RESEARCH ARTICLE

Inhibition of neointimal formation by *trans*-resveratrol: Role of phosphatidyl inositol 3-kinase-dependent Nrf2 activation in heme oxygenase-1 induction

Jung Woo Kim[1], Sung Chul Lim[2], Moo Yeol Lee[3], Jeong Woon Lee[1], Won Keun Oh[1], Sang Kyum Kim[4] and Keon Wook Kang[1]

[1] BK21 Project Team, College of Pharmacy, Chosun University, Gwangju, Republic of Korea
[2] Department of Pathology, College of Medicine, Chosun University, Gwangju, Republic of Korea
[3] College of Pharmacy, Chonnam National University, Gwangju, Republic of Korea
[4] College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea

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Neointima, defined as abnormal growth of the intimal layer of blood vessels, is believed to be a critical event in the development of vascular occlusive disease. Although resveratrol's inhibitory effects on proliferation and migration of vascular smooth muscle cells has been reported, its activity on neointimal formation is still unclear. Oral administration of *trans*-resveratrol significantly suppressed intimal hyperplasia in a wire-injured femoral artery mouse model. In cultured vascular smooth muscle cells, *trans*-resveratrol inhibited platelet-derived growth factor-stimulated DNA synthesis and cell proliferation with down-regulation of cyclin D and pRB. Moreover, platelet-derived growth factor-induced production of reactive oxygen species was inhibited by *trans*-resveratrol and the compound induced heme oxygenase-1 (HO-1). The anti-proliferative activity of *trans*-resveratrol was reversed by an HO-1 inhibitor, ZnPPIX. Subcellular fractionation and reporter gene analyses revealed that *trans*-resveratrol increased the level of nuclear Nrf2 and antioxidant response element reporter activity, and that these were essential for the induction of HO-1. *Trans*-resveratrol also enhanced the activities of phosphatidyl inositol 3-kinase and extracellular signal regulated kinase, and phosphatidyl inositol 3-kinase was required for Nrf2/antioxidant response element-dependent HO-1 induction. These data have significant implications for the elucidation of the pharmacological mechanism by which *trans*-resveratrol prevents vascular occlusive diseases.

Keywords:
Heme oxygenase-1 / Neointima / Nrf2 / Phosphatidyl inositol 3-kinase
Trans-resveratrol

1 Introduction

Resveratrol is a natural polyphenolic stilbene that is frequently found in grapes and other food products [1, 2]. The compound is the principal active component of red wine, and its intake is inversely correlated with the incidence of chronic cardiovascular diseases such as atherosclerosis and vascular thrombosis [3, 4]. Many studies have found that *trans*-resveratrol prevents the progress of cardiovascular diseases. *Trans*-resveratrol attenuates cardiac hypertrophy in spontaneously hypertensive rats *via* AMP kinase activation [5] and decreases blood pressure in

Correspondence: Dr. Keon Wook Kang, BK21 Project Team, College of Pharmacy, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501-759, Republic of Korea
E-mail: kwkang@chosun.ac.kr
Fax: +82-62-222-5414

Abbreviations: ARE, antioxidant response element; **BrdU,** 5-bromo-2′-deoxy-uridine; **DA,** diacetate; **DCFH,** 2′, 7′-dichlorodihydrofluorescein; **ERK,** extracellular signal regulated kinase; **HO-1,** heme oxygenase-1; **iNOS,** inducible nitric oxide synthase; **MEF,** mouse embryonic fibroblast; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **NO,** nitric oxide; **PDGF,** platelet-derived growth factor; **PI3-kinase,** phosphatidyl inositol 3-kinase; **PPAR,** peroxisome proliferator-activated receptor; **ROS,** reactive oxygen species; **VSMC,** vascular smooth muscle cell

hypertensive rats [6]. The compound also suppresses development of myocarditis [7] and atherogenic lesion formation [8].

Neointima, defined as abnormal growth of the intimal layer of blood vessels, is believed to be a critical event in the development of vascular occlusive disease. In particular, neointima is responsible for both restenosis following angioplasty and formation of atherosclerotic lesions [9]. Synthetic phenotype acquisition of vascular smooth muscle cells (VSMC) is a key event during neointimal formation in restenotic areas. Because the initial pathogenesis of neointimal formation is mediated through VSMC proliferation, suppression of VSMC proliferation could be a useful therapeutic intervention for attenuating the incidence of vascular occlusive diseases. In fact, paclitaxel, a potent cell proliferation inhibitor, has been clinically used as a coating material for stents. Although several studies found that *trans*-resveratrol suppressed proliferation and migration of VSMC [10, 11], direct evidence showing an inhibitory effect of *trans*-resveratrol on neointimal formation has not been reported. In the present study, we tried to determine whether *trans*-resveratrol inhibits intimal hyperplasia in guide-wire injured femoral arteries of mice. Resveratrol has also been shown to modulate diverse cell cycle regulatory genes (*e.g.* p53, Rb and cyclins) and these are related with its anti-cancer or anti-proliferation effect [12, 13]. Because diverse agents blocking cell cycle progression are effective to suppress intimal hyperplasia [14], we also tested the effect of *trans*-resveratrol on the expression of cell cycle regulatory proteins.

