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Piperlongumine inhibits atherosclerotic plaque formation and vascular smooth muscle cell proliferation by suppressing PDGF receptor signaling

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ABSTRACT

Piperlongumine (piplartine, PL) is an alkaloid found in the long pepper (*Piper longum* L.) and has well-documented anti-platelet aggregation, anti-inflammatory, and anti-cancer properties; however, the role of PL in prevention of atherosclerosis is unknown. We evaluated the anti-atherosclerotic potential of PL in an *in vivo* murine model of accelerated atherosclerosis and defined its mechanism of action in aortic vascular smooth muscle cells (VSMCs) *in vitro*. Local treatment with PL significantly reduced atherosclerotic plaque formation as well as proliferation and nuclear factor-kappa B (NF-κB) activation in an *in vivo* setting. PL treatment in VSMCs *in vitro* showed inhibition of migration and platelet-derived growth factor BB (PDGF-BB)-induced proliferation to the *in vivo* findings. We further identified that PL inhibited PDGF-BB-induced PDGF receptor beta activation and suppressed downstream signaling molecules such as phospholipase Cγ1, extracellular signal-regulated kinases 1 and 2 and Akt. Lastly, PL significantly attenuated activation of NF-κB—a downstream transcriptional regulator in PDGF receptor signaling, in response to PDGF-BB stimulation. In conclusion, our findings demonstrate a novel, therapeutic mechanism by which PL suppresses atherosclerosis plaque formation *in vivo*.

1. Introduction

Atherosclerosis is an inflammatory disease of the blood vessel wall and the primary cause of cardiovascular disease [1]. Atherosclerosis is marked by abnormal proliferation of vascular smooth muscle cells (VSMCs) in the medial layer of blood vessels with subsequent intimal thickening leading to atherosclerotic lesions development [2,3]. Thus, inhibition of VSMC proliferation offers a potential therapeutic strategy for atherosclerosis prevention or treatment [4].

One of the principal mitogenic regulators of VSMC proliferation whose increased expression leads to atherosclerotic lesions is platelet derived growth factor (PDGF) [5]. Further, PDGF-BB is the isoform most characterized in this setting [6–8]. More specifically, the binding of PDGF-BB to PDGF-receptor (PDGF-R) leads to phosphorylation of tyrosine residues in the PDGF-Rβ chain and ultimately activation. Activated PDGF-Rβ associates with a number of downstream signaling proteins, including phospholipase C (PLC)-γ1, phosphatidylinositol 3-kinase (PI3K)/Akt, and extracellular-regulated kinases 1 and 2 (ERK1/2) [5,9]. Additionally, PDGF-Rβ signaling is known to activate nuclear factor kappa B (NF-κB), which has an established causative role in the development and maintenance of atherosclerosis [10,11]. Therefore, the regulatory mechanism of PDGF-Rβ signaling to inhibit VSMC proliferation is a key therapeutic strategy for atherosclerosis prevention.

Piperlongumine (piplartine, PL) is an alkaloid found in members of the Piper species and is well-characterized structurally [12] (Supplementary Fig. 1). Previously, we reported that PL inhibits mycotoxins biosynthesis [13] and platelet aggregation [14,15] consistent with other studies [16–20]. Also, other studies show that PL inhibits tumor cell growth [21–26] and induces cell cycle arrest

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[27,28]. Interestingly, a recent study reported the anti-tumorigenic effect of PL by selectively killing cancer cells with increasing reactive oxygen species (ROS) levels [29]. However, PL's function in preventing atherosclerosis and VSMC proliferation has not been studied.

We, therefore, evaluated PL's effect on atherosclerosis development and VSMCs proliferation using a mouse partial carotid ligation model, which is a newly developed and more physiologically relevant model as compared to the complete carotid ligation or arterial injury models [30–32]. The anti-atherosclerotic property of PL was evaluated on the basis of lesion development including atherosclerotic plaque formation, cell proliferation, and NF-κB activation.

