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Pathophysiological Regulation of Vascular Smooth Muscle Cells by Prostaglandin $F_{2\alpha}$-dependent Activation of Phospholipase C-β3

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Atherosclerosis is an obstructive vessel disease mainly caused by chronic arterial inflammation to which the proliferation and migration of vascular smooth muscle cells (VSMCs) is the main pathological response. In the present study, the primary responsible inflammatory cytokine and its signaling pathway was investigated. The proliferation and migration of VSMCs was significantly enhanced by the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), while neither was affected by tumor necrosis factor α. Prostacyclin I_2 was seen to enhance the proliferation of VSMCs while simultaneously suppressing their migration. Both prostaglandin D_2 and prostaglandin E_2 significantly enhanced the migration of VSMCs, however, proliferation was not affected by either of them. The proliferation and migration of VSMCs stimulated by $PGF_{2\alpha}$ progressed in a dose-dependent manner; the EC_{50} value of both proliferation and migration was 0.1 μM. VSMCs highly expressed the phospholipase isoform C-β3 (PLC-β3) while others such as PLC-β1, PLC-β2, and PLC-β4 were not expressed. Inhibition of the PLCs by U73122 completely blocked the $PGF_{2\alpha}$-induced migration of VSMCs, and, in addition, silencing PLC-β3 significantly diminished the $PGF_{2\alpha}$-induced proliferation and migration of VSMCs. Given these results, we suggest that $PGF_{2\alpha}$ plays a crucial role in the proliferation and migration of VSMCs, and activation of PLC-β3 could be involved in their $PGF_{2\alpha}$-dependent migration.

Key words : Atherosclerosis, migration, proliferation, prostaglandin, VSMC

Introduction

Atherosclerosis is a chronic inflammatory disease which is a major leading cause of sudden death in worldwide [17]. Protruding of plaques into the arterial lumen or rupture followed by thrombosis results in clinical complications such as myocardial infarction and stroke [16]. The mechanistic pathway of initiation and progression of atherosclerosis is still unknown but hypertension and metabolic stress such as obesity, diabetes, high low-density lipoprotein (LDL), high cholesterol, and high triacylglycerol levels have been reported to be associated with atherogenesis [19]. It also has been reported that metabolic stress is closely associated with chronic inflammation [13]. Thus, atherosclerosis, metabolic stress, and chronic inflammation seem to have vicious relation each other. Currently, how the burden of inflammation affect the progression of atherosclerosis remains to be elucidated.

Initiation of atherosclerosis is started with adhesion of monocytes to endothelial layer and infiltration into the intima layer of arterial walls [17]. Circulating LDL is oxidized and consistently taken up by the monocytes. Meanwhile monocytes differentiate into the macrophage and massive loading of lipid leads to apoptosis of macrophage which is shown as fatty streak in atherosclerotic lesions. The thickness of arterial walls during the progression of atherosclerosis is mainly acquired by vascular smooth muscle cells (VSMCs) [3]. For example, VSMCs in medial layer migrate into intimal layer and rapidly proliferate and secrete extracellular matrix proteins such as collagen and fibronectin. Since the stability of plaque is maintained by these extracellular matrix proteins, viability of VSMCs is regarded as crucial target to protect cardiovascular embolism. It is also notable that VSMCs in medial layer do not proliferate, however, unlike cardiac or skeletal muscle cells, VSMCs have phenotypic plasticity [27]. Therefore, contractile phenotype of VSMCs could be

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converted into synthetic phenotype. For example, contractile phenotype of VSMCs could be converted into synthetic type VSMCs by tumor necrosis factor and platelet-derived growth factor [1, 11]. Thus, unveiling the responsible factors that modulate phenotypic change, migration, and proliferation of VSMCs might be important for the understanding the pathogenesis of atherosclerosis.

