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Cytosolic Phospholipase A2 Alpha is Required for Human Smooth Muscle Cell Proliferation and Not Migration to Platelet Derived Growth Factor BB

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Abstract

Platelet derived growth factor BB (PDGF BB) has an important influence on smooth muscle cell migration and 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 A2 (cPLA2) in smooth muscle cells to PDGF BB with the production of arachidonic acid and prostaglandin E2. In this study, we first investigated the role of cPLA2α on human aortic smooth muscle cell (HASMC) migration using modified Boyden chamber assay and under agarose migration studies. AACOCF3 (cPLA2 and iPLA2 inhibitor), 1,2,4-trisubstituted pyrrolidine derivative (cPLA2 inhibitor), and Bromoenol lactone (iPLA2 inhibitor) had no effect on HASMC chemotaxis to PDGF-BB in a modified Boyden chamber. These results were confirmed with specific inhibition of cPLA2α using small interfering RNA (siRNA) showing that HASMC migration was not inhibited in modified Boyden chamber or under agarose migration studies. Using the same siRNA to cPLA2 alpha had very significant inhibition to PDGF-BB dependent HASMC proliferation. These data demonstrate there is a distinct role especially for cPLA2α on human aortic HASMC proliferation and not migration to PDGF-BB.

Keywords: Muscle cell; Cell proliferation; Vascular smooth muscle cells

Introduction

Migration and proliferation of vascular smooth muscle cells (VSMCs) are important initial steps in the progression of atherosclerotic lesions, restenosis, and transplant vasculopathy [1,2]. The common characteristics of vascular responses to balloon injury are proliferation and migration of VSMCs and neointima formation in the injured arteries [3-6]. Neointimal hyperplasia and restenosis are the major problems limiting the long-term efficacy of percutaneous transluminal angioplasty with or without stent placement [7,8]. 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 VSMCs [9,10]. Denudation of endothelial cells after balloon angioplasty results in the release of PDGF and other growth factors, which stimulates VSMC proliferation and migration into the intima resulting in intimal hyperplasia.

PDGF is a growth factor that plays a role in embryonic development, cell proliferation, cell migration, angiogenesis, atherosclerosis and restenosis [11]. It is 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 [12]. 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 [13,14]. Accumulating data suggests that although this molecule is generally part of growth signaling complex, it plays a more profound role in controlling cell migration and proliferation. PDGF-BB is the highest-affinity ligand for the PDGFR-beta and is the most potent stimulator of VSMC migration [15]. Stimulation of VSMC with PDGF-BB produces a rapid release of arachidonic acid and prostaglandin E2 through the activation of cPLA2 [16].

The direct products of PLA2 action, lysophospholipids and fatty acids, can themselves act as cellular mediators or serve as precursors for the formation of mediators such as platelet-activating factor and eicosanoids [17,18]. Phospholipase A2 specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing fatty acid and lysophospholipid [19]. PLA2 includes a number of different phospholipase enzymes that include secretory forms (sPLA2), cPLA2 which is dependent on calcium (Group IV), calcium independent PLA2 or iPLA2 (Group VI), and lipoprotein-associated PLA2s (lp-PLA2), also known as platelet activating factor acetylhydrolase [20,21]. There are six enzymes classified as Group IV PLA2s: cPLA2α(GIVA), cPLA2β (GIVB), cPLA2γ (GIVC), cPLA2δ (GIVD), cPLA2ε (GIVE), and cPLA2ζ (GIVF) [22]. These enzymes contain a conserved Ser/Asp active site dyad and an Arg residue, which are critical for catalytic activity. cPLA2α has been studied extensively because it is the only PLA2 that exhibits specificity for hydrolysis of sn-2 arachidonic acid from membrane phospholipids [23,24]. Once arachidonic acid is liberated in the cytosol, it can be further metabolized into eicosanoids such as prostaglandins and leukotrienes which are lipid mediators of inflammation [25]. The exact role these PLA2s play in smooth muscle migration and proliferation to PDGF-BB is currently limited.

Arachidonic acid and its metabolites are involved in the regulation

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of a variety of physiological processes including vascular tone [26,27]. It was reported that arachidonic acid and its eicosanoid metabolites play a role in cell migration [28-31]. This study investigates the role of cPLA2 and iPLA2 through pharmacologic inhibition in migration of HASMC to PDGF-BB *in vitro* and further the investigation of targeted suppression of cPLA2α in multiple migration assays to PDGF-BB. It also investigated targeted suppression of cPLA2α in PDGF-BB stimulated HASMC proliferation.

Methods

Tissue culture and SMC isolation

Human arterial vascular smooth muscle cell lines (HAVSMC) which were obtained from an 11 month old female aorta were purchased from ATCC (CLR-1999). The cells were cultured in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) containing 10 mM of L-glutamine, 1500 mg/L sodium bicarbonate, 5 ng/mL Endothelial Cell Growth Supplement, 5 µg/mL insulin, 10 Units/mL Penicillin, 10 µg/mL Streptomycin, 25 ng/mL Amphotericin B, and 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. Cells were grown to >80% confluence and non-adherent cells were removed.

Pharmacological inhibitors

HAVSMC were washed once in PBS and resuspended in F-12K without FBS. The cells were treated with the pharmacologic inhibitors and incubated for 1 h at 37°C with 5% CO_2 before performing the chemotaxis or proliferation assays. All inhibitors were reconstituted in F-12K. Varying concentrations of Bromoenol lactone (iPLA2 inhibitor), 1,2,4-trisubstituted pyrrolidine (cPLA2 inhibitor) or AACOCF3 (cPLA2 and iPLA2 inhibitor) were added in less than 1/200 inhibitor volume to total volume of culture media. Unstimulated control cells were only exposed to PBS in F-12K without FBS.

Chemotaxis assay

HAVSMC chemotaxis was evaluated using a microchamber technique [32]. 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 2×10^6 cells/ml; 1×10^5 cells/well) was added to the upper compartment of the chamber that had been precoated with collagen type 1 dissolved in 10% acetic acid for 2 h. The two compartments were separated by a 8-µm pore size, polyvinylpyrrolidone-free polycarbonate filter. The chamber was incubated at 37°C in air with 5% CO_2 for 48 h. 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 nonmigrated cells. The side of the filter with the migrated cells was fixed in 100% methanol for 1 min and stained with Hema 3 Stain Set (Biochemical Science, distributed by Fisher Scientific, Pittsburgh, PA). All migrated SMC were counted in in each well by light microscopy using an Axioskop 2 plus Zeiss Microscope. All samples were tested in triplicate, and the data are expressed as the mean ± SD.

