Original Paper

Zedoarondiol Inhibits Platelet-Derived Growth Factor-Induced Vascular Smooth Muscle Cells Proliferation *via* Regulating AMP-Activated Protein Kinase Signaling Pathway

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Key Words

Zedoarondiol • Vascular smooth muscle cell • Proliferation • AMP-activated protein kinase

Abstract

Background/Aims: Vascular smooth muscle cells (VSMCs) proliferation contributes significantly to atherosclerosis and in-stent restenosis. Platelet-derived growth factor-BB (PDGF-BB) plays a vital role in VSMCs proliferation. Zedoarondiol, a sesquiterpene lactone compound, has an anti-inflammatory activity. However, the role of zedoarondiol in PDGF-BB-mediated VSMCs proliferation remains unclear. In this study, we investigated the effects of zedoarondiol on PDGF-BB-induced VSMCs proliferation and explored the possible mechanisms. **Methods:** The inhibitory effects of zedoarondiol on PDGF-BB-induced VSMCs proliferation were evaluated by direct cell counting and the Cell Counting Kit-8 (CCK-8) assay. DNA synthesis was examined by bromodeoxyuridine (BrdU) incorporation assay. Cell cycle was assessed by propidium iodide staining. Western blotting was performed to determine the expression of cyclin-dependent kinase 2 (CDK2), cyclin E, p53, p21, total and phosphorylated adenosine monophosphate-activated protein kinase (AMPK), acetyl CoA carboxylase (ACC), mammalian target of rapamycin (mTOR), and p70 ribosomal protein S6 kinase (p70S6K). **Results:** Zedoarondiol suppressed PDGF-BB-induced VSMCs proliferation and DNA synthesis, and induced cell cycle arrest in G0/G1 phase. In addition, zedoarondiol activated AMPK and ACC, inhibited the phosphorylation of mTOR and p70S6K, increased the expression of p53 and p21, and decreased the expression of CDK2 and cyclin E. Compound C (an AMPK inhibitor) abrogated, whereas 5-aminoimidazole-4-carboxamide 1- β -ribofuranoside (AICAR, an AMPK activator) enhanced zedoarondiol-mediated inhibition of VSMCs proliferation and DNA synthesis. **Conclusion:** Zedoarondiol inhibits PDGF-BB-induced VSMCs proliferation *via* AMPK-mediated down-regulation of the mTOR/p70S6K pathway and up-regulation of the p53/p21 pathway. These findings suggest that zedoarondiol might be a promising compound against atherosclerosis and in-stent restenosis.

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Introduction

Vascular smooth muscle cells (VSMCs) proliferation and migration contribute significantly to the development of atherosclerosis (AS) [1] and restenosis (RS) after percutaneous coronary intervention [2]. VSMCs proliferation and migration can be induced by cytokines and growth factors released after vascular injury, including platelet-derived growth factors (PDGFs) [3, 4], interleukin-1 [5], tumor necrosis factor (TNF)- α [6], and angiotensin II [7]. PDGFs, especially PDGF-BB, are considered as the most potent mitogens stimulating VSMCs proliferation [8]. PDGF-BB initiates a series of biological effects associated with VSMCs proliferation via activating intracellular signal transduction pathways, including mitogen-activated protein kinase (MAPK) [9], phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) [10], Rho kinase (ROCK) [11] and mammalian target of rapamycin (mTOR) [12] pathways. Hence, inhibition of PDGF-stimulated VSMCs proliferation is a promising strategy to attenuate AS and RS.

Zedoariae rhizoma (*Curcuma zedaria* Roscoe, e'zhu in Chinese) is a herbal drug belonging to the Zingiberaceae family. The main bioactive constituents of *Zedoariae rhizoma* are diarylheptanoids and sesquiterpenoids [13], which have pleiotropic pharmacological effects, including anti-inflammatory, antioxidant, and anti-proliferative activities [14-16]. Our previous study showed that stents coated with *Zedoariae rhizoma* essential components (zedoary essential components, ZEC), mainly including sesquiterpenoids, inhibited neointimal hyperplasia in a porcine coronary arterial balloon-injury model [17], indicating that essential components of *Zedoariae rhizoma* play an important role in regulating VSMCs proliferation. Zedoarondiol (Fig. 1), a sesquiterpene lactone, is the main bioactive compound extracted from ZEC and has been reported to attenuate lipopolysaccharide (LPS)-induced inflammation in murine macrophages by suppressing nuclear factor-kappa B (NF- κ B) pathway [18] and reduce D-galactosamine-induced liver injury in mice [19, 20]. However, little is known about the role of zedoarondiol in regulating VSMCs proliferation.

