Biochemical and Biophysical Research Communications 423 (2012) 116–121

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Protocatechuic aldehyde inhibits migration and proliferation of vascular smooth muscle cells and intravascular thrombosis

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ARTICLE INFO

Article history:
Received 15 May 2012
Available online 26 May 2012

Keywords:
Protocatechuic aldehyde
Anti-oxidant
Atherosclerosis
Migration
Proliferation
Anti-platelet
PDGF

ABSTRACT

The migration and proliferation of vascular smooth muscle cells (VSMCs) and formation of intravascular thrombosis play crucial roles in the development of atherosclerotic lesions. This study examined the effects of protocatechuic aldehyde (PCA), a compound isolated from the aqueous extract of the root of *Salvia miltiorrhiza*, an herb used in traditional Chinese medicine to treat a variety of vascular diseases, on the migration and proliferation of VSMCs and platelets due to platelet-derived growth factor (PDGF). DNA 5-bromo-2′-deoxy-uridine (BrdU) incorporation and wound-healing assays indicated that PCA significantly attenuated PDGF-induced proliferation and migration of VSMCs at a pharmacologically relevant concentration (100 μM). On a molecular level, we observed down-regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK) pathways, both of which regulate key enzymes associated with migration and proliferation. We also found that PCA induced S-phase arrest of the VSMC cell cycle and suppressed cyclin D2 expression. In addition, PCA inhibited PDGF-BB-stimulated reactive oxygen species production in VSMCs, indicating that PCA's antioxidant properties may contribute to its suppression of PDGF-induced migration and proliferation in VSMCs. Finally, PCA exhibited an anti-thrombotic effect related to its inhibition of platelet aggregation, confirmed with an aggregometer. Together, these findings suggest a potential therapeutic role of PCA in the treatment of atherosclerosis and angioplasty-induced vascular restenosis.

1. Introduction

A variety of natural products with antioxidant properties have been used as traditional remedies for atherosclerosis. The herb *Salvia miltiorrhiza* Bunge (Labiatae) has shown beneficial effects in coronary heart disease, cerebrovascular disease, liver cirrhosis, and insomnia [1]. Although the mechanisms by which *S. miltiorrhiza* may improve atherosclerosis remain unclear, both the extract itself and isolated compounds of *S. miltiorrhiza* have demonstrated multiple antioxidant mechanisms [2–5].

The abnormal migration and proliferation of vascular smooth muscle cells (VSMCs) in arterial walls are important factors in the development of atherosclerosis as well as restenosis after angioplasty [2]. Although these processes are triggered by multiple cytokines and growth factors, one of the principal regulators of chemoattraction and mitogenesis of VSMCs is platelet-derived growth factor (PDGF)-BB, the expression of which is increased in atherosclerotic lesions [3–5]. The PDGF-induced mitogenesis signaling pathway has already been well characterized. For example, phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) are the two major PDGF signaling pathways and are linked to numerous cellular process, including proliferation and migration [2]. ERK 1/2 and PI3K pathways have also been shown to be important for PDGF-BB-induced cell cycle progression in VSMCs.

Several studies have reported that reactive oxygen species (ROS) generation occurs during restenosis after angioplasty [6–8] and that antioxidants attenuate neo-intimal hyperplasia [7,9–11]. Furthermore, recent evidence indicates that PDGF itself stimulates ROS production in VSMCs [6,12]. Though ROS were previously thought to be destructive to cells, numerous studies have shown them to be crucial messengers in the transduction of VSMCs' responses to PDGF, especially in terms of activating MAPK and Akt pathways. However, ROS can induce mitochondrial DNA damage, which has been reported to be associated with atherosclerosis [13]. These findings suggest that ROS may play a central role in

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http://dx.doi.org/10.1016/j.bbrc.2012.05.092

VSMC proliferation and migration, and indicate that antioxidant substances may have beneficial effects in atherosclerosis.

Platelets also play an important role in thrombus formation at the site of damaged blood vessels, resulting in many cases of cardiovascular and cerebrovascular diseases [14]. Collaborative meta-analysis of randomized trials has shown that anti-platelet therapy prevents serious vascular events in a wide range of patients with atherosclerosis.