Under agarose assay

HASMC migration was evaluated under agarose (2%) using a 2-chamber slide (LabTek II, Rochester, NY) preparation. One gram of ultra-pure agarose powder was mixed in 25 ml of 2X sterile PBS and heated in a microwave until fully dissolved. 25 ml of 10% FBS and F-12K media warmed to 37°C was fully mixed with the agarose/PBS solution and poured into the 2 chamber slides until three quarter full and allowed to cool at room temperature for one hour in a laminar flow hood. The holes were punched 6 mm in diameter and placed 5 mm apart with carful removal of all agarose from wells. 1×10^4 cells were added to one well and allowed to migrate under agarose for 48 h to the opposing well with 25 ng/ml of PDGF BB at 37°C with 5% CO_2. There was an additional feed of PDGF-BB in the same concentration in the chemoattractant well after 24 h. Agarose was removed carefully and slides were dried at room temperature then fixed in 100% methanol for 30 s. Slides were cover slipped with DAPI, and all cells between wells were counted under ultraviolet light using an Axioskop 2 plus Zeiss Microscope.

Immunofluorescence assay

For immunofluorescence, human aortic smooth muscle cells were grown to at least 70% confluence in eight chamber slides with two wells stimulated with 25 ng/ml of PDGF BB for 2 h. Slides were fixed in HistoChoice (Ameresco, Solon, Ohio) until stained. Cells were permeabilized 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 5 µg/mL, and 5 µg/mL of goat anti-rabbit IgG was used for vimentin staining. Negative control chambers were stained with secondary antibodies alone. The slides were cover slipped with a DAPI mounting media. This experiment was repeated in triplicate with representative micrographs. Slides were analyzed with an Axioskop 2 plus Zeiss Microscope and using Image Pro Plus 9.0 software (Media Cybernetics, Silver Spring, Md).

Western Blot/SDS-Page

HAVSMC were washed three times with PBS to remove traces of DMEM and 10% BCS. The six well plates 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 150 µg of total protein/well loaded on a 7.5% SDS-PAGE gel. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 µm; Bio-Rad) using a semi-dry 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. cPLA2 protein was detected with a 1/1000 dilution of anti-mouse recombinant cPLA2 mAb sc-454 (Santa Cruz), followed by incubation with anti-mouse IgG HRP (1/1000 dilution; Transduction Laboratories, Lexington, KY) at 37°C for one hour each. The blots were stripped and reprobed with smooth muscle α-actin (mouse monoclonal antibody, clone 1A-4) in the concentration of 1:1000 followed by the same concentration of secondary antibody above at 37°C for one hour each.

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 and cPLA2 siRNA (Santa Cruz Biotechnology, Inc, CA) was carried out according to manufacturer's protocol. Briefly,

varying concentrations of siRNA tested from 60 to 120 nM were each mixed with the transfection reagent for 45 min at room temperature, and then added to cells and allowed to incubate 7 h 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 18-24 h at 37°C with 5% CO_2. Toluene blue staining was used to check for toxicity. This was replaced with 1X normal growth media, and cells were prepared for either chemotaxis assay or under agarose migration respectively.

Proliferation assays

HASMC were serum starved for 24 h and 1×10^4 cells were plated in 96 well plates and treated with 120 nM of cPLA2 siRNA (Santa Cruz Biotechnology, Inc, CA) then cultured 37°C with 5% CO_2 for 24 h in serum free F-12K media. Cells were stimulated with 25 ng/ml human recombinant PDGF BB in serum free F-12K with 0.1% BSA for 72 h at 37°C with 5% CO_2. The number of cells were quantified using CyQUANT™ NF Cell Proliferation Assay Kit (Molecular Probes, OR) according manufacturers protocol outlined in adherent cells. Plates were then read using a Synergy™ Mx microplate reader (BioTek, VT) with excitation at 485 nm and emission detection at 530 nm.

Statistics

All the experiments were repeated three times with similar results. Data are presented as Mean ± S. D. The treatment effects were analyzed by Student's t-test. p values<0.05 were statistically significant.

Results

PDGF BB induces cPLA2 protein expression which doubles after 2 h of stimulation in HASMC (Figure 1). cPLA2 expression after stimulation with PDGF-BB continues to increase slightly over 10 h just

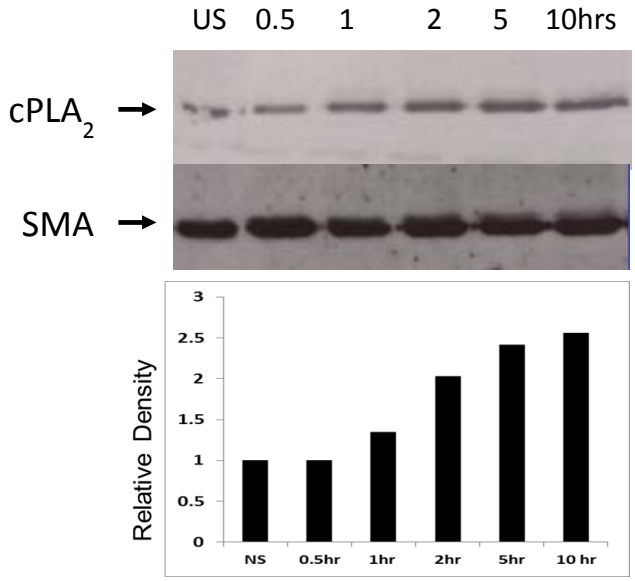

Figure 1: cPLA2 Protein doubles at two hours in HASMC after stimulation with PDGF-BB. VSMCs were unstimulated (US) or incubated with 25 ng/ml of PDGF-BB for 0.5, 1, 2, 5, and 10 h. 150 µg of total cytoplasmic protein was evaluated by Western Blot for cPLA2 protein (upper panel). The same blot was stripped and reprobed with an antibody to Smooth Muscle Actin (SMA–lower panel). Relative density was performed comparing cPLA2 protein to the corresponding SMA protein at the corresponding time point. This experiment was performed in triplicate and a representative blot is shown above.