Adenosine monophosphate-activated protein kinase (AMPK), consisting of a catalytic subunit (α) and two regulator subunits (β and γ), plays a key role in energy metabolism and acts as a cellular fuel sensor [21]. AMPK is activated by cellular stress including ischemia, hypoglycemia, hypoxia, and oxidative stress, which is associated with an increased adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio [22]. Accumulating evidence shows that AMPK activation suppresses VSMCs proliferation and attenuates neointima formation in vascular injury models [23-25], indicating AMPK as a novel therapeutic target for AS and RS [26]. Cell growth inhibition by AMPK activation is mainly attributed to activation of the p53/p21 pathway followed by cell cycle arrest in G0/G1 phase [27] and inhibition of the mTOR signaling pathway [28].

In the current study, we hypothesized that zedoarondiol inhibits PDGF-BB-induced VSMCs proliferation via AMPK activation. To test this hypothesis, we investigated the effects of zedoarondiol on PDGF-BB-induced VSMCs proliferation and AMPK pathway, to provide the evidence for utility of zedoarondiol in preventing and treating AS and RS.

Materials and Methods

Materials

Zedoarondiol was kindly provided by Dalian Institute of Chemical Physics, Chinese Academy of Sciences (Dalian, Liaoning, China). Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN, USA). Compound C was obtained from CalBiochem (San Diego, CA, USA). 5-aminoimidazole-4-carboxamide 1- β -ribofuranoside (AICAR) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Sijiqing Biochemistry Technique Co., Ltd. (Hangzhou, Zhejiang, China). The Cell Counting Kit-8 (CCK-8) kit was purchased from Dojindo Laboratories (Kumamoto, Japan). The Bromodeoxyuridine (BrdU) Cell Proliferation ELISA Kit was obtained from Roche Diagnostics (Mannheim, Germany). The Cell

Cycle and Apoptosis Analysis Kit was purchased from Beyotime Biotechnology Company (Shanghai, China). The Annexin V/fluorescein isothiocyanate (FITC) Apoptosis Direction Kit was purchased from KeyGen Biological Engineering Company (Nanjing, Jiangsu, China). The enhanced chemiluminescence (ECL) kit was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against AMPK α (2532), phospho-AMPK α ^{Thr172} (2535), acetyl CoA carboxylase (ACC) (3676), phospho-ACC (11818), mTOR (2972), phospho-mTOR^{Ser2481} (2974), p70S6K (2708), phospho-p70S6K^{Thr389} (9234), p53 (2524), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against cyclin-dependent kinase (CDK) 2 (sc-6248), cyclin E (sc-481) and p21 (sc-397) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody for SM α -actin (ab5694) was purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG were obtained from Epitomics (Burlingame, CA, USA).

Cell culture and experimental protocols

Primary VSMCs were obtained from thoracic aortas of male Sprague-Dawley rats (120 to 160 g), as described previously [29]. Briefly, the aorta was isolated and the adventitias were stripped off. Tunica media was isolated from the aorta, minced, and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, MD, USA) in a humidified atmosphere with 5% CO₂ at 37°C. The identity and purity of VSMCs were confirmed by positive staining of SM α -actin. Passages 4–8 of VSMCs were used in this study. To assess the effect of zedoarondiol on VSMCs proliferation in the absence of PDGF-BB, cells were treated with zedoarondiol (5, 10, 20, and 40 μ g/mL) for 24 h in serum-free media. To evaluate the effect of zedoarondiol on PDGF-BB-induced VSMCs proliferation, VSMCs were pretreated with zedoarondiol (5, 10, and 20 μ g/mL) for 24 h in serum-free media and then stimulated with 20 ng/mL PDGF-BB for another 24 h. To explore the role of AMPK in zedoarondiol-mediated anti-proliferative effects in VSMCs, cells were divided into six groups: (1) control group: VSMCs were cultured routinely for 48 h; (2) Zed group: VSMCs were treated with zedoarondiol (20 μ g/mL) in serum-free media for 48 h; (3) PDGF group: VSMCs were cultured routinely for 24 h, and then stimulated with PDGF-BB (20 ng/mL) for another 24 h; (4) PDGF+Zed group: VSMCs were pretreated with zedoarondiol (20 μ g/mL) in serum-free media for 24 h, and then stimulated with PDGF-BB (20 ng/mL) for another 24 h; (5) PDGF+Zed+compound C (an AMPK inhibitor) group: VSMCs were pretreated with zedoarondiol (20 μ g/mL) and compound C (10 μ M) for 24 h, and stimulated with PDGF-BB (20 ng/mL) for another 24 h; (6) PDGF+Zed+AICAR (an AMPK activator) group: VSMCs were pretreated with zedoarondiol (20 μ g/mL) and AICAR (0.5 mM) for 24 h, and stimulated with PDGF-BB (20 ng/mL) for another 24 h. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital, and complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (Document 55, 2001).

