Resveratrol, a Polyphenolic Compound Found in Wine, Inhibits Tissue Factor Expression in Vascular Cells

A Possible Mechanism for the Cardiovascular Benefits Associated With Moderate Consumption of Wine

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Abstract—A number of studies suggest that moderate consumption of red wine may be more effective than other alcoholic beverages in decreasing the risk of coronary heart disease mortality. The phytochemical resveratrol found in wine, derived from grapes, has been thought to be responsible for cardiovascular benefits associated with wine consumption because it was shown to have antioxidant and antiplatelet activities. In the present investigation, we examined the effect of resveratrol on induction of tissue factor (TF) expression in vascular cells that were exposed to pathophysiological stimuli. The data presented herein show that resveratrol, in a dose-dependent manner, inhibited the expression of TF in endothelial cells stimulated with a variety of agonists, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα) and lipopolysaccharide (LPS). A similar inhibition of TF induction was also seen in LPS stimulated monocytes that were pretreated with resveratrol before their stimulation with LPS. In addition, resveratrol was shown to inhibit the LPS-induced expression of TNFα mRNA in endothelial cells and of TNFα and IL-1β mRNA in monocytes. Nuclear run-on analysis in endothelial cells showed that resveratrol inhibited TF expression at the level of transcription. However, resveratrol did not significantly alter the binding of the transcription factors c-Fos/c-Jun and c-Rel/p65, the transcription factors required for the induction of TF promoter in both endothelial cells and monocytes. Similarly, resveratrol had no significant effect on the binding of NF-κB in endothelial cells stimulated with IL-1β, TNFα, and LPS. Overall, our data show that resveratrol could effectively suppress the aberrant expression of TF and cytokines in vascular cells, but it requires further investigation to understand how resveratrol exerts its inhibitory effect. (Arterioscler Thromb Vasc Biol. 1999;19:419-426.)

Key Words: tissue factor • resveratrol • endothelial cell • monocytes • cardioprotective effect

Several epidemiological studies suggest that coronary heart disease mortality is lowered by moderate consumption of alcohol, particularly red wine.1–5 Resveratrol (3,5,4’-trihydroxystilbene), a polyphenol present in wine, has been thought to be responsible for cardiovascular benefits associated with moderate wine consumption.6–8 Resveratrol, a phytoalexin, is produced in grapes and a variety of other plants in response to fungal infection or other types of stress.9–12 Because of its high concentration in grape skin, most of the red wines contain significant amounts of resveratrol.13 Cardiovascular benefits of moderate wine consumption have been thought to stem, at least partly, from antioxidant6 and antiplatelet activities of resveratrol.8,14 Resveratrol has been shown to protect human LDL against copper-catalyzed oxidation8 and to inhibit human platelet aggregation and thromboxane B2 synthesis.8 Recent studies in which humans consumed resveratrol-enriched grape juice showed that trans-resveratrol can be absorbed from grape juice in biologically active quantities and in amounts that are likely to cause reduction in the risk of atherosclerosis.15

Thrombosis plays a critical role in the development, progression, and clinical sequelae of atherosclerosis. Tissue factor (TF), a cell-surface glycoprotein, is the primary initiator of the coagulation cascade in both hemostasis and pathogenesis.16–18 TF is constitutively expressed in several extravascular cells, such as fibroblasts and pericytes, but not in cells within the vasculature, such as endothelial cells and monocytes.19,20 Little or no TF is found in the intima or media of normal arteries,19,21 whereas abundant TF is found in atherosclerotic plaques.21 TF antigen in human atherosclerotic plaques is localized within macrophages, smooth muscle cells, and endothelial cells and in the acellular lipid-rich core.21,22 Accumulation of TF in atherosclerotic plaques is thought to play a major critical role in determining plaque thrombogenicity.17

Because thrombosis is the integral part of the atherosclerosis and coronary artery disease, it is possible that the decreased risk of coronary heart disease associated with consumption of red wine, in part, could be caused by its...
ability to suppress the expression of TF in the arterial wall. In the present study, we have examined the effect of resveratrol on induction of TF expression in both endothelial cells and monocytes. The data show that resveratrol inhibits the induction of TF in these vascular cells exposed to various pathophysiological stimuli.