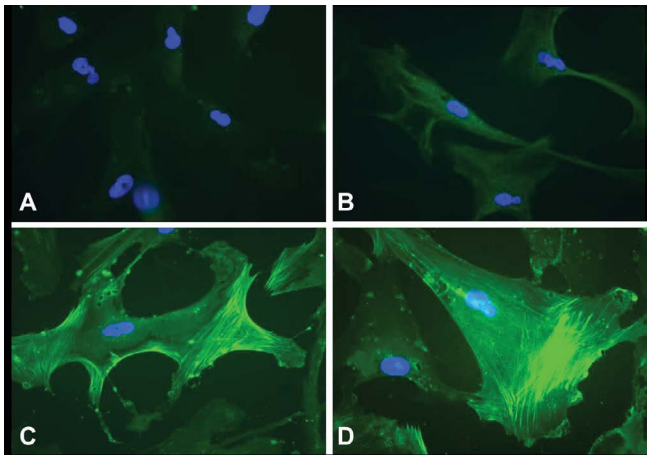

Figure 2: PDGF-BB produces actin polymerization at the leading edge of HASMC. Immunofluorescence of unstimulated HASMC stained with no primary antibody (A), vimentin (B), and smooth muscle actin (C). Stimulation of HASMC with 25 ng/ml of PDGF-BB for 2 h resulted in polymerization of smooth muscle actin fibers at the leading edge of cells (D). This experiment was performed in duplicate.

below 2.5 times unstimulated cells, however, this was maintained up to about 16 h and returned to unstimulated levels after 24 h (data not shown). Increased levels of smooth muscle actin polymerization were observed in the PDGF-BB stimulated cells at the leading edge of cells (Figure 2). The negative control was made up of unstimulated HASMC 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, and found to be largely negative for vimentin staining (Figure 2B). Normally, un-stimulated HASMC stained with SMA show actin staining around areas of anchoring points to the slide surface (Figure 2C). However, after stimulation with PDGF BB SMA filaments congregated at the leading edge of HASMC after 2h of exposure with PDGF-BB (Figure 2D). The cells became polarized to indicate movement with the HASMC making up part of the lamellipodia of the polarized cell. This actin polymerization was observed in about 65% of the cells within the culture colony. This demonstrates that PDGF BB plays a role in HASMC movement.

Inhibition of cPLA2 or iPLA2 with pharmacological inhibitors had no effect on smooth muscle cell chemotaxis to PDGF BB in a modified Boyden chamber (Figure 3). AACOCF3 has been reported to inhibit both cPLA2 and iPLA2, but not sPLA2 [18,19]. AACOCF3 did not cause significant, dose-dependent inhibition of HASMC chemotaxis to PDGF BB from 0.5 µM to 50 µM concentration (Figure 3A). 1,2,4-trisubstituted pyrrolidine derivative (cPLA2 INH) is a specific inhibitor of cPLA2 which had no significant effect on HASMC chemotaxis to PDGF BB from doses of 1 nM to 1 µM (Figure 3B). This was also seen for the iPLA2 inhibitor, Bromoenol lactone (BEL), using concentrations of up to 10 µM (Figure 3C). Pictures of the filter at 400X with controls and the highest concentration of pharmacologic inhibitors is shown after staining with HEMA 3 (Figures 3D-3I). Negative control had no PDGF-BB in bottom chamber and showed few cells migrating (Figure 3D) and the positive control showed many more untreated HASMC migrating to 25 ng/ml of PDGF-BB on the opposite side of the membrane after 48 h (Figure 3E). The highest concentration of pharmacologic inhibitors and the solvent (DMSO) used to dilute the inhibitors show an equal number of cells migrating to the other side of the filter after 48 h (Figure 3F, 50 µM AACOCF3; Figure 3G, 1