Protocatechuic aldehyde (PCA), a water-soluble compound isolated from the root of the herb *S. miltiorrhiza*, has been reported to protect against TNF-α-induced endothelial cell injury by suppressing ICAM-1 and VCAM-1 expression in human umbilical vein endothelial cells [15], as well as stimulating free radical scavenging activity in RAW264.7 macrophages [16]. To the best of our knowledge, however, no studies have been published that examine the influence of PCA on VSMC migration and proliferation.

In this study, we investigated the effects of PCA's antioxidant properties on PDGF-induced VSMC proliferation and migration. Furthermore, we examined PCA's influence on PI3K/Akt and ERK 1/2 pathways, which regulate cellular proliferation and migration. Finally, we evaluated the anti-thrombotic effect of PCA through inhibition of ADP-induced platelet aggregation.

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with the Institutional Animal Care and Use Committee of Yonsei University Health System based on the Laboratory Animal Manual and the "Guide for the care and use of laboratory animals" edited by the National Research Council of the National Academies (permit number 2010-0187). All animal studies were performed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Reagents

We purchased recombinant rat PDGF-BB from R&D System (Minneapolis, MN, USA) and the BrdU (colorimetric) proliferation assay kit from Roche (Indianapolis, IN, USA). Protocatechuic aldehyde (3,4-Dihydroxybenzaldehyde) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. VSMC isolation and culture

Thoracic aortic smooth muscle cells were isolated from Sprague–Dawley rats (200–250 g; ORIENT-Charles River Technology, Seoul, Korea) as described previously [17]. This study utilized cells of passages 5–7. VSMCs were grown in Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum containing 1% penicillin to subconfluence and synchronized by serum deprivation for 24 h. Syncronized cells were treated with PCA (100 μM) for 24 h prior to PDGF-BB (20 ng/ml) stimulation.

2.4. BrdU proliferation assay

The rate of DNA synthesis was determined from the incorporation of 5-bromo-2′-deoxy-uridine (BrdU) into cells according to the manufacturer's instructions. Briefly, VSMCs were seeded at 1×10^4 on a 96-well microplate, treated with PCA for 24 h, and then cultured with or without PDGF-BB (20 ng/ml) for the subsequent 24 h. Cells were labeled with BrdU labeling reagent for 2 h. After fixation, cells were incubated with anti-BrdU antibody for 90 min. After washing, 100 μl substrate (tetramethylbenzidine) was added to each well and incubated at room temperature for 30 min. The absorbance at dual wavelengths of 370 nm was determined using an ELISA reader (Synergy H4, Biotek, VT. USA).

2.5. Cell migration analysis

Wound healing assays were performed using 6-well plates. When cells reached 90% confluence, synchronized cells were pretreated with PCA (100 μM) in serum-free medium for 24 h. After 24 h of PDGF-BB (20 ng/ml) stimulation, a single wound was created in the center of the cell monolayers by gentle removal of the attached cells with a sterile plastic pipette tip. After 24 h of incubation, the cells that migrated into the wounded area or pro-

