Published in final edited form as:

Biochem Biophys Res Commun. 2012 October 19; 427(2): 349–354. doi:10.1016/j.bbrc.2012.09.061.

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

Dong Ju Son^{a,b}, Soo Yeon Kim^c, Seong Su Han^d, Chan Woo Kim^{a,b,h}, Sandeep Kumar^{a,b}, Byeoung Soo Park^e, Sung Eun Lee^f, Yeo Pyo Yun^g, Hanjoong Jo^{a,b,h,i,j}, and Young Hyun Park^{i,j}

^aWallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA, USA

^bDivision of Cardiology, Department of Medicine, Emory University, Atlanta, GA, USA

^cDivision of Life Science, Korea Basic Science Institute, Daejeon, South Korea

^dUniversity of Iowa Carver College of Medicine, Department of Pathology, Iowa City, IA, USA

^eNanotoxtech Co., Ansan, South Korea

^fDivision of Applied Biology and Chemistry, Kyungpook National University, Daegu, Korea

^gCollege of Pharmacy, Chungbuk National University, Cheongju, South Korea

^hDepartment of Bioinspired Science, Ehwa Womans University, Seoul, South Korea

ⁱDepartment of Food Science and Nutrition, College of Natural Sciences, Soonchunhyung University, Asan, South Korea

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- κ B) 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 C1, extracellular signal-regulated kinases 1 and 2 and Akt. Lastly, PL significantly attenuated activation of NF- κ B—a downstream transcriptional regulator in PDGF receptor

© 2012 Elsevier Inc. All rights reserved.

[†]Corresponding Authors: Hanjoong Jo, Ph. D., Mailing address: Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia 30322, USA, Telephone: 1-404-712-9654, Fax number: 1-404-727-3330, hjo@emory.edu. Young Hyun Park, Ph. D., Mailing address: Department of Food Science and Nutrition, College of Natural Sciences, Soonchunhyang University, Asan, Chungnam 336-745, South Korea, Telephone: 82-41-530-1259, Fax number: 82-41-530-1264, pyh012@sch.ac.kr.

[‡]These authors contributed equally to the work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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*.

Keywords

Piperlongumine; atherosclerosis; vascular smooth muscle cells; PDGF receptor; NF- κ B

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 [6,7,8]. More specifically, the binding of PDGF-BB to PDGF-receptor (PDGF-R) leads to phosphorylation of tyrosine residues in the PDGF-R β chain and ultimately activation. Activated PDGF-R β associates with a number of downstream signaling proteins, including phospholipase C (PLC)- γ 1, phosphatidylinositol 3-kinase (PI3K)/Akt, and extracellular-regulated kinases 1 and 2 (ERK1/2) [5,9]. Additionally, PDGF-R β signaling is known to activate nuclear factor kappa B (NF- κ B), which has an established causative role in the development and maintenance of atherosclerosis [10,11]. Therefore, the regulatory mechanism of PDGF-R β 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 [16,17,18,19,20]. Also, other studies show that PL inhibits tumor cell growth [21,22,23,24,25,26] and induces cell cycle arrest [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 [30,31,32]. The anti-atherosclerotic property of PL was evaluated on the basis of lesion development including atherosclerotic plaque formation, cell proliferation, and NF- κ B 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 μ g 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- κ B 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 g/mL streptomycin, 8 mM HEPES, and 2 mM L-glutamine at 37°C in a humidified incubator atmosphere of 95% air and 5% CO₂. VSMCs were cultured in serum-free medium containing PL (1–5 μ M) or DMSO. Cell sprouting and cell proliferation was assessed by Matrigel-embedded cell sprouting assay, cell counting and [³H]-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-R β , phospho-PDGF-R β , PLC- γ 1, phospho-PLC- γ 1, ERK1/2, phospho-ERK1/2, Akt, phospho-Akt, NF- κ B p65 and phospho-NF- κ B 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- κ B 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 1B). More specifically, the plaque size was increased (Fig. 1E left panel and 1C), 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 $77.44\pm2.34\%$ and $92.73\pm1.93\%$ respectively (Fig. 1A right panel, 1B and 1C), 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- κ B 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- κ B activation, a protein known to regulate both processes, by analyzing phosphorylated-p65 subunit staining. As shown in Fig. 1G, NF- κ B 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- κ B 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 2B, 1 μ M and 5 μ M PL treatment significantly inhibited FBS-induced sprouting of VSMCs by $52.73 \pm 2.68\%$ and $89.72 \pm 1.62\%$ 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 to 5 μ M) 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 μ M) 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-R β 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-R β signaling pathway activation. Activated PDGF-R β is known to stimulate a number of downstream signaling proteins, including PLC- γ 1, ERK1/2, and PI3K/Akt [5,9,36] which are implicate VSMC hypertrophy and migration. To examine whether PL blocks PDGF-R β -mediated signaling, PDGF-BB-stimulated phosphorylation of PDGF-R β , PLC- γ 1, ERK1/2, and Akt was determined in VSMCs. As shown in Fig. 3A, PL treatment prevented PDGF-BB-induced phosphorylation of PDGF-R β . Treatment with PL also inhibited PLC- γ 1 (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-R β signaling.