Heme oxygenase is an anti-oxidant enzyme that catalyzes the degradation of heme to carbon monoxide, free iron, and biliverdin. Heme oxygenase-1 (HO-1) can be induced in VSMC and endothelial cells and functions as an endogenous inhibitor of VSMC proliferation [15]. Biliverdin/bilirubin, the final product from HO-1 catalysis, diminishes injury-induced VSMC proliferation and narrowing of the vascular lumen. Moreover, HO-1-null mice showed exacerbated neointimal formation and myocardial ischemia/reperfusion injury [16, 17]. In the present study, we further examined whether *trans*-resveratrol's inhibitory effect on VSMC proliferation is associated with its HO-1 inducing effect, and we studied its molecular mechanism, focusing on cellular signaling pathways and transcription factors for *HO-1* gene expression induced by *trans*-resveratrol in VSMC.

2 Materials and methods

2.1 Materials

Anti-HO-1 antibody was obtained from Assay Designs (Ann Arbor, MI). Horseradish peroxidase-conjugated donkey anti-rabbit and alkaline phosphatase-conjugated donkey anti-mouse IgGs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). 5-Bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium was purchased from Life Technologies (Gaithersburg, MD), and anti-Nrf2 and anti-p65 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). pGL3-antioxidant response element (ARE) reporter plasmid containing three copies of the quinone oxidoreductase ARE was donated by Dr. Kwak M. K. (Yeungnam University, Korea). Most reagents including *trans*-resveratrol for the molecular studies were obtained from Sigma (St. Louis, MO).

2.2 Mouse femoral artery injury model and immunohistochemistry

The institutional animal care and utilization committee of Chosun University approved all the animal procedures used in this study. Transluminal mechanical injury of bilateral femoral arteries was induced by introducing a large wire, as previously reported [18]. In brief, the left femoral artery was exposed by blunt dissection, and was looped proximally and distally with 6-0 silk suture for temporal control of blood flow during the procedure. A straight spring wire, 0.38 mm in diameter (Cook, Bloomington, IN), was carefully inserted into the femoral artery and advanced toward the iliac artery and then left in place for 1 min. *Trans*-resveratrol (50 mg/kg) was dissolved in solubilization solvent (PEG400, Tween 80, ethanol, and sterile water) and orally administered three times a week, from 1 day before femoral artery injury to the day of sacrifice. The solubilization solvent was administered to mice in sham-operated and wire-injured groups as vehicle. At 21 days after injury, mice were sacrificed and pressure-perfused at 100 mm Hg with 0.9% sodium chloride solution followed by pressure-fixation with a 4% paraformaldehyde solution. The femoral artery was then carefully excised and paraffin-embedded and stained with hematoxylin and eosin for the determination of intimal and medial thickness.

2.3 Cell culture

VSMC were isolated from rat thoracic aorta as described previously [18]. Briefly, the aortas were removed, cleaned of connective tissue, fat, and endothelium, and digested with collagenase and elastase to remove the adventitia and to dissociate the VSMC. Mouse embryonic fibroblast (MEF) cells were obtained from Dr. Choi HS (Chosun University, Gwangju, Korea). Isolated VSMCs and MEF cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. For the cell culture studies, *trans*-resveratrol powder was dissolved in dimethylsulfoxide immediately before use.

2.4 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay

Viable adherent cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 2 mg/mL) for 4 h. Media were then removed and the formazan crystal-stained cells were dissolved in 200 μL dimethylsulfoxide. Absorbance was assayed at 540 nm using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

2.5 5-Bromo-2′-deoxy-uridine assay

Viable adherent cells were incubated with 5-bromo-2′-deoxy-uridine (BrdU) labeling solution (10 μM) for 2 h. Cells were fixed with fixation solution for 30 min at room temperature and incubated with 100 μL anti-BrdU peroxidase-labeled antibody for 90 min. After three washings, substrate solution for colorimetric quantification was added at a final concentration of 100 μL/mL and left at room temperature for 5–30 min until color development was sufficient for photometric detection. The absorbance was assayed at 405 nm.

2.6 Preparation of nuclear extracts

Cells in dishes were washed with ice-cold PBS, scraped, transferred to microtubes, and allowed to swell after adding 100 μL of hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. The lysates obtained were incubated for 10 min on ice and centrifuged at 7200 × *g* for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μL of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride and then incubated for 30 min on ice. The samples were centrifuged at 15 800 × *g* for 10 min to obtain supernatants containing nuclear fractions, which were stored at −80°C until required.

