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Original Article

Involvement of estrogen receptor-β in farrerol inhibition of rat thoracic aorta vascular smooth muscle cell proliferation

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Aim: To investigate the effect of farrerol, a major active component isolated from a traditional Chinese herb "Man-shan-hong" (the dried leaves of *Rhododendron dauricum* L) on fetal bovine serum (FBS)-induced proliferation of cultured vascular smooth muscle cells (VSMCs) of rat thoracic aorta.

Methods: VSMCs proliferation, DNA synthesis and cell cycle progression were studied using the MTT assay, bromodeoxyuridine (BrdU) incorporation and flow cytometry, respectively. The mRNA levels of cell cycle proteins were quantified using real-time RT-PCR, and the phosphorylation of ERK1/2 was determined using Western blotting. Reporter gene and receptor binding assays were employed to study the interaction between farrerol and estrogen receptors (ERs).

Results: Farrerol (0.3–10 μmol/L) inhibited VSMC proliferation and DNA synthesis induced by 5% FBS in a concentration-dependent manner. The effects were associated with G_1 cell cycle arrest, down-regulation of cell cycle proteins and reduction in FBS-induced ERK1/2 phosphorylation. Using a reporter gene, it was found that farrerol (3 μmol/L) induced 2.1-fold transcription of ER. In receptor binding assays, farrerol inhibited the binding of [^{3}H]estradiol for ERα and ERβ with IC_{50} values of 57 μmol/L and 2.7 μmol/L, respectively, implying that farrerol had a higher affinity for ERβ. Finally, the inhibition of VSMC proliferation by farrerol (3 μmol/L) was abolished by the specific ERβ antagonist PHTPP (5 μmol/L).

Conclusion: Farrerol acts as a functional phytoestrogen to inhibit FBS-induced VSMC proliferation, mainly via interaction with ERβ, which may be helpful in the treatment of cardiovascular diseases related to abnormal VSMCs proliferation.

Keywords: farrerol; phytoestrogen; estrogen receptor; vascular smooth muscle cells; cell proliferation; cell cycle; ERK1/2; receptor binding assay

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Introduction

Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays a critical role in intimal formation in the early stage of atherosclerosis and restenosis[1]. Several reports have indicated that reduction of VSMC proliferation using therapeutic agents (*eg*, estrogen) would give rise to vasoprotective effects[2]. Estrogen exhibits a variety of actions on the vascular wall that could be implicated in its athero-protective properties[3]. These include, but are not limited to, the inhibition of VSMC proliferation[4, 5].

As we know, earlier studies supported the widespread belief in the hypothesis of cardiovascular benefit of hormone replacement therapy (HRT) in postmenopausal women, but recent Women's Health Initiative (WHI) trials found no coronary heart disease (CHD) benefit among women aged 50 to 79 and HRT increased risk of stroke[6, 7]. However, WHI also provided evidence that CHD risks are reasonably low for short-term use by younger postmenopausal women[8, 9]. Although "timing hypothesis" and "critical window theory" for estrogen intervention in preventing atherosclerosis were developed, there is still a need to better understand the relationship between circulating estrogen levels and aging of the cardiovascular system.

It has been suggested that steroidal hormones used in HRT may lead to unacceptable adverse effects such as carcinogenicity in the endometrium[10]. Tamoxifen and raloxifene[11, 12], the

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nonsteroidal and selective estrogen receptor modulators, have shown cardioprotective benefits with reduced side effects, indicating that alternative estrogen-like agents may possess better therapeutic index. Therefore, interests have been directed to phytoestrogens derived from natural products, especially isoflavones present in soya beans[13]. An increasing number of studies suggest that some phytoestrogens could be promising substitutes for estrogen in preventing cardiovascular diseases through inhibiting VSMC proliferation and migration[14-16].

The flavonoid farrerol (Figure 1) is abundant in a traditional Chinese herb "Man-shan-hong", the dried leaves of *Rhododendron dauricum* L. It is considered to be the main bioactive ingredient of this plant and has been used as an antibechic in China[17]. In recent years, farrerol has attracted considerable interests due to its anti-inflammatory, antibacterial, and antioxidant activities exerted via scavenging radicals and inhibiting a variety of enzymes[18, 19]. However, to our knowledge, the effects of farrerol on cardiovascular system have not been reported to date. In view of the structural similarities between farrerol and estrogenic isoflavones (*eg*, genistein and daidzein), it would be tempting to learn if farrerol could act as a phytoestrogen in VSMCs. Thus, this study was designed and carried out to investigate the impact of farrerol on VSMC proliferation and its interaction with estrogen receptors.