3.5. Piperlongumine suppresses NF- κ B activation

After establishing that PL inhibits NF- κ B activation in atherosclerosis lesion *in vivo* (Fig. 1G, Supplementary Fig. 4) and the PDGF-BB is known to be a potent stimulator of NF- κ B activation [37], we examined the effect of PL on PDGF-BB-induced NF- κ B activation in VSMCs. As shown in Fig. 4A, exposure of quiescent cells to PDGF-BB increased NF- κ B p65 phosphorylation in cell cytosol, and treatment of PL (1–5 μ M) strongly inhibited PDGF-BB-induced p65 phosphorylation in a concentration-dependent manner. To study the effect of PL on PDGF-BB-induced NF- κ B 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 4C). Moreover, PDGF-BB-induced strong NF- κ B 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- κ B in VSMCs.

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- κ B 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-R β signaling and suppresses NF- κ B activation by down-regulating NF- κ B 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-1E) 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 2B), 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 [40,41,42]. Our results show that PL significantly inhibited PDGF-BB-induced VSMC proliferation (Fig. 2C and 2D) 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-R β , such as ERK1/2, Akt and PLC1, appears to be essential for the VSMC proliferation [10,11,43]. PL potently inhibited PDGF-R β phosphorylation (Fig. 3A), and also inhibited the PLC1 (Fig. 3B), ERK1/2 (Fig. 3C) and Akt (Fig. 3D) phosphorylation. These results suggest that PL may directly target PDGF-R β phosphorylation leading to the inhibition of VSMC proliferation. Various studies have implicated that NF- κ B 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- κ B activation [10,11] which is associated with VSMC phenotypic switch. PL potently attenuated NF- κ B 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- κ B 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-R β mediated NF- κ B 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-R β signaling and subsequently blocking downstream NF- κ B 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Korea Research Foundation Grant (KRF-2007-357- E00032) funded by the Korean Government (MOEHRD) to Dong Ju Son, NIH grants HL095070, HL70531, HHSN268201000043C, and a World Class University Project (R31-2008-000-10010-0) from the Ministry of Science, Technology and Education of Korea (MEST) to Hanjoong Jo.