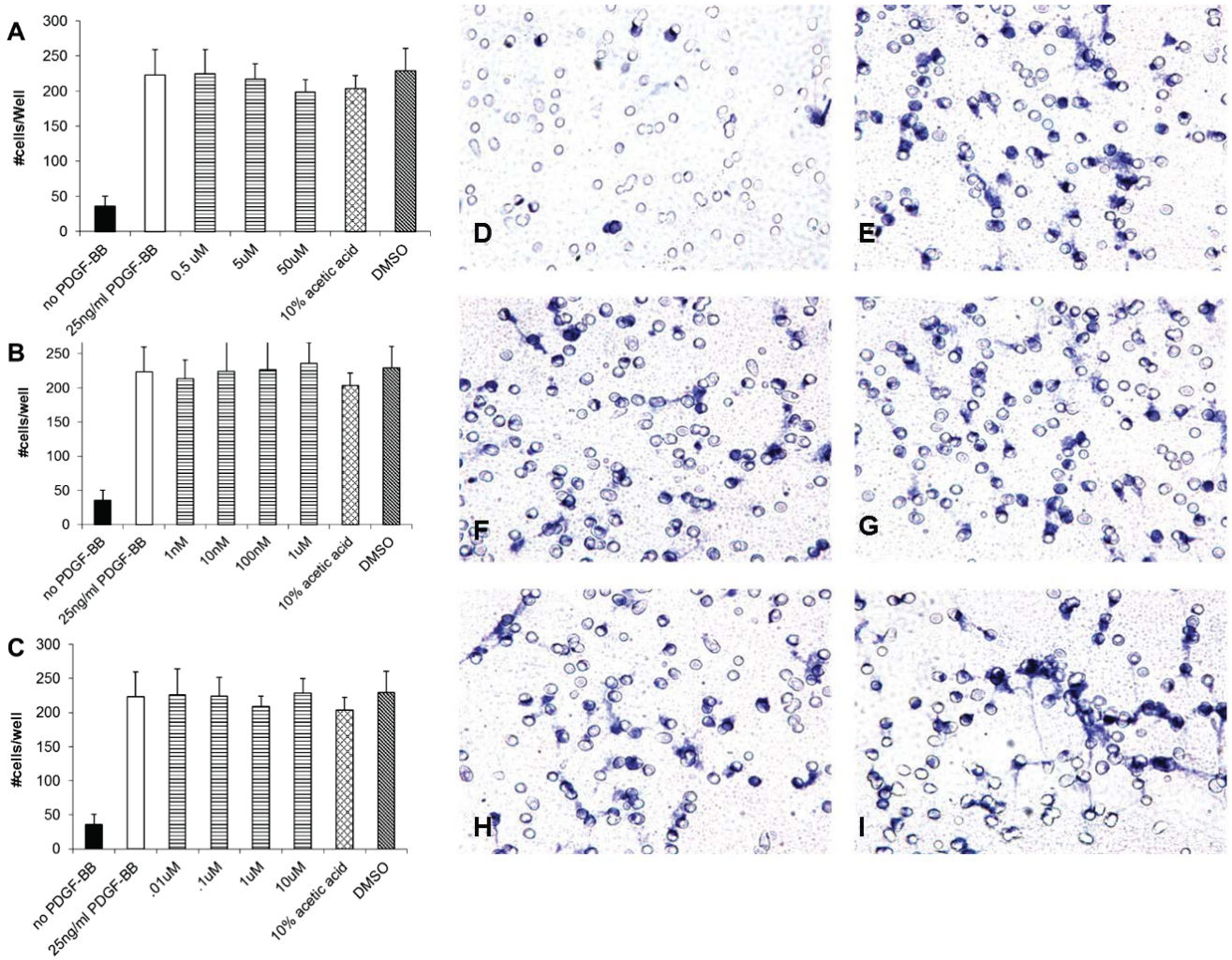

Figure 3: Pharmacological inhibitors of PLA 2 do not inhibit HASMC migration to PDGF-BB by modified Boyden chamber chemotaxis assay. HASMCs were cultured in F12K media without serum and in the presence or absence of various concentrations of AACOCF3 (A), 1,2,4-trisubstituted pyrrolidine derivative (cPLA2 INH - B), or BEL (C), for 1hr. Two thousand HASMCs underwent chemotaxis across a type 1 collagen coated polycarbonate filter in response to 25 ng/ml of PDGF-BB in a modified Boyden Chamber at 37°C in 5% CO_2 for 48 h and then counted by light microscopy. 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. Pictures of polycarbonate filter at 400X of cells migrating without PDGF-BB(D), 25 ng/ml of PDGF-BB(E), treated with 50 μM AACOCF3 (F), 1 μM cPLA2 INH (G), 10 μM BEL (H), or 0.2% DMSO (I) which are the highest concentrations of pharmacologic inhibitors are shown. 10% acetic acid was used to dissolve the type 1 collagen to precoat membrane and DMSO was the diluent for the pharmacological inhibitors. These were tested as controls in their appropriate concentrations. Student's t-test *p<0.05

μM cPLA2 INH; Figure 3H, 10 μM BEL and Figure 3I, 0.2% DMSO). Therefore, the data shows that pharmacologic inhibition of cPLA2 and iPLA2 does not significantly affect HASMC chemotaxis in a modified Boyden chamber migration assay.

A more specific inhibitor of cPLA2 using siRNA to target the suppression of this particular protein in HASMC was characterized using western blot (Figure 4). After 24 h of exposure of siRNA to cPLA2 in HASMC there was a about a 55% inhibition using 60 nM of siRNA and about 90% inhibition using 120 nM siRNA to cPLA2. These experiments were repeated over three times and had about a 50% inhibition using 60 nM of siRNA and range of 85-95% inhibition using 120 nM siRNA to cPLA2 measured by densitometry (data not shown). These reagents would be used in different migration studies of HASMC to PDGF-BB.

To confirm cPLA2 lack of effect in HASMC chemotaxis we turned to more targeted inhibition in modified Boyden chamber experiments using siRNA of cPLA2α. Targeted inhibition of cPLA2α had no effect on PDGF-BB stimulated chemotaxis on HASMC in modified Boyden chamber (Figure 5). The cells were transfected for 7 h and allowed to rest for a minimum of 18 h before use in chemotaxis assay. Toluene blue staining showed on evidence of toxicity in Control A scramble sequence at 12 nM siRNA (Con A) or in 6 nM and 12 nM of cPLA2α siRNA which was checked at the end of each experiment (data not shown). There was no inhibition from either concentration of cPLA2 siRNA treated cells compared to the untreated HASMC migrating to PDGF-BB which were run in triplicate, and this experiment was repeated four times. Trypan blue staining at the end of the timepoint showed similar staining in both concentrations of siRNA and Con A treated HASMC as in the NC or PC (data not shown).

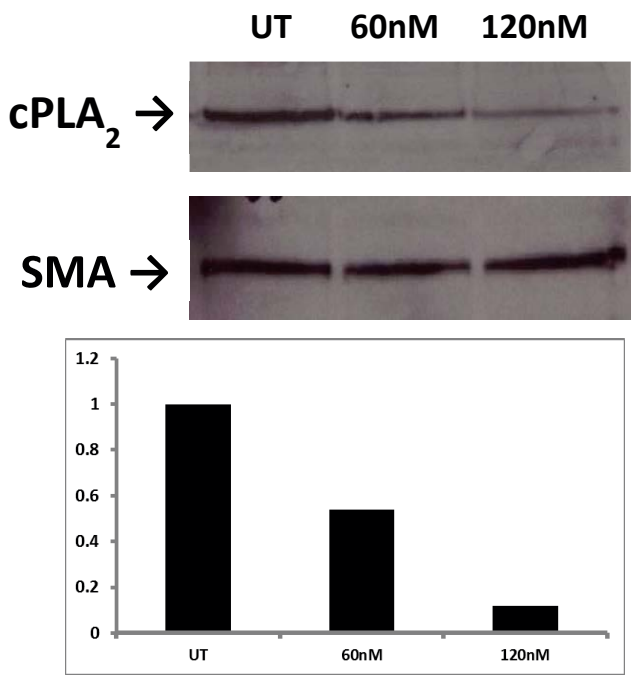

Figure 4: cPLA2 protein expression after 24 h of transfection with cPLA2 siRNA of human aortic smooth muscle cells. Representative western blot of HASMC's that were untreated (UT), 60 nM and 120 nM of cPLA2 siRNA treated for 24 h at 37°C in 5% CO_2. The same blot was stripped and reprobed with an antibody to Smooth Muscle Actin (SMA). Relative densitometry of western blot results from the above blot is shown below.

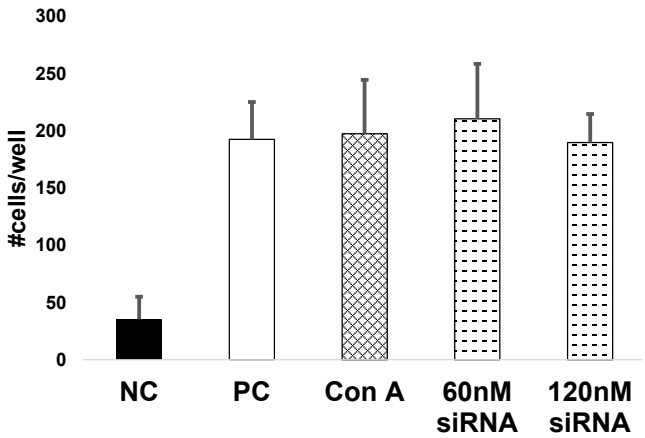

Figure 5: cPLA$_2$ siRNA does not inhibit HASMC chemotaxis to PDGF-BB by modified Boyden chamber chemotaxis assay. HASMCs were cultured in F12K media without serum for 24 h before being treated with 60 nM & 120 nM of cPLA$_2$ siRNA or 120 nM of control A scrambled siRNA (Con A) for 24 h. Two thousand HASMCs underwent chemotaxis across a type 1 collagen coated polycarbonate filter in response to 25 ng/ml of PDGF-BB in a modified Boyden Chamber at 37°C in 5% CO_2 for 48 h and then counted by light microscopy. Cells that did not migrate to PDGF-BB were negative control (NC), and untreated cells migrating to 25 ng/ml of PDGF-BB were the positive control (PC). All samples were performed in triplicate, and experiments were repeated four times. The results are expressed as the total number of migrated cells per well pulse standard deviation and are from a representative experiment. Student's t-test *, p<0.05

