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# Resveratrol Protects Vascular Endothelial Cell from Ox-LDL-induced Reduction in Antithrombogenic Activity

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## Abstract

**Dietary antioxidants are thought to be beneficial in reducing the incidence of coronary heart disease. In this study, the antithrombogenic endothelial cells (EC) defense was investigated in an experiment model in which cultured endothelial cells were incubated with aggregating platelets in the aggregometer. We examined the possible protective effect of *trans*-resveratrol (RSV) on oxidized low density lipoprotein (ox-LDL)-induced insults on the antithrombogenic activity of the vascular EC. EC were treated with ox-LDL (25 - 100 μg/ml) for 1 h with or without a 30 min-preexposure to RSV. The antiplatelet property of the endothelial cells was then shown by measuring platelet aggregation, $[Ca^{2+}]_i$ and cGMP contents in the platelets and EC. Exposure of EC to ox-LDL reduced the antiplatelet aggregating property of EC, and this effect was attenuated by pretreatment with RSV. Further studies revealed that exposure of EC to ox-LDL reduced the protein contents of endothelial nitric oxide synthase (NOS). The effect of ox-LDL on the NOS protein content was abrogated by pretreating EC with RSV. The results suggest that ox-LDL acts *via* reducing the endothelial NOS activity to suppress the antithrombogenic activity of the EC.**

**Key Words: *trans*-resveratrol, ox-LDL, endothelial cells, nonthrombogenecity, nitric oxide synthase.**

## Introduction

Oxidation of low-density lipoproteins (LDL) in the arterial walls induces endothelial dysfunction and facilitates atherogenesis (13). The atherogenic effects elicited by ox-LDL include the inhibition of endothelium-dependent vasodilation and modulation of the expressions of proatherogenic cytokines, growth factors and adhesion molecules (5, 38). Recent work suggested that ox-LDL might exert its detrimental effects on endothelium by a peroxidation mechanism (31, 36). In this regards, dietary antioxidants have been recommended to be an important factor in the prevention of the development and progression of atherosclerosis (6, 31). Several epidemiological studies also support a protective effect of antioxidants against atherosclerosis (7-9). Phenolic substances in red wine have been shown to retard or inhibit metal ion-induced LDL oxidation (7). Among them, *trans*-resveratrol (3, 4', 5-trihydroxystilbene, RSV) has been found to be the major active component (25). Recently, it has been postulated that RSV may reduce the risk of major vascular thrombotic events and may reduce the incidence of coronary heart disease (CAD) (32); however, the underlying mechanisms remain unclear. RSV was shown to have anti-inflammatory

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and anti-coagulatory properties and induce nitric oxide synthase (NOS) in pulmonary artery EC, an enzyme responsible for the biosynthesis of NO (14).

Vascular EC play a crucial role in the control of vascular tone and blood cell activation. The endothelium-derived NO is a potent inhibitor of platelet activation and a potent vasorelaxant. Acting as an antioxidant, NO can also retard the progression of LDL oxidation and abate the oxidation chemistry mediated by reactive oxygen species such as $H_2O_2$ and $O_2^-$ that occurs at physiological levels of NO (25, 37). Conversely, it has been shown that ox-LDL, through interaction with NO, can reduce the anti-platelet aggregating properties of the EC (27). Here we show that treatment of cultured EC with ox-LDL reduces its anti-platelet activation property (included platelet aggregation activity, $[Ca^{2+}]_i$ and cGMP contents) and pretreatment of EC with RSV attenuates the ox-LDL effect. Further study reveals that the antioxidant effect is correlated with an increased NOS expression and NO production by EC.

## Materials and Methods

*Chemicals*

Medium MDCB 107 was purchased from JRH Biosciences Inc. (Lenexa, KS, USA). Calcium (Fura-2 AM) probes were purchased from Molecular Probes Inc. (Eugene, OR, USA). Antibodies to NOS, $cPLA_2$ (cytoplasmic phospholipiase $A_2$) and $COX_2$ (cyclooxygenase 2) were purchased from Santa Cruz Biotech. Inc., (Santa Cruz, CA, USA). RSV, 3-isobutyl-1-methyl-xanthine and ADP were purchased from Sigma Chemicals Inc. (St. Louis, MO, USA).

