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Resveratrol inhibits angiotensin II-induced endothelin-1 gene expression and subsequent proliferation in rat aortic smooth muscle cells

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Abstract

Resveratrol is a phytoestrogen naturally found in grapes and is the major constituent of wine thought to have a cardioprotective effect. The aims of this study were to examine whether resveratrol alters angiotenisn II-induced cell proliferation and endothelin-1 gene expression and to identify the putative underlying signaling pathways in rat aortic smooth muscle cells. Cultured rat aortic smooth muscle cells were preincubated with resveratrol then stimulated with angiotensin II, after which [^{3}H]thymidine incorporation and endothelin-1 gene expression were examined. The intracellular mechanism of resveratrol in cellular proliferation and endothelin-1 gene expression was elucidated by examining the phosphorylation level of angiotensin II-induced extracellular signal-regulated kinase (ERK). The inhibitory effects of resveratrol (1–100 μM) on angiotensin II-induced DNA synthesis and endothelin-1 gene expression were demonstrated with Northern blot and promoter activity assays. Measurements of 2′7′-dichlorofluorescin diacetate, a redox-senstive fluorescent dye, showed a resveratrol-mediated inhibition of intracellular reactive oxygen species generated by the effects of angiotensin II. The inductive properties of angiotensin II and H_2O_2 on ERK phosphorylation and activator protein-1-mediated reporter activity were found reversed with resveratrol and antioxidants such as *N*-acetyl-cysteine. In summary, we speculate that resveratrol inhibits angiotensin II-induced cell proliferation and endothelin-1 gene expression, and does so in a manner which involves the disruption of the ERK pathway via attenuation of reactive oxygen species generation. Thus, this study provides important insight into the molecular pathways that may contribute to the proposed beneficial effects of resveratrol on the cardiovascular system.

Keywords: Endothelin-1; Resveratrol; Angiotensin II; Smooth muscle cell; Reactive oxygen species; ERK (extracellular signal-regulated kinase)

1. Introduction

Many epidemiologic studies show a correlation between a low incidence of coronary heart disease and atherosclerosis and a moderate consumption of red wine (German and Walzem, 2000; Mukamal et al., 2003). The vasoprotective effect of red wine, also known as the "French paradox," is currently best exemplified by *trans*-resveratrol (*trans*-3,5,4′-hydroxystilbene) (Sato et al., 2002; Sun et al., 2002). Resveratrol possesses many biologic activities, including protection from or reduction of the incidence of coronary heart disease. Resveratrol also has been found to protect the heart from ischemic-reperfusion injury (Ray et al., 1999). Antioxidant properties of resveratrol seem to be partly responsible for this activity (Hung et al., 2002; Jang and Surh, 2001; Olas and Wachowicz, 2002; Ray et al., 1999; Stivala et al., 2001). Moreover, resveratrol was shown to relax aortic rings in rats through an endothelium-mediated

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enhancement of the nitric oxide (NO)-cGMP cascade (Fitzpatrick et al., 1993). Subsequent pharmacologic studies indicate a direct relaxant effect of resveratrol on vascular smooth muscle, which may exert beneficial effects in cardiovascular disease, though the mechanism underlying these effects is not known (Chen and Pace-Asciak, 1996).

Recently, Orallo et al. (2002) demonstrated that the characteristic endothelium-dependent vasorelaxant effect of resveratrol in the rat aorta seemed to be caused by the inhibition of vascular NADH/NADPH oxidase and the subsequent decrease in the generation of basal cellular superoxide anions and, therefore, of NO biotransformation. However, little is known about the cellular/molecular mechanisms whereby resveratrol could protect against coronary heart disease. Indeed, whether resveratrol could inhibit the production of endogenous vasoconstrictors and thereby regulate vasomotion remains an intriguing possibility.