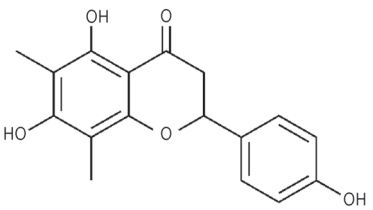

Figure 1. Chemical structure of farrerol.

Materials and methods

Reagents

Farrerol is a reference compound (purity ≥99.0%) supplied by the Division of Chinese Materia Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Ministry of Health, China. β-Estradiol 17-acetate (E_2), methyl-piperidino-pyrazole (MPP), 4-[2-[phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), ICI182,780, BrdU, and 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN) were obtained from Sigma (St Louis, MO, USA). Polyclonal anti-β-actin, monoclonal anti-phospho-ERK1/2 and polyclonal anti-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies to ERα and ERβ were procured from Abcam Inc (Cambridge, MA, USA).

Cell culture

Primary VSMCs were obtained from the thoracic aorta of 3-month old female Sprague-Dawley rats using the tissue explant method described elsewhere[20]. More than 98% of the cells were positive for staining with smooth muscle-specific α-actin, and exhibited the typical hill-and-valley morphology of VSMCs. Cells between passages 2 and 5 were used in this study to ensure the genetic stability of the culture. Cells grown to 80%−95% confluence were made quiescent by starvation (0.1% FBS) for 24 h.

Cell proliferation

The cell proliferation assay was performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Briefly, growth-arrested VSMCs were incubated with or without farrerol for 2 h prior to stimulation with 5% FBS for 48 h. They were then incubated with 0.5 mg/mL MTT for 4 h at 37 °C. Finally, the culture medium was removed and the formazan salt crystals was dissolved with 200 μL dimethylsulfoxide (DMSO) followed by shaking at room temperature for 10 min. The absorbance was read at a wavelength of 570 nm using a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

BrdU incorporation

DNA synthesis in VSMCs was examined using the BrdU incorporation assay previously described[21]. Quiescent VSMCs were treated with or without farrerol for 2 h prior to stimulation with 5% FBS for 24 h. For inhibition experiments, cells were pretreated with MPP or PHTPP for 30 min before addition of farrerol or DMSO.

Subsequently, 10 μmol/L BrdU was added to the cells and incubated for additional 24 h. To immunostain BrdU, the cells were washed with PBS, fixed in 4% polyformaldehyde and permeabilized with 0.1% Triton X-100. After DNA denaturation with 4 mol/L HCl, non-specific binding sites were blocked with 5% non-fat milk. The cells were then stained with an antibody to BrdU (Invitrogen, Carlsbad, CA, USA) followed by incubation with Alexa Flour 568 labeled goat anti-mouse IgG (Invitrogen) secondary antibodies. The cell nuclei were stained with Hoechst 33342 and evaluated by fluorescence microscopy with appropriate fluorescent filters. Results are presented as mitotic index, and defined as the percentage of BrdU-positive nuclei observed in a cell population.

Flow cytometry

Quiescent VSMCs were preincubated with or without farrerol for 2 h, followed by 5% FBS treatment for 24 h. They were then trypsinized, collected, and washed twice with cold PBS. Cell pellets were fixed in 70% ethanol and stored at 4 °C until use. The fixed cells were reacted with RNase A (10 μg/mL), DNA was stained with propidium iodide (50 μg/mL) for 30 min at 37 °C, and 1×10^4 cells were analyzed by flow cytometry. The rates of G_0/G_1, S, and G_2/M phases were determined using the software program ModiFit LT (BD, Franklin Lakes, NJ, USA).

Quantitative real-time PCR

Total RNA from VSMCs was extracted with the TRIzol reagent

(Invitrogen) and reverse transcribed (RT) using Superscript II First-strand Synthesis SuperMix (Invitrogen). Primers and real-time PCR conditions were described previously[22, 23]: β-actin: 5′-ATG GAT GAC GAT ATC GCT GCG-3′ (forward) and 5′-CAG GGT CAG GAT GCC TCT CTT-3′ (reverse); cyclin D1: 5′-TGC ATC TAC ACT GAC AAC TCT AT-3′ (forward) and 5′-GCA TTT TGG AGA GGA AGT GTT C-3′ (reverse); cyclin E: 5′-TGA AAT TGG TGT CGG TGC CTA T-3′ (forward) and 5′-TGC TCC TCC ATT AGG AAC TCT CAC-3′ (reverse); cyclin-dependent kinase 2 (CDK2): 5′-CTT AAG AAA ATC CGG CTC GAC-3′ (forward) and 5′-ATC CAG CAG CTT GAC GAT GTT A-3′ (reverse); CDK4: 5′-GAC TCC CAC AAC ATC CAG ACC-3′ (forward) and 5′-ACT CGG AGG AGG AGA AAT CCT-3′ (reverse); ERα: 5′-CTA AGA AGA ATA GCC CCG CC-3′ (forward) and 5′-CAG ACC AGA CCA ATC ATC AGG-3′ (reverse); ERβ: 5′-CGA CTG AGC ACA AGC CCA AAT G-3′ (forward) and 5′-ACG CCG TAA TGA TAC CCA GAT G-3′ (reverse). For RT-PCR, SYBR® Premix Ex Taq™ (Takara Bio Inc, Dalian, China) was used. Final PCR products were subjected to graded temperature-dependent dissociation to verify that only one product was amplified. Reactions with no RT sample and no template were included as negative controls. Relative quantitative evaluation of target gene levels was performed by the comparative CT (cycle threshold) method[24] and performed in triplicates.

