Inhibition of Cyclic Strain–Induced Endothelin-1 Gene Expression by Resveratrol

Ju-Chi Liu, Jin-Jer Chen, Paul Chan, Ching-Feng Cheng, Tzu-Hurng Cheng

Abstract—Resveratrol is a phytoestrogen naturally found in grapes and is among the major constituents of wine thought to have a cardioprotective effect. Endothelin-1 (ET-1) is a potent vasopressor synthesized by endothelial cells both in culture and in vivo. The aims of this study were to test the hypothesis that resveratrol may alter strain-induced ET-1 gene expression and to identify the putative underlying signaling pathways in endothelial cells. We show that resveratrol indeed potently inhibits strain-induced ET-1 secretion, ET-1 mRNA level, and ET-1 promoter activity. Resveratrol also inhibits strain-increased NADPH oxidase activity, reactive oxygen species formation, and extracellular signal–regulated kinases1/2 (ERK1/2) phosphorylation. Furthermore, pretreating cells with resveratrol or antioxidant N-acetyl-cysteine decreases strain-increased or hydrogen peroxide–increased ET-1 secretion, ET-1 promoter activity, and ET-1 mRNA and ERK1/2 phosphorylation. Using both the electrophoretic mobility shift assay and a reporter gene assay, resveratrol and N-acetyl-cysteine also attenuated the strain-stimulated activator protein-1 binding activity and activator protein-1 reporter activity. In summary, we demonstrate for the first time that resveratrol inhibits strain-induced ET-1 gene expression, partially by interfering with the ERK1/2 pathway through attenuation of reactive oxygen species formation. Thus, this study provides important new insights in the molecular pathways that may contribute to the proposed beneficial effects of resveratrol in the cardiovascular system. (Hypertension. 2003;42:1198-1205.)

Key Words: endothelin  gene expression  atherosclerosis  endothelium  oxygen  kinase

Many epidemiological studies show a correlation between a low incidence of coronary heart disease and atherosclerosis and a moderate consumption of red wine.1,2 The vasoprotective effect of red wine, also known as the “French paradox,” is currently best exemplified by trans-resveratrol (trans-3,5,4′-hydroxystilbene).3,4 Resveratrol has many biological activities, including protection from or reduction of the incidence of coronary heart disease. Resveratrol also has been found to protect the heart from ischemia-reperfusion injury.5 Antioxidant properties of resveratrol appear to be partly responsible for this activity.5–9 Moreover, resveratrol was shown to relax aortic rings in rats through an endothelium-mediated enhancement of the nitric oxide (NO)-cGMP cascade.10 Subsequent pharmacological studies indicate a direct relaxant effect of resveratrol on vascular smooth muscle that may exert beneficial effects in cardiovascular disease, though the mechanism of these effects is not known.11 Recently, Orallo et al12 demonstrated that the characteristic endothelium-dependent vasorelaxant effect of resveratrol in the rat aorta appeared 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.

Among the earliest indications of vascular dysfunction in atherosclerosis is an impaired regulation of vasomotion, representing disturbed homeostasis of vascular cells.13 Key regulators of vasomotor function are the vasodilator NO and the vasoconstrictor endothelin-1 (ET-1). Among the endogenous mediators of cardiovascular disorders, ET-1, a 21–amino acid peptide, is a primary antecedent in coronary heart disease.14–17 Such effects are mediated by extremely potent vasopressors and mitogenic responses for ET-1 in the vasculature.18,19 Results from preclinical studies in humans as well as animal studies showed that plasma ET-1 levels are consistently elevated in many spasm-related cardiovascular diseases19,20 and that blockers for ET receptors can substantially alleviate complications of such diseases.20,21

ET-1 was originally isolated from a culture of porcine endothelial cells.21 Endothelial cells are constantly under the influence of mechanical forces, including cyclic strain, as a consequence of vessel contraction and relaxation. Recently,
numerous studies have shown that oxidative stress, represented by reactive oxygen species (ROS), is capable of significantly altering vascular function.22–24 Thus, oxidative stress appears to be causally linked to the pathogenesis of atherosclerosis.22–24 Previous studies demonstrated that intracellular ROS levels are elevated in endothelial cells after cyclic strain treatment.25–26 Our previous study demonstrated that intracellular ROS mediate cyclic strain-induced ET-1 expression through the Ras/Raf/extracellular signal–regulated kinases1/2 (ERK1/2) signaling pathway.27 However, no studies exist that address the interference of resveratrol on ET-1 gene induction in vascular endothelial cells.