There is increasing evidence that the renin–angiotensin system may contribute to the pathogenesis of chronic vascular disease. Angiotensin II is an important component of the renin–angiotensin system and a vasoactive peptide (Vaughan, 2000). For a review of the literature, it would appear that angiotensin II is able to "turn on" the synthesis of endothelin-1 for different vascular cell types, including cultured vascular smooth muscle cells (Hahn et al., 1990; Ito et al., 1993; Sung et al., 1994). Endothelin-1 was shown to mediate the growth-promoting effect of angiotensin II and thus play an important role in cardiovascular disease and vascular remodeling (Hahn et al., 1990; Ito et al., 1993; Sung et al., 1994; Rossi et al., 1999). Angiotensin II has also been shown to stimulate membrane-bound NAD(P)H oxidase, which generates reactive oxygen species in vascular smooth muscle cells (Seshiah et al., 2002; Touyz and Schiffrin, 2001). Reactive oxygen species including superoxide anions and hydrogen peroxide (H_2O_2) are recognized as important signaling molecules for cardiovascular tissues. Excess reactive oxygen species generation is considered to be a likely initiator of atherosclerotic events, resulting in the increased synthesis of numerous mitogenic factors that contribute to the hyperproliferation of vascular smooth muscle cells and vascular plaque formation (Berliner and Heinecke, 1996). Reactive oxygen species may also act as second messengers that regulate various intracellular signal transduction cascades and the activity of various transcription factors such as activator protein-1 (AP-1) (Sen and Packer, 2000; Wung et al., 1997). We have previously reported that reactive oxygen species mediate endothelin-1 gene induction within cardiac fibroblasts and vascular endothelial cells and smooth muscle cells (Cheng et al., 2001, 2003; Hong et al., 2004a,b; Hsu et al., 2004; Liu et al., 2003). Although the significance of angiotensin II-induced endothelin-1 gene expression in vascular tissues is determined (Hahn et al., 1990; Moreau et al., 1997), whether resveratrol inhibits angiotensin II-induced cell proliferation and endothelin-1 gene induction via attenuation of reactive oxygen species generation in vascular smooth muscle cells remains unspecified.

Our study was conducted to examine whether resveratrol inhibits angiotensin II-induced endothelin-1 gene expression and to identify signaling protein kinase cascades that may be responsible for the alleged cardioprotective effects of resveratrol. In the present study, we demonstrate that resveratrol inhibits a number of angiotensin II-induced events, including cellular proliferation, endothelin-1 gene expression, reactive oxygen species generation, ERK phosphorylation and activator protein-1 (AP-1)-mediated reporter activity in vascular smooth muscle cells. Given the known pathophysiological influence of angiotensin II on the progression of cardiovascular disease, this study provides important insight into the molecular mechanisms underlying the actions of resveratrol and supports its proposed benefits in the cardiovascular system.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum, and tissue culture reagents were from Life Technologies, Inc. A rat endothelin-1 cDNA probe (accession no. M64711) was obtained as previously described (Deng et al., 1994). A full length of the endothelin-1 promoter region (4.4 kb) was fused to the chloramphenicol acetyltransferase reporter gene (Cheng et al., 2001). PBLCAT2 (containing chloramphenicol acetyltransferase reporter gene with its promoter) and PBLCAT3 (containing the chloramphenicol acetyltransferase gene only) were constructed as previously described (Cheng et al., 1999). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR, USA). H_2O_2 was purchased from Acros Organics (Pittsburgh, PA, USA). Resveratrol, *N*-acetyl-cysteine and all other reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The plasmid AP-1-Luc containing the firefly luciferase reporter gene was driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 binding element, which were obtained from Stratagene (La Jolla, CA, USA).

2.2. Culture of rat aortic smooth muscle cells

The investigation conduct conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of Taipei Medical University. Thoracic aortae from male Sprague–Dawley rats were excised and rapidly immersed in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Connective tissue and adherent fat were cleansed from the