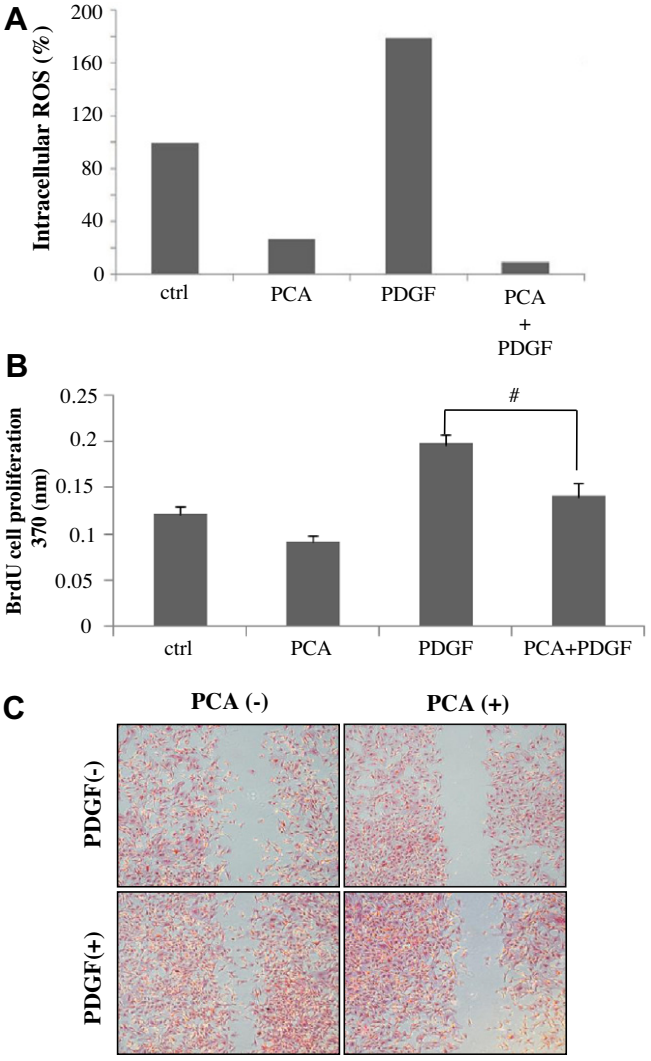

Fig. 1. Effects of PCA on ROS production and on PDGF-BB-induced VSMC proliferation and migration. (A) Reactive oxygen species scavenging effect of PCA in PDGF-induced VSMCs. Relative CM-H_2DCF-DA emission was recorded using a fluorescence cytometer. Relative fluorescence intensities were calculated using untreated control cells as a standard. (B) Proliferation activities were measured by BrdU incorporation assay in the absence or presence of PDGF-BB (20 ng/ml). Relative proliferation activities were expressed using untreated control cells as a standard. Results are mean ± S.E.M. from three independent experiments performed in triplicate. $^*P < 0.001$ compared with PDGF-BB (+) control. (C) in vitro wound-healing assay showed that PCA inhibited PDGF induced VSMC migration. Confluent cultures were scrape wounded and allowed to migrate for 24 h. Photomicrographs are fixed and Hematoxyline and Eosin stained cells imaged at ×10 magnification.

truded from the border of the wound were visualized and photographed under an inverted microscope.

2.6. Immunoblot analysis

Immunoblot analysis was performed as described previously [18]. Cell lysates (pretreated with or without 100 μM PCA and 20 ng/ml PDGF) were separated on 10% SDS–polyacrylamide gels, transferred to PVDF membrane, and analyzed with specific antibodies. Primary antibodies against phosphorylated Akt (Ser473) and ERK 42/44 were purchased from Cell Signaling Technology, Beverly, MA, USA. We also used beta-actin and cyclin D2 antibodies, as well as the secondary antibodies peroxidase-conjugated anti-mouse and anti-rabbit (Santa Cruz Biotechnology). An enhanced chemifluorescent labeling kit (Amersham, Buckinghamshire, UK), was used to visualize immunoreactive bands.

2.7. PI staining for cell cycle analysis

Cells were pretreated with or without PCA (100 μM) in serum-free media for 24 h and then treated with PDGF-BB (20 ng/ml) for 24 h. Next, 1×10^6 VSMCs were collected and re-suspended in 0.3 ml PBS containing 10% FBS, to which 0.7 ml of 100% ethanol (pre-chilled to −20 °C) was added dropwise and gently mixed. After being stored at −20 °C for 2 h, cells were washed twice with PBS/10% FBS, re-suspended in 500 ml PI staining solution, and incubated at room temperature for 30 min. Cell cycle analysis was performed using a flow cytometer (Becton Dickinson, FACS Calibur).