Prostanoid is active lipid compound consisting of prostaglandins, thromboxane, and prostacyclin that regulates variety of cellular physiologies [24]. It is generated from arachidonic acid by sequential enzymatic activities. Cyclooxygenase (COX) is involved in catalyzing the rate limiting step of prostanoid biosynthesis. Prostaglandin H_2 (PGH_2) synthesized by COX is converted to thromboxane A_2 (TXA_2), prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostacyclin I_2 (PGI_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by different enzymes. Prostanoids regulate variety of cellular pathophysiologies. For example, PGE_2 and $PGF_{2\alpha}$ strongly induce the endometrial smooth muscle contraction thereby being used for the termination of pregnancy [5, 21]. Recently, it has been reported that PGD_2 is involved in the inflammatory responses of airway smooth muscle cells and the allergic responses [22, 25]. Prostanoids activate five basic types of G protein coupled receptor (GPCR) named type D, E, F, I, and T prostanoid receptor (DP, EP, FP, IP, and TP, respectively) [12]. DP and EP receptors consist of subtypes. For example, there are two subtypes of DP receptors such as DP1 and DP2, and four subtypes of EP receptors such as EP1, EP2, EP3, and EP4. DP1, EP2, EP4 and IP receptors are coupled with G_s protein thereby elevate intracellular cAMP level whereas DP2 and EP3 receptors are coupled with G_i protein thereby reduce intracellular cAMP level. EP1, FP, and TP receptors are coupled with G_q protein thereby elevate the intracellular Ca^{2+} level through the activation of phospholipase C (PLC)-β family of enzyme.

There are three major isoforms of PLC enzymes, *i.e.*, PLC-β, PLC-γ, and PLC-δ [9]. PLC-β is activated by GPCR whereas PLC-γ is mainly activated by growth factors that activate receptor tyrosine kinases. PLC-β has four different subtypes such as PLC-β1, -β2, -β3, and -β4. The expression and function of each subtype of PLC-β show differences in tissues and organs. For example, PLC-β1 and PLC-β4 are mainly expressed in the brain and shows epilepsy and ataxia in mice lacking PLC-β1 and PLC-β4, respectively [15]. Although the role of PLC-β3 in the activation of Stat3 transcriptional factor in immune cells has been elucidated [14], the role of PLC-β3 in vasculature is largely unknown. In the present study, we have explored the responsible inflammatory cytokines that mediates VSMC proliferation and migration and their mechanistic pathways.

Materials and Methods

Materials

Dulbecco's modified eagle medium (DMEM) culture medium, fetal bovine serum (FBS), trypsin EDTA, and antibiotics were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Pan-PLC inhibitor (U73122) was obtained from Merck Millipore (Billerica, MA, USA). Human TNFα was purchased from Koma Biotech (Seoul, Korea). PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-PLC-β1, - β2, - β3, and -β4 were kind gifts from Dr. Pann-Ghill Suh (UNIST, Ulsan, Korea). To obtain brain extract as a control, 3-week old rat brain was isolated and homogenized in the lysis buffer containing 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 5 mM EGTA/EDTA, and 150 mM NaCl. Established vascular smooth muscle cells (A10) were obtained from American Type Cell Culture Inc., and cultured under DMEM medium supplemented with 10% FBS. Cells were lysed with lysis buffer as mentioned above and used for control. DAPI and all other high quality reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Preparation of primary vascular smooth muscle cells from rat aorta

VSMCs were isolated from 4-week-old male Sprague-Dawley rats using a tissue explanting method as described previously [29]. Briefly, rats were euthanized by intravenous ketamine (100 mg/kg) injection and perfused with PBS for 5 min. Thoracic aorta was aseptically isolated and the surrounding fat and connective tissues were discarded. Vessels were longitudinally cut and the lumen sides were scraped with a razor blade to remove intima. Vessels were cut into 3-5 mm lengths and explanted lumen side down on collagen-coated culture dishes. Seven days after explanting, tissue fragments were discarded and sprouted VSMCs were collected. All the animal use was permitted by Pusan National University Institutional Animal Care and Use Committee (approval number: PNU-2016-1960).

Lentiviral knockdown

For gene silencing, HEK293-FT packaging cells were grown to ~70% confluence in 6-well plates. The cells were triple transfected with 5 µg of pLKO.1 lentiviral construct carrying shRNA of PLC- β3, 1 µg of Δ8.9 expressing Gag and Pol gene of virus, and 1 µg of pVSV-G expressing envelope protein of viral particle using the calcium phosphate method. The medium was replaced with a fresh medium at 8 hr post-transfection. Lentiviral supernatants were harvested at 24 hr post-transfection and passed through 0.45 µm filters. Cell-free viral culture supernatants were used to infect the contractile VSMCs in the presence of 8 µg/ml of polybrene. An additional round of infection was done at 48 hr and 72 hr post-transfection. The infected cells were isolated by 10 µg/ml puromycin for 2 days.