specimens. Isolated arteries were cut open, and the endothelium was removed by gently scraping the intimal surface with the flat edge of a pair of sharp scissors. Denuded aortae were cut into ~3-mm pieces and placed with the intimal face down into three 35-mm culture dishes (Iwaki, Osaka, Japan). DMEM containing 10% fetal calf serum and penicillin/streptomycin was gently added to the dishes to cover the tissues without disturbing the orientation of the explants. Vascular smooth muscle cells were allowed to proliferate from the tissue (7–10 days), and the tissues were removed using sterilized fine forceps and washed with culture medium. After reaching confluence in three 35-mm dishes, cells were harvested by brief incubation with trypsin and subsequently cultured in T-75 flasks (Iwaki) (passage 1). Cells were routinely propagated in culture dishes to 75–95% confluence and used between passages 3 and 12. The purity of smooth muscle cells was evaluated by staining the cells with monoclonal antibodies to α-smooth muscle actin. Vascular smooth muscle cells were grown in DMEM without phenol red containing antibiotics and 10% fetal calf serum until 24 h prior to experimentation, at which time cells were incubated in a defined serum-free medium containing insulin (0.5 μM) and transferrin (5 mg/ml) for all experiments. Cells were then preincubated with different concentrations of resveratrol for 30 min and then with or without angiotensin II (100 nM) for different incubation times as indicated, followed by harvesting. Cellular viability under all treatment conditions was determined by cell count, morphology and trypan blue exclusion.

2.3. DNA synthesis

To measure synthesis of new DNA, cells (1×10^5/well) were plated in six-well (35-mm) dishes 24 h before experiments as previously described (Liu et al., 2003). Cells were incubated with [^{3}H]thymidine (5μ Ci/ml). Following the treatment as indicated, cells were harvested by incubation at 4 °C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH, and radioactivity was determined by scintillation counting. Data are presented as the mean ± S.E.M. of six different cell preparations and normalized to the untreated sample × 100 (i.e., percentage of control).

2.4. Assay of intracellular reactive oxygen species

The level of reactive oxygen species were measured using a previously described method (Cheng et al., 2001). Prior to the chemical or angiotensin II treatment, smooth muscle cells were incubated in culture medium containing a fluorescent dye, 2′ 7′-dichlorofluorescin diacetate (DCF-DA) (Molecular Probes, Eugene, OR, USA) at a concentration of 30 μM for 1 h to establish a stable intracellular level for the probe. The same concentration of DCF-DA was maintained during either chemical or angiotensin II treatment. Subsequently, the cells were washed with PBS, removed from petri dishes by brief trypsinization, and then assessed for their 2′,7′-dichlorofluorescein (DCF) fluorescence intensity. The DCF fluorescence intensity of the cells is an index of intracellular levels of ROS, and it can be determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively. For counting cell numbers, cells were harvested and counted in an automatic cell counter (S.ST.II/ZM, Coulter Electronics Ltd., Miami, FL, USA). The cell number in each sample was counted and utilized to normalize the DCF fluorescence intensity.

2.5. RNA isolation and Northern blot analysis

Total RNA was isolated from cells by the guanidine isothiocyanate/phenol chloroform method as previously described (Cheng et al., 2001). The RNA (10 g/lane) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher and Schuell, Inc., Germany) by a vacuum blotting system (VacuGene XL, Pharmacia, Sweden). Following hybridization with the [^{32}P]-labeled endothelin-1 cDNA probes, the membrane was washed with 0.1× standard saline citrate (SSC) containing 1% sodium dodecyl sulfate (SDS) at 42 °C for 30 min and then exposed to X-ray film at −70°C. Blots of specific mRNA bands were detected by autoradiography and analyzed with a densitometer (Computing Densitometer 300S, Molecular Dynamics). Blots were stripped and reprobed for 18S cDNA probe (obtained from American Type Culture Collection) to control for loading. The level of expression of endothelin-1 mRNA was quantified and was normalized to the 18S signal.

2.6. Transfection and chloramphenicol acetyltransferase assays

For the transient transfections, cells were transfected with different expression vectors by the calcium phosphate method (Cheng et al., 1999). DNA concentrations for all samples were adjusted to equal amounts with empty vector pSRα in each experiment. To correct for variability in transfection efficiency, 5 μg of pSV-β′-galactosidase plasmid DNA was cotransfected in all the experiments. The chloramphenicol acetyltransferase and β′-galactosidase assays were performed as previously described (Cheng et al., 1999). The relative chloramphenicol acetyltransferase activity was corrected by normalizing the respective chloramphenicol acetyltransferase value to that of β-galactosidase activity. Cotransfected β-galactosidase activity varied by <10% within a given experiment and was not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) were included in each assay.