2. Material and methods

Detailed methods can be referred to in the online-only Supplementary material. For the effects of PL on atherosclerosis development, Apolipoprotein E knockout (ApoE KO) mice (Jackson Laboratory, Bar Harbor, ME) underwent partial ligation of left carotid artery (LCA) to induce atherosclerotic lesion formation as previously described [30,31]. The contralateral right carotid artery (RCA) was left intact as an internal control. Following ligation, Pluronic gel solution containing 50 μg of PL (Indofine, Hillsborough, NJ) (or vehicle as control) was applied to the exposed adventitia of the ligated carotid artery ($n = 6$/group), and animals were fed Paigen's high-fat diet [33] (Research Diets, New Brunswick, NJ). Animals were sacrificed 2 weeks post-surgery and arteries were isolated and atherosclerotic plaque lesion was determined. Vascular cell proliferation and NF-κB activation in atherosclerotic lesion was assessed on frozen sections by immunohistochemical staining methods. All animal protocols were conducted with approval of the Emory University Institutional Animal Care and Use Committee according to NIH guidelines.

To define the mechanism of action of PL in aortic VSMCs activation, primary VSMCs were obtained from the thoracic aorta of Sprague–Dawley rats by enzymatic dispersion as previously described [34], and used for *in vitro* studies as indicated. Cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES, and 2 mM L-glutamine at 37 °C in a humidified incubator atmosphere of 95% air and 5% CO_2. VSMCs were cultured in serum-free medium containing PL (1–5 μM) or DMSO. Cell sprouting and cell proliferation was assessed by Matrigel-embedded cell sprouting assay, cell counting and [^{3}H]-thymidine incorporation, respectively. Following experimental treatment, whole cell lysates, cytosolic extract, and nuclear extract were obtained, and immunoblotting analysis was performed with primary antibodies against PDGF-Rβ, phospho-PDGF-Rβ, PLC-γ1, phospho-PLC-γ1, ERK1/2, phospho-ERK1/2, Akt, phospho-Akt, NF-κB p65 and phospho-NF-κB p65, followed by incubation with alkaline phosphatase-conjugated secondary antibodies. The β-actin or Lamin A/C was used as loading control. Protein expression was detected by chemiluminescence. Nuclear translocation and DNA binding activity of NF-κB were determined by immunofluorescence analysis and Electrophoretic mobility shift analysis. Statistical analysis was conducted using appropriate test with $P < 0.05$ considered significant.

3. Results

3.1. Piperlongumine prevents plaque formation in partial ligated carotid artery of ApoE KO mice

We first determined whether PL prevents atherosclerotic plaque formation using the partial carotid artery ligation atherosclerosis model in ApoE KO mice. Control group (Pluronic gel without PL) developed robust plaque lesions in the LCA compared to non-ligated RCA (Fig. 1A left panel and B). More specifically, the plaque size was increased (Fig. 1E left panel and C), the lumen size was reduced (Supplementary Fig. 2B), and the overall vessel size was increased compared to non-ligated control RCA (Supplementary Fig. 2A). Treatment with PL significantly reduced the lesion area and plaque size by 77.44 ± 2.34% and 92.73 ± 1.93% respectively (Fig. 1A right panel, B and C), leading to prevention of lumen occlusion (Fig. 1E right panel, Supplementary Fig. 2). Furthermore, the vessel intimal-medial thickness (IMT) was increased in the control mice LCA compared to the non-ligated RCA (Supplementary Fig. 1A). This increase was significantly reduced by PL treatment (Fig. 1D). These findings clearly demonstrate that PL inhibits atherosclerosis development.