2.7 Immunoblot analysis

After washing the VSMC twice with sterile PBS, they were scraped into microtube and lysed with lysis buffer (50 mM Tris, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, pH 7.6). SDS-PAGE and immunoblot analysis were performed as previously described [19]. Samples were separated by 10% gel electrophoresis, electrophoretically transferred to nitrocellulose paper, incubated with primary antibodies, then incubated with a horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibody, and developed using an ECL® chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK) or 5-bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium solution. Equal protein loading was verified by immunoblotting with actin antibody.

2.8 Determination of the production of reactive oxygen species

Reactive oxygen species (ROS) production in VSMC was monitored spectrofluorometrically using 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorescent dye [20]. VSMCs were incubated with or without *trans*-resveratrol for 12 h in serum-free DMEM, rinsed twice with sterile PBS, and incubated in phenol red-free DMEM for an additional 30 min. Then, DCFH-DA was added to VSMC (final concentration = 5 μM) and the dye-loaded cells were further incubated with 30 ng/mL platelet-derived growth factor (PDGF) for 30 min following measurement of the initial fluorescence. Oxidation of DCFH by peroxides yielded the fluorescent derivative dichlorofluorescein. Fluorescence was monitored using an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence plate reader (Varioskan, Thermoeletron, USA). Data were expressed as changes relative to the initial fluorescence.

2.9 Reporter gene assay

MEF cells were cultured in a 12-well plate and transiently transfected with pGL3-ARE plasmid, NF-κB-Luciferase or PPRE-Luc and the phRL-SV plasmid (*hRenilla* luciferase expression for normalization)(Promega, Madison, WI) using Hillymax® reagent (Dojindo Molecular Tech., Gaithersburg, MD). The cells were then incubated in culture medium without serum for 18 h, and the firefly and *hRenilla* luciferase activities in the cell lysates were measured using a luminometer (Berthold Tech.). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to *hRenilla* luciferase.

2.10 Scanning densitometry and statistics

Scanning densitometry was performed using an image scan and analysis system of LAS-3000mini (Fujifilm, Tokyo, Japan). One way analysis of variance procedures were used to assess significant differences between treatment groups. When treatment was found to have a significant effect, the Newman–Keuls test was used to compare multiple group means. Statistical significance was accepted at either $p < 0.05$ or $p < 0.01$.

3 Results

3.1 *Trans*-resveratrol inhibits neointimal formation

We first measured the effect of *trans*-resveratrol on neointimal formation induced by guide-wire artery injury.

Oral administration of *trans*-resveratrol (50 mg/kg, three times a week for 3 wk) significantly inhibited intimal hyperplasia compared with the vehicle-treated group (Fig. 1).

3.2 *Trans*-resveratrol inhibits PDGF-stimulated VSMC proliferation

PDGF, a key mediator in the proliferation of VSMC, plays an important role in neointima formation [21]. Hence, we then assessed the effect of *trans*-resveratrol on PDGF-induced VSMC proliferation. MTT and BrdU assays revealed that incubation of serum-deprived VSMC with PDGF (30 ng/mL) for 48 h increased both DNA synthesis and cell number, compared with the untreated control group (Figs. 2A and B). Pretreatment with *trans*-resveratrol (3–100 μM) for 8 h significantly suppressed PDGF-induced DNA synthesis and proliferation in a concentration-dependent manner (Figs. 2A and B).

3.3 *Trans*-resveratrol changes the expression of cell cycle regulators

The effect of *trans*-resveratrol on the expression of cell cycle regulators was examined to assess whether the compound caused growth inhibition *via* cell cycle regulation. The expression levels of cyclin D1, cyclin D3, and pRb were suppressed by *trans*-resveratrol (Fig. 3). However, cyclin E and total levels of Rb were not distinctly changed by *trans*-resveratrol. These findings suggest that the inhibitory effect of *trans*-resveratrol on VSMC proliferation is related to cell cycle inhibition.

3.4 HO-1 induction is essential for the anti-proliferative effect of *trans*-resveratrol

The transition of non-proliferating VSMCs to proliferating ones is accompanied by the sustained production of ROS [22]. Given the role of ROS generation in VSMC proliferation, the expression levels of HO-1 in the injured vasculature may determine the extent of neointimal formation. In fact, HO-1 functions as a defensive factor during atherosclerosis or restenosis [23, 24]. Western blot analyses showed that incubation of VSMC with PDGF (30 ng/mL) for 12 h slightly induced HO-1, but co-treatment with *trans*-resveratrol (1–30 μM) concentration-dependently potentiated HO-1 protein expression (Fig. 4A). Moreover, PDGF-mediated peroxide production was efficiently inhibited in *trans*-resveratrol (10 and 30 μM)-treated VSMC (Fig. 4B). Thus, the inhibitory effects of *trans*-resveratrol on VSMC proliferation and intimal hyperplasia may be associated with its HO-1 inducing effect and subsequent ROS suppression.