2.7. Western blot analysis

Rabbit polyclonal anti-phospho-specific ERK antibodies were purchased from New England Biolabs (Beverly, MA, USA). Anti-ERK antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Western blot analysis was performed as previously described (Cheng et al., 2001).

2.8. Luciferase assay

Smooth muscle cells plated on six-well (35-mm) dishes were transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) (Stratagene, La Jolla, CA, USA). Following incubation for 24 h in serum-free DMEM, smooth muscle cells were cultured under various conditions as indicated for a period of 48 h. Smooth muscle cells were assayed for luciferase activity with a luciferase reporter assay kit (Stratagene). The specific firefly luciferase activity, as was the case for AP-1 transcriptional activity, was normalized for transfection efficiency to its respective β-galactosidase activity and expressed as activity relative to the control.

2.9. Statistical analysis

Results are expressed as mean±S.E.M. for six experiments. Statistical analysis was performed using analysis of variance (ANOVA) and Student's *t*-test as appropriate. A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of resveratrol on angiotensin II-induced cell proliferation of smooth muscle cells

The effects of resveratrol on angiotensin II-stimulated rat aortic smooth muscle cell proliferation were assessed by analyzing DNA synthesis with [^{3}H]thymidine incorporation. Preincubation of smooth muscle cells with resveratrol for 30 min (1–100 μM) followed by exposure to resveratrol with angiotensin II (100 nM) for 24 h resulted in a concentration-dependent decrease in angiotensin II-induced cell proliferation (Fig. 1). These data indicate clearly that resveratrol is able to inhibit the proliferation of smooth muscle cells induced by the activities of angiotensin II.

3.2. Effects of resveratrol on angiotensin II-induced endothelin-1 gene expression in smooth muscle cells

Examination of resveratrol on the inhibition of the angiotensin II-increased endothelin-1 mRNA levels in smooth muscle cells was completed with Northern blot analysis (Fig. 2A). Smooth muscle cells were preincubated with resveratrol (1–100 μM, 30 min) then treated with angiotensin II (100 nM) for 6 h and then assayed for the effect of resveratrol on angiotensin II-induced endothelin-1 mRNA expression. Preincubation with resveratrol (10, 100 μM, 30 min) significantly inhibited angiotensin II-induced endothelin-1 mRNA (Fig. 2A) expression. To determine whether the resveratrol-mediated inhibition of angiotensin II-induced endothelin-1 expression was regulated at the transcriptional level, an endothelin-1 promoter construct containing the endothelin-1 promoter region (−4.4 kb) and the reporter gene chloramphenicol acetyltransferase was constructed and transiently transfected into smooth muscle cells. Smooth muscle cells exposed to angiotensin II (100 nM) for a period of 24 h had significantly increased endothelin-1 promoter activity (Fig. 2B). On the other hand, preincubation with resveratrol (10, 100 μM, 30 min) prior to angiotensin II exposure showed decreased angiotensin II-induced endothelin-1 promoter activity (Fig. 2B). These results suggests strongly that resveratrol inhibits angiotensin II-induced endothelin-1 gene expression in smooth muscle cells, and does so at a transcriptional level.

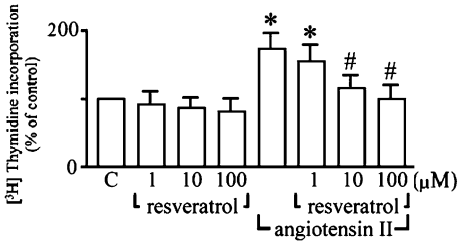

Fig. 1. Effects of resveratrol on the angiotensin II-induced cell proliferation in smooth muscle cells. Cells were preincubated with the indicated doses of resveratrol, and then treated with angiotensin II (100 nM) for a period of 24 h, following which, the level of [^{3}H]thymidine incorporation was then assayed. All experiments were performed by way of the incorporation of [^{3}H]thymidine into DNA. Increases in [^{3}H]thymidine incorporation are each expressed relative to the [^{3}H] content (100%) in their respective controls (C). All data are shown as the mean±S.E.M. for triplicate determinations for six cell preparations. *$P<0.05$ vs. control. $^{\#}P<0.05$ vs. angiotensin II alone.