Cell counting

The cells were seeded in 6-well plates at a density of 2×10^5 cells/well. After experimental treatment, cells were harvested and stained with 0.4 % trypan blue dye solution. The number of cells not stained with trypan blue was determined using a hemocytometer.

CCK-8 assay

The CCK-8 assay was conducted according to the manufacturer's instructions to determine VSMCs proliferation. VSMCs were seeded in 96-well plates at a density of 5×10^3 cells/well. After experimental treatment, 10 μ L of CCK-8 was added to each well for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (Tecan Infinite f200 Pro, Tecan Group Ltd, Männedorf, Switzerland).

DNA synthesis assay

DNA synthesis was assessed by BrdU incorporation according to the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. BrdU (10 μ L/well) was added for the last 4

Fig. 1. Chemical structure of zedoarondiol.

h of the experiment. After removing the culture medium, FixDenat (200 μ L/well) was added, followed by incubation with anti-BrdU-POD (100 μ L/well) for 90 min at room temperature. The fixed cells were washed twice with PBS and incubated with 100 μ L/well substrate solution for 15 min. Absorbance was measured at 492 nm using an ELISA reader (Tecan Infinite f200 Pro, Tecan Group Ltd, Männedorf, Switzerland).

Cell cycle analysis

Cell cycle was analyzed according to the manufacturer's protocol of Cell Cycle and Apoptosis Analysis Kit. VSMCs were seeded in 6-well plates at a density of 2×10^5 cells/well. Cells were harvested and fixed in cold 70% methanol overnight at -20°C . Afterwards, the cells were washed twice with PBS and incubated with propidium iodide (PI) staining buffer (100 μ g/mL PI and 100 μ g/mL RNase A) for 30 min at 37°C . FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used in the analysis.

Annexin V and PI double-staining assay

VSMCs were seeded in 6-well plates at a density of 1×10^5 cells/well. After treatment, cells were harvested with 0.25% trypsin and washed twice with cold PBS. Cells were subjected to an apoptosis assay, using an Annexin V and PI apoptosis kit according to the manufacturer's instructions. Cell preparations were resuspended in 500 μ L binding buffer, labeled with Annexin V and PI, and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis

Cells were collected and lysed in lysis buffer, as previously described [30]. After quantifying protein concentration using the bicin-choninic acid (BCA) method [31], 80 μ g of protein was resolved on 8-12% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis, and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5 % bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, incubated with primary antibodies against CDK2, cyclin E, p21 (all 1:200 diluted), AMPK, p-AMPK, ACC, p-ACC, mTOR, p-mTOR^{Ser2481}, p70S6K, p-p70S6K^{Thr389}, p53, and GAPDH (all 1:1000 diluted) overnight at 4°C , and finally probed with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Protein bands were detected using an ECL kit and the optical density of bands was quantified using Image-Pro Plus (Roper Industries, New York, NY, USA). Protein expression was normalized to GAPDH.

Statistical analyses

SPSS 17.0 software (IBM, Chicago, IL, USA) was applied for statistical analyses. Data were expressed as mean $\pm$ standard deviation (SD). Differences were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A value of $P < 0.05$ was considered statistically significant.