To test whether an HO-1 increase is required for the anti-proliferative effect of *trans*-resveratrol, we then assessed whether ZnPPIX, an HO-1 inhibitor, reverses the inhibitory effect of *trans*-resveratrol on PDGF-mediated cell proliferation. MTT and BrdU assays revealed that co-treatment with ZnPPIX (1 and 5 nM) significantly reversed the inhibitory effect of *trans*-resveratrol (30 μM) on VSMC proliferation (Figs. 4C and D).

3.5 Nrf2/ARE activation is required for HO-1 induction by *trans*-resveratrol

Expression of the *HO-1* gene depends on several transcription factors such as Nrf2, NF-κB, and peroxisome proliferator-activated receptor (PPAR) in the gene promoter

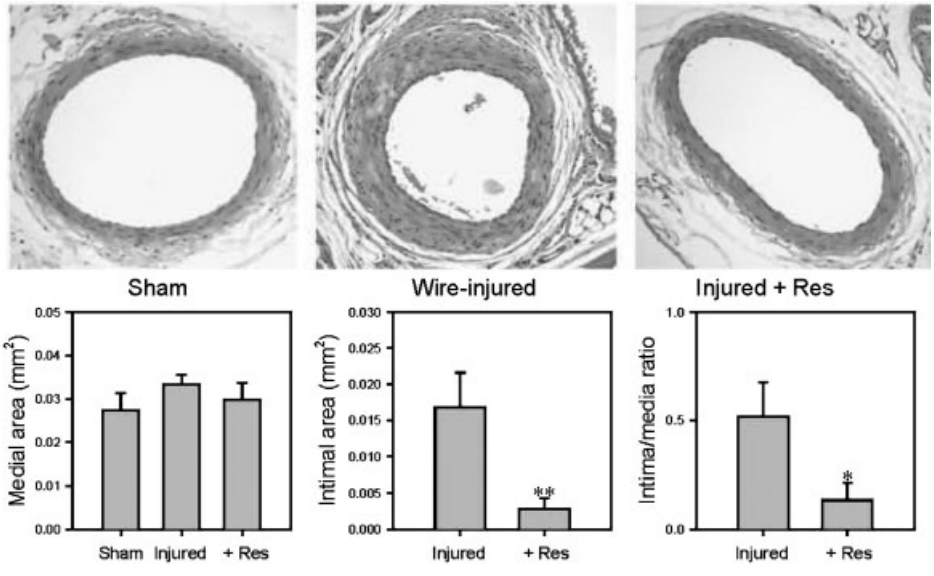

Figure 1. Suppression of neointimal formation and VSMC proliferation by *trans*-resveratrol. Representative pictures (magnification × 200) of H&E (hematoxylin and eosin)-stained femoral arteries obtained from guide wire injured mice (3 wk). Data represent the mean ± SD of three to six different samples (significant as compared with wire-injured group, $^{*}p < 0.05$; $^{**}p < 0.01$).