3.3. Effects of resveratrol on angiotensin II-increased intracellular reactive oxygen species levels

We and others have demonstrated that angiotensin II stimulates reactive oxygen species production in various cell types, including cardiomyocytes, cardiac fibroblasts and smooth muscle cells (Laufs et al., 2003; Nakamura et al., 1998; Sano et al., 2001; Seshiah et al., 2002; Shih et al., 2001; Touyz and Schiffrin, 2001). Consequently, we examined resveratrol for possible preventive effects against angiotensin II-increased generation of intracellular reactive oxygen species. To do so we pretreated smooth muscle cells with resveratrol (1–100 μM, 30 min)

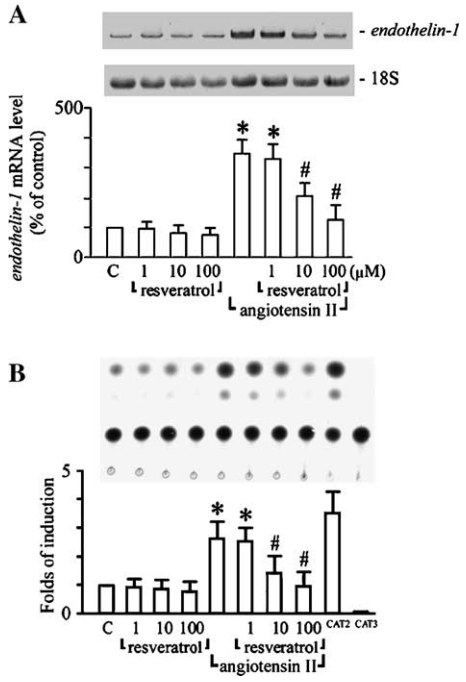

Fig. 2. Resveratrol down-regulates angiotensin II-induced endothelin-1 gene expression in smooth muscle cells. The results are shown as the mean±S.E.M. ($n=6$). *$P<0.05$ vs. control. $^{\#}P<0.05$ vs. angiotensin II alone. (A) Down-regulation of angiotensin II-induced endothelin-1 mRNA by resveratrol. Cells were preincubated with resveratrol (1–100 μM) and then stimulated with angiotensin II (100 nM) for 6 h or its absence. Total RNA was extracted and Northern hybridization was performed with [^{32}P]-labeled endothelin-1 as a probe. 18S RNA was used to normalize the RNA applied in each lane. Data were presented as percentage changes of experimental groups compared to untreated controls. (B) Resveratrol inhibits angiotensin II-induced endothelin-1 promoter activity. Cells were transfected with chimeric chloramphenicol acetyltransferase fusion genes and preincubated with resveratrol (1–100 μM) and then stimulated with angiotensin II (100 nM) for 24 h or not. Cells were harvested, and chloramphenicol acetyltransferase activities were measured. Chloramphenicol acetyltransferase activities after normalizing to that of β-galactosidase activities are shown as relative activity as compared to control groups. C indicates control (no drugs). CAT2 and CAT3 are positive and negative controls, respectively.

followed by treatment with angiotensin II (100 nM). The preincubation of cultured smooth muscle cells with resveratrol (1–100 μM) significantly decreased angiotensin II-induced reactive oxygen species levels as measured after angiotensin II treatment for 1 h (Fig. 3A). It should be noted that pretreatment of the cells with antioxidants *N*-acetyl-cysteine (10 mM) and diphenylene iodonium (DPI; 10 μM) also significantly reduced the generation of angiotensin II-induced reactive oxygen species to levels comparable with resveratrol (Fig. 3B). On the other hand, exogenous oxidative stress (e.g., H_2O_2, 100 μM) in the absence of angiotensin II elicited a significant increase in intracellular reactive oxygen species levels (Fig. 3B). These findings substantiate our earlier suspicions that resveratrol inhibits angiotensin II-increased intracellular reactive oxygen species levels in smooth muscle cells.

