. Cytosolic phospholipase A2-alpha: a potential therapeutic target for prostate cancer. Clin Cancer Res. 2008 Dec 15;14(24):8070-9.

[42] Song Y, Wilkins P, Hu W, Murthy KS, Chen J, Lee Z, Oyesanya R, Wu J, Barbour SE, Fang X. Inhibition of calcium-independent phospholipase A2 suppresses proliferation and tumorigenicity of ovarian carcinoma cells. Biochem J. 2007 Sep 15;406(3):427-36.

[43] Masuda S, Yamamoto K, Hirabayashi T, Ishikawa Y, Ishii T, Kudo I, Murakami M. Human group III secreted phospholipase A2 promotes neuronal outgrowth and survival. Biochem J. 2008 Jan 15;409(2):429-38.

[44] Balsinde J, Balboa MA. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A2 in activated cells. Cell Signal. 2005 Sep;17(9):1052-62.

[45] Devin JK, Young PP. The effects of growth hormone and insulin-like growth factor-1 on the aging cardiovascular system and its progenitor cells. Curr Opin Investig Drugs. 2008 Sep;9(9):983-92.

[46] Ger R, Abrahams P, Essentials of Clinical Anatomy. Pitman Publishing Limited, 1986; pp44.

[47] Anderson JE, Grant's Atlas of Anatomy, Eighth Edition. Williams and Wilkins, 1983; pp 2-100, 4-11.

[48] Sternberg SS, Histology for Pathologists. Raven Press, 1992; pp195-198.

[49] Faury G. Function-structure relationship of elastic arteries in evolution: from microfibrils to elastin and elastic fibres. Pathol Biol. 2001 May;49(4):310-25.

[50] Kampmann A, Fernández B, Deindl E, Kubin T, Pipp F, Eitenmüller I, Hoefer IE, Schaper W, Zimmermann R. The proteoglycan osteoglycin/mimecan is correlated with arteriogenesis. Mol Cell Biochem. 2009 Feb;322(1-2):15-23.

[51] Qiao X, McConnell KR, Khalil RA. Sex steroids and vascular responses in hypertension and aging. Gend Med. 2008;5 Suppl A:S46-64.

[52] Watts SW, Priestley JR, Thompson JM. Serotonylation of vascular proteins important to contraction. PLoS One. 2009 May 25;4(5):e5682.

[53] Halka AT, Turner NJ, Carter A, Ghosh J, Murphy MO, Kirton JP, Kielty CM, Walker MG. The effects of stretch on vascular smooth muscle cell phenotype in vitro. Cardiovasc Pathol. 2008 Mar-Apr;17(2):98-102.

[54] Orr AW, Lee MY, Lemmon JA, Yurdagul A Jr, Gomez MF, Bortz PD, Wamhoff BR. Molecular mechanisms of collagen isotype-specific modulation of smooth muscle cell phenotype. Arterioscler Thromb Vasc Biol. 2009 Feb;29(2):225-31.

[55] Hilgers RH, Webb RC. Molecular aspects of arterial smooth muscle contraction: focus on Rho. Exp Biol Med. 2005 Dec;230(11):829-35.

[56] Pfitzer G, Lubomirov LT, Reimann K, Gagov H, Schubert R. Regulation of the crossbridge cycle in vascular smooth muscle by cAMP signalling. J Muscle Res Cell Motil. 2006;27(5-7):445-54.

[57] Ajtai K, Halstead MF, Nyitrai M, Penheiter AR, Zheng Y, Burghardt TP. The myosin C-loop is an allosteric actin contact sensor in actomyosin. Biochemistry. 2009 Jun 16;48(23):5263-75.

[58] Gunning P. Emerging issues for tropomyosin structure, regulation, function and pathology. Adv Exp Med Biol. 2008;644:293-8.

[59] Yang Z, Yamazaki M, Shen QW, Swartz DR. Differences between cardiac and skeletal troponin interaction with the thin filament probed by troponin exchange in skeletal myofibrils. Biophys J. 2009 Jul 8;97(1):183-94.

[60] Singh A, Hitchcock-Degregori SE. A peek into tropomyosin binding and unfolding on the actin filament. PLoS One. 2009 Jul 24;4(7):e6336.