Western blot analysis

Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose membranes, which were immunoblotted using indicated primary antibodies and IRDye-conjugated secondary antibodies (Li-COR biosciences). Western blots were developed using Odyssey (Li-COR biosciences).

Measurement of migration and proliferation

VSMCs were grown and starved serum for 6 hrs before plating on ChemoTx chamber. Cells were detached with trypsin-EDTA and washed with serum-free DMEM. For migration assay, bottom side of ChemoTx membrane was coated with type I collagen for 30 min and 2×10^4 serum-starved cells in 100 µl volume were placed on top side of ChemoTx membrane. Migration was induced by placing the cell over-laid ChemoTx membrane on top of serum-free medium either in the presence or absence of inflammatory cytokines for 3 hr. ChemoTx membrane was fixed with 4% paraformaldehyde and non-migrated cells on top side of membrane were removed by gently wiping with cotton swab. Membrane was stained with DAPI and migrated cells were counted under the fluorescent microscope at 20× magnitude (Axiovert 200). For proliferation assay, VSMCs (2×10^4) were plated on a six-well plate and stimulated with the inflammatory cytokines for four days. Cells were fixed with 4% paraformaldehyde, and the nuclei were stained with DAPI. Stained cells were captured with a fluorescence microscope at ×20 magnification.

Statistical analysis

Results are expressed as means ± SEM of multiple experiments. When comparing two groups, an unpaired Student's *t*-test was used to assess differences. *P*-values less than 0.05 were considered significant and indicated by *.

Results

$PGF_{2\alpha}$ stimulates both proliferation and migration of VSMCs

To identify responsible inflammatory cytokines that induce VSMC migration and proliferation, we treated VSMCs with maximum dose of various inflammatory cytokines. As shown in Fig. 1A, stimulation of VSMCs with $PGF_{2\alpha}$ (10 µM) and PGI_2 (10 µM) significantly induced proliferation whereas TNFα (50 ng/ml), PGD_2, (10 µM) and PGE_2 (10 µM) had no effect on the proliferation. As shown in Fig. 1B, stimulation of VSMCs with $PGF_{2\alpha}$, PGD_2, and PGE_2 significantly induced migration, however, TNFα had no effect on the migration. Stimulation of VSMCs with PGI_2 markedly suppressed proliferation of VSMCs. Therefore, $PGF_{2\alpha}$ significantly induced both proliferation and migration whereas other inflammatory cytokines didn't have stimulatory effects on both proliferation and migration.

$PGF_{2\alpha}$ stimulates proliferation and migration of VSMCs in a dose-dependent manner

Since the pathophysiological concentration of inflammatory cytokine is important for the biological relevance, we examined the dosage effect of $PGF_{2\alpha}$ on the proliferation and

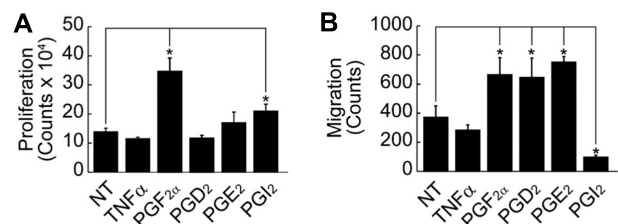

Fig. 1. Regulation of VSMC proliferation and migration by prostaglandins. (A) VSMCs were stimulated with the indicated inflammatory cytokines and prostaglandins for 4 days. Cells were fixed and stained with DAPI, and number of cells were counted under the microscopic fields (10×). (B) VSMCs were stimulated with the indicated inflammatory cytokines and prostaglandins for 3 hr. Migrated cells were stained with DAPI, and number of cells were counted under the microscopic fields (10×). Data are means ± S.D. of three independent experiments (n=3). Asterisks indicate statistical significance (p<0.05).