References

1. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. C. American Heart Association Statistics, S. Stroke Statistics. Heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation*. 2012; 125:e2–e220. [PubMed: 22179539]
2. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993; 362:801–809. [PubMed: 8479518]
3. Ross R. Cell biology of atherosclerosis. *Annu Rev Physiol*. 1995; 57:791–804. [PubMed: 7778883]
4. Jung F, Haendeler J, Goebel C, Zeiher AM, Dimmeler S. Growth factor-induced phosphoinositide 3-OH kinase/Akt phosphorylation in smooth muscle cells: induction of cell proliferation and inhibition of cell death. *Cardiovasc Res*. 2000; 48:148–157. [PubMed: 11033117]
5. Tallquist M, Kazlauskas A. PDGF signaling in cells and mice. *Cytokine Growth Factor Rev*. 2004; 15:205–213. [PubMed: 15207812]
6. Myllarniemi M, Frosen J, Calderon Ramirez LG, Buchdunger E, Lemstrom K, Hayry P. Selective tyrosine kinase inhibitor for the platelet-derived growth factor receptor in vitro inhibits smooth muscle cell proliferation after reinjury of arterial intima in vivo. *Cardiovasc Drugs Ther*. 1999; 13:159–168. [PubMed: 10372232]
7. Banai S, Wolf Y, Golomb G, Pearle A, Waltenberger J, Fishbein I, Schneider A, Gazit A, Perez L, Huber R, Lazarovich G, Rabinovich L, Levitzki A, Gertz SD. PDGF-receptor tyrosine kinase blocker AG1295 selectively attenuates smooth muscle cell growth in vitro and reduces neointimal formation after balloon angioplasty in swine. *Circulation*. 1998; 97:1960–1969. [PubMed: 9609090]
8. Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN, Schwartz SM. PDGF ligand and receptor gene expression during repair of arterial injury. *J Cell Biol*. 1990; 111:2149–2158. [PubMed: 2172262]
9. Claesson-Welsh L. Platelet-derived growth factor receptor signals. *J Biol Chem*. 1994; 269:32023–32026. [PubMed: 7798193]
10. Obata H, Biro S, Arima N, Kaieda H, Kihara T, Eto H, Miyata M, Tanaka H. NF-kappa B is induced in the nuclei of cultured rat aortic smooth muscle cells by stimulation of various growth factors. *Biochem Biophys Res Commun*. 1996; 224:27–32. [PubMed: 8694825]
11. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature*. 1999; 401:86–90. [PubMed: 10485711]
12. Chatterjee A, Dutta CP. Alkaloids of Piper longum Linn. I. Structure and synthesis of piperlongumine and piperlonguminine. *Tetrahedron*. 1967; 23:1769–1781. [PubMed: 6047519]