3.2. Piperlongumine inhibits cell proliferation and NF-κB activation in atherosclerosis lesions

Because cell proliferation is a well-known contributing factor in atherosclerotic lesion development, we next determined local cell proliferation by Ki-67 staining [35]. Increased cell proliferation was observed in the LCA of control group ApoE KO mice compared to non-ligated RCA and was significantly reduced in PL-treated LCA (Fig. 1F, Supplementary Fig. 3). After establishing that PL inhibits atherosclerosis and cell proliferation, we next measured PL's effect on NF-κB activation, a protein known to regulate both processes, by analyzing phosphorylated-p65 subunit staining. As shown in Fig. 1G, NF-κB activation in the media as well as plaque lesion area was increased compare to non-ligated control RCA (Supplementary Fig. 4), and this increase was markedly reduced by PL treatment. These results suggest that PL may inhibit vascular cell proliferation through suppression of NF-κB activation in the atherosclerotic lesion development.

3.3. Piperlongumine inhibit VSMC migration and proliferation in vitro

To examine the functional role and effect of PL on VSMCs migration, matrigel-embedded cell sprouting assays were conducted. As shown in Fig. 2A and B, 1 μM and 5 μM PL treatment significantly inhibited FBS-induced sprouting of VSMCs by 52.73 ± 2.68% and 89.72 ± 1.62% of control cells, respectively. The inhibitory effect of PL on proliferation was further confirmed by direct cell counting and DNA synthesis assay. PDGF-BB stimulation increased cell proliferation of the control group, and this increase was significantly reduced by PL (1–5 μM) treatment in a concentration-dependent manner (Fig. 2C). PL also significantly inhibited PDGF-BB-induced [^{3}H]-thymidine incorporation at the same concentrations (Fig. 2D). Additionally, cell cycle analysis showed that PL significantly blocks cell cycle progression thereby reducing the percentage of cells in the S phase (Supplementary Fig. 5A). Importantly, an MTT assay using VSMCs treated with PL alone (up to 10 μM) revealed that cell viability was not significantly altered (Supplementary Fig. 5B). These results demonstrate that PL can inhibit mitogen induced cell migration, proliferation and cell cycle progression.

3.4. Piperlongumine reduces PDGF-BB-induced PDGF-Rβ phosphorylation and its downstream signaling in VSMCs

To determine the underlying anti-atherogenic and anti-proliferative effect of PL, we examined whether PL affects PDGF-BB-induced PDGF-Rβ signaling pathway activation. Activated PDGF-Rβ is known to stimulate a number of downstream signaling proteins, including PLC-γ1, ERK1/2, and PI3K/Akt [5,9,36] which are implicate VSMC hypertrophy and migration. To examine whether PL blocks PDGF-Rβ-mediated signaling, PDGF-BB-stimulated