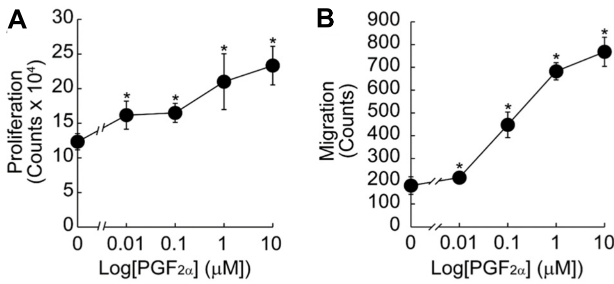

Fig. 2. Dose-dependent proliferation and migration of VSMCs by $PGF_{2\alpha}$. VSMCs were stimulated with $PGF_{2\alpha}$ with the indicated doses. Proliferation (A) and migration (B) were measured as described in "Materials and Methods". Data are means ± S.D. of three independent experiments (n=3). Asterisks indicate statistical significance (p<0.05).

migration of VSMCs. As shown in Fig. 2A, $PGF_{2\alpha}$ stimulated VSMC proliferation in a dose-dependent manner. The approximate EC_{50} was about 0.1 μM and the proliferation was induced at minimum dose of 0.01 μM. In addition, $PGF_{2\alpha}$ stimulated VSMC migration in a dose-dependent manner and the EC_{50} (0.1 μM) was similar to that of proliferation. Therefore, $PGF_{2\alpha}$ significantly enhances the proliferation and migration of VSMCs at the pathophysiological concentration.

Inhibition of PLC attenuates $PGF_{2\alpha}$-induced VSMC migration

Since $PGF_{2\alpha}$ receptor is coupled with PLC-β family of enzymes, we next examined the expressions of PLC-β subtypes in VSMCs. As shown in Fig. 3A, PLC-β1, -β2, and -β4 were

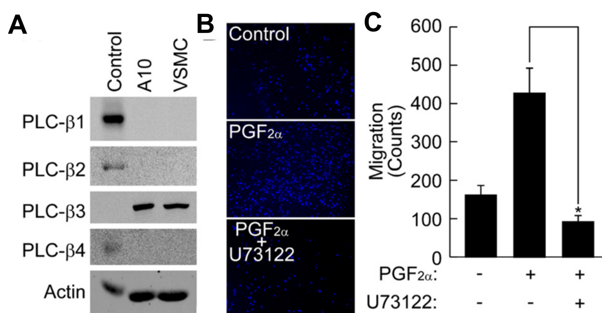

Fig. 3. Inhibition of $PGF_{2\alpha}$-induced migration of VSMCs by blocking of PLC activity. (A) Rat brain (control), established smooth muscle cells line (A10), and VSMCs lysates were performed Western blot analysis. Each PLC-β isoform was visualized by the indicated antibodies. (B and C) VSMCs were pretreated with U73122 (2 μM) and migration was measured as described in "Materials and Methods". Data are means ± S.D. of three independent experiments (n=3). Asterisks indicate statistical significance (p<0.05).

expressed in rat brain (control) whereas only PLC-β3 subtype was expressed in established vascular smooth muscle cells lines (A10) as well as VSMCs. In addition, PLC-β3 was not expressed in the brain. As shown in Figs. 3B and 3C, inhibition of PLC by U73122 (2 μM) significantly blocked $PGF_{2\alpha}$-induced VSMC migration. Therefore, PLC-β3 is exclusively expressed in VSMCs and the inhibition of PLC completely blocks $PGF_{2\alpha}$-induced VSMC migration.

Silencing of PLC-β3 attenuates $PGF_{2\alpha}$-induced VSMC proliferation and migration

Since only PLC-β3 was expressed in VSMCs and $PGF_{2\alpha}$-induced VSMC migration was completely blocked by pan-PLC inhibitor, we next examined the effect of PLC-β3 knockdown on the VSMC proliferation and migration. As shown in Fig. 4A and Fig. 4B, lentiviral expression of short hairpin loop of PLC-β3 (shPLC-β3) significantly reduced the expression of PLC-β3. In addition, knockdown of PLC-β3 sig-