We turned to another method of migration to investigate PDGF-BB stimulated movement of HASMC with targeted inhibition of cPLA2α

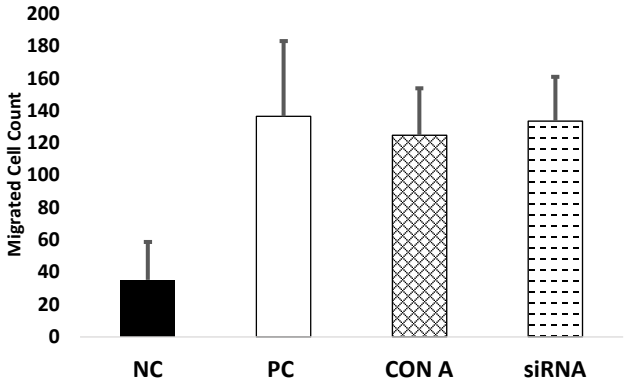

Figure 6: cPLA$_2$ siRNA does not inhibit HASMC migration to PDGF-BB by under agarose migration assay. HASMCs were cultured in F12K without serum and in the presence or absence of and 120 nM of control A scrambled siRNA (Con A) and 120 nM of cPLA$_2$ siRNA(siRNA) for 24 h. Ten thousand HASMCs migrated under 2% agarose in response to 25 ng/ml of PDGF-BB at 37°C in 5% CO_2 for 48 h total with one additional feed at 24 h. Untreated cells that had no PDGF-BB in the opposing well were negative control (NC), and untreated cells migrating to 25 ng/ml of PDGF-BB in the opposing well were the positive control (PC). The cells were stained with DAPI and then counted under ultraviolet light using an Axioskop 2 plus Zeiss Microscope. The results are expressed as the average number of migrated cells outside the area of original placement towards the chemoattractant well pulse standard deviation in triplicate experiments. Student's t-test *, p<0.05

through siRNA. We used under agarose migration method which can evaluate longer migration distances towards a chemoattractant gradient. In figure 6 we showed targeted suppression of cPLA2α had no effect on the amount of HASMC migrating outside their place of origin towards PDGF-BB in under agarose migration assay compared to controls. This is further evidence that cPLA2α is not involved in PDGF-BB stimulated migration of HASMC.

Smooth muscle migration together with proliferation are features relevant to the pathogenesis of atherosclerosis and restenosis [1,2]. Therefore, we investigated targeted inhibition of cPLA2α using siRNA on PDGF-BB stimulated proliferation of HASMC. This resulted in a very significant decrease in HASMC proliferation *in vivo* measured after 72 h of stimulation with PDGF-BB (Figure 7). Trypan blue staining at the end of the time point had similar staining in siRNA treated HASMC as in the NC or PC (data not shown). These results show that cPLA2α is required for PDGF-BB stimulated HASMC proliferation and not migration using *in vivo* assays.

Discussion

Pathogenic mechanisms leading to smooth muscle migration in atherosclerosis and restenosis are incompletely understood. Many growth factors including epidermal growth factor, fibroblast growth factor, insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), transforming growth factor, vascular endothelial growth factor and angiotensin II are involved, however, PDGF-BB is one of the most potent to stimulate migration in vascular smooth muscle cells [33,34]. It has been shown that PDGF-BB stimulates arachidonic acid (AA) and prostaglandin E2 release in human smooth muscle cells [16]. cPLA2α that has a selectivity for sn-2 AA cleavage within phospholipids, and plays an important role in AA release which form potent eicosanoids [35-37]. The activation of cPLA2 has been recognized in both vascular and tracheal smooth muscle cells [38-41]. Our studies show that the stimulation of HASMC with PDGF-BB induces the production

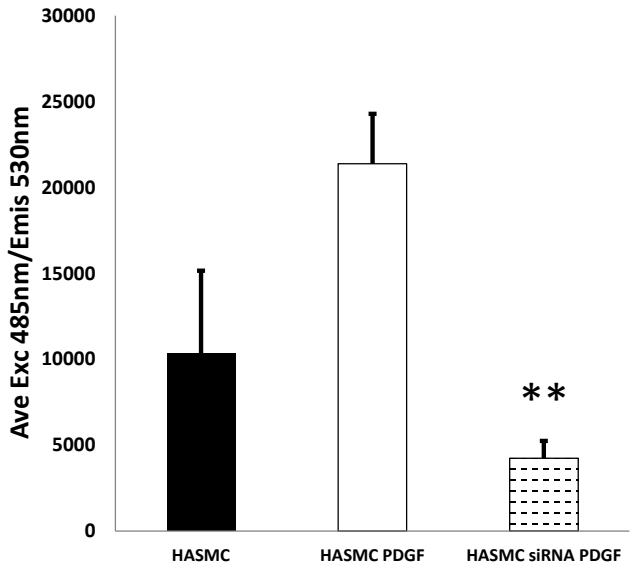

Figure 7: cPLA$_2$ siRNA does significantly inhibit to HASMC proliferation after stimulation with PDGF-BB. HASMCs were cultured in F12K media without serum in the presence or absence of 120 nm siRNA to cPLA2 for 24 h and then stimulated with 25 ng/ml PDGF-BB and incubated for 72 h at 37°C in 5% CO_2. The number of cells were quantified by CyQuant NF adherent cell assay (Molecular Probes). All samples were performed in quadruplicate, and the experiment was repeated twice. The results are expressed as average OD 485 nm excitation/530 nm emission from both experiments and Student t-test analysis compared to wells stimulated with PDGF-BB alone. **p<0.01

of cPLA2 within an hour and peaks by 5 h after stimulation while remaining elevated for multiple h after initial stimulation (Figure 1).