Results

Zedoarondiol inhibits PDGF-BB-induced VSMCs proliferation

Previous studies have indicated that zedoarondiol inhibits D-galactosamine-induced cytotoxicity in primary cultured rat hepatocytes and attenuates inflammation by suppressing NF- κ B pathway in macrophages [18-20]. In the present study, we first evaluated the effect of zedoarondiol on VSMCs in the absence of PDGF-BB. Zedoarondiol (5, 10, 20, and 40 μ g/mL) did not decrease VSMCs viability in the absence of PDGF-BB when compared with that in the control group ($P > 0.05$; Fig. 2A), indicating that zedoarondiol was not cytotoxic at the concentrations tested. Next, we examined the effect of zedoarondiol on PDGF-BB-induced VSMCs proliferation by direct cell counting. PDGF-BB (20 ng/mL) significantly increased the number of cells up to 1.5 fold ($P < 0.05$ vs. control). Pretreatment with zedoarondiol decreased the number of cells in a dose-dependent manner ($P < 0.05$ vs. PDGF; Fig. 2B). We further investigated the inhibitory effect of zedoarondiol on VSMCs by the CCK-8 assay. Compared with control group, PDGF-BB (20 ng/mL) treatment significantly increased VSMCs proliferation up to 1.3 fold ($P < 0.05$). Compared with PDGF group, zedoarondiol pretreatment at 5 and 10 μ g/mL had no effect on PDGF-BB-induced VSMCs proliferation ($P > 0.05$). However, zedoarondiol at 20 μ g/mL decreased PDGF-BB-induced VSMCs proliferation

Fig. 2. Effects of zedoarondiol (Zed) on VSMCs viability and proliferation. (A) VSMCs were treated by zedoarondiol (5, 10, 20, and 40 $\mu\text{g/mL}$) for 24 h. (B) VSMCs were treated with zedoarondiol (5, 10, 20, and 40 $\mu\text{g/mL}$) for 24 h and incubated with PDGF-BB (20 ng/mL) for another 24 h. VSMCs proliferation was assessed by direct cell counting. (C) VSMCs were treated as in B. VSMCs proliferation was assessed by the CCK-8 assay. (D) VSMCs were pretreated with Zed (20 $\mu\text{g/mL}$), Zed+compound C (10 μM) or Zed+AICAR (0.5 mM) for 24 h and incubated with/without PDGF-BB (20 ng/mL) for a further 24 h. VSMCs proliferation was assessed by direct cell counting. (E) VSMCs were treated as in D. VSMCs proliferation was assessed by the CCK-8 assay. Data are expressed as mean $\pm$ SD. n = 6. * P < 0.05 vs. control; # P < 0.05 vs. PDGF; & P < 0.05 vs. PDGF+Zed.

by 17% (P < 0.05 vs. PDGF; Fig. 2C). Our results indicated that zedoarondiol showed an inhibitory effect on PDGF-BB-induced VSMCs proliferation.

To determine whether the anti-proliferative effects of zedoarondiol on VSMCs were mediated by AMPK activation, cells were co-treated with AICAR (an AMPK activator) or compound C (an AMPK inhibitor). Compared with PDGF + Zed group, compound C (10 μM) dramatically reversed zedoarondiol-mediated anti-proliferative effects on PDGF-BB-stimulated VSMCs proliferation, resulting in a 63% increase in cell number (P < 0.05). Notably, pretreatment with zedoarondiol (20 $\mu\text{g/mL}$) and AICAR (0.5 mM) further decreased the number of cells by 14 % (P < 0.05 vs. PDGF + Zed; Fig. 2D). Similar results were obtained from the CCK-8 assay. Compared with PDGF + Zed group, the addition of compound C increased VSMCs proliferation by 25 % (P < 0.05), whereas the additional AICAR treatment decreased VSMCs proliferation by 19 % (P < 0.05; Fig. 2E).

Zedoarondiol inhibits PDGF-BB-induced DNA synthesis in VSMCs

We evaluated the effect of zedoarondiol on PDGF-BB-induced DNA synthesis in VSMCs by BrdU incorporation assay. PDGF-BB treatment increased DNA synthesis in VSMCs up to 1.57 fold (P < 0.05 vs. control), whereas zedoarondiol pretreatment decreased PDGF-BB-induced DNA incorporation in a concentration-dependent manner. When compared

Fig. 3. Effects of zedoarondiol (Zed) on PDGF-BB-induced DNA synthesis in VSMCs, measured by BrdU incorporation assay. (A) VSMCs were treated with zedoarondiol (5, 10, 20, and 40 µg/mL) for 24 h and incubated with PDGF-BB (20 ng/mL) for another 24 h. (B) VSMCs were pretreated with Zed (20 µg/mL), Zed+compound C (10 µM) or Zed+AICAR (0.5 mM) for 24 h and incubated with/without PDGF-BB (20 ng/mL) for a further 24 h. Data are expressed as mean ± SD. n = 6. **P* < 0.05 vs. control; #*P* < 0.05 vs. PDGF; &*P* < 0.05 vs. PDGF+Zed.

