Activity in vitro of resveratrol on granulocyte and monocyte adhesion to endothelium1,2

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ABSTRACT
Background: Resveratrol is a phytoalexin present in red wine. It has been shown to protect LDL from peroxidative degradation.
Objective: In consideration of the low plasma concentration of orally adsorbed resveratrol (which is insufficient for antioxidant protection of LDL), we studied another effect of the compound.
Design: Because resveratrol is a tyrosine kinase inhibitor like other members of the tyrphostin family, we hypothesized that it has the ability to modify intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression by stimulated endothelial cells. We studied the ability of resveratrol to inhibit such adhesion molecule expression and to block the adhesion of monocytes and granulocytes to endothelial cells.
Results: We showed that resveratrol, at concentrations as low as 1 μmol/L and 100 nmol/L, significantly inhibited ICAM-1 and VCAM-1 expression by tumor necrosis factor α (TNF-α)-stimulated human umbilical vein endothelial cells and lipopolysaccharide-stimulated human saphenous vein endothelial cells (HSVEC), respectively. In addition, we showed that resveratrol induced a significant inhibition in the adhesion of U937 monocytes to lipopolysaccharide-stimulated HSVEC. Such inhibition was comparable with that obtained when anti–VCAM-1 monoclonal antibody was used instead of resveratrol. Resveratrol also significantly inhibited the adhesion of neutrophils to TNF-α-stimulated NIH/3T3 ICAM-1-transfected cells, whereas neutrophils activated by formyl-methionyl-leucyl-phenylalanine did not significantly modify adhesion to NIH/3T3 ICAM-1-transfected cells.

KEY WORDS Endothelium, granulocytes, grapes, monocytes, resveratrol, wine, LDL, lipid peroxidation, regulation of expression

INTRODUCTION
Lipid oxidation in LDLs, or lipid peroxidation, has been proposed as a potential mechanism in the pathogenesis of atherosclerotic processes. In fact, oxidized LDLs favor the transformation of macrophages into foam cells (1). In addition, oxidized LDLs stimulate endothelial cells to produce monocyte chemotactic protein 1 and granulocyte and macrophage colony-stimulating factors (2), and they have direct chemotactic activity for monocytes (3). Finally, oxidized LDLs promote the binding of monocytes to endothelium (4).

The possibility of inhibiting LDL oxidation has been investigated by using antioxidants [probucol (5, 6), vitamin E (7), α-tocopherol (8), vitamin C, and β-carotene (9)]. However, it is difficult to attribute a reduction of atherosclerotic processes and consequently protection from coronary artery disease to only the inhibition of LDL oxidation because many vascular effects of antioxidants are not related to the resistance of LDL to oxidation (9).

Intake of flavonols has been related to a reduced rate of coronary artery disease (10). In addition, phenolic substances contained in red wine have been found to inhibit oxidation of human LDL (11). In particular, such activity has been shown for resveratrol, a phytoalexin (3,4,5-trihydroxystilbene) present in red wine (12). Recently, resveratrol, like the flavonoids, was found to protect porcine chelating and free radical–scavenging mechanisms, thus protecting LDL from peroxidative degradation (13). Tea flavonoids reduce the oxidizability of LDL in vitro and in vivo (14), and red wine consumption reduces the susceptibility of human plasma and LDL to lipid peroxidation (15).

We showed previously that resveratrol [which is present in grape juice and wine and has anticancer (16), antiinflammatory, and antiaggregating (17) properties] is adsorbed after oral ingestion, quickly enters the bloodstream, and is detectable in different organs (18). Because plasma concentrations of orally adsorbed resveratrol are 10–100-fold lower than those useful for...
any antioxidant activity on LDL (18), and in consideration of the small contribution of resveratrol to the total antioxidant activity of red wine (19), we hypothesized a possible mechanism of action for the compound other than antioxidative functions. In fact, resveratrol has a chemical structure similar to that of the tyrphostin family (20) and possesses, like such compounds, protein kinase inhibitory activity (21).