Membrane protrusions at the leading edge of cells, known as lamellipodia, drive cell migration in many normal and pathological situations. Lamellipodial protrusion is powered by actin polymerization, which is mediated by the actin-related protein 2/3 induced nucleation of branched actin networks and the elongation of actin filaments [42]. After 2 h of stimulation with PDGF-BB formation of an actin meshwork at the leading edge of HASMC were seen (Figure 2D) in over half of the cultured cells. This demonstrates that PDGF-BB stimulation of HASMC promotes actin polymerization necessary for lamellipodial protrusion which drives chemotaxis.

In past studies cPLA2α is specifically recruited to the leading edge at sites of actin and membrane remodeling in mouse fibroblasts after stimulation with PDGF-BB and in primary human endothelial cells migration assays using scratch assays [43]. This was achieved by pharmacologic inhibition of cPLA2α of Pyrrolidine-2 or Wyeth-1, which are selective inhibitors for cPLA2α and not cPLA2β. The ability of PDGF-BB to induce chemotaxis of HASMC coincides with their ability to stimulate phosphatidylinositol turnover, diacylglycerol formation, and intracellular Ca2+ flux which is found to be more potent in PDGF-BB stimulation than IGF-1 [44]. cPLA2 is an intracellular, 85-kDa protein that requires nanomolar to micromolar concentrations of calcium to become activated. Protein kinase C and mitogen activated kinase regulates the activity of cPLA2 by phosphorylating the 505 and 727 serine sites before traveling to the plasma membrane to enzymatically act on phospholipids [45]. To test if cPLA2 or iPLA2 is required for PDGF-BB dependent HASMC chemotaxis we used a series of pharmacologic inhibitors which resulted in no significant reduction in chemotaxis to PDGF-BB after inhibiting with AACOCF 3(cPLA2 and iPLA2 inhibitor-Figure 3A), 1,2,4-trisubstituted pyrrolidine derivative (cPLA2 only inhibitor–Figure 3B), or BEL (iPLA2 only inhibitor–Figure 3C) in a modified Boyden chamber assays. In past studies rat thoracic smooth muscle cells treated with palmitoyl trifluoromethyl ketone (PACOCF3) resulted in decreased migration to PDGF-BB in a cell wound assay [46]. PACOCF3 is a general PLA2 inhibitor and alters Ca2+ signaling. Since there are inconsistent results and types of assays in the role of PLA2 in smooth muscle migration to PDGF-BB with pharmacologic inhibitors, we moved to specific inhibition using targeted siRNA to cPLA2α inhibition in modified Boyden chamber and under agarose migration assays.

The cPLA2 siRNA is 25-nucleotide sequence designed to knock down gene expression of human PLA2G4A (cPLA2α) mapping to chromosome 1q31.1. This provided significant inhibition of cPLA2 protein in HASMC at 120 nM concentration after treating for 24 h (Figure 4). Knockdown of cPLA2α in HASMC with this siRNA had no effect on migration to PDGF-BB in modified Boyden assays (Figure 5) or under agarose migration assays (Figure 6). It has been reported that during vascular injury in rats, the exposure of balloon injured carotid arteries to retroviral vectors containing cPLA2 or PLD2 siRNA diminished the increase in neointimal growth elicited by ANG II in an Akt kinase dependent manner [47]. However, this *in vivo* model could possibly effect smooth muscle cell proliferation in addition to migration because Akt activation promotes vascular smooth muscle proliferation and migration [48]. After rat carotid artery balloon injury, antibody neutralization of PDGF caused a 27% decrease in smooth muscle proliferation [49]. Conversely, our studies suggest that cPLA2α is not involved in PDGF-BB directed migration of HASMC *in vitro*. The exact role of other forms of cPLA2 after stimulation with PDGF-BB dependent migration would need further investigation in HASMC. It is possible it plays more of a role in proliferation than migration.

iPLA2 has been suggested to function active participant in signal transduction pathways regulating chemotaxis in different cells [50-52]. Moon et al. showed that mouse iPLA2β knockout mesenteric arterial SMC inhibited migration and proliferation in *in vitro* arterial explant assays [53]. The inhibition was rescued through the addition of AA and subsequently re-inhibited by the COX-2-specific inhibitor NS-398 which was rescued by PGE2. This demonstrates that iPLA2β is important in murine VSMC migration and proliferation in an AA and PGE2 dependent manor. They showed iPLA2 has not been associated with PDGF-BB, however, it does produce AA in a calcium independent way in murine vascular smooth muscle cells. Our findings showed no significant inhibition to chemotaxis with cells treated specified iPLA2 BEL in HASMC to PDGF-BB (Figure 3C). This finding partially oppose the Moon et al. [53] studies, however, our studies were performed in a modified Boyden chamber testing chemotaxis to PDGF-BB over 2 days where Moon's experiments were done with excised artery on glass slides which counted migration and proliferation together after 21 days with no chemoattractant which makes this more of a chemokinesis assay. However, in an artery ligation study, overexpression of iPLA2β in smooth muscle cells carotid artery ligation in a transgenic mouse model results in increased neointimal formation, macrophage infiltration, and early vascular inflammation with overexpression of IL-6 and TNF-α [54]. iPLA2 plays a role in neointimal formation, but the exact role in smooth muscle cell migration to PDGF-BB remains unknown.

In VSMC from rabbit aorta stimulated with norepinephrine, cPLA2 required phosphorylation at serine-515 and 505 to release AA which was blocked after amino acid substitution at these sites [55]. It also has been reported that a dominant negative mutant-dependent suppression

of Jak-2 and STAT-3 blocked PDGF-BB-induced rat aortic VSMC motility. PDGF-BB induced the expression of cPLA2 in Jak-2/STAT-3-dependent manner and pharmacological inhibitor PACOCF3 of cPLA2 prevented PDGF-BB induced VSMC motility. Furthermore, either exogenous addition of AA or forced expression of cPLA2 rescued PDGF-BB-induced VSMC motility from inhibition by blockade of Jak-2 and STAT-3 activation [46]. These experiments were done as a wound healing assay and used general inhibitors of cPLA2 which may have had other effects. The restoration with AA shows promise as it may be required for VSMC motility, but the exact cPLA2 isoform responsible for its release will require further research. Lastly, 20-hydroxyeicosatetraenoic acid (20-HETE) has been shown to stimulates PDGF-BB mediated rat VSMC chemotaxis by modified Boyden chamber assays acting through pathways that involve MEK and PI3K [56]. 20 HETE is a cytochrome P4504A derived metabolite of AA endogenously produced by VSMC. All these experiments had aortic VSMC from different species with differences in cell migration assays. Our findings imply that cPLA2α is not required for HASMC to PDGF-BB. The exact eicosanoid responsible and the way AA is being released and activated after stimulation with PDGF-BB in HASMC migration warrants further investigation.

