Biochemical and Biophysical Research Communications 427 (2012) 349–354

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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,1,*], Young Hyun Park [i,1,*]

[a] Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA, USA
[b] Division of Cardiology, Department of Medicine, Emory University, Atlanta, GA, USA
[c] Division of Life Science, Korea Basic Science Institute, Daejeon, South Korea
[d] University of Iowa Carver College of Medicine, Department of Pathology, Iowa City, IA, USA
[e] Nanotoxtech Co., Ansan, South Korea
[f] Division of Applied Biology and Chemistry, Kyungpook National University, Daegu, South Korea
[g] College of Pharmacy, Chungbuk National University, Cheongju, South Korea
[h] Department of Bioinspired Science, Ehwa Womans University, Seoul, South Korea
[i] Department of Food Science and Nutrition, College of Natural Sciences, Soonchunhyang University, Asan, South Korea

ARTICLE INFO

Article history:
Received 24 August 2012
Available online 17 September 2012

Keywords:
Piperlongumine
Atherosclerosis
Vascular smooth muscle cells
PDGF receptor
NF-κB

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

* Corresponding authors. Addresses: Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30322, USA. Fax: +1 404 727 3330 (H. Jo). Department of Food Science and Nutrition, College of Natural Sciences, Soonchunhyang University, Asan, Chungnam 336-745, South Korea. Fax: +82 41 530 1264 (Y.H. Park).
E-mail addresses: hjo@emory.edu (H. Jo), pyh012@sch.ac.kr (Y.H. Park).
[1] These authors contributed equally to the work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.09.061

[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