Fig. 4. Zedoarondiol arrests VSMCs cell cycle in G0/G1 phase. VSMCs were pretreated with zedoarondiol (5, 10, and 20 µg/mL) for 24 h followed by PDGF-BB (20 ng/mL) stimulation for another 24 h. Then, cells were stained with PI to determine DNA fluorescence and cell cycle distribution. (A) Representative cell cycle. (B) VSMCs in G0/G1 phase. (C) VSMCs in S phase. Data are expressed as mean ± SD. n = 3. **P* < 0.05 vs. control; #*P* < 0.05 vs. PDGF.

with PDGF group, zedoarondiol at 5, 10, and 20 µg/mL decreased PDGF-BB-induced DNA synthesis in VSMCs by 18.5%, 24.4%, and 26.2%, respectively (*P* < 0.05; Fig. 3A).

Next, we explored the role of AMPK in zedoarondiol-mediated inhibition of DNA synthesis. As expected, pretreatment with zedoarondiol and compound C abolished zedoarondiol-induced inhibition of DNA synthesis under PDGF-BB conditions, showing a 51 % increase in DNA synthesis, as compared with PDGF+Zed group (*P* < 0.05). In contrast, pretreatment with zedoarondiol and AICAR displayed a greater inhibitory effect on DNA synthesis, revealing a further 15.59 % decrease (*P* < 0.05 vs. PDGF+Zed; Fig. 3B).

Fig. 5. Zedoarondiol does not induce PDGF-BB-stimulated VSMCs apoptosis. VSMCs were double-stained with annexin V and PI to analyze apoptosis by flow cytometry. (A) Representative scatter plots of apoptosis. (B) Apoptotic rate was determined using the flow cytometry data. Data are expressed as mean $\pm$ SD. $n = 3$. * $P < 0.05$ vs. control; # $P < 0.05$ vs. PDGF.

Zedoarondiol arrests VSMCs cell cycle in G₀/G₁ phase without accelerating apoptosis

Cell proliferation is tightly controlled by cell cycle progression. Thus, we investigated the effect of zedoarondiol on cell cycle progression by flow cytometry. As shown in Fig. 4, PDGF-BB significantly decreased the number of cells in G₀/G₁ phase from 88.85 % to 71.79% ($P < 0.05$ vs. control), and increased the proportion of cells in S phase from 9.0% to 20.9% ($P < 0.05$ vs. control). Compared with PDGF group, Zedoarondiol pretreatment at 5 and 10 μ g/mL had no effect on cell cycle ($P > 0.05$). However, pretreatment with 20 μ g/mL zedoarondiol, followed by PDGF-BB stimulation, increased the proportion of cells in G₀/G₁ phase to 79.1% ($P < 0.05$ vs. PDGF) and decreased the proportion of cells in S phase to 13.2% ($P < 0.05$ vs. PDGF).

The reduction in cell number may result from inhibition of cell growth or induction of apoptosis. To investigate whether zedoarondiol induced PDGF-BB-stimulated VSMCs apoptosis, flow cytometry analysis was performed. The results showed no significant difference between groups ($P > 0.05$; Fig. 5), indicating that the inhibitory effect of zedoarondiol on PDGF-BB-stimulated VSMCs was not associated with VSMCs apoptosis.

Zedoarondiol induces the activation of AMPK

To further illustrate the role of AMPK activation in zedoarondiol-mediated anti-proliferative effects on VSMCs, the phosphorylation level of AMPK was examined. Compared with control group, zedoarondiol treatment alone did not affect AMPK phosphorylation ($P > 0.05$). Although PDGF-BB activated AMPK phosphorylation by 46% ($P < 0.05$ vs. control), its phosphorylation level was significantly lower than that observed in PDGF + Zed group ($P < 0.05$). However, compound C abolished this action, showing a 40.71 % decrease in AMPK phosphorylation compared with PDGF + Zed group ($P < 0.05$). The pretreatment with zedoarondiol and AICAR further increased AMPK activation by 7.5% ($P < 0.05$ vs. PDGF + Zed; Fig. 6A). In addition, we investigated the phosphorylation of ACC, a downstream target of AMPK. In accordance with AMPK activation, ACC phosphorylation was significantly activated in PDGF+Zed group, compared with PDGF group ($P < 0.05$).