Western blotting

VSMCs were lysed and equal amounts of protein from each sample were subjected to SDS-PAGE and blotted on PVDF membrane, which was incubated for 2 h at room temperature with blocking buffer (5% non-fat milk, 0.1% Tween 20, in TBS, pH 7.6) and then probed with primary antibodies overnight at 4 °C. After incubation with the appropriate secondary antibodies, the immunoreactive band was detected by an ECL Western blotting detection system (GE Healthcare, Bucks, UK) and subsequently photographed by a LAS-3000 luminescent image system (Fujifilm, Tokyo, Japan).

Receptor binding assays

For ER binding assay, an appropriate amount of estrogen receptor α/β (ERα/β) was loaded into each well of an Isoplate™ (PerkinElmer, Boston, MA, USA) containing the assay buffer [10% glycerol (*v/v*), NaH_2PO_4 25 mmol/L, $MgCl_2$ 0.5 mmol/L, DTT 1 mmol/L, edetic acid 1 mmol/L, CHAPS 5 mmol/L, aprotinin 2 mg/L and leupeptin 100 μmol/L] followed by addition of [^{3}H]estradiol (140 Ci/mmol, 5 nmol/L; PerkinElmer). Various concentrations of E_2 were added thereafter and incubated overnight at 4 °C. Twenty-five μL of hydroxyapatite (25%, *v/v*) was added to each well the next morning and the plates were gently agitated twice for 5 min each. Following centrifugation for 3 min at 2500 r/min (4 °C), the supernatant was decanted and 100 μL of assay buffer was added to each well. This washing procedure was repeated twice before the addition of 150 μL scintillation liquid (PerkinElmer), gentle agitation of the plates to resuspend hydroxyapatite and measurement of radioactivity with a MicroBeta counter (PerkinElmer).

For peroxisome proliferator-activated receptor γ (PPARγ) binding assay, biotinylated PPRE was mixed with the assay buffer containing fish sperm DNA (Shanghai Sangon Biotechnology, Shanghai, China) and 4 mg streptavidin-coated microbeads (GE Healthcare) in a conical polypropylene centrifuge tube, and incubated overnight at 4 °C. The mixture was centrifuged for 10 min at 2500 r/min. The supernatant was then removed followed by washing three times with 10 mL assay buffer. Reaction solution (10 mL) containing 700 μg PPARγ extract protein, 47 μg retinoid X receptor α (RXRα) extract protein, 10 nmol/L [^{3}H]BRL49653 (American Radiolabeled Chemicals, Inc, St Louis, MO, USA), and various concentrations of BRL49653 or farrerol were distributed to each well of an Isoplate™ (PerkinElmer) and incubated at 4 °C for 4 h before counting by a MicroBeta counter (PerkinElmer).

Reporter gene assay

pERE-Luc reporter (0.5 μg/well of 24-well plate) or pGL3-Luc (negative control) plasmids were transfected into VSMCs with Lipofectamine™ (Invitrogen) and the procedure was carried out as directed by the manufacturer. pERE-Luc contains tandems of ERE linked to a downstream luciferase reporter gene. pGL3-Luc is an identical plasmid but without the ERE. The pSV-β-galactosidase plasmid (pCH110) was co-transfected as an internal control. After transfection for 24 h, cells were treated in conditioned medium and harvested following further 24 h incubation. Luciferase activity was assayed according to the manufacturer's protocol using the luciferase assay system (Promega, Madison, WI, USA). Relative luciferase units (RLU) normalized to transfection efficiency were calculated as the ratio of luciferase activity to β-galactosidase.

Gal-ERα and Gal-ERβ were constructed by transferring the coding sequences for the ligand-binding domain (LBD) of ERα (amino acids 282–595) or ERβ (amino acids 234–530) to yeast transcription activator protein Gal4, respectively. Gal-ERα or Gal-ERβ, together with the 5×UAS-Luc reporter and pSV-β-galactosidase plasmids, were co-transfected into HEK293 cells according to the manufacturer's instructions. Luciferase activity and β-galactosidase activity were determined as described above.