3.4. Effects of resveratrol on angiotensin II-activated ERK phosphorylation in smooth muscle cells

Angiotensin II has been previously shown to activate ERK in a manner that is redox sensitive (Sano et al., 2001). We recently reported that the generation of reactive oxygen species is involved in the activation of the ERK pathway and culminates in endothelin-1 gene expression (Liu et al., 2003). To gain insight into the actions of resveratrol with regards to this pathway in smooth muscle cells, we examined the phosphorylation of ERK in smooth muscle cells exposed to resveratrol (1–100 μM) in the absence or presence of angiotensin II (100 nM) treatment. As shown in Fig. 4A, smooth muscle cell exposure to angiotensin II treatment for 30 min rapidly activated phosphorylation of ERK. However, smooth muscle cells pretreated with resveratrol (1–100 μM) showed significantly decreased angiotensin II-induced ERK phosphorylation. Pretreatment with the antioxidant *N*-acetyl-cysteine (10 mM, 30 min) also significantly decreased angiotensin II-induced ERK phosphorylation to a level consistent with

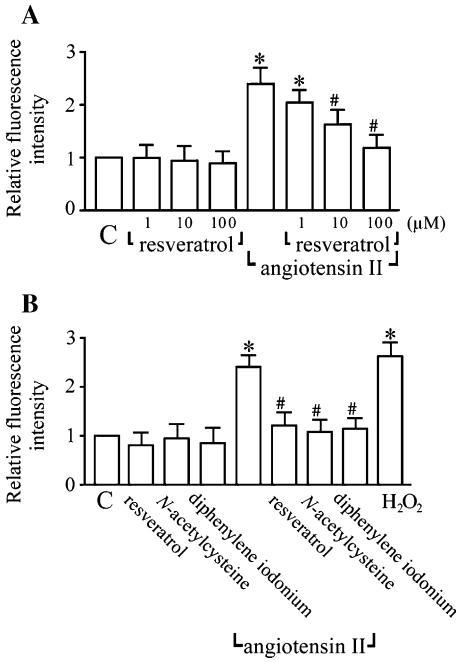

Fig. 3. Effect of resveratrol on angiotensin II-induced reactive oxygen species generation in smooth muscle cells. Intracellular reactive oxygen species levels were revealed by fluorescent intensities of DCF. Fluorescence intensities of cells are shown as relative intensity of experimental groups compared to untreated control cells. The results are shown as the mean±S.E.M. ($n=6$). *$P<0.05$ vs. control. $^{\#}P<0.05$ vs. angiotensin II alone. (A) Effect of resveratrol (1–100 μM) on angiotensin II-induced reactive oxygen species generation. Cells were preincubated with resveratrol (1–100 μM) for 1 h and then stimulated with angiotensin II (100 nM) for 1 h or not. (B) Effects of resveratrol, or antioxidants on angiotensin II-induced reactive oxygen species generation. Cells were preincubated with resveratrol (100 μM), the antioxidants *N*-acetyl-cysteine (10 mM), or DPI (10 μM) and then stimulated with angiotensin II (100 nM) for 1 h or not. H_2O_2 (100 μM) was used as a positive control.

resveratrol (10 μM, 30 min). These findings suggest that resveratrol inhibits the angiotensin II-activated ERK signaling pathway in an anti-oxidative manner in smooth muscle cells.

3.5. Effects of resveratrol on angiotensin II-increased AP-1-mediated reporter activity in smooth muscle cells

We then evaluated the effect of resveratrol on angiotensin II-elicited AP-1 activation, which in turn is involved in endothelin-1 gene induction (Cheng et al., 1999; Hirotani et al., 2002; Liu et al., 2003). The effects of resveratrol on angiotensin II-or H_2O_2-induced AP-1 functional activity were assessed in a reporter gene assay. We found that both resveratrol (100 μM) and *N*-acetyl-cysteine (10 mM) significantly attenuated angiotensin II- or H_2O_2-induced AP-1-mediated reporter activation (Fig. 5), indicating that angiotensin II-induced AP-1 activation is inhibited by resveratrol in a fashion that is consistent with its anti-oxidative actions in smooth muscle cells.