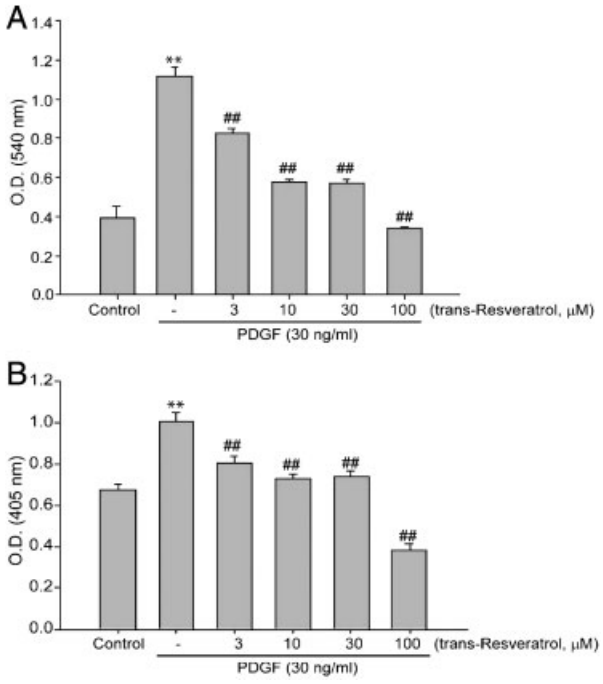

Figure 2. Inhibitory effects of *trans*-resveratrol on PDGF-induced VSMC proliferation and DNA synthesis. (A) VSMC proliferation. VSMC were treated with or without PDGF (30 ng/mL) in the presence or absence of *trans*-resveratrol (3–100 μM) for 48 h. *Trans*-resveratrol was treated 8 h before PDGF treatment. Data represent the mean ± SD of eight different samples (significant as compared with control group, $^{**}p<0.01$; significant as compared with PDGF alone treated group, $^{\#\#}p<0.01$). (B) DNA synthesis. VSMC were treated same as described in (A) and BrdU assays were performed. Data represent the mean ± SD of six different samples (significant as compared with control group, $^{**}p<0.01$; significant as compared with PDGF alone-treated group, $^{\#\#}p<0.01$).

[25–27]. In particular, it has been shown that resveratrol at low concentrations (1–10 μM) increased HO-1 in human aortic smooth muscle cells in which NF-κB binding at the *HO-1* promoter is actively involved [26]. However, we found that nuclear translocation of p65, the active subunit of NF-κB, was not altered by 30 μM *trans*-resveratrol (30 μM is the most active concentration for the induction of HO-1 by *trans*-resveratrol in rat VSMCs). Because transfection efficiency in rat VSMC is extremely low, NF-κB-dependent reporter activity was performed using MEF cells [28]. NF-κB reporter activity was not significantly changed in MEF cells treated with 3–30 μM *trans*-resveratrol (Fig. 5A). These results imply that NF-κB is not an active transcription factor for HO-1 induction by *trans*-resveratrol.

Nuclear localization of Nrf2 in response to diverse insults causes Nrf2 to bind to its target sequence, ARE, and regulate HO-1 expression [25, 29, 30]. Subcellular fractionation and Western blot analyses of VSMCs showed that *trans*-resveratrol increased the level of Nrf2 in the nuclear fraction at 1–12 h, and maximal band intensity was found at 12 h

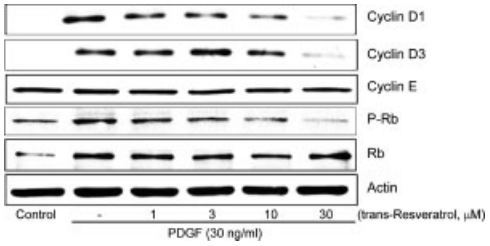

Figure 3. Effect of *trans*-resveratrol on cell cycle regulators. VSMC were treated with 30 ng/mL PDGF in the presence or absence of *trans*-resveratrol (1–30 μM) for 12 h. Total cell lysates were subjected to immunoblotting.

(Fig. 5B, upper panel). MEF cells were transfected with pGL3-ARE, a minimal ARE reporter containing three copies of the quinone oxidoreductase ARE sequence [31]. *Trans*-resveratrol (3–30 μM) concentration-dependently increased the reporter activity of pGL3-ARE in transiently transfected MEF cells (Fig. 5B, lower panel). Hence, HO-1 induction by *trans*-resveratrol may result from Nrf2/ARE activation.

Treatment of vascular endothelial and smooth muscle cells with PPAR ligands leads to expression of HO-1 [27] and a recent study has shown that HO-1 induction by PPAR ligands is not mediated through PPAR-γ binding [32]. We further determined PPAR-dependent transcription activity using a PPRE-Luc reporter in *trans*-resveratrol-treated MEF cells. *Trans*-resveratrol did not change PPRE reporter activity (Fig. 5C), which suggests that *trans*-resveratrol does not function as a PPAR ligand.

3.6 Phosphatidyl inositol 3-kinase activation is required for the induction of HO-1 by *trans*-resveratrol

Transcription factors can be phosphorylated by kinases in response to stimuli [33]. It has been reported that phosphatidyl inositol 3-kinase (PI3-kinase), extracellular signal regulated kinase (ERK), and p38 kinase regulate HO-1 expression [34–36]. Hence, we measured levels of phosphorylated Akt, ERK, and p38 kinase to determine activation levels of these kinases in *trans*-resveratrol-treated VSMCs. *Trans*-resveratrol (30 μM) potently increased phosphorylated levels of Akt and ERK from 30 min after treatment. However, the compound failed to activate p38 kinase in VSMCs (Fig. 6A). We then assessed the role of each kinase in Nrf2-mediated HO-1 induction in VSMCs. Pretreatment of VSMCs with LY294002 (30 μM), a specific inhibitor of PI3-kinase, completely blocked the induction of HO-1 and the nuclear accumulation of Nrf2 in response to 30 μM *trans*-resveratrol (Figs. 6B and C). However, PD98059 (30 μM, ERK inhibitor) or SB203580 (10 μM, p38 kinase inhibitor) only marginally decreased HO-1 induction by *trans*-resveratrol (Figs. 6B and C), which shows that *trans*-resveratrol-stimulated PI3-kinase activation is involved in Nrf2/ARE-dependent induction of HO-1.