The release of AA and PGE2 has been detected during unstimulated VSMC proliferation implicating a role of cPLA2 in this process [57]. Both insulin and thrombin induced VSMC proliferation requires a partial role for cPLA2 during its progression [58,59]. In the case of insulin, it was shown through pharmacologic inhibition that PI3K/Akt, cPLA2 and ERK1/2 signaling pathways were important to VSMC proliferation [59]. Our finding show that cPLA2α is important for PDGF-BB induced HASMC proliferation (Figure 7). It has been suggested in models of smooth muscle proliferation that PDGF-induced MAPK activation leads to cytosolic phospholipase A2 activation, PGE2 release, and subsequent activation of the cAMP-dependent protein kinase (PKA), which acts as a strong inhibitor of VSMC proliferation [60]. Our data shows the essential role of targeted inhibition to cPLA2α in HASMC proliferation and not migration after stimulation with PDGF-BB.

References

1. Orlandi A, Bennett M (2010) Progenitor cell-derived smooth muscle cells in vascular disease. Biochem Pharmacol 79: 1706-1713.
2. Bennett MR, Sinha S, Owens GK (2016) Vascular Smooth Muscle Cells in Atherosclerosis. Circ Res 118: 692-702.
3. Lao KH, Zeng L, Xu Q (2015) Endothelial and smooth muscle cell transformation in atherosclerosis. Curr Opin Lipidol 26: 449-456.
4. Wang G, Jacquet L, Karamariti E, Xu Q (2015) Origin and differentiation of vascular smooth muscle cells. J Physiol 593: 3013-3030.
5. Lacolley P, Regnault V, Nicoletti A, Li Z, Michel JB (2012) The vascular smooth muscle cell in arterial pathology: A cell that can take on multiple roles. Cardiovasc Rev 95: 194–204.
6. Lekshmi KM, Che HL, Cho CS, Park IK (2017) Drug- and Gene-eluting Stents for Preventing Coronary Restenosis. Chonnam Med J 53: 14-27.
7. Al-Bawardy RF, Waldo SW, Rosenfield K (2017) Advances in Percutaneous Therapies for Peripheral Artery Disease: Drug-Coated Balloons. Curr Cardiol Rep 19: 99.
8. Byrne RA, Stone GW, Ormiston J, Kastrati A (2017) Coronary balloon angioplasty, stents, and scaffolds. Lancet 390: 781-792.
9. Marmur JD, Poon M, Rossikhina M, Taubman MB (1992) Induction of PDGF-responsive genes in vascular smooth muscle. Implications for the early response to vessel injury. Circulation 86: S53-S60.
10. Zhao J, Jian L, Zhang L, Ding T, Li X, et al. (2016) Knockdown of SCARA5 inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and migration through suppression of the PDGF signaling pathway. Mol Med Rep 13: 4455-4460.
11. Heldin CH, Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79: 1283-1316.
12. Kazlauskas A (2017) PDGFs and their receptors. Gene 614: 1-7.
13. Campbell M, Allen WE, Silversides JA, Trimble ER (2003) Glucose-induced phosphatidylinositol 3-kinase and mitogen-activated protein kinase-dependent up regulation of the platelet-derived growth factor-beta receptor potentiates vascular smooth muscle cell chemotaxis. Diabetes 52: 519-526.
14. Gao BB, Hansen H, Chen HC, Feener EP (2006) Angiotensin II stimulates phosphorylation of an ectodomain-truncated platelet-derived growth factor receptor-beta and its binding to class IA PI3K in vascular smooth muscle cells. Biochem J 397: 337-344.
15. Sachinidis A, Locher R, Vetter W, Tatje D, Hoppe J (1990) Different effects of platelet-derived growth factor isoforms on rat vascular smooth muscle cells. J Biol Chem 265: 10238–10243.
16. Graves LM, Bornfeldt KE, Sidhu JS, Argast GM, Raines EW, et al. (1996) 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 271: 505-511.
17. Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G (2011) Phospholipase A2 Enzymes: Physical Structure, Biological Function, Disease Implication, Chemical Inhibition, and Therapeutic Intervention. Chem Rev 111: 6130–6185.
18. Burke JE, Dennis EA (2009) Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res 50 Suppl: S237-242.
19. Zhou F, Schulten K (1996) Molecular dynamics study of phospholipase A2 on a membrane surface. Proteins 25: 12-27.
20. Kudo I, Murakami M (2002) Phospholipase A2 enzymes. Prostaglandins Other Lipid Mediat 68-69: 3-58.
21. Six DA, Dennis EA (2000) The expanding superfamily of phospholipase A2 enzymes: classification and characterization. Biochimica et Biophysica Acta 1488: 1-19.
22. Ghosh M, Tucker DE, Burchett SA, Leslie CC (2006) Properties of the Group IV phospholipase A2 family. Prog Lipid Res 45: 487-510.
23. Balsindea J, Winstead MV, Dennis EA (2002) Phospholipase A2 regulation of arachidonic acid mobilization. FEBS Letters 531: 2-6.
24. Clark JD, Schievella AR, Nalefski EA, Lin LL (1995) Cytosolic phospholipase A2. J Lipid Mediat Cell Signal 12: 83-117.
25. Leslie CC (2004) Regulation of arachidonic acid availability for eicosanoid production. Biochem Cell Biol 82: 1-17.
26. Smith WL (1989) The eicosanoids and their biochemical mechanisms of action. Biochem J 259: 315-324.
27. Lin L, Balazy M, Pagano PJ, Nasjletti A (1994) Expression of prostaglandin H2-mediated mechanism of vascular contraction in hypertensive rats. Relation to lipoxygenase and prostacyclin synthase activities. Circ Res 74: 197-205.
28. Ott VL, Cambier JC, Kappler J, Marrack P, Swanson BJ (2003) Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. Nature Immunol 10: 974-981.
29. Honig SM, Fu S, Mao X, Yopp A, Gunn MD, et al. (2003) FTY720 stimulates multidrug transporter- and cysteinyl leukotriene-dependent T cell chemotaxis to lymph nodes. J Clin Invest 111: 627-637.
30. Stockton RA, Jacobson BS (2001) Modulation of cell-substrate adhesion by arachidonic acid: lipoxygenase regulates cell spreading and ERK1/2-inducible cyclooxygenase regulates cell migration in NIH-3T3 fibroblasts. Mol Biol Cell 12: 1937-1956.
31. Maddox JF, Colgan SP, Clish CB, Petasis NA, Fokin VV, et al. (1998) Lipoxin B4 regulates human monocyte/neutrophil adherence and motility: design of stable lipoxin B4 analogs with increased biologic activity. FASEB J 12: 487-494.
32. Goncharova EA, Goncharov DA, Krymskaya VP (2006) Assays for in vitromonitoring of human airway smooth muscle (ASM) and human pulmonaryarterial vascular smooth muscle (VSM) cell migration. Nat Protoc 1: 2933–2939.
33. De Donatis A, Comito G, Buricchi F, Parenti A, Caselli A, et al. (2008) Proliferation versus migration in platelet-derived growth factor signaling: The key role of endocytosis. J Biol Chem 283: 19948–19956.