Methods

Reagents
Resveratrol, phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS, Escherichia coli serotype O111:B4), human recombinant tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), endothelial cell growth supplement (ECGS), fibronectin, and Histopaque were obtained from Sigma Chemical Co. A stock concentration (100 mM) of resveratrol was made in ethanol. RPMI and F-12 culture media were obtained from Gibco-BRL, Life Technologies. Fetal bovine serum, trypsin-versene mixture, penicillin-streptomycin, and l-glutamine were obtained from Bio-Whittaker. TRI reagent was from Molecular Research Center Inc. [γ-32P]ATP (3000 Ci/mmol) and [α-32P]CTP (3000 Ci/mmol) were from Dupont NEN. cDNA probes for TNFα, IL-1β, and others were obtained from American Type Culture Collection. Most of the molecular biology-grade chemicals were obtained from either Boehringer Mannheim or United States Biochemicals.

Coagulant Proteins
Recombinant VIIa was a gift from Novo-Nordisk. Human plasma factor X23 and factor Xa24 were purified as described earlier or purchased from Enzyme Research Laboratories Inc.

Human Umbilical Vein Endothelial Cells
Primary cultures of human umbilical vein endothelial cells (HUVEC) were obtained from Cell Systems and cultured at 37°C under 5% CO2 in T-75 flasks that were coated with Matrigel (BD Biosciences). Fetal bovine serum, trypsin-versene mixture, penicillin-streptomycin and l-glutamine were obtained from GIBCO-BRL, Life Technologies. F-12 culture media were obtained from GIBCO-BRL, Life Technologies.

Monocytes
Mononuclear cells were isolated from blood as described earlier.25 Briefly, 20 to 50 mL of blood was drawn from healthy volunteers into a plastic syringe with a 19-gauge needle and immediately added to a plastic tube containing heparin (a final concentration of 10 U/mL). Mononuclear cells were isolated by applying blood, which was diluted with an equal volume of sterile saline, on top of Histopaque 1077 (1.5 mL of Histopaque for 1 mL of blood) followed by density gradient centrifugation at 500g for 15 minutes at room temperature. The mononuclear cell band was collected and washed with sterile 0.15 mol/L NaCl for 2 hours with a control vehicle (ethanol) or varying concentrations of resveratrol for 2 hours and then stimulated with LPS (100 ng/mL) for 5 hours. The cells were continuously mixed (200 rpm) in a shaker incubator at 37°C during the incubation period. At the end of the incubation period, cells were sedimented by centrifugation and resuspended in RPMI medium. The cell suspension contained approximately 2.5 × 106 cells/mL. The cell suspension was frozen at −20°C until tested to determine TF activity.

Measurement of TF Procoagulant Activity
TF activity was measured as the ability of cell lysates to support the activation of factor X with the addition of VIIa and CaCl2. Measurement of TF activity was as follows: cell lysates (45 μL) were incubated with a reagent mixture (5 μL) containing factor VIIa (0.5 μg/μL), factor X (10 μg/μL), and CaCl2 (5 mol/L; all concentrations were final concentrations) in a 96-well plate. At the end of 15 minutes, 50 μL of Chromozym X (Boehringer Mannheim) containing 25 mol/L EDTA was added to each well, and the initial rate of color development in mOD/min at 405 nm was measured with a microplate reader ( Molecular Devices). This initial rate was converted to micrograms per milliliter of Xa from a standard curve prepared by adding 50 μL of Chromozym X to 50-μL serial dilutions of a 1-μg/mL sample of purified human factor Xa. Cell lysates from PMA treatments were diluted 100 times, whereas cell lysates from other treatments were diluted 10 times before they were used in the assay.

Analysis of TF mRNA
Total RNA was prepared from 2 to 3 × 106 cells by the acid phenol method using TRI reagent according to the manufacturer's technical bulletin. Ten micrograms of total RNA was size fractionated by gel electrophoresis in 1% agarose/6% formaldehyde gels and transferred onto the nitrocellulose membrane by a capillary blot method. Northern blots were prehybridized at 42°C with a solution containing 50% formamide, 5% SSC, 50 mol/L Tris HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 1% polyvinylpyrrolidone, 1% Ficoll, 25 mol/L EDTA, 100 μg/mL denatured salmon sperm DNA, and 1% BSA and hybridized with 32P-labeled TF cDNA probe and other cDNA probes (105 cpm/mL) as described earlier.27 The filters were exposed to either Dupont NEN or Fuji RX x-ray film.