2.8. Reactive oxygen species assay

The effect of PCA on intracellular ROS was examined as previously described [2]. Briefly, synchronized VSMCs were pretreated with PCA (100 μM) for 24 h and then treated with PDGF-BB (20 ng/ml) for 24 h. Cells were then incubated for 30 min with CM-H_2DCF-DA (Invitrogen Molecular Probes, Eugene, Oregon, USA) in Dulbecco's phosphate buffered saline (DPBS). Finally, CM-H_2DCF-DA emission was recorded using a fluorescence cytometer (Becton Dickinson, FACS Calibur).

2.9. Inhibition of platelet aggregation

Platelet aggregation was evaluated with an impedance aggregometer (Chrono-log model 700, Chronolog Corporation, Havertown, PA, USA). Whole blood (9 ml) was obtained from male SD rats weighing 250–300 g. Whole blood was collected in a plastic syringe containing1.5% heparin (JW Pharmaceutical, Seoul, Korea) to avoid premature aggregation. Single-use cuvettes containing a silicon-coated stirrer (1200 rpm, Chronolog Corporation) were filled with 500 μl physiological saline and 500 μl whole blood. The mixture was pretreated with PCA (100, 500, 750 μg/ml) for 10 min and incubated for 15 min at 37 °C, after which platelet aggregation was initiated by adding 20 μM ADP (Sigma–Aldrich Co., MO, USA).

2.10. Statistical analysis

All data are expressed as mean ± standard error unless otherwise stated. Statistical significance was determined by one-way ANOVA with a Bonferroni post hoc correction for comparison. Statistical analysis was performed with SPSS (PASW) for Windows (version 18.0; SPSS, Chicago, IL). A *P*-value less than 0.05 was considered statistically significant.

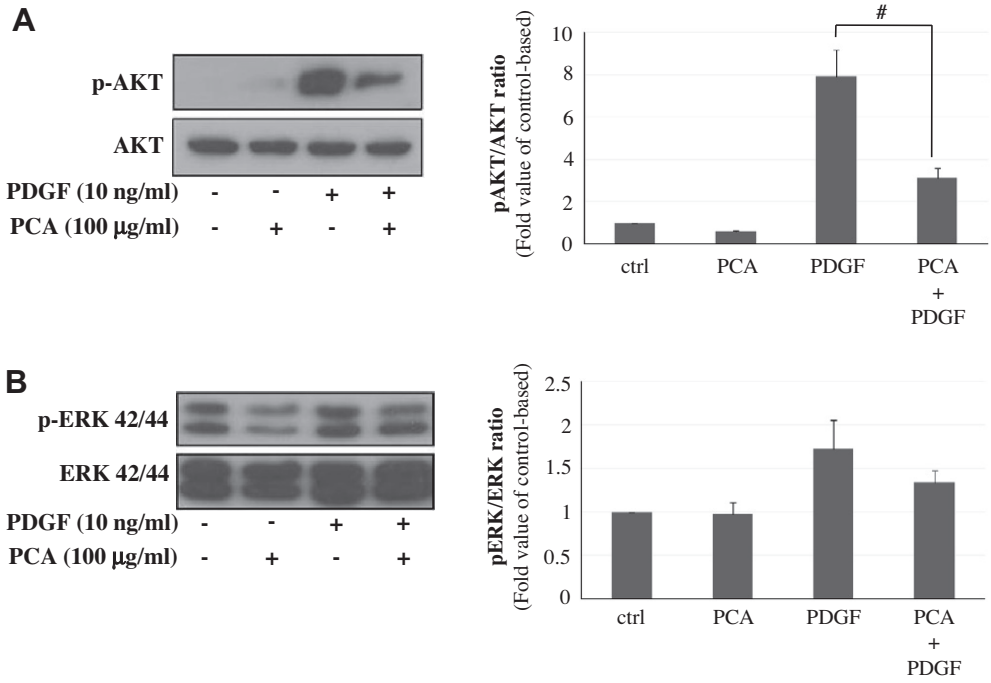

Fig. 2. PCA inhibition of PDGF-BB stimulated AKT and ERK 1/2 pathways in VSMCs. (A) p-Akt and Akt. (B) p-ERK 1/2 and ERK 1/2. VSMCs were serum starved for 24 h in the absence or presence of PCA and stimulated with PDGF-BB for 24 h. Whole cell lysates were prepared and subsequently used for detection of p-Akt, Akt, p-ERK 1/2, or ERK 1/2 by western blot. The bar graph shows the ratio of the densities of phosphoproteins to total proteins for the blots. Data are expressed as mean ± S.E.M from three independent experiments. $^{\#}p < 0.05$ vs. PDGF-BB (+) control.