The present study was undertaken to identify potential signaling components underlying the protective actions of resveratrol in the vascular system. Because of the pathophysiological influence of endothelin on the cardiovascular system, the aims of this study were to investigate the effect of resveratrol on the strain-induced ET-1 gene expression and to identify signaling protein kinase cascades that may be responsible for the putative effect of resveratrol.

Methods

Materials

Imubind ET-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biochemica (ENDOTHELIN, Biomedica). ET-1 cDNA was obtained from a human endothelial cell cDNA library, as described previously.28 A full length of the ET-1 promoter region (4.4 kb) was fused to the chloramphenicol acetyltransferase (CAT) reporter gene.27 PBLCAT2 (containing CAT reporter gene with its promoter) and PBLCAT3 (containing CAT gene only) were constructed as described previously.29 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Serva Co. Hydrogen peroxide (H2O2) was purchased from Acros Organics. Resveratrol, N-acetyl-cysteine (NAC), and all other chemicals of reagent grade were obtained from Sigma.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords, as described previously.27 All procedures involving human samples were conducted according to the Guidelines for Animal and Human Experimentation of Taipei Medical University. The transformed human cell line, ECV304 (ATCC CRL-1998), was purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) before they were subcultured.

In Vitro Cyclic Strain on Cultured Endothelial Cells

Endothelial cells cultured on the flexible membrane base were subjected to cyclic strain produced by a computer-controlled application of sinusoidal negative pressure, as described previously.27

Measurement of ET-1 Concentration

ET-1 concentrations in culture media were assayed with Imubind-ET-1 ELISA kits (Biomedica), as described previously.27

Northern Blot Analysis

Preparation of total RNA and Northern blot analyses of ET-1 and 18S RNA were performed as described previously.27

Transfections

ECV304 were transiently transfected with different expression vectors by the calcium phosphate method, as described previously.27

CAT and β-Galactosidase Assays

The CAT and β-galactosidase assays were performed as described previously.27

NADPH Oxidase Activity Assay

NADPH oxidase was measured in HUVECs, as described previously.30

Detection of Intracellular ROS

Measurement of intracellular ROS formation in HUVECs was recorded by monitoring changes in dichlorofluorescein (DCF) fluorescence, as described previously.27 Chemiluminescence assay of superoxide production was measured as described previously.28

Western Blot Analysis

Rabbit polyclonal antiphosphospecific extracellular signal–regulated kinases1/2 (ERK1/2) antibody was purchased from New England Biolabs. Anti-ERK antibody was purchased from Santa Cruz Biotechnology. Western blot analysis was performed as described previously.27

Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay was performed as described previously.31

Luciferase Assay

ECV304 plated on 3-cm-diameter culture dishes were transfected with the luciferase reporter construct having consensus AP-1 (AP-1-Luc) binding sites (Stratagene). After incubation for 24 hours in 2% serum DMEM, ECV304 were cultured under different treatments, as indicated for 48 hours. ECV304 were assayed for luciferase activity with a luciferase reporter assay kit (Stratagene). The firefly luciferase activities at AP-1 transcriptional activity were normalized for transfection efficiency to its respective β-galactosidase activity and expressed as relative activity to control.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis was performed with the use of the Student t test and ANOVA, followed by a Dunnett multiple comparison test, with Prism version 3.00 for Windows (GraphPad Software). Probability values <0.05 were considered to be statistically significant.

Results

Effect of Resveratrol on Strain-Induced ET-1 Gene Expression in Endothelial Cells

HUVECs cultured on flexible membrane bases were subjected to deformation to produce an average strain of 20%. ET-1 released into the culture media was measured. HUVECs under cyclic strain for 24 hours increased their ET-1 mRNA level by 6 hours of cyclic strain. HUVECs under cyclic strain for 6 hours showed a 3.7-fold increase in ET-1 mRNA level compared with unstrained endothelial cells. We then examined the effect of resveratrol on strain-increased ET-1 secretion. As shown in Figure 1B, treatment with cyclic strain for 24 hours increased ET-1 secretion. Resveratrol (1 to 100 μmol/L) significantly inhibited strain-increased ET-1 secretion (Figure 1B). These data indicate that resveratrol inhibits strain-increased ET-1 secretion in endothelial cells.