Statistical analysis

Results are expressed as mean±SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA). The inter-group comparisons (*post hoc* analysis) among the data with equal variances were made by the LSD method, while Tamhane's T2 method was used for the data with unequal variances. A P value of less than 0.05 is considered significant.

Results

Farrerol inhibits FBS-induced VSMC proliferation and DNA synthesis

In this study, we first investigated the effect of farrerol on the proliferation of VSMCs using the MTT assay. When growth-arrested cells were treated with farrerol (0.3, 1, 3, and

10 μmol/L) in the presence of 0.1% FBS, no significant difference was observed in cell viability (Figure 2A, left panel), suggesting that farrerol did not show significant cytotoxicity up to 10 μmol/L. The absence of cytotoxicity was further confirmed with a trypan blue exclusion assay (data not shown). However, we found that 5% FBS treatment led to a 2.65-fold increase in VSMC proliferation, while incubation of the cells with farrerol for 2 h prior to FBS stimulation reduced cell proliferation in a dose-dependent manner (Figure 2A, right panel). The influence of farrerol on DNA synthesis was also studied: BrdU incorporation was markedly increased in VSMCs following exposure to 5% FBS for 48 h, indicative of elevated DNA synthesis; this effect was abolished by pretreatment of VSMCs with farrerol in a dose-dependent manner and the complete blocking was achieved at the highest concentration (10 μmol/L) used (Figure 2B).

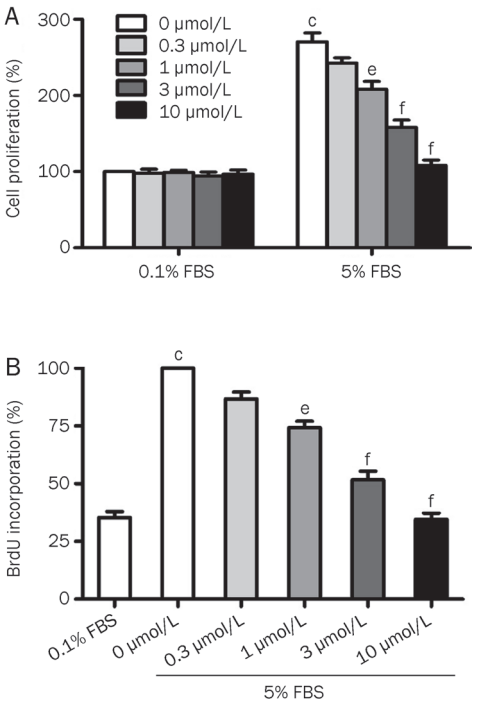

Figure 2. Effects of farrerol on FBS-induced proliferation and DNA synthesis in VSMCs. A, proliferation was measured by the MTT assay in the absence (left) or presence (right) of 5% FBS. Relative proliferation (%) was displayed using untreated control cells as a standard (n=6). B, DNA synthesis was measured by BrdU incorporation. The left part shows BrdU incorporation of quiescent and FBS-stimulated VSMCs. On the right side, a concentration-dependent decrease of BrdU incorporation in farrerol-treated VSMCs is shown (n=6). Values are presented as mean±SEM. ^{c}P<0.01 *vs* control; ^{e}P<0.05, ^{f}P<0.01 *vs* FBS induction.

Farrerol arrests FBS-stimulated VSMCs in G_1 phase and abrogates cell cycle protein transcription

Proliferative cells pass through several cell cycle checkpoints, mainly the G_1 to S and G_2 to M transitions. The former checkpoint is considered to be the most important step in DNA replication. Accordingly, flow cytometric assessment was performed to determine the effect of farrerol on cell cycle progression. As shown in Figure 3, the percentage of G_0/G_1 or S phase cells in the 5% FBS-stimulated group were 59.32%±2.73% and 18.30%±2.62%, respectively. Farrerol at concentrations of 3 and 10 μmol/L effectively increased the proportion of cells in the G_0/G_1 phase and simultaneously decreased the S phase cell population.