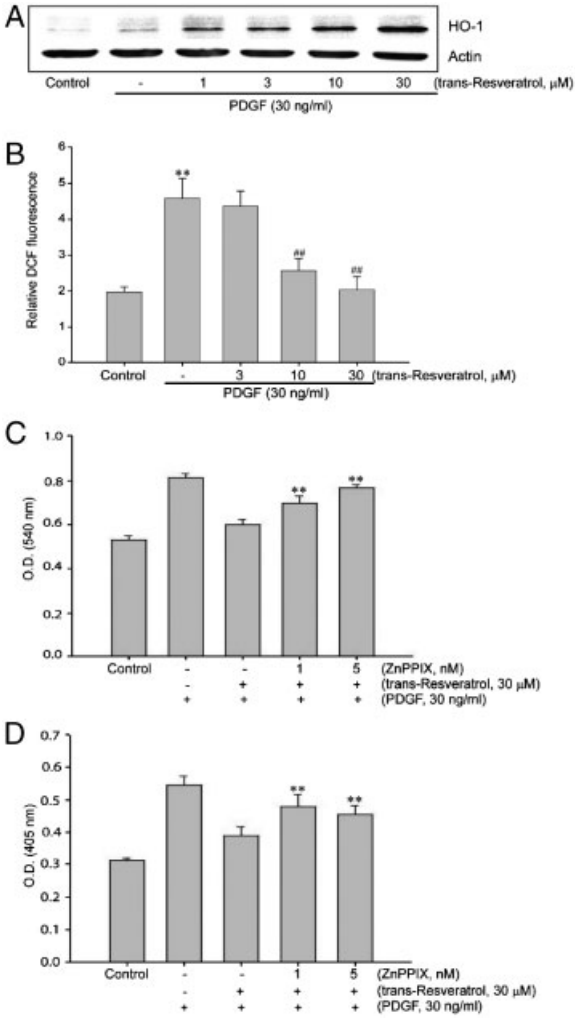

Figure 4. Role of HO-1 induction by *trans*-resveratrol in VSMC proliferation inhibition. (A) Concentration-dependent induction of HO-1 by *trans*-resveratrol in PDGF-treated VSMC. Representative immunoblot analysis shows the level of HO-1 in 30 ng/mL PDGF-treated VSMC coincubated with or without *trans*-resveratrol (1–30 μM) for 12 h. (B) Inhibition of ROS production by *trans*-resveratrol pretreatment. VSMC were treated with 3–30 μM *trans*-resveratrol for 12 h and exposed to DCFH-DA (5 μM) and 30 ng/mL PDGF for 30 min. Data were expressed as relative changes to the initial fluorescence. Data represent the mean ± SD of four different samples (significant as compared with control group, $^{**}p<0.01$; significant as compared with PDGF alone treated group, $^{\#\#}p<0.01$). (C) Reversal of *trans*-resveratrol's inhibitory effect on VSMC proliferation by HO-1 inhibition. VSMC were preincubated with or without 30 μM resveratrol in the presence or absence of 1 or 5 nM ZnPPIX, HO-1 inhibitor for 8 h and the cells were exposed to 30 ng/mL PDGF for 48 h. Data represent the mean ± SD of eight different samples (significant as compared with *trans*-resveratrol-treated group, $^{**}p<0.01$). (D) Reversal of *trans*-resveratrol's inhibitory effect on DNA synthesis by HO-1 inhibition. VSMC were treated same as described in (C) and BrdU assays were performed. Data represent the mean ± SD of six different samples (significant as compared with *trans*-resveratrol-treated group, $^{**}p<0.01$).