To determine whether resveratrol inhibits the strain-induced ET-1 gene expression, HUVECs were pretreated with resveratrol (1 to 100 μmol/L) for 30 minutes followed by 6 hours of cyclic strain. HUVECs under cyclic strain for 6 hours showed a 3.7-fold increase in ET-1 mRNA level compared with unstrained controls (Figure 2A). Resveratrol...
(1 to 100 μmol/L) pretreatment significantly reduced the strain-induced ET-1 expression. To further investigate whether the effect of resveratrol on strain-induced ET-1 gene expression is a transcriptional event, a plasmid containing an ET-1 upstream sequence in a CAT reporter construct (−4.4 kCAT) was cotransfected with pSV-β-galactosidase into ECV304. The cell extract was subsequently used to determine CAT activity. As shown in Figure 2B, increased ET-1 promoter activity was observed in endothelial cells under cyclic strain for 24 hours. In endothelial cells pretreated with resveratrol (1 to 100 μmol/L), the strain-induced ET-1 promoter activity was significantly reduced (P<0.05) (Figure 2B). To verify that resveratrol-mediated effects on vascular endothelial cells are not a consequence of cytotoxicity, we determined lactate dehydrogenase (LDH) activity as a parameter of necrosis in supernatants of resveratrol (1 to 100 μmol/L) and strain-treated HUVECs. Consistent with light microscopic observations, LDH activity in supernatants of treated and untreated cells remained unchanged (data not shown).

**Resveratrol Inhibits Strain-Increased NADPH Oxidase Activity and ROS Formation**

Previous studies showed that cyclic strain increases NADPH oxidase activity and ROS formation in endothelial cells, which is involved in ET-1 induction. We next examined whether resveratrol prevents the strain-increased NADPH oxidase activity and ROS formation. HUVECs were treated with resveratrol (0.1 to 100 μmol/L) in the absence or presence of strain treatment. The addition of resveratrol (1 to 100 μmol/L) to cultured HUVECs significantly inhibited strain-induced NADPH oxidase activity and ROS formation as measured after strain treatment for 1 hour (Figure 3, A through C). The pretreatment of resveratrol (100 μmol/L) or NAC (10 mmol/L) to cultured HUVECs also significantly inhibited strain- or H2 O2-induced ROS formation (Figure 3D). These findings clearly demonstrate that resveratrol inhibits strain-increased NADPH oxidase activity and intracellular ROS levels in endothelial cells.

**Figure 1.** Resveratrol inhibits strain-induced ET-1 secretion. A, Increased ET-1 secretion from HUVECs subjected to cyclic strain. Culture media containing ET-1 from cells treated or not treated with cyclic strain (60 Hz; 20%) for various times were collected and analyzed by enzyme immunoassay. B, Resveratrol inhibits strain-induced ET-1 release. HUVECs were pretreated with resveratrol (0.1 to 100 μmol/L) 30 minutes before strain treatment. After strain (20%) for 24 hours, culture media were collected and concentrations were analyzed by enzyme immunoassay. Results are presented as mean ± SEM (n=6). *P<0.05 vs unstrained control cells, #P<0.05 vs strained cells (ANOVA).

**Figure 2.** Resveratrol inhibits strain-induced ET-1 gene expression in endothelial cells. A, Inhibition of strain-induced ET-1 mRNA by resveratrol. HUVECs were either untreated controls (C, lane 1), or treated with different concentrations of resveratrol (1 to 100 μmol/L) in the absence (lanes 2 to 4) or presence of strain treatment (lanes 6 to 8). Total RNA was extracted and Northern hybridization was performed with 32P-labeled ET-1 as probe. 18S RNA was used to normalize the RNA applied in each lane. Data are presented as percentage changes of experimental groups compared with untreated controls. B, Resveratrol inhibits strain-induced ET-1 promoter activity. ECV304 cells were pretreated with resveratrol (1 to 100 μmol/L) for 30 minutes and then treated with cyclic strain for 24 hours. C (control), no drugs; CAT2 and CAT3 are shown as positive and negative control. CAT activities after normalizing to that of α-galactosidase activities are shown as relative activity compared with control groups. Results are shown as mean ± SEM (n=6). *P<0.05 vs control, #P<0.05 vs strain.

