Resveratrol, a Polyphenolic Phytoalexin Present in Red Wine, Enhances Expression and Activity of Endothelial Nitric Oxide Synthase

Thomas Wallerath, PhD; Göran Deckert; Thomas Ternes, PhD; Henrik Anderson; Huige Li, MD; Klaus Witte, MD; Ulrich Förstermann, MD, PhD

Background—Estrogens can upregulate endothelial nitric oxide synthase (eNOS) in human endothelial cells by increasing eNOS promoter activity and enhancing the binding activity of the transcription factor Sp1. Resveratrol, a polyphenolic phytoalexin found in grapes and wine, has been reported to act as an agonist at the estrogen receptor. Therefore, we tested the effect of this putative phytoestrogen on eNOS expression in human endothelial cells.

Methods and Results—Incubation of human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 cells with resveratrol for 24 to 72 hours upregulated eNOS mRNA expression in a time- and concentration-dependent manner (up to 2.8-fold). eNOS protein expression and eNOS-derived NO production were also increased after long-term incubation with resveratrol. Resveratrol increased the activity of the eNOS promoter (3.5-kb fragment) in a concentration-dependent fashion, with the essential trans-stimulated sequence being located in the proximal 263 bp of the promoter sequence. In addition, eNOS mRNA was stabilized by resveratrol. The effect of resveratrol on eNOS expression was not modified by the estrogen receptor antagonists ICI 182780 and RU 58668. In electrophoretic mobility shift assays, nuclear extracts from resveratrol-incubated EA.hy 926 cells showed no enhanced binding activity of the eNOS promoter–relevant transcription factors Sp1, GATA, PEA3, YY1, or Elf-1. In addition to its long-term effects on eNOS expression, resveratrol also enhanced the production of bioactive NO in the short-term (after a 2-minute incubation).

Conclusions—in concert with other effects, the stimulation of eNOS expression and activity may contribute to the cardiovascular protective effects attributed to resveratrol. (Circulation. 2002;106:1652-1658.)

Key Words: endothelium • nitric oxide synthase • cardiovascular diseases

Coronary artery disease is the leading overall cause of mortality. For women, the risk increases significantly after menopause; this is facilitated by a state of estrogen deficiency and is reduced by estrogen replacement therapy. 1

Estrogens have been shown to upregulate the expression of endothelial nitric oxide synthase (eNOS) mRNA and protein in various tissues of laboratory animals and in human endothelial cells. 2,3 Endothelial NO can be considered a vasoprotective and antiatherosclerotic agent on the basis of a number of mechanisms. 4

The phytoalexin resveratrol (3,4',5-trihydroxy-trans-stilbene) has structural similarity to the synthetic estrogen diethylstilbestrol. Resveratrol has been shown to bind to and activate gene transcription by the estrogen receptor subtypes α and β in estrogen-sensitive tissues and cell lines. 5,6

Resveratrol is found mainly in grape wines (and in a few other fruits and vegetables). Given that it is present in grape berry skins but not in grape flesh, white wine contains small amounts of resveratrol compared with red wine. 7 As an important antioxidant in red wine, resveratrol is likely to contribute to the potential of red wine to prevent human cardiovascular disease. 7

There is some evidence that resveratrol interacts with the vascular NO system. Resveratrol caused relaxation of the phenylephrine-precontracted rat aorta, an effect that was endothelium dependent and mediated by NO. 8,9

NO production from the endothelium may be regulated by changes in eNOS gene expression and/or activity. The present study was designed to investigate the effects of resveratrol on these parameters. The study demonstrates that resveratrol produces a concentration- and time-dependent upregulation of eNOS expression and of eNOS-derived NO production.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and HUVEC-derived EA.hy 926 cells were grown in Dulbecco’s modified Eagle...
medium, as described previously. The cells were incubated for 12 to 72 hours with resveratrol (1 to 100 \( \mu \text{mol/L} \); Sigma Chemical) or were left untreated (control). In experiments with the estrogen receptor antagonists ICI 182780 (Biotrend) or RU 58668 (Aventis), the antagonists were added 30 minutes before resveratrol. Then, RNA or protein was extracted or eNOS activity of intact cells was determined.