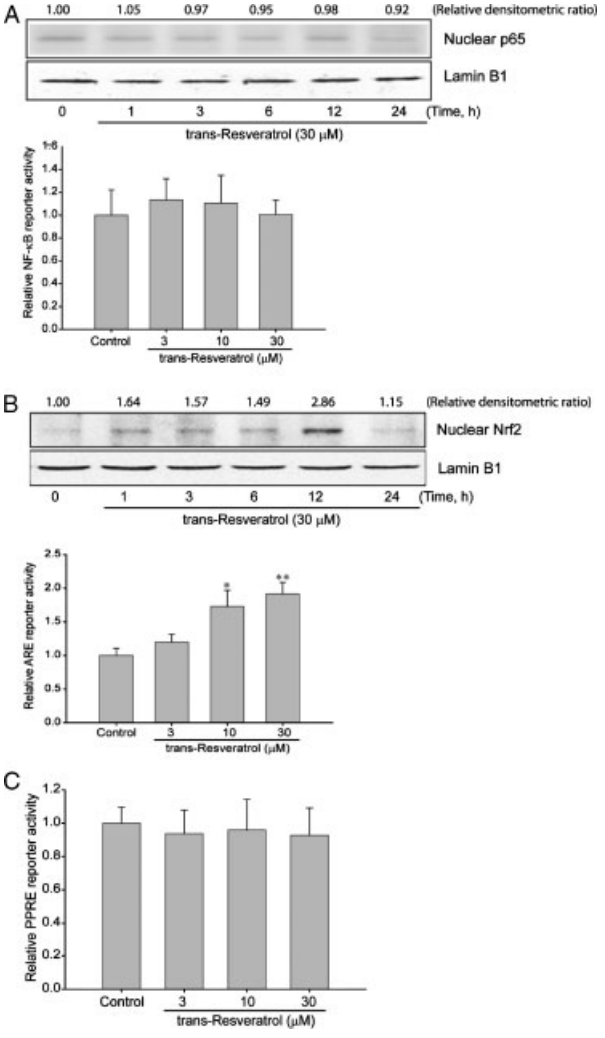

Figure 5. Selective Nrf2/ARE activation by *trans*-resveratrol. (A) NF-κB activity. Upper: nuclear levels of p65 in *trans*-resveratrol-treated VSMC. The nuclear p65 was immunochemically assessed in VSMC treated with *trans*-resveratrol (30 μM) for 0–24 h. Lower: NF-κB reporter activity. Dual luciferase reporter assays were performed on lysed MEF cells co-transfected with NF-κB-luciferase and phRL-SV after exposure to *trans*-resveratrol (3–30 μM) for 18 h. Data represent the mean ± SD with four different samples. (B) Nrf2/ARE activity. Upper: nuclear levels of Nrf2 in *trans*-resveratrol-treated VSMC. Lower: ARE reporter activity. Reporter transfection and *trans*-resveratrol treatment schedule was same as described in (A). Data represent the mean ± SD with four different samples (significant as compared with control, $^{*}p<0.05$; $^{**}p<0.01$). (C) PPAR activity. Activation of PPARs by *trans*-resveratrol was assessed by determining PPRE-Luc reporter activity in MEF cells. *Trans*-resveratrol treatment schedule was same as described in (A). Data represented the mean ± SD with four different samples.

4 Discussion

Resveratrol is a naturally occurring, biologically active phytochemical commonly found in grapes, berries, and red

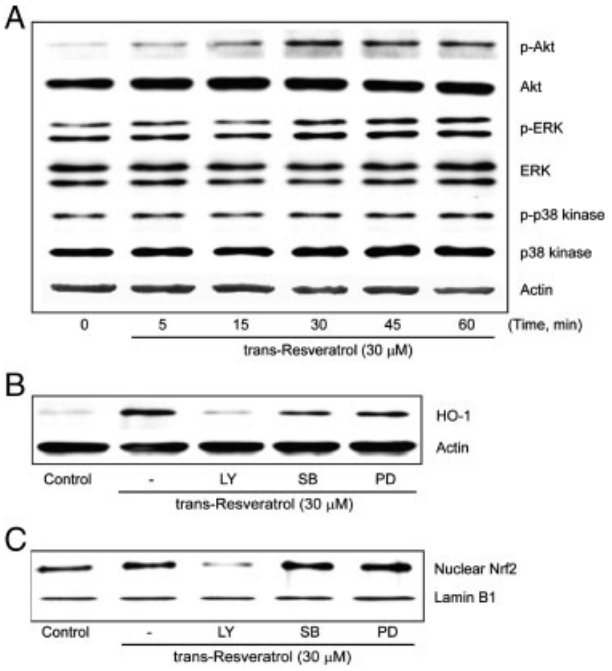

Figure 6. Key role of PI3-kinase in HO-1 induction by *trans*-resveratrol in VSMC. (A) *Trans*-resveratrol-inducible activation of PI3-kinase, ERK, and p38 kinase. VSMC were treated with *trans*-resveratrol (30 μM) for 5 min to 1 h. (B) Effect of each kinase inhibitor on HO-1 induction by *trans*-resveratrol. VSMC were cultured with 30 μM *trans*-resveratrol in the presence or absence of 30 μM LY294002 (LY, PI3-kinase inhibitor), 10 μM SB203580 (SB, p38 kinase inhibitor), or 30 μM PD98059 (PD, ERK inhibitor) for 12 h. (C) Effect of each kinase inhibitor on nuclear translocation of Nrf2 by *trans*-resveratrol. *Trans*-resveratrol treatment schedule was same as described in (B).