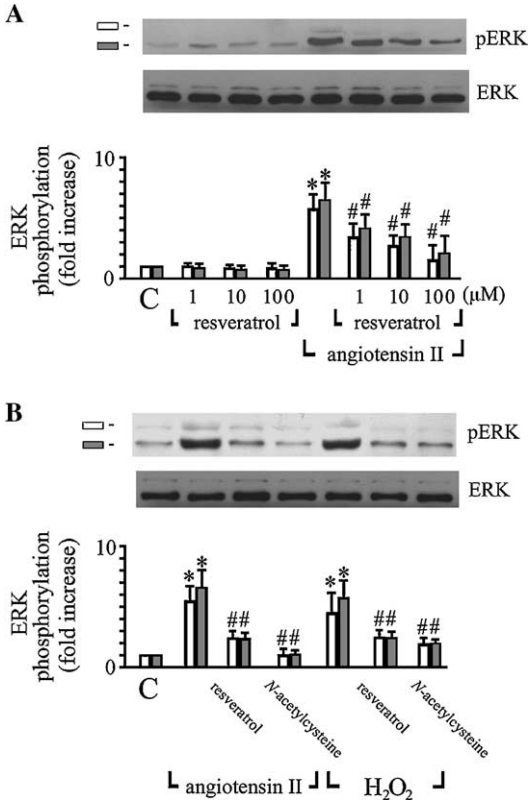

Fig. 4. Inhibitory effect of resveratrol on angiotensin II-increased ERK phosphorylation in smooth muscle cells. Phosphorylation of ERK was detected by Western blotting using anti-phospho-ERK antibody. Phosphorylation of ERK was detected, and densitometric analyses were performed. Data are represented as fold increase relative to control groups. The results are shown as the mean ± S.E.M. ($n=6$). *$P<0.05$ vs. control. #$P<0.05$ vs. angiotensin II alone. (A) Effect of resveratrol (1–100 μM) on angiotensin II-activated ERK phosphorylation. (B) Effect of resveratrol on angiotensin II- or H_2O_2-induced phosphorylation of ERK. Cells were preincubated with either the resveratrol (100 μM), or *N*-acetyl-cysteine (10 mM) for 30 min and stimulated with angiotensin II (100 nM) or H_2O_2 (25 μM) for 30 min.

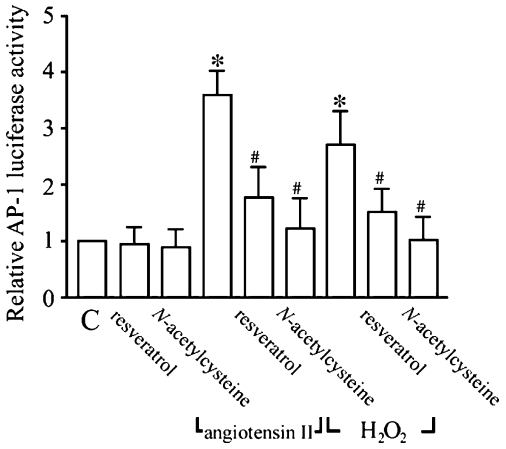

Fig. 5. Effects of resveratrol, or *N*-acetyl-cysteine on angiotensin II- or H_2O_2-increased AP-1-mediated reporter activity. Smooth muscle cells transfected with AP-1-Luc were incubated for 24 h with either no drug, 10 μM resveratrol, or 10 mM *N*-acetyl-cysteine in the absence or presence of angiotensin II (100 nM) or H_2O_2 (25 μM). Luciferase activity was expressed as relative activity to untreated control (C). The results are shown as the mean ± S.E.M. ($n=6$). *$P<0.05$ vs. control. #$P<0.05$ vs. angiotensin II alone.

4. Discussion

It has been clearly demonstrated that the proliferation of vascular smooth muscle cells contributes to the pathophysiology of hypertension, atherosclerosis and coronary artery restenosis following angioplasty and stent placement (Ross, 1993). The production of reactive oxygen species in the blood vessel wall, likewise, is increased in models associated with vascular remodeling such as hypercholesterolemia, hypertension, diabetes and balloon injury to the coronary arteries (Grunfeld et al., 1995; Langenstroer and Pieper, 1992; Ohara et al., 1993). Thus, an inhibition of the reactive oxygen species-mediated mechanisms involved may lead to additional treatments for these diseases. In our study, we demonstrated that the compound resveratrol exerts an antioxidant-like inhibitory effect on smooth muscle cellular proliferation and endothelin-1 gene expression induced by angiotensin II, an agent shown to stimulate both hyperplasia and hypertrophy in vascular smooth muscle cells (Touyz et al., 1999). Past studies in animals have shown that this angiotensin II-induced vascular hypertrophy is associated with increased blood pressure and an increased level of endothelin-1, both preventable effects when these animals were pretreated with an endothelin receptor antagonist (Moreau et al., 1997). The effects of endothelin receptor antagonism has similarly been characterized in other studies to be successful in blocking angiotensin II-induced vasoconstriction in certain in vitro blood vessel preparations (Chen et al., 1995a,b) and in preventing angiotensin II-mediated in vivo effects, which