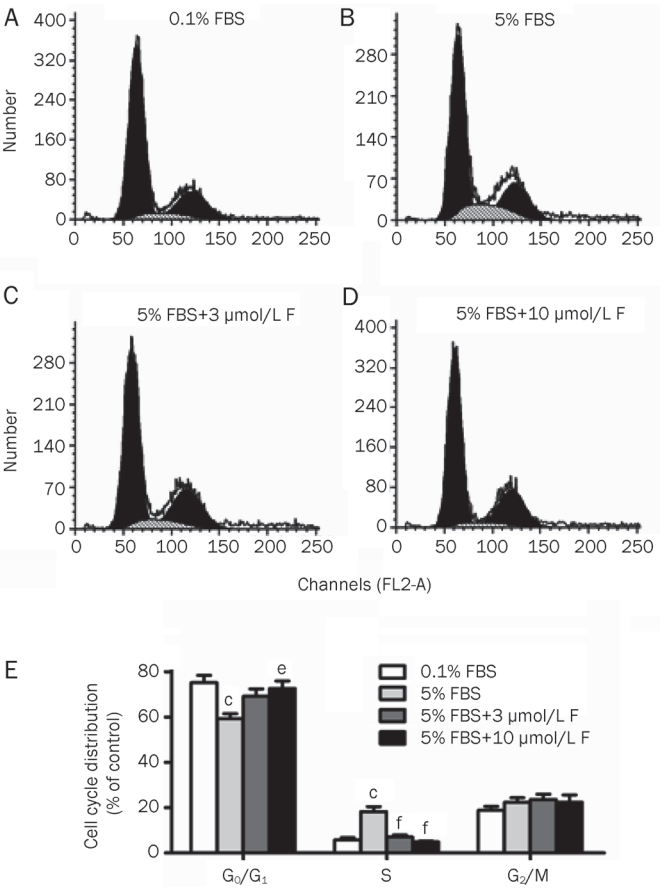

Figure 3. Cell cycle distribution of (A) quiescent and (B) FBS-stimulated VSMCs. C and D show VSMCs treated with 3 μmol/L and 10 μmol/L farrerol (F), respectively, in the presence of FBS. It demonstrates farrerol-induced cell cycle arrest at the G_0/G_1 phase. G_0/G_1 phase is represented by the first peak, S phase in diagonal and G_2/M by the second peak. E, results are expressed as a percentage of the total number of cells in G_0/G_1, S, or G_2/M phases of the cell cycle. Values are presented as mean±SEM. ^{c}P<0.01 *vs* control; ^{e}P<0.05, ^{f}P<0.01 vs FBS induction.

Then, we examined the mRNA levels of cell cycle-related proteins using real-time PCR to determine whether farrerol induced changes in this pathway. The results were analyzed by the comparative CT method. Treatment with farrerol not only produced a significant down-regulation in cyclin D1 and E gene transcription, but also markedly decreased the expression of CDK2 and CDK4 genes (Figure 4A).