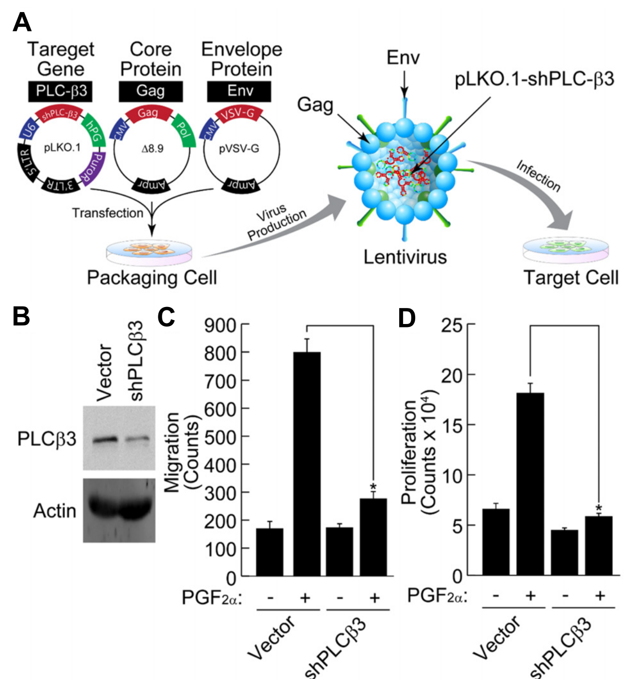

Fig. 4. Attenuation of $PGF_{2\alpha}$-induced VSMC proliferation and migration by silencing of PLC-β3. (A) Schematic representation of knockdown strategy using lentivirus. (B) VSMCs were infected by lentivirus carrying shRNA of PLC-β3. Expression of PLC-β3 was verified by Western blot analysis as described in "Materials and Methods". After silencing of PLC-β3, $PGF_{2\alpha}$-induced proliferation (C) and migration (D) of VSMCs were measured as described in "Materials and Methods". Data are means ± S.D. of three independent experiments (n=3). Asterisks indicate statistical significance (p<0.05).

nificantly suppressed the $PGF_{2α}$-induced proliferation and migration of VSMCs (Fig. 4C, Fig. 4D). Therefore, these results suggest that PLC-β3 plays an essential role in $PGF_{2α}$ -dependent proliferation and migration of VSMCs.

Discussion

Atherosclerosis is a disease that is associated with complex pathophysiological conditions. Especially, chronic inflammation seems to be an important risk factor during the disease progression [17]. In addition, proliferation and migration of VSMCs is the key pathogenic responses of occlusive blood vessel diseases [3]. In the present study, we provide several lines of evidences that link inflammation and VSMC physiologies. First, among the inflammatory prostanoids that are generated by COX enzyme, $PGF_{2α}$ could induce the proliferation and migration of VSMCs. Second, VSMC uniquely expressed PLC-β3 subtypes and other subtypes scarcely expressed. Third, inhibition or silencing of PLC-β3 completely blocked $PGF_{2α}$-induced proliferation and migration of VSMCs. Therefore, we suggest that PLC-β3 is the major responsible enzyme that medicates occlusive vessel disease by chronic inflammation.

It has been known that COX which produces various prostanoids is deeply involved in the progression of atherosclerosis [7]. For example, COX-2 expression is restricted to atherosclerotic plaque lesions and is not observed in the normal arteries [6]. In addition, COX-2 is highly expressed in symptomatic lesions rather than asymptomatic lesion [8]. It has also been reported that low-dose of aspirin which suppresses COX enzymatic activity has beneficial effect on the atherothrombosis [20]. Therefore, it is clear that COX and its enzymatic products might be involved in the disease progression of atherosclerosis.

COX produces several key inflammatory cytokines such as PGD_2, PGE_2, PGI_2, and $PGF_{2α}$ [24]. Each of these cytokines has pro-inflammatory or anti-inflammatory properties, and the balance of these cytokines production affects many pathophysiological processes. For example, PGE_2 induces inflammation whereas the metabolites of PGD_2 such as $\triangle^{12}PGJ_2$ and 15-deoxy-$\triangle^{12,14}$-PGJ_2 (15-d-PGJ_2) have anti-inflammatory properties [23]. PGI_2 has beneficial effect on the cardiovascular system, *i.e.*, it enhances vascular dilatation and inhibits blood clotting. $PGF_{2α}$ has detrimental effects on the cardiovascular system by protecting the action of 15-d-PGJ_2. Indeed, our results also showed that each of these prostanoids differentially affected VSMC proliferation and migration (Fig. 1). It is also notable that only the $PGF_{2α}$ activated VSMCs proliferation and migration. It has been reported that the level of $PGF_{2α}$ is elevated in patients with various arthritis [2]. In line with this, our results also showed that $PGF_{2α}$ was able to induce VSMC proliferation and migration at the pathological concentration (Fig. 2). In addition, deletion of $PGF_{2α}$ receptor diminishes inflammation-induced pulmonary fibrosis [18]. Furthermore, ablation of $PGF_{2α}$ receptor results in the low blood pressure and blockade of atherogenesis [28]. Therefore, it is reasonable to suggest that $PGF_{2α}$ plays an essential role in occlusive vascular disease.