In previous studies we showed that in vivo administration of another natural tyrosine kinase inhibitor, lavendustin A, prevents platelet endothelial cell adhesion molecule 1 (PECAM-1) expression in vitro and its surface distribution on endothelium (22). Consequently, we hypothesized a role for resveratrol in the regulation of endothelial adhesion molecule expression, in view of the possibility that its antiatherogenic activity is not necessarily related to the protection of LDL from oxidation (9). We previously tested the in vitro efficacy of resveratrol regulation on the expression of intercellular cell adhesion molecule-1 (ICAM-1) by endothelial cells. ICAM-1 promotes the adhesion of monocytes, neutrophils, and lymphocytes to endothelium and has been detected in atherosclerotic lesions in humans (23).

In the present study, we evaluated the influence of resveratrol on ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) endothelial expression. Moreover, we studied the modifications induced by resveratrol on the adhesion of monocytes and neutrophils to endothelial cells and on neutrophil phagocytic activity. The aim of the study was to show the possible activity of resveratrol in modulating endothelial cell adhesion molecule expression and in modifying cell adhesion to endothelium.

MATERIALS AND METHODS

Addition of resveratrol to cells

Resveratrol (Sigma–Aldrich Co, Milan, Italy) was solubilized first in dimethyl sulfoxide and subsequently in 199 medium (Gibco, Paisley, United Kingdom). Successive dilutions removed the dimethyl sulfoxide. The concentrations of resveratrol used, 100 nmol/L and 1 μmol/L, were shown to be nontoxic by measuring cell viability with the trypan blue dye exclusion test. In a previous dose-response experiment on the activity of resveratrol on ICAM-1 endothelial expression, we found that 100 nmol/L and 1 μmol/L were the most effective concentrations for lowering ICAM-1 expression by tumor necrosis factor α (TNF-α)–stimulated human umbilical vein endothelial cells (HUVEC). We chose to use such concentrations for successive experiments. Resveratrol concentrations >10 μmol/L affected cell viability.

Endothelial cell cultures

Primary cultures of HUVEC were derived from umbilical cords, maintained as described previously (24), and tested within the fourth passage. HUVEC were found to express endothelial-related antigens such as PECAM-1 and factor VIII (data not shown).

Human saphenous vein endothelial cells (HSVEC) were isolated and cultured from unused portions of saphenous veins harvested for coronary artery bypass surgery. The cells were harvested enzymatically with 0.1% type II collagenase (Sigma–Aldrich Co), as described previously (25), and maintained in 199 medium containing HEPES (25 mmol/L), heparin (1% Liquemin; Roche, Milan, Italy), endothelial cell growth factor (50 mg/L; Sigma–Aldrich Co), l-glutamine (1%; Sigma–Aldrich Co), antibiotics, and 5% fetal calf serum (Flow Laboratories, Irvine, United Kingdom). Once grown to confluence, the cells were replated on low-pyrogen fibronectin (1.5 μg/cm²; Sigma–Aldrich Co). HSVEC thus isolated formed a confluent monolayer of polygonal cells and expressed von Willebrand factor as determined by their content of specific messenger RNA and immunoreactive protein.

The permanent human EA.hy926 endothelial cell line was kindly provided by C Edgell (Pathology Department, University of North Carolina at Chapel Hill). EA.hy926 cells were maintained in Dulbecco’s modified Eagles medium (Gibco) supplemented with 10% fetal calf serum (Flow Laboratories), gentamicin (50 mg/L; Gibco), and nystatin (40 000 U/L; Gibco). The cell line has been shown to express factor VIII–related antigens with the same morphologic distribution as in primary human endothelial cells. NIH/3T3 ICAM-1–transfected cells were kindly supplied by R Pardi (Human Immunology Unit, Scientific Institute San Raffaele, Milan, Italy). ICAM-1 transfectants were obtained as described previously (26).