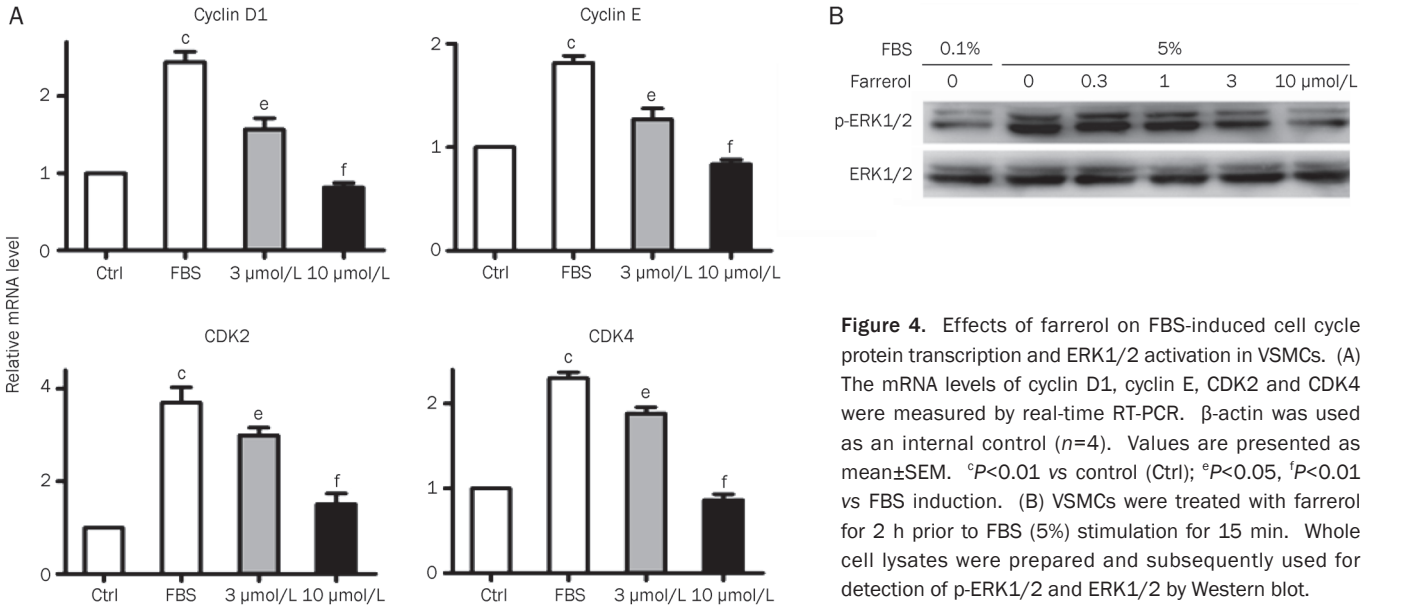

Figure 4. Effects of farrerol on FBS-induced cell cycle protein transcription and ERK1/2 activation in VSMCs. (A) The mRNA levels of cyclin D1, cyclin E, CDK2 and CDK4 were measured by real-time RT-PCR. β-actin was used as an internal control (n=4). Values are presented as mean±SEM. ^{c}P<0.01 *vs* control (Ctrl); ^{e}P<0.05, ^{f}P<0.01 *vs* FBS induction. (B) VSMCs were treated with farrerol for 2 h prior to FBS (5%) stimulation for 15 min. Whole cell lysates were prepared and subsequently used for detection of p-ERK1/2 and ERK1/2 by Western blot.

Farrerol suppresses FBS-induced ERK1/2 phosphorylation in VSMCs

It was reported that ERK1/2 activation plays a critical role in the proliferation of VSMCs[21]. Inhibition of this signaling protein proved to be a useful method for preventing intimal hyperplasia[25]. Therefore, we examined the effect of farrerol on ERK1/2 activation and the results indicate that exposure of VSMCs to FBS for 15 min enhanced ERK1/2 phosphorylation considerably. Conversely, addition of farrerol dose-dependently abrogated ERK1/2 activation in FBS-stimulated VSMCs: at a concentration of 10 μmol/L, farrerol completely blocked the phosphorylation of ERK1/2 (Figure 4B).

Farrerol is a functional ERβ ligand in VSMCs

To evaluate the possible role of ER in farrerol-induced antimitogenic activity, we first evaluated the expression of ERα and ERβ by both Western blot analysis and real-time PCR in VSMCs. Figure 5A depicts that protein and mRNA of both ER subtypes were present in the VSMCs derived from female Sprague-Dawley rats, as reported previously[26].

Thereafter, an ERE-luciferase reporter gene assay was performed to study whether farrerol acts as a phytoestrogen in VSMCs. As shown in Figure 5B, farrerol was able to activate ER transcription from a luciferase reporter gene (pERE-luc) under the control of a promoter containing two copies of the ERE in VSMCs, showing a 2.1-fold induction over control and the efficacy was comparable to that of E_2. The ERE-driven luciferase activity induced by farrerol was completely blocked by the ERα/β antagonist ICI182,780, but not by the specific ERα antagonist MPP, implying that farrerol may exert its phytoestrogen action via ERβ.

To obtain further evidence that farrerol activates ERβ directly, a heterologous system was employed. Chimeric protein consisting of the DNA binding domain of Gal4 and ERβ LBD, but not ERα LBD, responded to farrerol in a transient expression assay in HEK293 cells (Figures 5C and 5D), thereby pointing to a mechanism of direct interaction between farrerol and ERβ signaling.

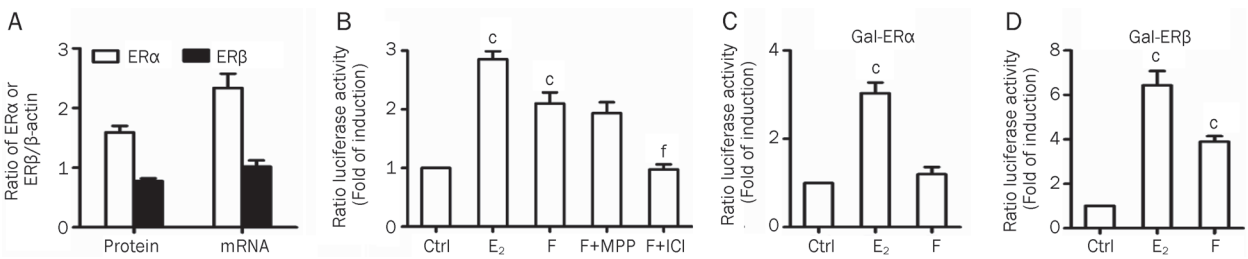

Figure 5. Activation of ERβ-mediated transcription by farrerol in reporter gene assays. A, expression of ERα and ERβ in VSMCs. Western blot and real-time PCR were performed on VSMCs for ERα and ERβ protein expression and mRNA quantification. β-actin was used as an internal control (n=3). B, farrerol activates ERE-mediated transcription in VSMCs. Cells cotransfected with pERE-luc and β-gal expression plasmids were treated with 3 μmol/L farrerol (F) with or without MPP (10 μmol/L) or ICI182,780 (ICI, 10 μmol/L). E_2 (100 nmol/L) was used as a positive control. Relative luciferase units (RLU) normalized to transfection efficiency were calculated as the ratio of luciferase activity to β-galactosidase (n=3). C, fusion protein consisting of ERβ and Gal4 DBD was activated by farrerol in transfected HEK293 cells (n=3). Values are presented as mean±SEM. ^{c}P<0.01 *vs* control (Ctrl); ^{f}P<0.01 *vs* farrerol treatment.