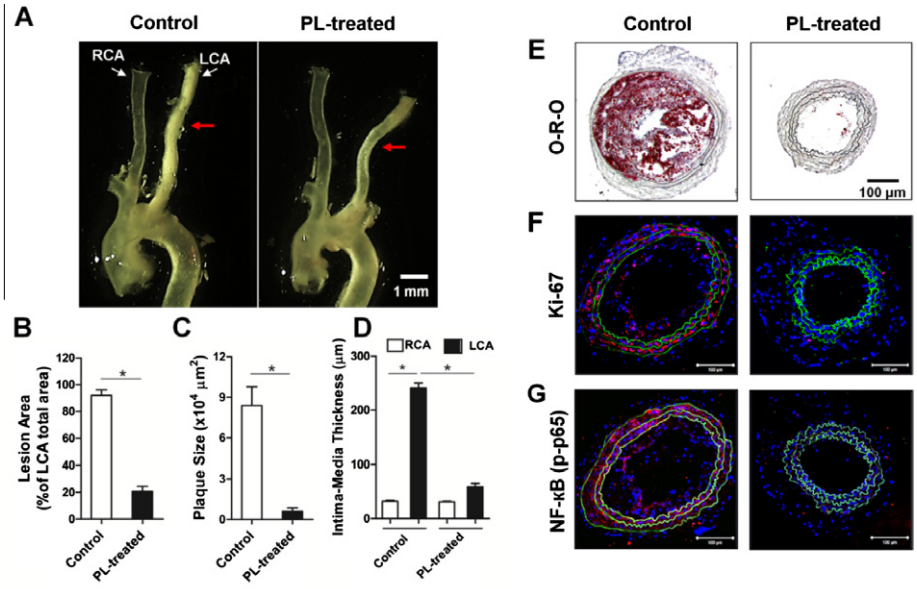

Fig. 1. Local treatment with PL prevents atherosclerotic plaque formation and inhibits cell proliferation and NF-κB activation in the partially ligated carotid artery in ApoE KO mice. ApoE KO mice were partially ligated and locally treated with pluronic gel containing either the vehicle alone or with PL (50 μg), and fed a high-fat diet to induce atherosclerosis in 2 weeks. Arteries were isolated and the lesion area was imaged by light microscopy (A) and quantified as the percent of total LCA area (B) (scale bar; 1 mm). Frozen sections of carotid tissues were stained with Oil Red O and hematoxylin, and then plaque size (C) and intimal-medial thickness (D) were measured by using NIH ImageJ software. Images of LCA are shown in (E) and represent the areas highlighted by arrows in (A). Histological analysis of cell proliferation (F) and NF-κB activation (G) were obtained by Ki-67 and phospho-NF-κB p65 staining in the LCAs (red), respectively. DAPI was used to visualize nuclei (blue). Autofluorescence highlighting the elastic lamina is shown in green (scale bar; 100 μm). Values are means ± S.E. of 6 mice per group. $^{*}P < 0.05$ indicates statistical significance between mice treated with PL and vehicle alone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

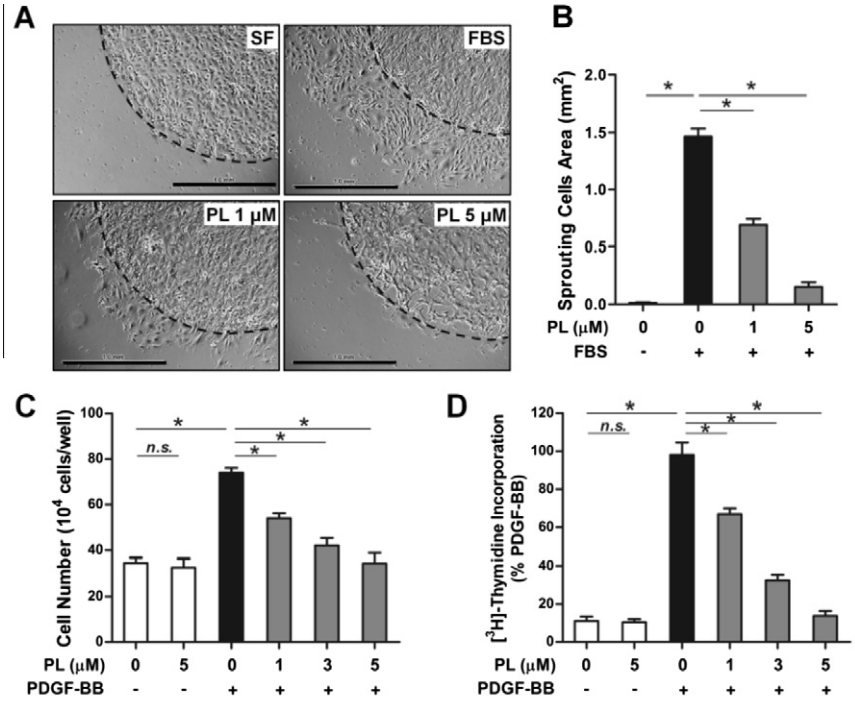

Fig. 2. PL inhibits VSMC migration and proliferation. VSMCs were pre-treated with PL (1 or 5 μM) and then stimulated with 20% FBS. Representative images of VSMCs in a matrigel-embedded cell sprouting assay are shown in (A) (scale bar; 1 mm). Sprouting cell area was quantified by ImageJ Software (B). VSMCs were pre-treated with PL (1–5 μM) and then stimulated with 25 ng/mL of PDGF-BB. Proliferation of VSMCs was examined by cell counting (C) and [^{3}H]-thymidine incorporation assay (D). Values are means ± S.E. of 3–4 experiments. $^{*}P < 0.05$ indicates statistical significance between the FBS- or PDGF-BB-treated cells, *n.s.* indicates not significant.