Fig. 6. Zedoarondiol induces the activation of AMPK. VSMCs were pretreated with/without Zed (20 μ g/mL), Zed+compound C (10 μ M) or Zed+AICAR (0.5 mM) for 24 h and stimulated with/without PDGF-BB (20 ng/mL) for a further 24 h. VSMCs were subjected to western blotting to determine the protein levels of (A) p-AMPK and AMPK (B) p-ACC and ACC. Protein levels were quantified by densitometry. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SD. n = 3. * P < 0.05 vs. control; # P < 0.05 vs. PDGF; & P < 0.05 vs. PDGF+Zed.

Fig. 7. Zedoarondiol down-regulates the mTOR/p70S6K signaling pathway. Western blotting was performed with antibodies specific for (A) p-mTOR and mTOR (B) p-p70S6K^{Thr389} and p70S6K. Protein levels were quantified by densitometry. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SD. n = 3. * P < 0.05 vs. control; # P < 0.05 vs. PDGF; & P < 0.05 vs. PDGF+Zed.

Additional treatment with compound C reversed zedoarondiol-mediated effects, resulting in a decrease in ACC phosphorylation. However, additional AICAR treatment further increased ACC phosphorylation (P < 0.05 vs. PDGF + Zed; Fig. 6B). Our findings indicate that AMPK activation is involved in the anti-proliferative effects of zedoarondiol on VSMCs.

Zedoarondiol down-regulates mTOR/p70S6K activity

The mTOR/p70S6K pathway, a key downstream target of AMPK, is involved in the regulation of mitogen-induced VSMCs proliferation [32]. Therefore, we investigated whether the activity of mTOR/p70S6K was altered by zedoarondiol under PDGF-BB conditions. As shown in Fig. 7, zedoarondiol alone had no effect on the phosphorylation of mTOR and p70S6K at Thr389 compared with control group (P > 0.05). PDGF-BB significantly increased the phosphorylation of mTOR and p70S6K up to 1.96 and 1.91 fold, respectively (P < 0.05 vs. control). However, compared with PDGF group, pretreatment with 20 μ g/mL zedoarondiol

Fig. 8. Zedoarondiol up-regulates the p53/p21 pathway. Western blotting was performed with antibodies specific for p53 and p21. Protein levels were quantified by densitometry. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SD. $n = 3$. * $P < 0.05$ vs. control; # $P < 0.05$ vs. PDGF; & $P < 0.05$ vs. PDGF+Zed.

induced a 34.44 % reduction in mTOR phosphorylation and a 23.81% reduction in p70S6K phosphorylation ($P < 0.05$). Compound C effectively reversed zedoarondiol-induced decrease in phosphorylation of mTOR and p70S6K by 89 % and 41 %, respectively ($P < 0.05$ vs. PDGF+Zed). Furthermore, in the presence of AICAR, the phosphorylation of mTOR and p70S6K at Thr389 further declined by 45.45 % and 21.87 %, respectively, compared with PDGF+Zed group ($P < 0.05$).

Zedoarondiol up-regulates the p53/p21 pathway

Cell cycle progression is controlled by cyclins/CDKs complexes, CDK inhibitors (CDKIs) and tumor suppressor genes. Recently, studies documented that AMPK activation is accompanied by the accumulation of p53, which, in turn, up-regulates p21 *via* a transcriptional mechanism [26, 33]. Therefore, we focused on the mechanisms of zedoarondiol-induced cell cycle arrest and determined whether the p53/p21 pathway was involved in the zedoarondiol-induced inhibition of VSMCs proliferation. As shown in Fig. 8, PDGF-BB stimulation did not affect the expression of p53 and p21 ($P > 0.05$ vs. control). When compared with PDGF group, zedoarondiol (20 μ g/mL) pretreatment increased the expression of p53 and p21 by 52 % and 18 %, respectively, under PDGF-BB conditions ($P < 0.05$). As expected, pretreatment with zedoarondiol and compound C abolished zedoarondiol-mediated increased expression of p53 and p21 under PDGF-BB conditions, resulting in a 39.27% and 12.28% decrease in p53 and p21 expression, respectively ($P < 0.05$ vs. PDGF + Zed). Conversely, pretreatment with zedoarondiol and AICAR further increased the expression of p53 and p21 by 7% and 22%, respectively ($P < 0.05$ vs. PDGF + Zed).