Next, a competitive receptor-binding assay was employed to study binding specificities of farrerol for ERα and ERβ. As shown in Figure 6, the IC_{50} values of farrerol for ERα and ERβ were 57 μmol/L and 2.7 μmol/L, respectively, implying that farrerol has a higher binding specificity for ERβ than ERα. Moreover, cross-reactivity check revealed that the binding affinity of farrerol to PPARγ is rather weak (IC_{50}=238 μmol/L; Figure 6). These data suggest that farrerol appears to be a selective ERβ agonist.

Farrerol exerts its action on DNA synthesis mainly via ERβ in VSMCs

Previous work has suggested the involvement of ERβ in the inhibitory effects of estrogen on VSMCs[27]. Our results also showed that the selective ERβ agonist 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN, 1 μmol/L) closely resembled E_2 in significantly reducing DNA synthesis in VSMCs (data not shown).

Having established that farrerol is an ERβ agonist, PHTPP, a specific ERβ antagonist was chosen to characterize the role of ERβ in farrerol-mediated effects. Figure 7 shows that blockade of ERβ signaling by PHTPP noticeably attenuated the inhibitory effect of farrerol on VSMC proliferation while MPP, a specific ERα antagonist was ineffective. Furthermore, neither PPARγ antagonist GW9662 nor PPARα antagonist GW6471 was able to reverse the inhibitory effects of farrerol on VSMCs (data not shown). Taken together, the results indicate that ERβ is involved in farrerol-mediated inhibition on VSMC proliferation.

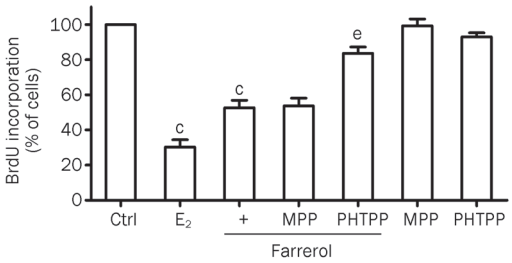

Figure 7. Inhibition on VSMC DNA synthesis by farrerol was reversed by a specific ERβ antagonist, PHTPP. Cells were treated with or without 3 μmol/L farrerol, in the absence or presence of PHTPP (5 μmol/L) or MPP (10 μmol/L). E_2 (100 nmol/L) was used as a positive control (n=3). Values are presented as mean±SEM. ^cP<0.01 *vs* control (Ctrl); ^eP<0.05 *vs* farrerol treatment.

Discussion

The development of advanced lesions associated with atherosclerosis and restenosis is highly dependent on VSMC proliferation. Thus, inhibition of VSMC proliferation has become one of the focal points in the prevention of atherosclerosis[3]. Farrerol, a naturally occurring flavonoid derivative, is found in various plants and more abundantly in *Rhododendron dauricum* L. Here we show that farrerol possesses estrogenic properties and could mimic E_2 to suppress VSMC proliferation *in vitro*. We also demonstrate that the effect exerted by farrerol on cell cycle involves the participation of ERβ, but not ERα. Our data thus provide a potential molecular target through which farrerol manifests itself as an anti-mitogenic agent.

In this study, we replaced a single growth factor with 5% FBS to induce cell proliferation. FBS contains a range of growth factors, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor, serotonin, and thrombin[18]. It was hoped that this would simulate the multiple factors environment *in vivo*. Indeed, addition of 5% FBS to the culture medium resulted in a 2.65-fold increase in VSMC proliferation that was blocked by farrerol in a concentration-dependent manner (Figure 2).