Isolation of Nuclei and Run-on Transcription Assay
Nuclei from 4 to 6 × 106 HUVEC were harvested as described earlier,27 and run-on assays were performed with [α-32P]UTP-labeled RNA as described previously.28

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay
Nuclear extracts were prepared from 4 to 6 × 106 HUVEC as described in our earlier publication.20 Protein concentration in nuclear extracts was determined using the Bio-Rad protein assay kit. Oligonucleotides for the
These experiments, resveratrol (100 μmol/L) to be an effective inhibitor of TF induction. For HUVEC monolayers were preincubated for 2 hours with resveratrol for 2 hours before the addition of LPS suppressed TF activity by 75%, and the inhibition was about 95% in cells that were incubated with resveratrol for 6 hours before the addition of LPS. Resveratrol also markedly suppressed the induction of TF activity in endothelial cells when it was added at the time of LPS addition, and the suppression was minimal when it was added 2 hours after the addition of LPS (data not shown). Additional experiments were performed to determine how long the suppressive effect of resveratrol lasts after its removal from the endothelial cell surface. HUVEC were treated with resveratrol for 2 hours, washed with medium to remove resveratrol, and then stimulated with PMA at defined times after the wash. The data showed that a continuous presence of resveratrol was required to suppress the induction of TF because more than 60% of the PMA-induced TF activity was recovered when resveratrol was removed from the cell surface immediately before the addition of PMA. A full PMA-induced TF activity was obtained when the cells were stimulated 1 to 2 hours after the removal of resveratrol (data not shown). In this respect, resveratrol-mediated suppression of TF was similar to that of salicylate-mediated suppression of TF induction.30

To test whether resveratrol can also inhibit the induction of TF activity in monocytes, mononuclear cell fraction was isolated by density gradient centrifugation from heparinized blood drawn from healthy human volunteers, and mononuclear cell fractions were preincubated with various concentrations of resveratrol (0 to 100 μmol/L) for 2 hours at 37°C before they were stimulated with LPS (100 ng/mL) for 5 hours to induce TF expression in monocytes. As shown in Figure 2, resveratrol inhibited the LPS-induced TF expression in monocytes in a dose-dependent manner. The half-maximal concentration of resveratrol required to inhibit the induction of TF activity in monocytes is approximately 10 μmol/L, similar to the concentration of resveratrol required to inhibit platelet aggregation and cyclooxygenase activity.11,31 One should note that resveratrol alone had no effect on the induction of TF, ie, the cells (both endothelial cells and monocytes) treated with resveratrol (up to 200 μmol/L), in the absence of the inducer, did not express TF activity. Further, the control vehicle, ethanol (0.1% vol/vol), had no effect on the agonist-induced TF expression in both endothelial cells and monocytes.

Although the concentrations of resveratrol used in the above experiments are similar or lower than the concentrations of resveratrol used by other investigators with different cell
Resveratrol Suppresses Induction of TF mRNA

We next examined whether resveratrol inhibits the induction of TF mRNA in endothelial cells. HUVEC monolayers were preincubated with various concentrations of resveratrol (0, 5, 20, 100, and 200 μmol/L) for 2 hours and then treated with PMA for 2 hours to induce TF mRNA. Northern blot analysis revealed a dose-dependent inhibition of TF mRNA accumulation in resveratrol-treated cells. A concentration of 100 μmol/L resveratrol inhibited the PMA-induced TF mRNA accumulation by more than 95%. Endothelial cells that were treated with resveratrol (200 μmol/L) but not stimulated with PMA expressed no measurable TF mRNA (data not shown). Resveratrol also suppressed LPS-, TNFα-, and IL-1β-induced TF expression in HUVEC (Figure 3). Pretreatment of HUVEC with resveratrol also inhibited LPS-induced accumulation of TNFα mRNA (Figure 4). Next, we investigated the effect of resveratrol on LPS-induced expression of TF, TNFα, and IL-1β mRNA in monocytes. As shown in Figure 5, pretreatment of mononuclear cells with resveratrol markedly inhibited the expression of TF, TNFα, and IL-1β mRNA in monocytes. (Because there was no significant induction of IL-1β mRNA in stimulated HUVEC under our experimental conditions, we could not test the effect of resveratrol on the induction of IL-1β mRNA in endothelial cells.)