*Endothelial Cell Culture*

Endothelial cells were isolated from human umbilical cord veins as previously described (2). Cells were cultured in medium MCDB 107 supplemented with 2% FBS and an FGF-enriched fraction of porcine brain extract (1 μg per ml). Cells were incubated in a humidified incubator with 5% $CO_2$/95% air at 37°C.

*Preparation of LDL and Oxidized LDL*

Low density lipoprotein (d = 1.019 – 1.069 g per ml) was prepared from fresh human plasma by sequential ultracentrifugal flotation (10). LDL fraction was dialyzed at 4°C against 0.9% NaCl containing 0.1 mM EDTA. LDL oxidation was performed by dialysis against EDTA-free isotonic saline containing 5 μM $CuSO_4$ at 37°C for 8 h. Oxidation was stopped by the addition of EDTA to a final concentration of 100 μM. The copper ion was removed by extensive dialysis against isotonic saline containing 0.1 mM EDTA at 4°C. LDL was sterilized by passage through a 0.22 μm filter. LDL oxidation was measured by the malondialdehyde (MDA) and thiobarbituric reactive substance (TBAR) contents of the ox-LDL. The thiobarbituric acid reactive substance was assayed using MDA as a standard, and the TBAR were recorded as equivalents of MDA per mg of LDL protein (24). In our hands, the unoxidized LDL had a TBAR value of around 0.4 nmol/mg. By adjusting the oxidation time, ox-LDL with a TBAR value of 3.6 to 4.9 was prepared. The MDA contents of ox-LDL was also measured directly using a colorimetic assay of lipid peroxidation (manufactured by Calbiochem, Merck, Darmstadt, Germany). The assay takes the advantage of a chromogenic reagent N-methyl-2-phenylindole (R1 reagent), which reacts with MDA at 45°C. Condensation of MDA with the R1 reagent yields a stable chromophore with maximal absorbance at 586 nm (15). In our hand, the unoxided LDL and ox-DL had a MDA content of 0.256 and 2.484 nmol, respectively, per mg protein. The protein concentration was determined by the method of Lowry *et al*. (19).

*Pretreatment of Vascular EC*

Vascular EC in 4% FBS were either preexposed to various concentrations of RSV for 30 min or untreated. The medium was then replaced with the same medium without antioxidant, and the desired amount of ox-LDL (or n-LDL) was added and the cells were further incubated for 1 h. Cells were washed twice with basal medium and resuspended at $2 \times 10^6$ per ml in 1 mM $CaCl_2$/PBS or in MDCB 107 medium.

*Platelet Aggregation Assay*

Venous blood was obtained from the informed volunteer donors, who have not received any medication for at least two weeks, by veinpuncture and collected in a syringe containing sodium citrate (3.8 g/dl; 1 vol. for 9 vol. of blood). Platelet-rich plasma (PRP) was prepared by centrifugation of the blood sample at 120 × g for 10 min at room temperature. The plasma fraction was removed, and platelet-poor plasma (PPP) was then prepared by centrifuging the residual blood at 1,600 × g for 10 min. Platelet aggregation was determined using an aggregometer (Payton, Series 10008). Three tenth ml PRP ($2.0 \times 10^8$ platelets per ml) and 0.3 ml EC ($2 \times 10^6$ cells per ml), were transferred to siliconized cuvettes, and the mixture was stirred at 900 rev./min at 37°C in the aggregometer for 1 min. Platelet aggregation was induced by 2 μM ADP in a light transmittion aggregometer as described elsewhere (20).

*Determination of $[Ca^{2+}]_i$*

Endothelial cells or platelets were washed with an albumin cushion by repeated centrifugation and labeled with a calcium-sensitive fluorescent dye, Fura-2 AM, as described (16). Basal and 2 μM ADP-evoked $[Ca^{2+}]_i$ levels were calculated from the ratio of fluorescenct intensities measured at excitation wavelengths of 340 nm and 380 nm using a Jasco Caf 110 fluorometer (34).