RNase Protection Assay of eNOS mRNA

eNOS mRNA was measured by an RNase protection assay, as previously described. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or \( \beta \)-actin mRNA were determined in parallel for normalization. The GAPDH probe was generated according to a previously described procedure using total RNA from human A673 rhabdomyosarcoma cells and oligonucleotide primers for GAPDH corresponding to positions 501 to 524 (sense) and 582 to 605 (antisense) of the human GAPDH cDNA (GenBank accession number, NM_002046). The resulting cDNA fragments (105 bp) were cloned into the pXcm I–TA-cloning vector (which was kindly provided by Dr A. Borovkov, Department of Plant Sciences, North Dakota State University, Fargo). The protected RNA fragments were 280 nt for eNOS and 108 nt for GAPDH or \( \beta \)-actin, respectively.

Detection of Phytoestrogens by Gas Chromatography Mass Spectrometry

To detect potential phytoestrogens (\( \beta \)-sitosterol, stigmasterol, and resveratrol), 50 mL of red wine was extracted by solid-phase extraction. In brief, 0.5 g of RP-C 18 (Merck) was placed in a glass cartridge and then conditioned by flushing with hexane, acetone, and methanol. The samples were spiked with 17\( \alpha \)-estradiol-17- \( \alpha \)-acetate (as a surrogate standard; Sigma) and passed through the packed glass cartridges (flow rate of 20 mL/min, pH 7). Subsequently, the solid phase was dried completely, and the samples were eluted with acetone. For detection by gas chromatography mass spectrometry, the preconcentrated extracts were further cleaned over a deactivated silica gel and derivatized with a mixture of N-methyl-N-trimethylsilyl-trifluoroacetamide/triethylsilylimidazole/dithioerythritol (1000:2:2, v/v/w; Sigma), as described previously. The derivatized sample extracts were measured by gas chromatography mass spectrometry (Hewlett Packard 5890 series II coupled with a Hewlett Packard 5971 mass selective detector).

Western Blotting of eNOS Protein

To measure eNOS protein expression, total protein was isolated and Western blots were performed as previously described. Densitometric analyses were performed with a Video-Imager (BioRad). The eNOS protein bands were normalized using the respective \( \beta \)-tubulin protein bands.

Determination of eNOS Activity

eNOS activity was determined by measuring NO\( _2 \) formation. EA.hy 926 cells were preincubated for 72 hours with resveratrol and then stimulated for 2 minutes with the calcium ionophore A23187 (10 \( \mu \text{mol/L} \)). NO\( _2 \) was measured in the supernatant by chemiluminescence using a NOA 280 Nitric Oxide Analyzer (Sievers). Total protein content was determined (Bradford), and NO\( _2 \) levels were normalized for protein.

Bioactive NO produced by eNOS in EA.hy 926 cells was assayed using the stimulation of soluble guanylyl cyclase in RFL-6 rat lung fibroblasts, as previously described.

Determination of eNOS Promoter Activity

EA.hy 926 cells stably transfected with p-eNOS-3500-Hu-Luc-neo (containing a 3.5-kb human eNOS promoter fragment cloned before a luciferase reporter gene) have been described previously. Extracts from resveratrol-treated and untreated cells were prepared using passive lysis buffer (Promega), and luciferase activities were determined.

Transient Transfection of EA.hy 926 Cells and Reporter Gene Assays

EA.hy 926 cells were transfected with luciferase constructs containing human eNOS promoter fragments of various lengths (0.9 \( \mu \)g of p-eNOS-1600-Hu-Luc, p-eNOS-1111-Hu-Luc, p-eNOS-633-Hu-Luc, or p-eNOS-263-Hu-Luc) using SuperFect (Qiagen). The plasmid pRL-SV40 (containing the renilla-luciferase gene driven by an SV40 promoter) was cotransfected for normalization. The luciferase and renilla-luciferase activities of the extracts were determined using the Dual-Luciferase System (Promega).

Electrophoretic Mobility Shift Assays

Binding activities of transcription factors GATA, SV40 virus promoter specific transcription proteins (Sp1/Sp3), polymavirus enhancer activator 3 (PEA3), Yin Yang 1 (YY1), and E74-like factor 1 (Elf-1) in nuclear extracts from resveratrol-treated and untreated cells were determined by electrophoretic mobility shift assays, as described previously. Double-stranded oligonucleotides were used; they contained the following transcription factor–binding motifs of the human eNOS promoter: Sp1-binding motif (positions –95 to –104), Sp1/Sp3-like–binding motif (positions –141 to –146), GATA-binding motif (positions –225 to –230), PEA3-binding motif (positions –24 to –40), YY1-binding motif (positions –117 to –121), and Elf-1–binding motif (positions –126 to –129). DNA-protein complexes were analyzed on polyacrylamide gels. The dried gels were autoradiographed on x-ray film.