Resveratrol Inhibits Induction of TF Gene Transcription

To determine whether the lower number of TF mRNA transcripts in stimulated endothelial cells that were treated with resveratrol was caused by a decrease in transcriptional activation of the TF gene, nuclear run-on experiments were performed. As shown in Figure 6, there was minimal transcription of the TF gene in unstimulated endothelial cells, and PMA treatment markedly enhanced the transcriptional rate. The data also showed that treatment of endothelial cells with resveratrol before the addition of PMA markedly suppressed the PMA-induced transcriptional activation of the TF gene. Quantitative analysis of the hybridization signal using a PhosphorImager showed that resveratrol suppressed the tran-
Resveratrol increases the transcriptional rate of the TF gene by more than 70%. Resveratrol also inhibited transcriptional activation of tissue plasminogen activator. In contrast, resveratrol had no effect on PMA-induced transcriptional activation of IL-8, plasminogen activator inhibitor (PAI)-2, and urokinase plasminogen activator (Figure 6). Transcription rate of the TF pathway inhibitor (TFPI) gene was unchanged with PMA treatment, and resveratrol had no effect on the transcription of the TFPI gene. These data illustrate that the resveratrol suppresses TF gene transcription by a specific mechanism and not by acting as a nonspecific inhibitor of transcription.

Resveratrol Does Not Affect DNA Binding of c-Fos/c-Jun and c-Rel/p65 Transcription Factors

Earlier studies showed that induction of TF gene transcription in endothelial cells and monocytes is regulated by the interaction of c-Fos/c-Jun and c-Rel/p65 bound to TF promoter, and electrophoretic mobility shift assays (EMSA) have been used to demonstrate the binding of these transcription factors to specific sequences in the TF promoter. To determine the mechanism by which resveratrol suppresses the induction of TF gene transcription in endothelial cells, we examined the binding of nuclear proteins to oligonucleotides containing sequences of the AP-1 site and the κB-like site of the TF promoter. HUVEC monolayers were treated with a control vehicle or resveratrol (100 μmol/L) for 2 hours and then stimulated for 1 hour with TNFα and IL-1β. Nuclear extracts were analyzed by EMSA using radiolabeled oligonucleotides containing AP-1 (proximal) and κB-like sites in the TF promoter. As shown in Figure 7, nuclear extracts from both unstimulated and stimulated endothelial cells formed a DNA–protein complex with the AP-1 site, and resveratrol treatment had no significant effect on AP-1 binding activity of both unstimulated and stimulated endothelial cells. Quantitative analysis of the data showed no statistically significant differences in the levels of AP-1 binding in nuclear extracts from cells pretreated with resveratrol or a control vehicle (probability value varied between 0.48 and 0.89 in different treatments). EMSA performed to examine the binding of nuclear proteins to an oligonucleotide containing the TF κB-like site showed a minimal DNA–protein complex in unstimulated cells, whereas a prominent DNA–protein complex was observed in TNFα- and IL-1β-stimulated cells (Figure 8). Treatment of cells with resveratrol (100 μmol/L) for 2 hours before the addition of stimuli had no effect or only a minimal effect on the formation of the inducible complex. Quantitative analysis of the data showed...
no statistically significant differences between the levels of c-Rel/p65 in nuclear extracts from cells treated with either TNF-α or IL-1β in the presence and absence of resveratrol (IL-1β treatment, \( P = 0.89 \); TNF-α treatment, \( P = 0.66 \); \( n = 4 \)). Similar results were also obtained using a κB site that binds to p50/p65, the heterodimers that regulate TNF-α gene transcription (Figure 9). No differences were found in their binding activity to the κB site between the nuclear extracts isolated from the cells treated with the control vehicle and the cells treated with resveratrol before they were stimulated with TNF-α, IL-1β, or LPS. However, a moderate inhibition was observed in binding activity to the κB site in cells that were pretreated with resveratrol and stimulated with PMA.

**Discussion**

Several epidemiological studies have suggested that coronary heart disease mortality can be decreased by moderate consumption of alcohol, particularly red wine.\(^1\)\(^-\)\(^3\) However, it is unclear how the moderate consumption of red wine provides the protective effect against heart disease. Although the cardioprotective effects of most alcoholic beverages are probably because of ethanol-induced elevation of HDLs\(^35\),\(^36\) and lowering of platelet aggregation,\(^37\),\(^38\) factors other than ethanol in alcohol-containing beverages may also be responsible for the protective effect of alcoholic beverages.\(^7\),\(^39\),\(^40\) Resveratrol, a phenolic compound found primarily in the skin of grapes and relatively abundant in red wine, has been thought to be one such factor because it has been shown to have antioxidant\(^6\) and antiplatelet\(^8\),\(^14\) activities. However, in-depth knowledge on whether and how resveratrol provides the protective effect against heart disease is lacking.