ICAM-1 expression

Immunofluorescence analysis of ICAM-1 expression was performed by using HUVEC. Confluent monolayers of HUVEC were exposed to human recombinant TNF-α (100 000 U/L; Genzyme, Boston) for 16 h in the absence and presence of resveratrol at 2 different concentrations. The monolayers were evaluated for the expression of ICAM-1 molecules by using a FACScan Plus equipped with an argon ion laser (Becton Dickinson, San Jose, CA).

VCAM-1 expression

VCAM-1 expression by HSVEC was evaluated by cell surface enzyme immunoassays using mouse anti-human monoclonal antibody against VCAM-1 (Ab E1/6). Enzyme immunoassays were carried out by incubating monolayers in 96-well plates, first with saturating concentrations of specific monoclonal antibody against the target molecule, then with biotinylated goat anti-mouse immunoglobulin (Ig) G, and finally with streptavidin alkaline phosphatase. Layers were washed 3 times between each incubation step and the integrity of the monolayer was monitored by phase contrast microscopy. The surface expression of each protein was quantified spectrophotometrically and the optical density of the wells (at 410 nm) was read 15–60 min after the addition of a chromogenic substrate (para-nitrophenylphosphate) as described previously (27). Cultured HSVEC were preincubated for 12 h or coincubated for 24 h with 2 different resveratrol concentrations and then stimulated with bacterial lipopolysaccharide from Escherichia coli (4 mg/L).

Monocyte adhesion assay

Monocytic U937 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and grown in RPMI medium 1640 (Gibco) containing 10% fetal calf serum. U937 cells were concentrated by centrifugation at 400 × g for 10 min at room temperature to 1 × 10⁷ cells/L. For adhesion assays, HSVEC were grown to confluence and resveratrol (1 μmol/L) was added to 6-well tissue culture plates, after which lipopolysaccharide (4 mg/L) or interleukin 1α (10 μg/L) was added for 24 h to induce the expression of VCAM-1. As a control, some monolayers were treated with Ab E1/6. The adhesion assay was performed by adding 1 mL of the concentrated U937
cell suspension to each monolayer under rotating conditions (63 rpm in an orbital shaker) at 21 °C (27, 28). After 10 min, nonadherent cells were removed by gentle washing with medium 199 and monolayers were fixed with 1% paraformaldehyde. The number of adherent cells was determined by counting 6 different fields using an ocular grid and a 20× objective (0.16 mm²/field). Fields for counting adherent leukocytes were randomly selected at half-radius distance from the center of the monolayers. Counts were performed by 2 independent, blinded observers, with an interobserver variability <8%.

Granulocyte adhesion assay

Venous blood was anticoagulated with 0.065 mol citric acid/L (Riedel-Hannover, Germany), 0.085 mol sodium citrate/L (Pharmacia, Milan, Italy), and 2% glucose monohydrate (Riedel) in a blood-anticoagulant ratio of 7:1. Granulocytes (polymorphonuclear cells, PMNs) were isolated by dextran (Sigma–Aldrich Co) sedimentation followed by Lymphoprep (Nycomed, Oslo) gradient and hypotonic lysis of erythrocytes, as described previously (26). For the granulocyte adhesion assay, EA.hy926 cells were incubated overnight with TNF-α and resveratrol. PMNs were incubated with formyl-methionyl-leucyl-phenylalanine (fMLP) and resveratrol for 30 min and then layered on NIH/3T3 ICAM-1–transfected cells.