3. Results

3.1. PCA inhibition of ROS production and PDGF-BB-induced VSMC proliferation and migration

Recent evidence supports the hypothesis that PDGF increases intracellular ROS levels in VSMCs and that both PDGF-induced VSMC proliferation and migration are ROS-dependent. As shown in Fig. 1A, 20 ng/mL PDGF-BB significantly increased ROS (75.06%) compared to control cells (42.06%). Furthermore, PCA considerably neutralized the PDGF-BB-induced ROS accumulated in VSMCs (from 75.06% to 4.07%). Quantitative analysis of VSMC proliferation was measured by BrdU incorporation (Fig. 1B). When VSMCs were stimulated by PDGF-BB (20 ng/ml), the number of cells incorporating BrdU increased by approximately 37.2% compared to unstimulated cells. Treatment with PCA (100 μM) for 24 h in PDGF-BB-stimulated VSMCs resulted in a significant 28.9% decrease in BrdU incorporation compared to the PDGF-BB stimulated group, without evidence of the cytotoxicity. Next, we examined the effect of PCA on PDGF-BB-induced migration. Wound-healing experiments were conducted using 20 ng/ml PDGF-BB with or without PCA (100 μM). Fig. 1C shows representative pictures after 24 h incubation with PDGF-BB, with and without PCA. PDGF-BB markedly induced VSMC migration, whereas 100 μM PCA was able to completely block PDGF-BB-induced migration. PCA significantly reduced PDGF-mediated migration based on the complied data of at least three independent wound-healing experiments.

3.2. PCA inhibition of PDGF-BB stimulated AKT and ERK 1/2 pathways in VSMCs

The two main PDGF signaling pathways, PI3K/Akt and MAPK/ERK, are linked to numerous cellular processes including proliferation and migration. Given the evidence of PDGF-BB's ability to stimulate phosphorylation of Akt and MAPK [19–21], we used PDGF-BB (10 ng/ml, 24 h) to induce VSMC proliferation. We investigated the effects of PCA on these signaling pathways in VSMCs. PCA (100 μM) significantly inhibited AKT phosphorylation induced by PDGF-BB by 48% compared to the untreated group ($p = 0.023$, $n = 3$) (Fig. 2A). PCA did not significantly suppress ERK 1/2 phosphorylation induced by PDGF-BB (22.5%, $n = 3$). These results suggest that PCA inhibited the upstream signaling cascade of PI3K/