include increases in total peripheral and renal vascular resistance as well as urinary protein excretion (Herizi et al., 1998). In keeping with these observations, our findings support the idea that the pathological effects attributed to angiotensin II in the vascular system are mediated, at least in part, by the production of vascular endothelin-1 and that by modulating endothelin-1, a certainty of these effects can be diminished or eradicated.

Recent studies have shown that a major source of reactive oxygen species in vascular tissues is NAD(P)H oxidase, and that the activity of NAD(P)H oxidase is increased by angiotensin II (Griendling et al., 2000). Thus, the source of reactive oxygen species in the present study can be attributed to the increased activity of NAD(P)H oxidase due to stimulation with angiotensin II. The elevated reactive oxygen species levels implicated in subsequent smooth muscle cell proliferation and endothelin-1 induction can be attenuated by pretreatment of these cells with antioxidants (Cheng et al., 2001; Liu et al., 2003). Furthermore, it has been demonstrated that the complete suppression of reactive oxygen species blocks endothelin-1 gene expression (Cheng et al., 2001, 2003; Hong et al., 2004a,b; Hsu et al., 2004; Juan et al., 2004; Liu et al., 2003).

Further indication linking reactive oxygen species generation with endothelin-1 expression includes the idea that the endothelin-1 promoter contains an AP-1 element that could be activated by reactive oxygen species (Lee et al., 1990). Evidence suggests that reactive oxygen species serve as messengers in AP-1 activation (Wung et al., 1997). The *cis*-acting AP-1 element binds the proto-oncogene products jun and fos (Paul et al., 1995) and it has been well documented that the genes for jun and fos are activated by reactive oxygen species (Guyton et al., 1996). Recently, we also found that the activation of AP-1 is redox-sensitive and might play a key role in endothelin-1 gene induction (Liu et al., 2003). The present results confirm that resveratrol inhibits angiotensin II-induced AP-1-mediated reporter activity.

Elevated levels of reactive oxygen species has also been shown to increase activation of the ERK pathway (Sano et al., 2001; Shih et al., 2001; Tanaka et al., 2001), a phenomenon which we and others were able to inhibit with resveratrol in cultured smooth muscle cells (Haider et al., 2002). In concert, the results of these studies suggest an irrefutable relationship between the generation of reactive oxygen species with an increased activation of ERK and possibly other components of the mitogen-activated phosphorylation (MAP) kinase pathway that ultimately lead to an increased expression of endothelin-1 gene. Thus, one possible explanation for the inhibitory effect of resveratrol on angiotensin II-induced cell proliferation and endothelin-1 gene expression may be its ability to attenuate reactive oxygen species formation and subsequently inhibit ERK phosphorylation in smooth muscle cells. However, further experiments will be necessary to identify the precise mechanisms by which resveratrol exerts its inhibitory effects on angiotensin II-induced endothelin-1 gene expression.

Our study provides insight into the molecular actions of resveratrol in vascular smooth muscle cells. Moreover, we show that resveratrol suppresses the ERK pathway and reduces angiotensin II-induced cell proliferation and endothelin-1 gene expression. It appears plausible that the angiotensin II-activated signaling pathway consists of a number of redox-sensitive steps and that resveratrol treatment could modulate the redox state of the cell through its antioxidant properties. In summary, our data show that resveratrol inhibits the angiotensin II-induced reactive oxygen species formation, ERK phosphorylation, endothelin-1 gene expression and cell proliferation in vascular smooth muscle cells. These data strongly support the proposed beneficial effects of resveratrol in the cardiovascular system.

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