CDK2 and cyclin E play a central role in regulating cell cycle transition from G1 to S phase. In addition, p21 down-regulates CDK2 and cyclin E, which is involved in cell cycle arrest [34]. We detected the protein levels of CDK2 and cyclin E by western blotting. PDGF-BB markedly increased the expression of CDK2 and cyclin E by 30 % and 84 %, respectively, compared with control group ($P < 0.05$). Zedoarondiol (20 μ g/mL) pretreatment caused a 25.8 % and 30.88 % decrease in CDK2 protein and cyclin E protein expression, respectively, under PDGF-BB conditions ($P < 0.05$ vs. PDGF). However, zedoarondiol-mediated reduction of CDK2 and cyclin E expression was abolished by additional pretreatment with compound C, as shown by a 29 % and 35 % increase in CDK2 and cyclin E expression, respectively

Fig. 9. Effects of zedoarondiol on expression of CDK2 and cyclin E. Western blotting was performed with antibodies specific for CDK2 and cyclin E. Protein levels were quantified by densitometry. GAPDH was used as an internal control. Data are expressed as mean $\pm$ SD. $n = 3$. * $P < 0.05$ vs. control; # $P < 0.05$ vs. PDGF; & $P < 0.05$ vs. PDGF+Zed.

($P < 0.05$ vs. PDGF+Zed). In contrast, additional AICAR pretreatment aggravated zedoarondiol-mediated inhibition of CDK2 and cyclin E expression ($P < 0.05$ vs. PDGF+Zed; Fig. 9).

Discussion

In this study, we investigated the effects of zedoarondiol on PDGF-BB-induced VSMCs proliferation and explored its possible mechanism. Our findings demonstrated that zedoarondiol suppressed VSMCs proliferation and DNA synthesis through AMPK activation-mediated inhibition of mTOR/p70S6K and up-regulation of p53/p21, followed by VSMCs cycle arrest.

VSMCs proliferation plays an essential role in AS and RS [1, 2]. PDGF-BB is a potent mitogen released from injured vessels, which contributes significantly to VSMCs proliferation. Therefore, it is a promising strategy to look for compounds which suppress PDGF-BB-mediated VSMCs proliferation [8]. Zedoarondiol is a sesquiterpenoid. Amounting evidence shows that sesquiterpenoids exhibit potent anti-tumor activities against HeLa cells, L615 cells, and hepatoma cells [35, 36]. Our study demonstrated that zedoarondiol, the main compound extracted from ZEC, suppressed PDGF-BB-induced VSMCs proliferation and DNA synthesis, and caused cell cycle arrest in G0/G1 phase. These results may explain the previous reports of ZEC attenuating neointimal hyperplasia in a porcine coronary arterial balloon-injury model [17].

AMPK is a stress-activated protein kinase which serves as a cellular energy sensor [37]. AMPK activation suppresses neointimal formation in vascular injury models [23], and activation of AMPK inhibits VSMCs proliferation *in vitro* [26]. As a result, AMPK is considered as a promising novel target in the treatment of AS and RS. Many plant natural products, including berberine [38], sodium tanshinone IIA silate [39], and protocatechuic acid [40] have shown anti-proliferative effects on VSMCs *via* activating AMPK pathway. In the present study, we showed that the inhibitory effects of zedoarondiol on VSMCs proliferation induced by PDGF-BB were accompanied by AMPK activation, as evidenced by increased phosphorylation of AMPK and ACC. However, compound C, an inhibitor of AMPK, significantly reduced the anti-proliferative effects of zedoarondiol on VSMCs, whereas AICAR, an activator

Fig. 10. Mechanisms of zedoarondiol-mediated inhibition of PDGF-BB-induced VSMCs proliferation. Zedoarondiol activates AMPK to inhibit the mTOR/p70S6K pathway, resulting in inhibition of protein synthesis. Activated AMPK up-regulates the p53/p21 pathway and induces cell cycle arrest. All effects described contribute to suppression of VSMCs proliferation.

of AMPK, enhanced the zedoarondiol-induced inhibition of VSMCs proliferation. Therefore, our findings indicate that the anti-proliferative activity of zedoarondiol on PDGF-BB-induced VSMCs is associated with AMPK activation.