Endothelial cells (detached by a brief exposure to 0.25% trypsin and 0.22% EDTA) were plated and grown to confluence in 24-well plates. PMNs were radiolabeled for 1 h at room temperature with Na₂[^51]CrO₄ (37 MBq/10⁷ cells; or 1 mCi/10⁷ cells; Amersham, Milan, Italy), washed twice, and resuspended at 5 × 10⁹ cells/L in HEPES-Tyrode buffer. Their viability was assessed by the trypan blue dye exclusion test. Radiolabeled PMN suspensions (100 μL) were layered on endothelial cells in the absence or presence of resveratrol (100 nmol/L or 1 μmol/L) and incubated for 30 min at 37°C. In resting conditions, neutrophil radioactivity was ~0.05 cpm/neutrophil. The loss of radioactivity from neutrophils during the course of the experiment was <5%. Leukocyte activation was obtained by preincubation (3 min) with 10⁻⁷ mol fMLP/L, whereas endothelial cell activation was obtained by an overnight treatment with 50 000 U TNF-α/L (Endogen, Boston). At the end of the incubation, the wells were washed 3 times to remove nonadherent cells, the remaining bound cells were lysed with Triton X-100 (1%; BDH, Poole, United Kingdom), and the individual lysates were counted in a gamma counter (model 5000; Packard, Sterling, VA).

Phagocytosis and intracellular killing test

PMNs were separated as described previously (26). They were incubated with resveratrol for 5 min at different concentrations (10⁻⁵, 10⁻⁶, and 10⁻⁷ mol/L) at 37 °C. The phagocytosis rate was expressed as phagocytosis frequency (number of phagocytosing cells/total cells) and phagocytosis index (number of yeast phagocytic cells/% total cells). Intracellular killing was expressed as the percentage of yeast cells killed, ie, stained with methylene blue (0.01% in distilled water), from which the percentage of the control tube dead yeast cells (serum killing activity) was subtracted (29).

Statistical analysis

Data were analyzed by analysis of variance (UNISTAT 4.5; UNISTAT Limited, London). Significance was assumed for P < 0.05.

RESULTS

The effect of TNF-α and of TNF-α plus resveratrol on the expression of ICAM-1 by HUVEC after overnight incubation is shown in Figure 1. TNF-α significantly increased ICAM-1 expression (P < 0.05) with respect to controls, whereas treatment

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Effect of the addition of resveratrol to human umbilical vein endothelial cells, in the presence of tumor necrosis factor α (TNF-α), on the expression of intracellular adhesion molecule 1 (ICAM-1). The data represent a typical experiment. a, Control; b, resveratrol (100 nmol/L); c, resveratrol (1 μmol/L); d, TNF-α–stimulated; e, TNF-α–stimulated + resveratrol (100 nmol/L); f, TNF-α–stimulated + resveratrol (1 μmol/L).
with resveratrol alone did not alter such expression. Incubation with TNF-α plus resveratrol, at the concentrations of 100 nmol/L and 1 μmol/L, significantly reduced the expression of ICAM-1 compared with that obtained in the presence of TNF-α alone.

Shown in Figure 2 is the effect of lipopolysaccharide and of lipopolysaccharide plus resveratrol on the expression of VCAM-1 by HSVEC stimulated with lipopolysaccharide followed by preincubation for 12 h or coincubation for 24 h with resveratrol. Lipopolysaccharide significantly increased VCAM-1 expression with respect to controls, whereas treatment with resveratrol alone did not modify such expression. Preincubation for 12 h or coincubation for 24 h with resveratrol significantly decreased the expression of VCAM-1 induced by lipopolysaccharide. Similar results were obtained when interleukin 1α was used instead of lipopolysaccharide (data not shown).

In Figure 3 the adhesion of monocytoid U937 cells to HSVEC in the presence of resveratrol alone (1 μmol/L), lipopolysaccharide alone, lipopolysaccharide plus anti-VCAM-1 monoclonal antibody (at saturating concentrations), and lipopolysaccharide plus resveratrol (at the same concentration of 1 μmol/L), is shown. Lipopolysaccharide significantly increased monocytoid cell adhesion to HSVEC with respect to basal conditions. Coincubation of resveratrol with lipopolysaccharide caused a significant decrease in monocytoid cell adhesion to HSVEC with respect to lipopolysaccharide alone. The decrease was not significantly different from that achieved with the anti-VCAM-1 monoclonal antibody. Resveratrol used alone did not modify basal adhesion conditions.