13. Lee SE, Park BS, Bayman P, Baker JL, Choi WS, Campbell BC. Suppression of ochratoxin biosynthesis by naturally occurring alkaloids. *Food Addit Contam.* 2007; 24:391–397. [PubMed: 17454112]
14. Park BS, Son DJ, Park YH, Kim TW, Lee SE. Antiplatelet effects of acidamides isolated from the fruits of *Piper longum* L. *Phytomedicine.* 2007; 14:853–855. [PubMed: 17689230]
15. Park BS, Son DJ, Choi WS, Takeoka GR, Han SO, Kim TW, Lee SE. Antiplatelet activities of newly synthesized derivatives of piperlongumine. *Phytother Res.* 2008; 22:1195–1199. [PubMed: 18697182]
16. Vasques da Silva R, Navickiene HM, Kato MJ, da Bolzani VS, Meda CI, Young MC, Furlan M. Antifungal amides from *Piper arboreum* and *Piper tuberculatum*. *Phytochemistry.* 2002; 59:521–527. [PubMed: 11853747]
17. Navickiene HM, da Bolzani VS, Kato MJ, Pereira AM, Bertoni BW, Franca SC, Furlan M. Quantitative determination of anti-fungal and insecticide amides in adult plants, plantlets and callus from *Piper tuberculatum* by reverse-phase high-performance liquid chromatography. *Phytochem Anal.* 2003; 14:281–284. [PubMed: 14515999]
18. Tsai IL, Lee FP, Wu CC, Duh CY, Ishikawa T, Chen JJ, Chen YC, Seki H, Chen IS. New cytotoxic cyclobutanoid amides, a new furanoid lignan and anti-platelet aggregation constituents from *Piper aborescens*. *Planta Med.* 2005; 71:535–542. [PubMed: 15971125]
19. Iwashita M, Oka N, Ohkubo S, Saito M, Nakahata N. Piperlongumine, a constituent of *Piper longum* L., inhibits rabbit platelet aggregation as a thromboxane A(2) receptor antagonist. *Eur J Pharmacol.* 2007; 570:38–42. [PubMed: 17618620]
20. Fontenele JB, Leal LK, Silveira ER, Felix FH, Bezerra Felipe CF, Viana GS. Antiplatelet effects of piplartine, an alkamide isolated from *Piper tuberculatum*: possible involvement of cyclooxygenase blockade and antioxidant activity. *J Pharm Pharmacol.* 2009; 61:511–515. [PubMed: 19298699]
21. Duh CY, Wu YC, Wang SK. Cytotoxic pyridone alkaloids from the leaves of *Piper aborescens*. *J Nat Prod.* 1990; 53:1575–1577. [PubMed: 2089124]
22. Bezerra DP, Militao GC, de Castro FO, Pessoa C, de Moraes MO, Silveira ER, Lima MA, Elmiro FJ, Costa-Lotufo LV. Piplartine induces inhibition of leukemia cell proliferation triggering both apoptosis and necrosis pathways. *Toxicol In Vitro.* 2007; 21:1–8. [PubMed: 16971088]
23. Bezerra DP, Castro FO, Alves AP, Pessoa C, Moraes MO, Silveira ER, Lima MA, Elmiro FJ, Costa-Lotufo LV. In vivo growth-inhibition of Sarcoma 180 by piplartine and piperine, two alkaloid amides from *Piper*. *Braz J Med Biol Res.* 2006; 39:801–807. [PubMed: 16751987]
24. Bezerra DP, Pessoa C, de Moraes MO, Silveira ER, Lima MA, Elmiro FJ, Costa-Lotufo LV. Antiproliferative effects of two amides, piperine and piplartine, from *Piper* species. *Z Naturforsch C.* 2005; 60:539–543. [PubMed: 16163826]
25. Golovine KV, Makhov PB, Teper E, Kutikov A, Canter D, Uzzo RG, Kolenko VM. Piperlongumine induces rapid depletion of the androgen receptor in human prostate cancer cells. *Prostate.* 2012
26. Lin Z, Liao Y, Venkatasamy R, Hider RC, Soumyanath A. Amides from *Piper nigrum* L. with dissimilar effects on melanocyte proliferation in-vitro. *J Pharm Pharmacol.* 2007; 59:529–536. [PubMed: 17430636]
27. Kong EH, Kim YJ, Kim YJ, Cho HJ, Yu SN, Kim KY, Chang JH, Ahn SC. Piplartine induces caspase-mediated apoptosis in PC-3 human prostate cancer cells. *Oncol Rep.* 2008; 20:785–792. [PubMed: 18813819]
28. Jyothi D, Vanathi P, Mangala Gowri P, Rama Subba Rao V, Madhusudana Rao J, Sreedhar AS. Diferuloylmethane augments the cytotoxic effects of piplartine isolated from *Piper chaba*. *Toxicol In Vitro.* 2009; 23:1085–1091. [PubMed: 19501152]
29. Raj L, Ide T, Gurkar AU, Foley M, Schenone M, Li X, Tolliday NJ, Golub TR, Carr SA, Shamji AF, Stern AM, Mandinova A, Schreiber SL, Lee SW. Selective killing of cancer cells by a small molecule targeting the stress response to ROS. *Nature.* 2011; 475:231–234. [PubMed: 21753854]
30. Nam D, Ni CW, Rezvan A, Suo J, Budzyn K, Llanos A, Harrison D, Giddens D, Jo H. Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. *Am J Physiol Heart Circ Physiol.* 2009; 297:H1535–1543. [PubMed: 19684185]