mTOR is an evolutionarily conserved serine/threonine kinase and a key downstream target of AMPK. The mTOR integrates nutrient and mitogen signals and acts as a vital regulator of protein synthesis, cell growth, cell division, and autophagy [41]. The most characterized downstream effector of mTOR is p70S6K, which is essential in activating several transcriptional factors, thereby regulating protein synthesis necessary for cell growth [42]. Increasing evidence indicates that the mTOR/p70S6K signaling pathway in VSMCs could be suppressed by AMPK activation, subsequently inhibiting VSMCs proliferation [43]. Our study revealed that zedoarondiol-mediated AMPK activation inhibited phosphorylation of mTOR and p70S6K at Thr389. As expected, compound C abolished zedoarondiol-mediated inhibition of mTOR and p70S6K phosphorylation, whereas AICAR further inhibited it, indicating that AMPK-mediated mTOR/p70S6K pathway inhibition is involved in the anti-proliferative effects of zedoarondiol on PDGF-BB-stimulated VSMCs.

The cell cycle is governed positively by cyclins/CDKs complexes, and negatively by CDKIs and tumor suppressor genes [44]. The cyclin D/CDK4 and cyclin E/CDK2 complexes are required for cell cycle progression from G1 phase to the S phase [45]. CDKIs, such as p21^{cip} and p27^{kip1}, inhibit cyclin/CDKs activity, resulting in cell cycle arrested in G0/G1 phase [46]. Another vital negative regulator is tumor suppressor p53, which is an essential downstream target of AMPK. Increased expression of p53 induces cell cycle arrest and/or apoptosis by modulating CDKs and CDKIs [47, 48]. Previous studies have shown that AMPK activation up-regulates the expression of p53 and p21 and down-regulates the expression cyclin E/CDK2, thus preventing cell cycle progression into S phase [49]. In this study, zedoarondiol up-regulated the protein expression of p53 and p21 and down-regulated the protein expression of cyclin E and CDK2 under PDGF-BB conditions, which was abrogated by compound C but aggravated by AICAR. ElKeeb et al. reported that increased p53 expression induced cell cycle arrest and apoptosis [50]. However, in this study, zedoarondiol-induced up-regulated p53 did not trigger apoptosis in VSMCs. Taken together, these results indicate that AMPK-mediated up-regulation of p53/p21 pathway is associated with the anti-proliferative effects of zedoarondiol on PDGF-BB-stimulated VSMCs.

Although the present study indicates the anti-proliferative effects of zedoarondiol on VSMCs, several limitations should be pointed out. First, AICAR is a non-selective activator of AMPK [51], which might elicit AMPK-independent effects. A more specific AMPK activator, such as A769662, or metabolic/endogenous stimulation should be adopted to activate AMPK in future studies, to further determine the role of AMPK in zedoarondiol-mediated effects. Second, in the present study, GAPDH was used to normalize protein expression in western blotting. Zedoarondiol is a sesquiterpenoid compound. Numerous studies evaluating sesquiterpenoids on VSMCs indicate that GAPDH is widely used as a normalization control in western blotting and that GAPDH expression appears unchanged with sesquiterpenoids treatment [52, 53]. Based on these findings, we chose GAPDH to normalize protein expression in our study. Given that zedoarondiol has been untested in VSMCs before, whether GAPDH expression remains unchanged with zedoarondiol needs to be investigated. Third, we did not evaluate the effect of compound C alone on VSMCs proliferation and AMPK phosphorylation. Previous studies have indicated that compound C alone does not affect VSMCs proliferation and inhibits AMPK phosphorylation in VSMCs [38, 54]. To further solidify the results from our study, the effect of compound C alone on VSMCs proliferation and AMPK phosphorylation should be incorporated in our future studies. Moreover, the time-dependent effects of zedoarondiol on AMPK phosphorylation were not investigated in our study. Thus, the present study did not clarify which time point after zedoarondiol treatment has the most favorable effect on the phosphorylation of AMPK. In the future, we will conduct the time-dependent study on AMPK phosphorylation, in order to provide more precise information of the role of zedoarondiol in VSMCs proliferation induced by PDGF-BB.

In conclusion, our study demonstrated that zedoarondiol, a sesquiterpene lactone compound extracted from *Zedoariae rhizoma*, inhibits VSMCs proliferation induced by PDGF-BB. The underlying mechanisms of zedoarondiol-mediated anti-proliferative effects are associated with AMPK activation, subsequently inhibiting the mTOR/p70S6K signaling pathway and up-regulating the p53/p21 pathway (Fig. 10). Our findings suggest that zedoarondiol might be a promising compound against VSMCs proliferation-related diseases, such as AS and RS.

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Disclosure Statement

The authors report no conflicts of interest.

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