The effect of overnight incubation with TNF-α alone and TNF-α plus resveratrol on the adhesion of granulocytes to EA.hy926 cells is shown in Figure 4. Activation of endothelial cells with TNF-α induced a significant increase in granulocyte adhesion, whereas treatment with resveratrol alone (at the concentrations of 1 μmol/L or 100 nmol/L) did not increase such adhesion. Incubation of cells with resveratrol (at both concentrations) plus TNF-α significantly reduced the TNF-α–stimulated granulocyte adhesion.

The effect on the adhesion of granulocytes, incubated for 30 min at 37°C in the presence of fMLP with resveratrol, to NIH/3T3 ICAM-1–transfected cells is shown in Figure 5. Resveratrol alone (at either concentration) did not modify granulocyte adhesion. Treatment with fMLP significantly increased the adhesion of granulocytes to transfected cells. Addition of resveratrol to fMLP did not significantly modify granulocyte adhesion with respect to the use of fMLP alone. Finally, the use of resveratrol at different concentrations did not significantly enhance granulocyte phagocytosis frequency, the phagocytic index, or intracellular killing (data not shown).

**DISCUSSION**

Our results show that overnight incubation of HUVEC in the presence of TNF-α plus resveratrol significantly reduced ICAM-1 expression compared with that obtained with TNF-α alone. Note that resveratrol concentrations in the experiments were similar to those detected in plasma (18). We also showed that preincubation or coincubation for 24 h with the same concentration of resveratrol significantly decreased the expression of another endothelial adhesion molecule, VCAM-1, induced on HSVEC by lipopolysaccharide. Because resveratrol possesses protein kinase inhibitory activity like the compounds of the tyrphostin family (20, 21), it is possible that it also has their capacity to modify adhesion molecule expression. We also showed previously that lavendustin A (a natural protein kinase inhibitor)

**FIGURE 2.** Effect of the addition of resveratrol to human saphenous vein endothelial cells before addition of lipopolysaccharide (LPS, 4 mg/L for 12-h preincubation, hatched columns) or in the presence of LPS at the same dose (for 24-h coincubation, solid columns) on the expression of vascular cell adhesion molecule 1 (VCAM-1). a, Control; b, resveratrol (100 nmol/L); c, resveratrol (1 μmol/L); d, LPS-activated; e, LPS-activated + resveratrol (100 nmol/L); f, LPS-activated + resveratrol (1 μmol/L). # Significantly different from LPS added alone, P < 0.05. x ± SD of 4 experiments. OD, optical density.
was able to prevent PECAM-1 expression on endothelium (22). Such data indicate that resveratrol may play a significant role in preventing pathologic events related to endothelial activation. Such activity could explain its protection against the risk of coronary artery disease, as recent epidemiologic studies have suggested for phenolic compounds (30).

Moreover, in the present study we assayed the capacity of resveratrol to modify the adhesion of mononuclear cells and neutrophils to endothelium. We first showed that coincubation of resveratrol with HSVEC and lipopolysaccharide caused a significant decrease in mononuclear cell adhesion to the endothelium compared with that obtained with lipopolysaccharide alone. The degree of inhibition was not significantly different from that obtained with interleukin 1α as a stimulus or with preincubation with resveratrol for ≤3 h before the addition of lipopolysaccharide (data not shown).

We subsequently showed that stimulation of EA.hy926 endothelial cells by overnight treatment with TNF-α significantly increased neutrophil adhesion. Coincubation with TNF-α plus resveratrol significantly reduced the increased TNF-α–dependent neutrophil adhesion. When neutrophils were activated by FMLP, treatment with resveratrol did not significantly modify neutrophil adhesion. The latter data were obtained by using NIH/3T3 ICAM-1–transfected cells. In such a condition, the only adhesion system involved was ICAM-1/lymphocyte function-associated molecule-1, an integrin located on PMNs as well as on monocytes and lymphocytes. Possibly, resveratrol did not exert its action by interfering with the intracellular processes that lead to integrin modification. Such data suggest that resveratrol may exert its effect by modulating endothelial activation instead of neutrophil activation. In fact, the reduction of TNF-α–dependent ICAM-1 expression by endothelium due to resveratrol treatment is comparable with the same resveratrol-induced reduction of TNF-α–stimulated adhesion of neutrophils to endothelium. In basal conditions, resveratrol did not inhibit ICAM-1 expression or neutrophil adhesion to endothelial cells. Finally, resveratrol did not significantly vary the phagocytic activity or the killing of neutrophils measured by in vitro tests.