phosphorylation of PDGF-Rβ, PLC-γ1, ERK1/2, and Akt was determined in VSMCs. As shown in Fig. 3A, PL treatment prevented PDGF-BB-induced phosphorylation of PDGF-Rβ. Treatment with PL also inhibited PLC-γ1 (Fig. 3B), ERK1/2 (Fig. 3C) and Akt (Fig. 3D) phosphorylation induced by PDGF-BB. These results suggest that PL inhibits VSMC signaling by suppression of PDGF-Rβ signaling.

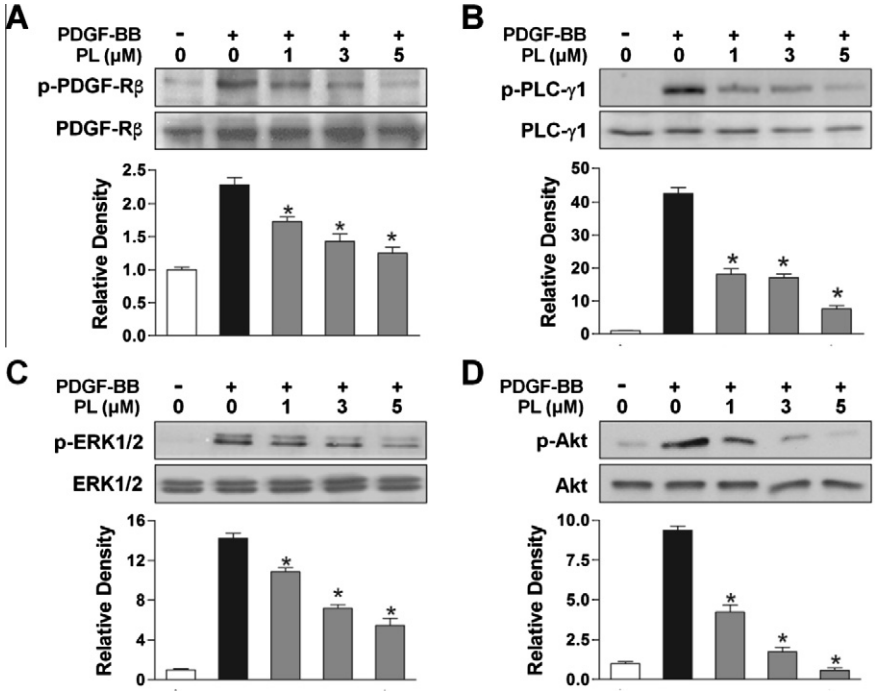

Fig. 3. PL inhibits PDGF-BB-induced PDGF-Rβ phosphorylation and its downstream signaling in VSMCs. Confluent cells were cultured in the presence or absence of the indicated concentration of PL. Cells were briefly stimulated with 25 ng/mL of PDGF-BB for different time periods (1 min for PDGF-Rβ tyrosine phosphorylation, 5 min for ERK1/2 and PLCγ1 phosphorylation and 15 min for Akt phosphorylation). Western blot analysis of PDGF-Rβ and its downstream PLC-γ1, ERK1/2, and Akt phosphorylation are shown in (A)–(D) respectively. After densitometric quantification, data are expressed as means ± S.E. of 3–4 experiments. $^{*}P < 0.05$ indicates statistical significance.