Statistics

Statistical differences between mean values were determined by ANOVA followed by the Fisher’s protected least significant difference test for comparison of means.

Results

Upregulation of eNOS mRNA Expression by Resveratrol in Human Endothelial Cells

As shown in Figure 1A, resveratrol enhanced the eNOS mRNA expression in HUVECs. To investigate the molecular mechanisms of this enhancement, subsequent experiments were performed in the HUVEC-derived cell line EA.hy 926. Incubation of these cells with resveratrol (1 to 100 \( \mu \text{mol/L} \)) for 24 hours or with 100 \( \mu \text{mol/L} \) resveratrol for different periods of time (12 to 48 hours) increased eNOS mRNA levels in a concentration- and time-dependent manner, as shown in Figure 1B and 1C.

Upregulation of eNOS Protein and Activity by Resveratrol in EA.hy 926 Cells

Incubation of EA.hy 926 cells with resveratrol (1 to 100 \( \mu \text{mol/L} \)) for 72 hours enhanced eNOS protein expression in a concentration-dependent fashion (Figure 2A). In the same cells, NO production (determined as NO\( _2 \) ) was increased in a concentration-dependent manner (Figure 2B).

Interestingly, short-term (2 minutes) incubation of EA.hy 926 cells with resveratrol (0.1 to 100 \( \mu \text{mol/L} \)) also increased the concentration of bioactive NO in the supernatant of EA.hy 926 cells (as measured by the activation of soluble guanylyl cyclase in RFL-6 reporter cells; Figure 2C).

Upregulation of eNOS Promoter Activity by Resveratrol in EA.hy 926 Cells

EA.hy 926 cells stably transfected with the plasmid p-eNOS-3500-Hu-Luc-neo showed significant eNOS promoter activ-
When the transfected cells were incubated for 24 hours with resveratrol (1 to 100 μmol/L; Figure 3A) or with 10 μmol/L resveratrol for different periods of time (3 to 48 hours; Figure 3B), promoter activity increased in a concentration- and time-dependent manner.

In further experiments, EA.hy 926 cells were transiently transfected with different pGL3–Basic–derived constructs containing fragments of 1.6 kb to 0.26 kb of the human eNOS promoter cloned before a luciferase reporter gene. The nonstimulated activities of the shorter promoter fragments did not differ significantly from those of p-eNOS-1600-Hu-Luc (n=3 for each). The transfected cells were incubated for 18 hours with 10 μmol/L resveratrol. Resveratrol increased the activities of all promoter fragments by 2-fold (Figure 3C).

Using electrophoretic mobility shift assays, we tested the effect of resveratrol (10 to 100 μmol/L) on the binding activities of transcription factors known to be important for the activity of the human eNOS promoter. We prepared nuclear extracts from resveratrol-treated or untreated cells and incubated them with oligonucleotides containing transcription factor–binding motifs of the human eNOS promoter for Sp1, Sp1/Sp3-like, GATA, PEA3, YY1, and Elf-1. However, no enhanced binding activity could be detected for any of these transcription factors (n=4 each; data not shown).

Resveratrol Increases eNOS mRNA Stability in EA.hy 926 Cells

EA.hy 926 cells were incubated for 24 hours with or without resveratrol (33 μmol/L). To inhibit transcription, actinomycin D was added and RNA was prepared at different times thereafter. As shown in Figure 4, the half-life of eNOS mRNA in untreated cells was ≈30 hours. Treatment of EA.hy 926 cells with resveratrol prolonged the eNOS mRNA half-life significantly.

Lack of Effect of the Estrogen Receptor Antagonists on Resveratrol-Induced eNOS Expression

The resveratrol-induced upregulation of eNOS promoter activity (Figure 5A) or eNOS mRNA expression (Figure 5B) in EA.hy 926 cells could not be prevented by the estrogen receptor antagonist ICI 182780 (10 μmol/L). The antagonist alone showed no significant effect on eNOS expression (Figure 5A and 5B). Similar effects were obtained with another pure antiestrogen, RU 58668 (10 μmol/L, n=4; data not shown).