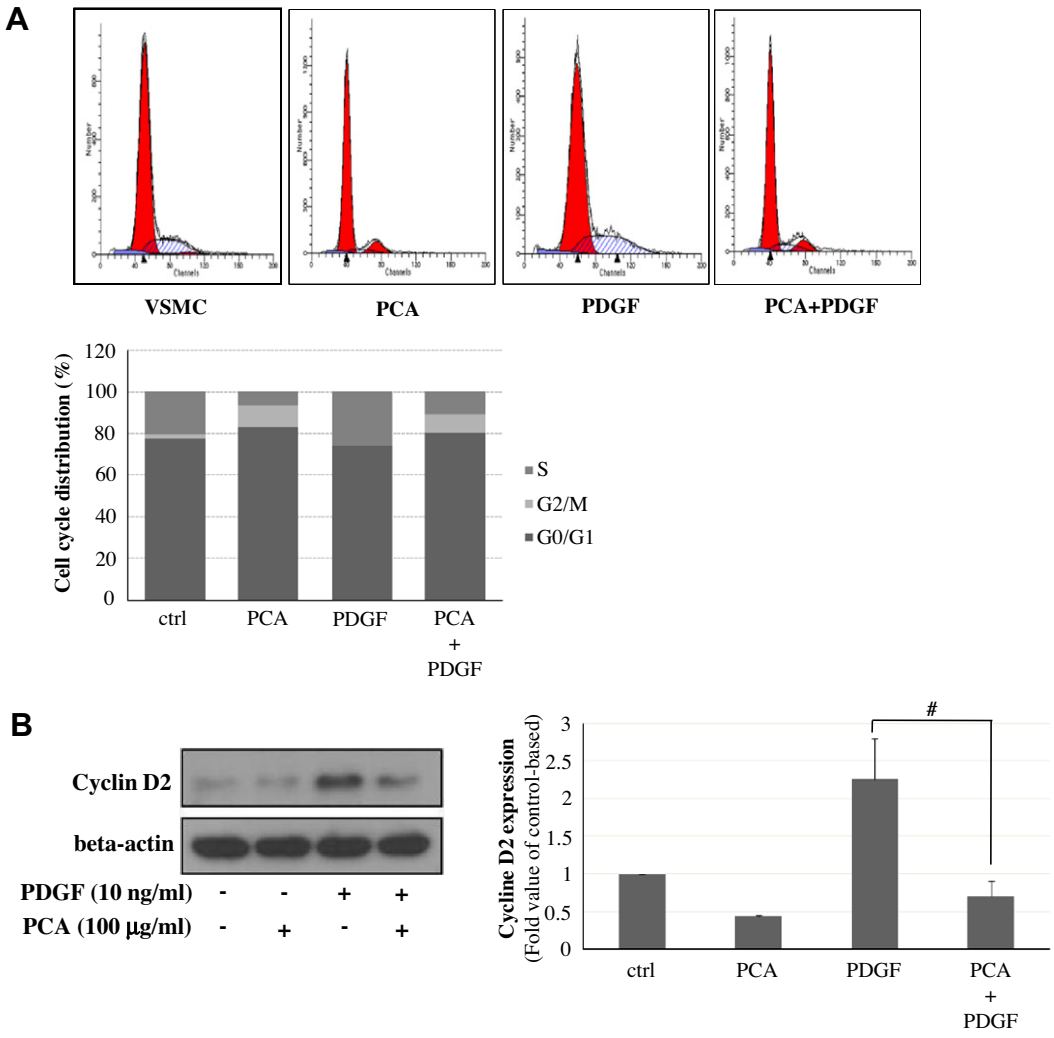

Fig. 3. Effect of PCA on cell cycle pregression in VSMC stimulated by PDGF-BB. VSMCs were treated as described in methods. (A) Representative DNA histograms of propidium iodide fluorescence in cells as assessed by flow cytometry. The profiles are representative experiments measured in the presence of PDGF, together with or without PCA (100 μM). (B) For evaluation of the expression of protein related to cell cycle progression, whole cell extract (50 μg) were subjected to western blot analysis for cyclin D2 or beta-actin. Data are expressed as mean ± S.E.M from three independent experiments. $^{\#}p < 0.05$ vs. PDGF-BB (+) control.

Akt and MAPK/ERK, perhaps by reducing PDGF-BB-induced intracellular ROS generation.

3.3. PCA induction of S-phase arrest of PDGF-BB-induced VSMC proliferation via cell cycle regulation

To evaluate PCA's effect on DNA synthesis and cell growth, we analyzed the cell cycle of VSMCs induced by PDGF-BB (20 ng/ml), with and without 24-h incubation with PCA (100 μM), using a flow cytometer after PI staining. Serum deprivation (for 24 h) of VSMCs led to approximately 77.8% synchronization in the G0/G1 cell cycle phase. PDGF-BB treatment alone led to cellular migration into the S phase with a concomitant decrease of cells in the G0/G1 phase. However, PCA diminished the PDGF-BB-stimulated cell cycle progression into S phase from approximately 12.52% to 10.83% (Fig. 3A). DNA is replicated in the S phase, which occurs between G1 and G2, leading to mitosis and cytokinesis. PCA treatment appeared to inhibit DNA synthesis in VSMCs in the presence of PDGF-BB. To characterize the mechanism of PCA-induced cell cycle arrest, the effects of PCA on cyclin D2 expression was determined. As shown in Fig. 3B, PCA significantly inhibited cyclin D2 expression. These results suggest that PCA treatment may cause G0/G1 cell cycle arrest.