**Resveratrol Inhibits Strain-Activated ERK1/2 Phosphorylation in Endothelial Cells**

To gain insight into the mechanism of action of resveratrol, we thus examined whether resveratrol affects intracellular...
protein kinase signaling pathways. Given that the ERK1/2 signaling pathway is involved in strain-induced ET-1 expression, we further investigated whether resveratrol inhibits the ERK1/2 pathway in strain-treated endothelial cells. We examined the phosphorylation of ERK1/2 in HUVECs exposed to resveratrol (1 to 100 μmol/L) in the absence or presence of strain treatment. As shown in Figure 4A, HUVEC exposure to strain treatment for 30 minutes rapidly activated phosphorylation of ERK1/2. However, HUVECs pretreated with resveratrol (1 to 100 μmol/L) showed significantly decreased strain-induced ERK1/2 phosphorylation. Moreover, HUVECs treated with H2O2 (25 μmol/L) showed increased...
ERK1/2 phosphorylation (Figure 4B). HUVECs pretreated with resveratrol (100 μmol/L) or NAC (10 mmol/L) showed significantly decreased strain- or H₂O₂-induced ERK1/2 phosphorylation. These findings imply that resveratrol inhibits the strain-activated ERK1/2 signaling pathway through attenuation of ROS formation in endothelial cells.

Resveratrol Inhibits Strain-Increased AP-1 Transcriptional Activity in Endothelial Cells

We next evaluated the effect of resveratrol on AP-1 activation, which is involved in ET-1 gene induction. With the use of the electrophoretic mobility shift assay, AP-1 binding to the consensus AP-1 binding sequence was assayed in HUVECs with strain treatment for 6 hours (Figure 5A). Pretreating HUVECs with resveratrol or NAC attenuated the strain-stimulated AP-1 binding activity. The effects of resveratrol on strain-induced AP-1 functional activity were also assessed in a reporter gene assay. Cyclic strain had no effect on luciferase activity of the background vector containing no AP-1 binding site (Figure 5B). On the contrary, cyclic strain significantly increased AP-1–luciferase activities in a time-dependent manner (Figure 5B). These results indicate that cyclic strain increases AP-1 transcriptional activity. We next examined the effects of resveratrol and NAC on strain-increased AP-1 transcriptional activity. Either resveratrol (10 μmol/L) or NAC (10 mmol/L) significantly attenuated strain- or H₂O₂-induced AP-1 reporter activation (Figure 5C). These results indicate that resveratrol inhibits strain-increased AP-1 transcriptional activation.

Resveratrol Inhibits Strain-Induced ET-1 Expression, Which Is Compatible With Antioxidant Action

To further determine whether resveratrol affects the strain-induced ET-1 gene expression through attenuation of ROS formation, the effects of resveratrol on ET-1 gene induction were examined under cyclic strain or H₂O₂ stimulation. As demonstrated in Figure 6, either resveratrol or NAC alone had no effect on the basal ET-1 mRNA level (Figures 6A and 6B). However, endothelial cells treated with strain or H₂O₂ (25 μmol/L) significantly increased ET-1 mRNA and promoter activity (Figures 6A and 6B). In the presence of resveratrol (or NAC), strain- or H₂O₂-increased ET-1 mRNA and promoter activity were also significantly inhibited. Furthermore, either resveratrol or NAC alone had no effect on the basal ET-1 secretion. However, HUVECs treated with either strain or H₂O₂ (25 μmol/L) for 24 hours significantly increased ET-1 secretion (Figure 6C). In the presence of resveratrol (or NAC), both strain- and H₂O₂-increased ET-1 secretion were significantly inhibited. These data imply that resveratrol affects the strain-induced ET-1 gene expression, which is compatible with antioxidant action in endothelial cells.

Discussion

The major new finding of this work is that resveratrol inhibits strain-induced ET-1 gene expression in endothelial cells. It is supported by the observations that resveratrol inhibits strain-induced ET-1 protein secretion, mRNA level, and promoter activity in part through attenuation of ROS formation in
endothelial cells. Previous studies, including ours, have shown that cyclic-strain treatment of endothelial cells can induce intracellular ROS generation. This ROS generation is sustained at an elevated level as long as the mechanical forces are maintained and returns to basal levels with the removal of the forces. Matsushita et al further demonstrated a pivotal role for NADPH oxidase in cyclic strain–induced endothelial ROS production. Elevated ROS levels are involved in the release of ET-1, and this gene induction can be attenuated by antioxidant pretreatment of cells.