Phytoestrogen Content of Red Wines and Their Effect on eNOS mRNA Expression in Human EA.hy 926 Cells

Resveratrol is a major polyphenolic constituent of red wine. Therefore, we determined the content of the phytoestrogens...
β-sitosterol, stigmasterol, and resveratrol in 2 red wines (Table). The content of β-sitosterol and stigmasterol were higher in the German red wine Dornfelder 1997 than in the French red wine Les Chevaliers de la Reine 1998; however, these compounds (10 μmol/L) had no effect on eNOS expression (data not shown). In contrast, resveratrol concentrations were 2.5-fold higher in the French wine than in the German wine (Table).

EA.hy 926 cells were incubated for 24 hours with these wines (10%, v/v) in the cell culture medium. The cells treated with the German red wine showed only a small enhancement of eNOS mRNA expression, whereas cells incubated with the French red wine showed a significant enhancement of eNOS mRNA (up to 2.3-fold; Figure 6). A similar increase in eNOS mRNA expression by the French red wine could be detected when primary HUVECs were used (n=5, data not shown).

Discussion

The present study demonstrates that resveratrol, in addition to its antioxidant, antiplatelet, antiinflammatory, and antitumor activities, is able to increase eNOS expression and NO production in vitro. This effect is concentration-dependent, with maximal enhancement observed at 10 μmol/L resveratrol. Furthermore, an increase in eNOS expression was also detected when primary HUVECs were treated with the French red wine. These findings are consistent with previous studies showing that resveratrol can increase eNOS expression and NO production in various cell types.

Figure 2. Resveratrol increases eNOS protein expression and NO production in a concentration-dependent manner. A, EA.hy 926 cells were incubated with different concentrations of resveratrol (Res) or left untreated (control, Co). Combined cytosolic and solubilized particulate protein fractions were prepared after 72 hours, and Western blot analysis was performed using a polyclonal anti-eNOS-antibody and a monoclonal anti-β-tubulin-antibody (β-Tub; for normalization). The blot shown in the left panel is representative of 5 independent experiments with similar results. The right panel shows densitometric analyses of the 5 different blots. Bars represent mean±SEM. **P<0.01. B, Cells were exposed for 72 hours to different concentrations of resveratrol (Res), as indicated. Then, cells were stimulated with 10 μmol/L calcium ionophore A23187 for 10 minutes. NO2⁻ was measured by chemiluminescence using a Nitric Oxide Analyzer. The NO2⁻ production of untreated cells (6.0±0.9 pmol/μg protein) was set at 100%. Data represent the mean±SEM. *P<0.05, **P<0.01. C, EA.hy 926 cells were exposed for 2 minutes to resveratrol. Conditioned medium from EA.hy 926 cells was then transferred to RFL-6 reporter cells and incubated for 2 minutes. Thereafter, cGMP content in RFL-6 cells was determined by radioimmunoassay. Basal cGMP content of the RFL-6 cells (2.1±0.17 pmol/10⁶ cells) was subtracted from all samples. The NO production of untreated EA.hy 926 cells (3.9±0.7 pmol cGMP/10⁶ RFL-6 cells) was set at 100%. Data represent the mean±SEM of 3 independent experiments. ***P<0.001.
nogenic effects, can upregulate the eNOS gene in HUVECs (Figures 1 and 2). The enhanced mRNA and protein expression resulted in an increased production of NO (Figure 2). An upregulated (and thus activated) eNOS may contribute to the vasoprotective properties attributed to resveratrol and, thus, to its beneficial effects in the cardiovascular system. Vascular NO conveys vasoprotective and antiatherosclerotic effects through a number of mechanisms.

Resveratrol is a major antioxidant and potential phytoestrogen contained in red wine (Table). However, resveratrol alone cannot be responsible for the eNOS upregulation produced by “good” red wine (eg, the French wine; Figure 6). The resveratrol concentration in the French wine was 74 μmol/L (Table), and thus 7.4 μmol/L in the cells in Figure 6. This concentration produced an increase in eNOS mRNA to -150% of control (Figure 1B), whereas the wine increased eNOS mRNA expression to -230% of control (Figure 6).