3.4. PCA's anti-platelet effects in ex vivo rat whole blood

To evaluate the *ex vivo* anti-platelet effect of PCA in rats, freshly isolated rat whole blood was incubated with PCA (100, 500, and 750 μg/ml) or vehicle (normal saline). The aggregation response was evaluated with an impedance aggregometer (Chronolog model 700). As shown in Fig. 4, PCA produced a dose-dependent anti-aggregation effect in ADP (20 μM)-induced platelet aggregation. Changes in both the slope and maximal ohms in the aggregometer were prevented after PCA treatment in a dose-dependent manner.

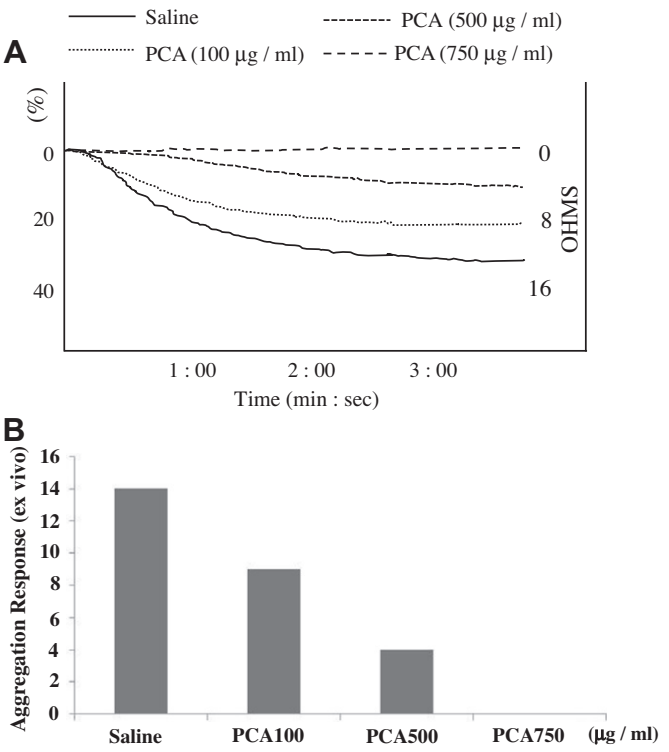

Fig. 4. Blood thinning effect of PCA. The impedence aggregometer revealed that PCA (100, 500, 750 μg/ml) prevented platelet aggregation in a dose-dependent manner *ex vivo*. The platelet aggregation that was induced by ADP (20 mM) recovered more quickly with increasing doses of PCA.

4. Discussion

PCA is a water-soluble antioxidant phenolic aldehyde isolated from the root of *S. miltiorrhiza*. In traditional Chinese medicine, this herb has been used to treat vascular diseases for centuries with no serious adverse effects reported in the scientific literature [1]. The herb's therapeutic utility in cardiovascular disease has been attributed to improved microcirculation, vasodilation, anti-coagulation, and anti-inflammation [21,22]. Recently, several chemical compounds have been isolated from the herb and divided into two classes: caffeic acid-derived phenolic acids and several tanshinones, a type of diterpene quinine [23]. These compounds can be further classified as hydrophilic or lipophilic compounds. Most studies that evaluate the role of *S. miltiorrhiza* on cardiovascular disease used a standard extract that contains abundant lipid-soluble compounds such as tanshinone IIA [24]. Although the beneficial effects of *S. miltiorrhiza* on cardiovascular disease have been reported in other studies, the underlying mechanisms and the role of water-soluble compounds have not been fully evaluated.