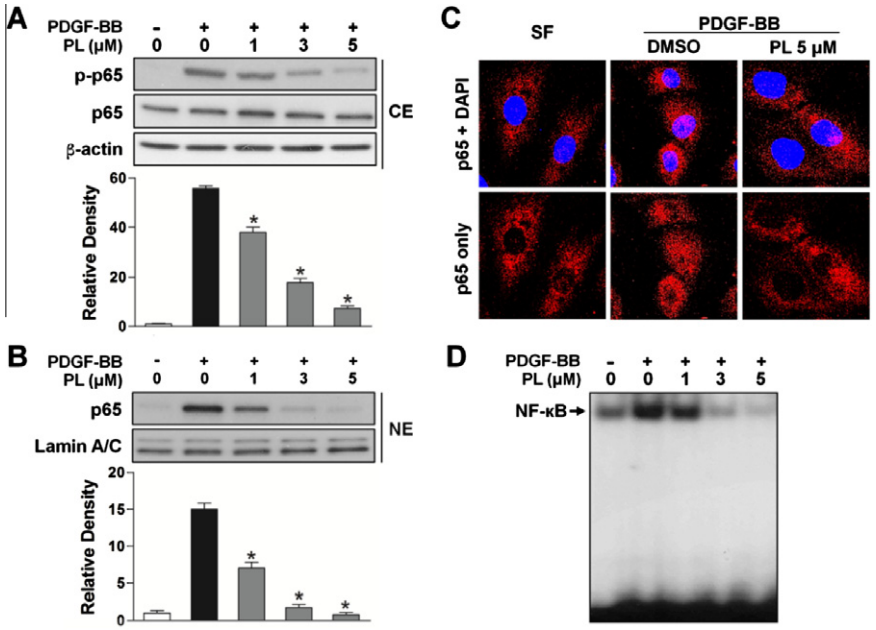

Fig. 4. PL suppresses PDGF-BB-induced NF-κB activation in VSMCs. Confluent cells were cultured in the presence or absence of PL. Cells were then briefly stimulated with 25 ng/mL of PDGF-BB for 1 h. Western blot analysis of NF-κB p65 subunit phosphorylation in the cytosol is shown in (A) and total NF-κB p65 expression in nuclear is shown in (B). After densitometric quantification, data are expressed as means ± S.E. of 3–4 experiments. $^{*}P < 0.05$ indicates statistical significance. (C) The nuclear translocation of NF-κB p65 was determined by immunofluorescence after stimulation with 25 ng/mL of PDGF-BB for 1 h. Total-NF-κB p65 (red) was visualized by a confocal laser scanning microscopy (magnification, 63×). DAPI (blue) was used to visualize nuclei. (D) For NF-κB DNA binding assay, cells were pretreated with PL (1–5 μM) and then stimulated with 25 ng/mL of PDGF-BB for 1 h. Nuclear extracts were used in a NF-κB DNA binding assay by EMSA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Piperlongumine suppresses NF-κB activation

After establishing that PL inhibits NF-κB activation in atherosclerosis lesion *in vivo* (Fig. 1G, Supplementary Fig. 4) and the PDGF-BB is known to be a potent stimulator of NF-κB activation [37], we examined the effect of PL on PDGF-BB-induced NF-κB activation in VSMCs. As shown in Fig. 4A, exposure of quiescent cells to PDGF-BB increased NF-κB p65 phosphorylation in cell cytosol, and

treatment of PL (1–5 μM) strongly inhibited PDGF-BB-induced p65 phosphorylation in a concentration-dependent manner. To study the effect of PL on PDGF-BB-induced NF-κB translocation, we next examined the nuclear expression level of p65 in VSMCs. PDGF-BB stimulation increased p65 subunit nuclear expression (Fig. 4B) and translocation to the nuclear (Fig. 4C) and PL strongly attenuated these responses in PDGF-BB-stimulated cells (Fig. 4B and C). Moreover, PDGF-BB-induced strong NF-κB DNA binding activity which was attenuated by PL treatment (Fig. 4D). These results suggest that PL suppresses the PDGF-BB-induced nuclear translocation of NF-κB in VSMCs.