$PGF_{2α}$ activates its cognate FP receptor [12]. The involvement of PLC in FP receptor signal transduction has been suggested. For example, pharmacological inhibition of FP receptor modifies calcium mobilization and phosphoinositide turnover [10]. Likewise our results also showed that inhibition of PLC by pharmacological inhibitor such as U73122 completely blocked $PGF_{2α}$-induced VSMC migration (Fig. 3). The involvement between $PGF_{2α}$ and PLC in VSMCs almost never reported. Our results provide novel key findings that PLC-β3 is a crucial downstream regulator of $PGF_{2α}$ receptor signaling pathway. First, VSMCs exclusively expressed PLC-β3 as judged by western blot analysis (Fig. 3A). Other report has shown that all four PLC-β subtypes are expressed in vascular smooth muscle cells isolated from pig aorta [4]. The discrepancy in the expression of each PLC isoform may be due to the species difference. It is also possible that methodological differences may lead to the contamination of adventitial fibroblast cells. Second, inhibition of PLC activity downregulated almost all the $PGF_{2α}$-induced VSMC migration (Fig. 3B, Fig. 3C). Likewise, it has been reported that calcium mobilization and phosphoinositide turnover is coupled with $PGF_{2α}$ receptor [10]. It is also known that $PGF_{2α}$ receptor is coupled with G_q family of G protein which is coupled with PLC-β family [26]. Third, selective silencing of PLC-β3 completely blocked the $PGF_{2α}$-induced VSMC proliferation and migration (Fig. 4). Since silencing of PLC-β 3 completely blocked $PGF_{2α}$-induced VSMC proliferation and migration, it is reasonable to suggest that PLC-β3 is the major leading isoform that regulates $PGF_{2α}$-induced proliferation and migration of VSMCs. In these regards, we suggest that inflammatory prostanoid such as $PGF_{2α}$ regulates pathophysiological response of VSMCs through the activation of PLC-β3. Targeting PLC-β3-specific pathway would provide beneficial therapeutic strategy for the cardiovascular

disease and others.

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초록 : Prostaglandin $F_{2\alpha}$ 의존적 phospholipase C-β3 활성화에 의한 혈관평활근세포의 병태생리 조절 연구

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죽상동맥경화는 대동맥의 만성염증에 의해 주로 발병되는 폐쇄동맥질환이다. 혈관평활근세포의 증식 및 이동은 죽상동맥경화 발병의 주된 병리적 반응이다. 본 연구에서는 죽상동맥경화 발병기전을 유도하는 표적 염증반응물질의 탐색 및 이들에 의한 신호전달 기전을 연구하였다. 혈관평활근세포의 증식 및 이동은 prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)에 의해 의미 있게 증가하였으나 tumor necrosis factor α (TNFα)에 의해서는 증가하지 않았다. Prostacyclin I_2 (PGI_2)는 혈관평활근세포의 증식은 촉진시켰으나 이동은 오히려 억제하였다. prostaglandin D_2 (PGD_2) 및 prostaglandin E_2 (PGE_2)는 혈관평활근세포의 증식을 촉진시켰으나 이동에는 영향을 미치지 않았다. $PGF_{2\alpha}$는 용량 의존적으로 혈관평활근세포의 증식 및 이동을 촉진시켰고 EC50는 약 0.1 μM로 관찰되었다. 혈관평활근세포에서 phospholipase C-β3 (PLC-β3) 아형의 발현은 매우 높았으나 PLC-β1, PLC-β2, 및 PLC-β4의 발현은 관찰되지 않았다. U73122 처리를 통해 PLC의 활성을 억제하면 $PGF_{2\alpha}$에 의한 혈관평활근세포의 이동이 억제되었다. 또한 PLC-β3의 발현을 억제하면 $PGF_{2\alpha}$에 의한 혈관평활근세포의 증식 및 이동이 억제되었다. 이러한 결과들을 바탕으로 $PGF_{2\alpha}$ 는 혈관평활근세포의 증식 및 이동에 중요한 역할을 수행하고, 여기에는 PLC-β3가 필수적인 역할을 담당하고 있음을 제안한다.