Phagocytosis is characterized by a dramatic increase in neutrophil respiratory bursts (oxygen consumption). Such metabolic activity promotes the generation of reactive oxygen intermediates after the activation of NADPH oxidase localized in the plasma membrane. This enzyme is subjected to inhibition by certain flavonoids (31). Phagocytosis and oxygen free radical production should thus be inhibited by compounds with antioxidant activity, such as flavonoids. However, in our experiments, resveratrol did not inhibit neutrophil phagocytic activity. In addition, resveratrol was less potent than epicatechin or quercetin as an antioxidant in inhibiting human LDL oxidation (32). Another
study (33) showed that the antioxidant activity of different commercial wines against LDL oxidation is not a property of a single phenol compound but that the activity is widely distributed among phytochemical substances, including resveratrol. The low activity of resveratrol as an antioxidant was also shown by the fact that it completely failed to prevent the decrease in rat liver state-3 mitochondrial respiration caused by the radical initiator 2,2'-azobis-(2-amidinopropane) dihydrochloride (34).

It was shown recently that red wine consumption in volunteers did not affect oxidizability of LDL (35), thereby suggesting that the activity of wine in reducing coronary artery disease may not be related to the antioxidant activity of wine compounds. The oxidative modification of LDL plays an important role in the development of early atherosclerotic lesions (9). However, after epidemiologic studies on the effect of antioxidant administration, linking the reduced oxidation of LDL to a reduction in atherosclerosis has been problematic. The activity of resveratrol may not be strictly related to the inhibition of LDL oxidation. Because our results indicated that resveratrol acts by modulating endothelial function, its function could be that of preserving endothelial function by inhibiting the impairment of release of nitric oxide or other vasodilators, such as prostaglandins. Our data favor the interpretation that resveratrol may be able to inhibit leukocyte adhesion and reduce cellular oxidative injury because it did not favor phagocytic activation and consequently oxygen free radical production. Note that both resveratrol isomers (trans and cis) could also be useful in reducing atherosclerotic processes (and consequent coronary artery disease) through the inhibition of platelet aggregation, which we showed previously (17, 36). In fact, the concentrations of resveratrol used in the present study (which were chosen because they did not affect cell viability) were the same as those used by us to decrease collagen-induced platelet aggregation and similar to those detected in plasma after oral administration of red wine.

In conclusion, we hypothesize a role for resveratrol different from that linked to its antioxidant activity. The preventive effects of resveratrol we observed on endothelial cell activation provide a new complementary and possibly alternative explanation for the beneficial activity of the compound and could support the conclusions of a recent clinical study (35).

REFERENCES
6. Carew TE, Schwenke DC, Steinberg D. Antithromogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci U S A 1987;84:7725–9.
11. Frankel FN, Kanner J, German JB, Parkes E, Kinsella JE. Inhibition of

FIGURE 5. Effect of addition of resveratrol in the presence of formyl-methionyl-leucyl-phenylalanine (fMLP: 10^{-9} \text{mol/L}) on neutrophil adhesion to NIH/3T3 ICAM-1–transfected cells during 30 min of neutrophil stimulation. a, Basal; b, resveratrol (100 nmol/L); c, resveratrol (1 \mu mol/L); d, fMLP; e, fMLP + resveratrol (100 nmol/L); f, fMLP + resveratrol (1 \mu mol/L). SEM of 4 experiments, each done in triplicate.