Thrombotic disorders are the main cause of myocardial infarction and stroke.\(^41\) Activation of the TF-mediated coagulation pathway not only plays a major role in determining plaque thrombogenicity but could also have other effects on the vessel wall.\(^17\) For example, thrombin\(^42\) and factor Xa,\(^43\) intermediary products generated in the TF-mediated pathway of coagulation, are shown to promote vascular smooth cell...
proliferation and thus may play a role in the development of intimal hyperplasia. Furthermore, recent studies show that TF may also function as a true receptor in generating intracellular signals and thus may participate in other biological effects, including cell adhesion and migration. Macrophages, smooth muscle cells, and endothelial cells in atherosclerotic arterial wall were shown to express TF. Therefore, our present observations that show resveratrol suppresses the induction of TF expression in both endothelial cells and monocytes suggest an alternative or an additional mechanism by which the moderate consumption of red wine could contribute to the decreased risk of coronary artery disease observed in epidemiological studies. However, we should add a caveat to the above interpretation that HUVEC, although commonly used as a model for endothelial cell function, are not representative of the vascular tree affected by atherosclerosis. Given the heterogeneity of endothelial cell function and response to agonists, it would be important to extend the above data with resveratrol to other endothelial cell types before reaching a generalized conclusion.

The half-maximal concentration of resveratrol required to inhibit the induction of TF activity in monocytes and endothelial cells (10 to 20 μmol/L) is similar to the reported concentrations of resveratrol required to inhibit platelet aggregation and eicosanoid synthesis. Goldberg and colleagues found that resveratrol inhibited thromboxane B2 synthesis and thrombin-induced platelet aggregation with IC50 of 7 μmol/L and 160 μmol/L, respectively. Bertelli et al. reported that resveratrol at the concentration of 10 μmol/L decreased collagen-induced platelet aggregation by 44%. Recent studies of Jang et al. showed that resveratrol inhibited cyclooxygenase activity with an IC50 of 15 μmol/L. It is not clear whether resveratrol could be present in this range in vivo during the consumption of red wine. We are not aware of any data on serum levels of resveratrol after wine consumption or its absorption and metabolism. In general, the concentration of trans-resveratrol in red wines is in the range 10 to 20 μmol/L, although as high as 100 μmol/L has been recorded. Furthermore, in addition to free trans-resveratrol, most red wines also contain high concentrations of cis-resveratrol and resveratrol glycosides that could contribute to the biologically available dose. Therefore, it is possible that moderate consumption of red wine could provide resveratrol in sufficient doses to influence the expression of TF in vascular cells. In this context, it may be important to point out that recent studies of Goldberg and colleagues with healthy human subjects who consumed red wine and grape juice enriched with resveratrol showed that trans-resveratrol can be absorbed in biologically active quantities and in amounts that are likely to reduce the synthesis of thromboxane B2 synthesis and probably thrombin-induced platelet aggregation.

Resveratrol-mediated inhibition of TF activity in endothelial cells is caused by inhibition of transcriptional activation of the TF gene. Suppression of TF activity is associated with the lower accumulation of TF mRNA. Nuclear run-on assays demonstrated that the rate of TF gene transcription is reduced by 70% in resveratrol-treated endothelial cells. Induction of TF gene transcription in both endothelial cells and monocytes is mediated by the functional interaction between c-Fos/c-Jun and c-Rel/p65 heterodimers. c-Fos/c-Jun heterodimers bind to two AP-1 sites, and c-Rel/p65 heterodimers bind to a κB-like site in the TF promoter. Although c-Fos/c-Jun heterodimers bind to AP-1 sites in both unstimulated and stimulated endothelial cells, the binding is an absolute requirement in stimulated endothelial cells for c-Rel/p65 heterodimers to facilitate activation of TF gene transcription. Therefore, either blocking of c-Fos/c-Jun binding or activation of c-Rel/p65 could downregulate TF transcription. However, our present data show that resveratrol had no significant effect on the DNA binding activity of c-Fos/c-Jun or c-Rel/p65 heterodimers. Therefore, it is unlikely that the resveratrol-mediated suppression of TF gene expression in endothelial cells is caused by reduced DNA binding of c-Fos/c-Jun or c-Rel/p65 in endothelial cells. Nonetheless, it is possible that the resveratrol-mediated suppression of TF gene expression involves the repression of AP-1- or NF-κB-mediated transcription, because resveratrol also suppressed the expression of TNFα and IL-1β, both of which are regulated by AP-1- and NF-κB-dependent transcription. It is possible that resveratrol may modulate NF-κB activity by altering phosphorylation of p65 or cofactors that are required for transactivation or inducing synthesis or activation of inhibitors of NF-κB-mediated transcription. Further work is required to elucidate how resveratrol suppresses the induction of TF expression in vascular cells.

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References

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