wine [37]. Although the diverse beneficial effects of resveratrol on the cardiovascular system have been elucidated in animal and clinical studies [38], direct evidence showing resveratrol's inhibitory activity against intimal hyperplasia has not been reported. In this study, we found that oral administration of *trans*-resveratrol suppressed neointima formation in wire-injured femoral arteries. This suppression may result from the inhibitory activity of *trans*-resveratrol on VSMC proliferation, since the compound efficiently blocked PDGF-mediated DNA synthesis and cell proliferation. It has been reported that cyclin/CDK inhibitor $p27^{Kip1}$ is a target molecule of resveratrol in prostate cancer cells [39]. We also tried to determine $p27^{Kip1}$, but the protein expression of $p27^{Kip1}$ was rather decreased by *trans*-resveratrol in VSMC (data not shown). However, the expression of cyclin D1, cyclin D3, or pRB was decreased by *trans*-resveratrol in PDGF-treated VSMCs. These results imply that *trans*-resveratrol-induced growth inhibition of VSMC is mediated through negative regulation of the G1/S phase cell cycle. Thus, *trans*-resveratrol-mediated inhibition of cyclin D expression may modulate the downstream pRb/E2F axis, which can trigger cell cycle arrest.

Production of ROS in VSMCs is believed to be an important contributor to the cell proliferation in response to PDGF [40]. In fact, ROS generation regulates expression of cyclin D1 [41]. HO-1 induction is a critical defense mechanism against ROS insult in the vasculature. Carbon monoxide and bilirubin produced by HO-1 enzyme responses efficiently scavenge ROS. Moreover, it has been reported that HO-1 overexpression itself suppresses neointima formation [23]. Here, we showed that *trans*-resveratrol potently enhanced the expression of HO-1 protein in VSMCs and HO-1 inhibition significantly reversed the inhibitory activity of *trans*-resveratrol on VSMC proliferation. These findings support the idea that HO-1 induction by *trans*-resveratrol is a critical event underlying its anti-intimal hyperplasia activity. Although several studies have already revealed that resveratrol prevents smooth muscle cell proliferation [10, 11], our data showing a direct relationship between HO-1 induction and an anti-VSMC proliferation effect should help elucidate the mechanistic basis for *trans*-resveratrol's inhibitory effect on vascular proliferative diseases.

Several studies have tried to identify the signaling pathways for resveratrol-mediated HO-1 induction. In human aortic smooth muscle cells, relatively low concentrations of resveratrol (1–10 μM) induce HO-1 in an NF-κB-dependent manner [26]. However, Chen *et al.* have shown that Nrf2/ARE is a crucial transcription factor for resveratrol-mediated HO-1 expression in PC12 cells [42]. In our study, we demonstrated that *trans*-resveratrol selectively activates Nrf2/ARE but not NF-κB and PPAR in VSMCs. In a series of studies, we demonstrated that PI3-kinase is essentially required for the induction of phase II anti-oxidant enzymes [20, 43, 44]. It has also been shown that MAP kinase family members, ERK and p38 kinase, are required for the activation of Nrf2/ARE-dependent HO-1 induction [36, 45]. Although *trans*-resveratrol functions as an efficient inhibitor of VSMC proliferation, VSMCs treated with *trans*-resveratrol showed sustained activation of cell survival signaling, PI3-kinase, and ERK. Using chemical inhibitors of each kinase, we demonstrated that PI3-kinase activation is a crucial event for Nrf2-dependent HO-1 induction in response to *trans*-resveratrol.

Nitric oxide (NO) production *via* activation of endothelial NO synthase or induction of inducible nitric oxide synthase (iNOS) in vasculatures also functions as a critical factor to regulate intimal hyperplasia [46]. It has been shown that resveratrol has bidirectional effects on NO production. Resveratrol stimulates NO production by increasing estrogen receptor α-dependent endothelial NO synthase phosphorylation in endothelial cells [47]. In contrast, role of resveratrol on iNOS expression seems to be dependent on experimental condition. Submicromolar ranges of resveratrol inhibit tumor necrosis factor-α-stimulated NF-κB activation and subsequent iNOS expression in coronary arterial endothelial cells [48]; *vice versa*, treatment of pulmonary arterial endothelial cells with resveratrol alone enhanced

iNOS expression [49]. Hence, inhibitory effect of resveratrol on neointima formation may be associated with its NO modulating activity in endothelial or smooth muscle cells and further studies will be required.

Taken together, the present study suggests that *trans*-resveratrol inhibits neointima formation in a guide-wire injured animal model through HO-1 induction. Nrf2 is activated by *trans*-resveratrol in VSMCs, and the PI3-kinase pathway plays a key role in the activation of Nrf2/ARE. Our findings have significant implications for the elucidation of the pharmacological mechanism of *trans*-resveratrol and the prevention of vascular occlusive diseases.

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