34. Ronnstrand L, Heldin CH (2001) Mechanisms of platelet-derived growth factor-induced chemotaxis. Int J Cancer 91: 757–62.

35. Shimizu T, Wolfe LS (1990) Arachidonic acid cascade and signal transduction. J Neurochem 55: 1-15.

36. Herschman HR (1996) Prostaglandin synthase 2. Biochim Biophys Acta 1299: 125-140.

37. Xie W, Herschman HR (1996) Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet-derived growth factor and serum. J Biol Chem 271: 31742-31748.

38. Pyne NJ, Pyne S (1998) PDGF-stimulated cyclic AMP formation in airway smooth muscle: assessment of the roles of MAP kinase, cytosolic phospholipase A2, and arachidonate metabolites. Cell Signal 10: 363-369.

39. Pilane CM, LaBelle EF (2004) cPLA2 activator peptide, PLAP, increases arachidonic acid release and apoptosis of vascular smooth muscle cells. J Cell Physiol 198: 48-52.

40. Liu Y,Taylor CW (2006) Stimulation of arachidonic acid release by vasopressin in A7r5 vascular smooth muscle cells mediated by Ca2+-stimulated phospholipase A2. FEBS Lett 580: 4114-4120.

41. Luo SF, Lin WN, Yang CM, Lee CW, Liao CH, et al. (2006) Induction of cytosolic phospholipase A2 by lipopolysaccharide in canine tracheal smooth muscle cells: involvement of MAPKs and NF-kappaB pathways. Cell Signal 18: 1201-1211.

42. Gimona M, Kaverina I, Resch GP, Vignal E, Burgstaller G (2003) Calponin repeats regulate actin filament stability and formation of podosomes in smooth muscle cells. Mol Biol Cell 14: 2482–2491.

43. Moes M, Boonstra J, Regan-Klapisz E (2010) Novel role of cPLA(2)alpha in membrane and actin dynamics. Cell Mol Life Sci 67: 1547-1557.

44. Bornfeldt KE, Raines EW, Nakano T, Graves LM, Krebs EG, et al. (1994) 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 93: 1266-1274.

45. Zhou H, Das S, Murthy KS (2003) Erk1/2- and p38 MAP kinase-dependent phosphorylation and activation of cPLA2 by m3 and m2 receptors. Am J Physiol Gastrointest Liver Physiol 284: G472-480.

46. Neeli I, Liu Z, Dronadula N, Ma ZA, Rao GN (2004) An essential role of the Jak-2/STAT-3/cytosolic phospholipase A(2) axis in platelet-derived growth factor BB-induced vascular smooth muscle cell motility. J Biol Chem 279: 46122-46128.

47. Li F, Zhang C, Schaefer S, Estes A, Malik KU (2005) ANG II-induced neointimal growth is mediated via cPLA2- and PLD2-activated Akt in balloon-injured rat carotid artery. Am J Physiol Heart Circ Physiol 289: H2592-2601.

48. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, et al. (2000) The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 6: 1004–1010.

49. Lewis CD, Olson NE, Raines EW, Reidy MA, Jackson CL (2001) Modulation of smooth muscle proliferation in rat carotid artery by platelet-derived mediators and fibroblast growth factor-2. Platelets 12: 352–358.

50. Carnevale KA, Cathcart MK (2001) Calcium Independent Phospholipase A2 is Required for Human Monocyte Chemotaxis to Monocyte Chemoattractant Protein 1. J Immunol 167: 3414-3421.

51. Mishra RS, Carnevale KA, Cathcart MK (2008) iPLA2beta: front and center in human monocyte chemotaxis to MCP-1. J Exp Med 205: 347-359.

52. Chen L, Iijima M, Tang M, Landree MA, Huang YE, et al. (2007) PLA2 and PI3K/PTEN pathways act in parallel to mediate chemotaxis. Dev Cell 12: 603-614.

53. Moon SH, Jenkins CM, Mancuso DJ, Turk J, Gross R (2008) Smooth Muscle Cell Arachidonic Acid Release, Migration, and Proliferation Are Markedly Attenuated in Mice Null for Calcium-independent Phospholipase A2ß. J Biol Chem 283: 33975–33987.

54. Liu S, Xie Z, Zhao Q, Pang H, Turk J, et al. (2012) Smooth muscle-specific expression of calcium-independent phospholipase A2ß (iPLA2ß) participates in the initiation and early progression of vascular inflammation and neointima formation. J Biol Chem 287: 24739-24753.

55. Pavicevic Z, Leslie CC, Malik KU (2008) cPLA2 phosphorylation at serine-515 and serine-505 is required for arachidonic acid release in vascular smooth muscle cells. J Lipid Res 49: 724-737.

56. Stec DE, Gannon KP, Beaird JS (2007) Drummond HA. 20-Hydroxyeicosatetraenoic acid (20-HETE) stimulates migration of vascular smooth muscle cells. Cell Physiol Biochem 19: 121-128.

57. Anderson KM, Roshak A, Winkler JD, McCord M, Marshall LA (1997) Cytosolic 85-kDa Phospholipase A2-mediated Release of Arachidonic Acid Is Critical for Proliferation of Vascular Smooth Muscle Cells. J Biol Chem 287: 30504–30511.

58. Gluck N, Schwob O, Krimsky M, Yedgar S (2008) Activation of cytosolic phospholipase A2 and fatty acid transacylase is essential but not sufficient for thrombin-induced smooth muscle cell proliferation. Am J Physiol Cell Physiol 294: C1597-1603.

59. Isenovic ER, Kedees MH, Tepavcevic S, Milosavljevic T, Koricanac G, et al. (2009) Role of PI3K/AKT, cPLA2 and ERK1/2 signaling pathways in insulin regulation of vascular smooth muscle cells proliferation. Cardiovasc Hematol Disord Drug Targets 9: 172-180.

60. Bornfeldt KE, Campbell JS, Koyama H, Argast GM, Leslie CC, et al. (1997) 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. 100: 875-885.