31. Nam D, Ni CW, Rezvan A, Suo J, Budzyn K, Llanos A, Harrison DG, Giddens DP, Jo H. A model of disturbed flow-induced atherosclerosis in mouse carotid artery by partial ligation and a simple method of RNA isolation from carotid endothelium. *J Vis Exp*. 2010
32. Rezvan A, Ni CW, Alberts-Grill N, Jo H. Animal, in vitro, and ex vivo models of flow-dependent atherosclerosis: role of oxidative stress. *Antioxid Redox Signal*. 2011; 15:1433–1448. [PubMed: 20712399]
33. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis*. 1987; 68:231–240. [PubMed: 3426656]
34. Travo P, Barrett G, Burnstock G. Differences in proliferation of primary cultures of vascular smooth muscle cells taken from male and female rats. *Blood vessels*. 1980; 17:110–116. [PubMed: 7362876]
35. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *Journal of cellular physiology*. 2000; 182:311–322. [PubMed: 10653597]
36. Heldin CH, Ostman A, Ronnstrand L. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta*. 1998; 1378:F79–113. [PubMed: 9739761]
37. Mehrhof FB, Schmidt-Ullrich R, Dietz R, Scheidereit C. Regulation of vascular smooth muscle cell proliferation: role of NF-kappaB revisited. *Circ Res*. 2005; 96:958–964. [PubMed: 15831813]
38. Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med*. 2002; 8:1249–1256. [PubMed: 12411952]
39. Sachinidis A, Locher R, Vetter W, Tatje D, Hoppe J. Different effects of platelet-derived growth factor isoforms on rat vascular smooth muscle cells. *J Biol Chem*. 1990; 265:10238–10243. [PubMed: 2162342]
40. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science*. 1991; 253:1129–1132. [PubMed: 1653454]
41. Maresta A, Balducelli M, Cantini L, Casari A, Chioin R, Fontanelli A, Monici Preti PA, Repetto S, Raffaghello S. The rapadil restenosis trial (STARC study): background, methods and clinical characteristics of the patient population. *Clin Trials Metaanal*. 1994; 29:31–40. [PubMed: 10150183]
42. Sirois MG, Simons M, Edelman ER. Antisense oligonucleotide inhibition of PDGFR-beta receptor subunit expression directs suppression of intimal thickening. *Circulation*. 1997; 95:669–676. [PubMed: 9024156]
43. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science*. 1999; 286:1738–1741. [PubMed: 10576741]
44. Brand K, Page S, Walli AK, Neumeier D, Baeuerle PA. Role of nuclear factor-kappa B in atherogenesis. *Experimental physiology*. 1997; 82:297–304. [PubMed: 9129944]

Highlights

- Anti-atherogenic effect of piperlongumine was examined using partial carotid ligation model in ApoE KO mice.
- Piperlongumine prevented atherosclerotic plaque lesion development, vascular smooth muscle cell proliferation, and NF- κ B activation.
- piperlongumine reduced vascular smooth muscle cell activation through PDGF-R β and NF- κ B-signaling.
- Piperlongumine may serve as a new therapeutic molecule for atherosclerosis treatment.

FIGURE 1. Local treatment with PL prevents atherosclerotic plaque formation and inhibits cell proliferation and NF- κ B 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- κ B activation (G) were obtained by Ki-67 and phospho-NF- κ B 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 $\pm$ S.E. of 6 mice per group. * $P < 0.05$ indicates statistical significance between mice treated with PL and vehicle alone.

FIGURE 2. PL inhibits VSMC migration and proliferation

VSMCs were pre-treated with PL (1 or 5 μ M) 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 μ M) and then stimulated with 25 ng/mL of PDGF-BB. Proliferation of VSMCs was examined by cell counting (C) and [³H]-thymidine incorporation assay (D). Values are means $\pm$ 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.

FIGURE 3. PL inhibits PDGF-BB-induced PDGF-R β 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-R β tyrosine phosphorylation, 5 min for ERK1/2 and PLC1 phosphorylation and 15 min for Akt phosphorylation). Western blot analysis of PDGF-R β and its downstream PLC- γ 1, ERK1/2, and Akt phosphorylation are shown in (A)–(D) respectively. After densitometric quantification, data are expressed as means $\pm$ S.E. of 3–4 experiments. * P <0.05 indicates statistical significance.

FIGURE 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.