4. Discussion

Although several roles of PL, especially its anti-cancer properties [29], have been discovered, the role of PL in atherosclerosis has never been evaluated and was the focus of this study. We demonstrated for the first time that PL prevents atherosclerotic plaque formation in the partial carotid ligation model and reduce vascular cell proliferation and NF-κB activation *in vivo* and inhibition of proliferation, migration, and cell cycle progression of VSMCs *in vitro*. Mechanistically, in an *in vitro* setting, we found that PL induces a concentration-dependent inhibition of various components of PDGF-Rβ signaling and suppresses NF-κB activation by down-regulating NF-κB p65 phosphorylation and nuclear translocation.

VSMCs, a main component of arterial wall, exist in a non-proliferative, contractile phenotype [2,3]. After vascular stress, there is a shift to a synthetic phenotype which is associated with proliferation and migration. This proliferation plays a central role in atherosclerosis by contributing arterial remodeling, neointimal formation, accelerating lipid accumulation or macrophage chemotaxis [3,38]. Our results demonstrated that PL treatment inhibited plaque lesion formation in the mouse partial carotid ligation model (Fig. 1A–E) and was associated with a significant decrease in local cell proliferation (Fig. 1F). To verify our findings *in vitro*, we performed cell sprouting assay, and showed that PL inhibits serum-induced migration of VSMCs (Fig. 2A and B), suggesting that the anti-atherosclerotic effect of PL might be due to suppression of VSMC activation.

PDGF plays an important role in VSMC proliferation and migration during vascular remodeling and atherosclerotic lesion development [8,36,39]. Whereas PDGF-R is expressed at low levels in the healthy arteries, its expression is up-regulated in injured arteries or in early stage atherosclerosis [8]. Targeted disruption of PDGF-R signaling has been shown to attenuate neointimal formation and atherosclerotic lesion development [40–42]. Our results show that PL significantly inhibited PDGF-BB-induced VSMC proliferation (Fig. 2C and D) and cell cycle progression (Supplementary Fig. 5A) without any decreases in cell viability (Supplementary Fig. 5B). Our results suggest that the anti-atherogenic and anti-proliferative properties of PL are associated with attenuation of proliferation and cell cycle progression of VSMCs induced by PDGF-BB. We therefore further investigated the inhibitory mechanism of the anti-proliferative effect exerted by PL on PDGF-BB-induced VSMC proliferation *via* PDGF-R signaling pathway. Activation of downstream signals of PDGF-Rβ, such as ERK1/2, Akt and PLCγ1, appears to be essential for the VSMC proliferation [10,11,43]. PL potently inhibited PDGF-Rβ phosphorylation (Fig. 3A), and also inhibited the PLCγ1 (Fig. 3B), ERK1/2 (Fig. 3C) and Akt (Fig. 3D) phosphorylation. These results suggest that PL may directly target PDGF-Rβ phosphorylation leading to the inhibition of VSMC proliferation. Various studies have implicated that NF-κB plays a crucial role in VSMC proliferation and inflammatory responses which are important mediators of atherosclerotic lesion development [37,44]. Also, PDGF-BB is known to be a potent stimulator of NF-κB activation [10,11] which is associated with VSMC phenotypic switch. PL potently attenuated NF-κB activation as demonstrated by reduction of PDGF-BB-induced p65 subunit nuclear translocation and DNA binding activity in VSMCs *in vitro* (Fig. 4). In support, immunohistological staining results clearly demonstrated that NF-κB activation in the plaque lesion as well as cell proliferation was increased, and that PL treatment reduced these phenomena (Fig. 1G, Supplementary Fig. 4). These results suggested that the suppression of PL may ultimately exert its anti-atherogenic effect by suppressing PDGF-Rβ mediated NF-κB activation of VSMCs.

In summary, we have provided the first evidence that PL prevents the development of advanced atherosclerotic plaques in partially ligated carotid artery of ApoE KO mice by inhibiting VSMC proliferation by impeding PDGF-Rβ signaling and subsequently blocking downstream NF-κB activation. This points to a novel mechanism of action of PL to inhibit abnormal cell proliferating progression and emphasizes a new therapeutic avenue to benefit atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.061.

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