Studies have shown that red wines strongly inhibit the synthesis of ET-1 and imply that components specific to red wine may help to prevent coronary heart disease. Resveratrol is a major component of the polyphenols from grapes and red wine. A number of studies have demonstrated the antioxidant effects of resveratrol. This amphipathic molecule is capable of scavenging lipid hydroperoxyl free radicals as well as hydroxyl and superoxide anion radicals. Resveratrol was found to protect the kidney, heart, and brains from ischemia-reperfusion injury through its antioxidant ability. Moreover, resveratrol is a lipophilic substance and has been shown to accumulate in tissues such as the heart, liver, and kidney. Therefore, Bertelli et al concluded that an average drinker of wine can, particularly in the long term, absorb a sufficient quantity of resveratrol to explain the beneficial effect of red wine on health. Indeed, the results of our present study demonstrated that resveratrol reduces the strain-increased NADPH oxidase activity and ROS formation in endothelial cells, suggesting a reduction in the generation of intracellular ROS caused by strain treatment. In particular, it has been demonstrated that activation of ERK1/2 is redox-sensitive and that suppression of ROS inhibits strain-induced ET-1 gene expression. In porcine coronary arteries, short-term treatment with resveratrol substantially inhibited mitogen-activated protein kinase activity and reduction in the phosphorylation of ERK1/2. Recently, Haider et al demonstrated that resveratrol inhibits angiotensin II–induced vascular smooth muscle cell hypertrophy, possibly by interfering mainly with the ERK1/2 signaling pathway. One possible explanation for the inhibitory effect of resveratrol on strain-induced ET-1 gene expression may be its ability to attenuate ROS formation. In our previous study, deletion mapping revealed constructs containing 143 bp of the ET-1 promoter sequence, allowing strain-induced transcription, and the presence of responsiveness elements for ROS- or strain-induced ET-1 expression located within 143 bp upstream of the transcription initiation site. Recently, we also found that the activation of AP-1 is redox-sensitive and might play a key role in ET-1 gene induction. Our present results indicate that resveratrol inhibits strain-induced AP-1 transcriptional activity. The inhibitory effect of resveratrol on strain-induced AP-1 transcriptional activation suggested that the attenuation of strain-induced ROS by resveratrol leads to inhibition of AP-1. Alternatively, resveratrol may inhibit strain-induced ET-1 gene expression by virtue of its increasing NO production. Resveratrol has been suggested to increase endothelial NO synthase gene expression in endothelial cells. Indeed, NO, acting through soluble guanylyl

Figure 6. Resveratrol modulates strain-induced ET-1 gene expression, which is compatible with antioxidant action. A, Resveratrol modulates strain-increased (or H2O2-increased) ET-1 mRNA. B, Resveratrol modulates strain-increased (or H2O2-increased) ET-1 promoter activity. Some cells were pretreated with resveratrol or NAC for 30 minutes. Endothelial cells were then treated with cyclic strain or H2O2 (25 μmol/L) for 24 hours. CAT2 (lane 10) and CAT3 (lane 11) are shown as positive and negative controls of CAT assay system. C, Resveratrol modulates strain-increased (or H2O2-increased) ET-1 secretion. Results are shown as mean±SEM (n=6). P<0.05 vs control, #P<0.05 vs strain (or H2O2) alone (ANOVA).
cyclooxygenase and cGMP formation, is a negative regulator of ET-1 gene induction.\textsuperscript{47–49} Thus, further experiments will be necessary to identify the mechanisms of the involvement of NO by which resveratrol exerts its inhibitory effects on strain-induced ET-1 gene expression.

The resveratrol-induced suppression of cyclic strain–induced ET-1 expression, as observed presently in cultures, if it does occur in vivo, for example, as a result of consumption of this agent, may have several consequences; that is, it is known that ET-1 is a primary antecedent in coronary heart disease and atherosclerosis.\textsuperscript{14–17} If indeed resveratrol consumed as a constituent of red wine is responsible for lowering the incidence of coronary heart disease and atherosclerosis, this may be one of its possible mechanisms of action. The effects of resveratrol on endothelial cells observed in the present study, for example, downregulation of ET-1 expression, suppression of ROS formation, and inhibition of ERK1/2 phosphorylation, all are compatible with its putative cardioprotective and vasoprotective effects.

Perspectives

Within the past decade, it has been repeatedly suggested that oxidative stress is related to and a cause of endothelial dysfunction in atherosclerosis. The present study delivers important new insights to the molecular mechanisms of action of resveratrol in endothelial cells. Our results for the first time clearly show that resveratrol remarkably influences important cyclic strain–activated pathways in endothelial cells. Moreover, they suggest that resveratrol acts partially by interfering with the ERK1/2 pathway to reduce strain-induced ET-1 gene expression. It is plausible that the strain-activated signaling pathway consists of redox-sensitive steps and that resveratrol treatment could modulate the redox state of the cell. In summary, our data show that resveratrol inhibits strain-induced ET-1 gene expression in part through attenuation of ROS formation and the suppression of the ERK1/2 pathway in endothelial cells. These findings support the proposed beneficial effects of resveratrol in the cardiovascular system.

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