Our results show that PCA, a potent ROS scavenger, significantly inhibited PDGF-induced VSMC proliferation and migration. In addition, PCA inhibited the PDGF-stimulated phosphorylation of Akt and ERK 1/2. These results suggest that PCA inhibits PDGF signaling by acting upstream of Akt and ERK 1/2, indicating that the PCA's antioxidant effect may be related to the inhibition of PDGF signal transduction. The proliferation and migration of VSMC are involved in vascular remodeling and intimal lesion formation, and these can be affected by growth factors such as PDGF and basic fibroblast growth factor [22,23]. Studies have shown that activation of MAPK/ERK 1/2 is implicated in PDGF-mediated DNA synthesis and proliferation [24]. In addition, PI3K/Akt has been correlated with its ability to induce migration of smooth muscle cells. The PDGF receptor transports its signal into the intracellular space by ROS generation, as in the regulation of the tyrosine phosphorylation of several of signaling proteins via intracellular H_2O_2 production [3,25,26]. Although we did not investigate the effect of PCA on H_2O_2-induced phosphorylation or protein tyrosine phosphatase activity in this study, our results support the hypothesis that PCA increases protein tyrosine phosphatase activity by scavenging PDGF-induced ROS, thereby blocking key players in the PDGF signaling cascade, such as PI3K and MAPK, which are responsible for VSMC proliferation and migration.

In the present study, we investigated the anti-proliferative effect of PCA on VSMC and the signal transduction mechanisms underlying this effect. We found that PCA inhibited proliferation and DNA synthesis of VSMCs. PCA treatment of PDGF-BB-stimulated VSMCs resulted in a significant decrease in BrdU incorporation compared control cells, without any evidence of cytotoxicity. BrdU can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication [27]. This result is likely related to the S-phase arrest of VSMCs. PDGF-BB treatment correlated with cellular migration into the S phase with concomitant decrease of cells in the G0/G1 phase, while PCA treatment diminished this migration into the S phase. Cell proliferation is controlled by a series of regulators that act as sequential points throughout the cell cycle. The G1 to S transition is controlled by several cyclin-dependent kinase (CDK) complexes [28,29]. The CDK/cyclin complex phosphorylates a large number of proteins, resulting in hyperphosphorylation of Rb, which promotes DNA synthesis [29]. The downregulation of cyclin D2 expression after PCA treatment was associated with the inhibition of PDGF-BB-stimulated cell proliferation, DNA synthesis, and cell cycle progression. Cyclin D2 expression was down-regulated when

VSMCs were treated with PCA (Fig 3B), demonstrating that the G0/G1 phase arrest might result from the down-regulation of CDK/cyclin complex expression. Taken together, these results suggest that PCA may contribute to S-phase arrest by inhibition of VAMC proliferation. In addition to the pathologic changes in VSMCs, thrombosis formation plays a critical role in cardiovascular disease [30]. Although there are many factors involved in thrombosis formation, the platelet acts as a main component in initiating and aggregating thrombosis [31,32]. In this study, PCA exhibited marked anti-platelet effects. We determined effects of PCA on platelet function by measuring *ex vivo* aggregation of isolated rat whole blood. The complex process of platelet aggregation is mediated primarily through platelet adhesion at the site of injury, as well as the action of endogenous agonists such as ADP, which stimulate platelet aggregation via specific receptors on the platelet membrane [33–35]. Platelet activation was induced by ADP in this study. Although other thrombosis inducers such as collagen were not evaluated, the inhibitory effect of PCA on ADP was supported by our *ex vivo* experiment. Concerning the physiologic role of ADP as a platelet activator, our results suggest PCA as a candidate for a pharmacologic anti-platelet agent. Further *in vivo* studies are needed to replicate PCA's anti-platelet effect.

In conclusion, our findings suggest that PCA is capable of preventing the proliferation and migration of VSMCs. Our findings suggest this effect may be due to PCA's ROS scavenging ability, which attenuates the signaling cascades of PDGF and other cytokines. Our results provide novel insights into the protective action of PCA on the vascular injury response, suggesting a potential therapeutic application of PCA in atherosclerosis and restenosis after angioplasty.

Acknowledgments

This work was supported by the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs (Grant numbers: A085136 [to E.J.L]).

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