Thus resveratrol, although a major antioxidant component of red wine, is unlikely to be the sole agent responsible for red wine’s beneficial effects on eNOS expression (and probably other cardiovascular parameters). Wine, especially red wine,
Phytoestrogens in Red Wines as Measured by Gas Chromatography Mass Spectrometry

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dornfelder 1997 (German)</th>
<th>Les Chevaliers de la Reine 1998 (French)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Sitosterol, nmol/L</td>
<td>7.7</td>
<td>4.3</td>
</tr>
<tr>
<td>Stigmasterol, nmol/L</td>
<td>36.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Resveratrol, nmol/L</td>
<td>29 800</td>
<td>73 600</td>
</tr>
</tbody>
</table>

contains a multitude of polyphenolic ingredients, many of which have antioxidant properties and could complement the effects of resveratrol.

However, there seems to be a correlation between the resveratrol content of red wine and its ability to upregulate eNOS expression. The German wine had a much lower resveratrol content than the French wine, and it had only a marginal effect on eNOS expression (Figure 6).

When taking into account the resveratrol content of a “good” red wine (=75 μmol/L, Table) and a reasonable wine consumption, it becomes apparent that effective concentrations are unlikely to be reached in plasma in vivo. However, resveratrol is a lipophilic substance and has been shown to accumulate in tissues such as the heart, liver, and kidney. 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.

Phytosterols contained in red wine, such as β-sitosterol and stigmasterol (Table), do not seem to contribute significantly to the eNOS upregulation produced by red wine. Both phytosterols were present in higher concentrations in the German than the French wine (Table), with the German wine having very little effect on eNOS expression (Figure 6). β-Sitosterol and stigmasterol are natural constituents of the human diet, but their phytoestrogenic properties are questionable. Interestingly, the eNOS-stimulating effect of resveratrol is unlikely to be mediated by estrogen receptors, because compounds ICI 182780 and RU 58668, antagonists at α and β estrogen receptors, failed to influence the stimulatory effect of resveratrol on eNOS mRNA expression and eNOS promoter activity (Figure 5).

The upregulation of eNOS expression by resveratrol involves transcriptional and post-transcriptional (mRNA-stabilizing) mechanisms (Figures 3 to 5). The predominant transcriptional component seems to involve an activation of rather proximal portions of the eNOS promoter (first 263 bp), because the transcriptional stimulation by resveratrol was largely preserved with this short promoter fragment (Figure 3C). This fragment is the binding target of a number of transcription factors, such as Sp1, GATA, PEA3, YY1, and Elf-1, whose functional relevance has been demonstrated previously. In electrophoretic mobility shift assays, no resveratrol-induced changes in protein-DNA binding were found with oligonucleotides containing the cognate Sp1, Sp1/Sp3-like, GATA-, PEA3-, YY1-, or Elf-1-binding motifs of the proximal human eNOS promoter. This suggests that the transcriptional activation caused by resveratrol is unlikely to be based on the activation of one of the above transcription factors alone. Rather, it may be a multifactorial process and/or involve yet unidentified transcription factor(s).

Various types of cardiovascular pathologies, such as hypertension, atherosclerosis, and diabetes, are associated with an overactivity of nicotinamide adenine dinucleotide (phosphate) oxidase. The resulting overproduction of superoxide inactivates eNOS-derived NO and can even lead to eNOS uncoupling. Recently, resveratrol has been shown to inhibit vascular nicotinamide adenine dinucleotide (phosphate) oxidase activity and subsequent superoxide generation (at the same concentrations as used here), thereby promoting endothelium-dependent, NO-mediated vasorelaxation. By slowing the degradation of NO, this additional mechanism could markedly enhance the biological effects of the eNOS stimulation observed here. Also in the present study, short-term exposure of endothelial cells to resveratrol (for 2 minutes) enhanced the release of eNOS-derived bioactive NO from endothelial cells (Figure 2C). The molecular mechanism behind this effect can be either a short-term enhancement of eNOS activity or a reduction of endothelial superoxide production similar to the findings of Orallo et al.

In conclusion, the present study demonstrates that resveratrol can stimulate the expression of the eNOS gene, thus leading to an enhanced production of bioactive NO. These effects could contribute to the cardiovascular protection attributed to resveratrol.

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References
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