Cell cycle is a highly regulated process that involves a complex cascade of events. Modulation of expression and function of cell cycle regulatory proteins provides an important approach to the control of cell growth. According to the flow cytometry analysis, farrerol profoundly decreased FBS-induced transition to the S phase (Figure 3). It is known that the move from G_1 to S phase is closely linked to the activation of cell cycle regulatory proteins such as CDKs[28]. In particular, the cyclin D1/CDK4 and cyclin E/CDK2 complexes are essential for entering the S phase[28]. As shown in Figure 4A, farrerol displayed a potent inhibitory effect on the expression of a number of cell cycle regulatory proteins (cyclin D1, cyclin

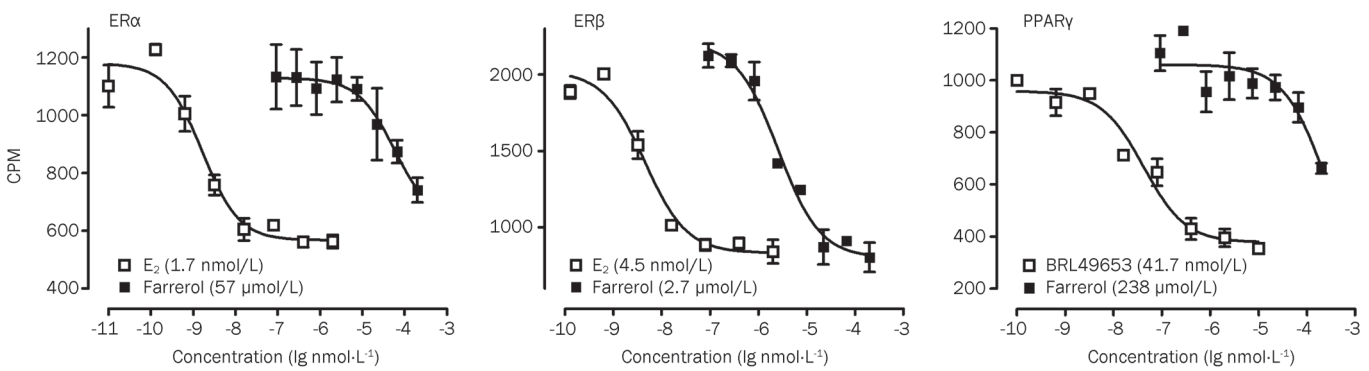

Figure 6. Specificity of farrerol for ERβ. Competitive receptor-binding assay was conducted to assess interaction between radiolabeled ligands and recombinant human ERα, ERβ, and PPARγ. Values in parentheses indicate respective IC_{50} (μmol/L) of farrerol for each receptor. Data are presented as mean±SEM.

E, CDK2, and CDK4). It appears that farrerol exerts its action through down-regulation of positive regulators in the cell cycle.

Cell cycle modulators such as cyclin D1 and cyclin E are regulated by ERK1/2 pathway, which plays a crucial role in serum-induced VSMC proliferation[28]. After mitogenic stimulation, ERK1/2 is phosphorylated by MEK kinase and enters the nucleus, where it regulates the expression of cell cycle regulators and subsequent DNA synthesis in VSMCs[29]. In our study, farrerol significantly decreased FBS-induced phosphorylation of ERK1/2 thereby suggesting that farrerol is a negative effecter for ERK1/2 activation leading to suppression on the expression of cell cycle regulators and eventual growth arrest.

An interesting observation relates to the fact that farrerol interacts directly with ERβ, rather than ERα, in VSMCs. In the ERE-reporter gene assay, farrerol was able to activate transcription from the consensus ERE motifs, while specific induction of Gal-ERβ-mediated transactivation and direct binding to ERβ confirmed the relationship between this phytoestrogen and ERβ signaling machinery.

The mechanism by which of estrogens exert inhibitory actions on VSMCs has been intensely investigated following the discovery of a second ER subtype, ERβ. Earlier studies in mice showed that estrogen inhibited VSMC proliferation in the medial area in response to vascular injury in both ERα[30] and ERβ knockout mice[31], indicating that both subtypes are responsible for the anti-mitogenic effect observed. Moreover, it was documented that ERβ was more potent than ERα in inhibiting VSMC proliferation[23, 32]. Taken together, it seems that ERβ behaves like a key mediator for estrogen-related vascular protection. Since isoflavones are capable of preferentially inducing ERβ expression, their beneficial effects on the cardiovascular system of postmenopausal women may be mediated via this pathway[33, 34].

In conclusion, our study provides new experimental evidence supporting the postulated property of farrerol as a functional phytoestrogen capable of inhibiting FBS-induced VSMC proliferation. The effects exerted by farrerol are cell cycle related and ERβ specific. Elucidation of underlying mechanisms of action may offer insights into a possible molecular target relevant to the therapeutic use of this class of natural products, represented by farrerol, in certain cardiovascular diseases, where excess proliferation plays a pivotal pathological role.

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Author contribution

Qun-yi LI, Li CHEN, and Meng ZHANG performed experiments; Qun-yi LI, Li CHEN, Yan-hui ZHU, Yi-ping WANG, and Ming-wei WANG analyzed the data; Qun-yi LI, Li CHEN, and Ming-wei WANG wrote the paper.

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