*Measurement of Cellular cGMP Contents*

Cellular cGMP levels in platelets, EC, and platelets plus EC were determined by enzyme-linked immunosorbent assay using a commercially available kit (Cayman, Ann Arbor, MI, USA). After challenging platelets, EC, or platelets plus EC with 2 μM ADP for 6 min, a phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine ($10^{-4}$ M), was added and the cell suspension was centrifuged at 6,500 × g at 4°C for 5 min. The pellet was vortexed with ice-cold trichloroacetic acid (TCA; 6%) for 2 min. After centrifugation (10,000 × g, 4°C, 15 min), TCA was removed by washing four times with five volumes of water - saturated ether. The extracted samples were stored at -80°C until measurement.

*Western Blot Analysis*

Control and treated cells were lysed in a buffer containing 10 mM Tris-HCl (pH 8), 120 mM NaCl, 1% nanodit P-40, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml pepstatin and 20 μg/ml leupeptein at 4°C for 20 min. Cell-free extracts were obtained by centrifugation in a microcentrifuge. The same amount of lysates equivalent to $6 \times 10^4$ cells were separated on 12% SDS-PAGE. After electrotransfer, the nitrocellulose membranes were incubated with the respective primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, Buckinghamshire, UK). The immunoreactive protein bands were visualized by chemiluminesence (ECL, Amersham International).

*Endothelial Cell Derived NO Metabolites (Nitrite Plus Nitrate)*

The sample solutions were obtained after centrifugation at 3,000 × g at 4°C for 10 min. Nitrite plus nitrate in plasma and platelets was measured by fluorometric assay kit (Cayman, Ann Arbor, MI, USA). The first step was the conversion of nitrate to nitrite by using nitrate reductase. The second step was the addition of 2, 3-diaminonaphthalene followed by NaOH which converted nitrite into a fluorescence of this compound in which nitrite concentration was precisely determined. The fluorescence intensity was measured using a microplate fluorometer, Fluoroskan II (Labsystems, Helsinki, Finland) with excitation at 375 nm and emission read at 415 nm.

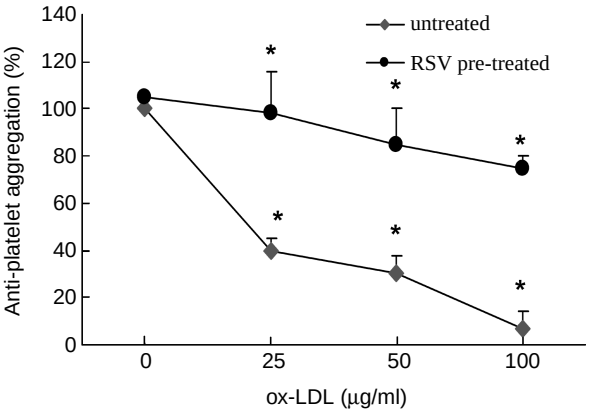


Fig. 1. *Trans*-resveratrol attenuates ox-LDL-induced reduction of the anti-platelet aggregation property of the endothelial cells. EC were treated with ox-LDL (25 - 100 μg/ml) for 1 h with or without a 30 min-preexposure to RSV (20 μM) before they were coincubated with platelets and ADP (4 μM). *$P$ < 0.05 for resveratrol treated *vs.* untreated cells (n = 4).

*Statistics*

All data were presented as mean ± S.E. Data were analyzed by one-way AVONA-Dunnett's test and $P$ value of 0.05 or less was considered significant.

## Results

The possible protective effect of RSV against ox-LDL-induced attenuation of the nonthrombogenic nature of the cultured vascular EC was first examined. EC were treated with ox-LDL (25 - 100 μg/ml) for 1 h with or without a 30 min-preexposure to RSV (20 μM) before they were coincubated with platelets and ADP (4 μM). Then, platelet aggregation was determined using an aggregometer (Fig. 1) and ADP-evoked $[Ca^{2+}]_i$ levels in platelets was calculated from the ratio of fluorescenct intensities (Fig. 2). Ox-LDL dose-dependently reduced the ability of EC to stabilize platelets from ADP-induced aggregation (Fig. 1). RSV not only attenuates ox-LDL-induced reduction of the anti-platelet property (Fig.1) but also inhibits the $[Ca^{2+}]_i$ rise in platelets (Fig. 2). The presences that coincubation of platelets with EC effectively inhibits ADP-induced platelet aggregation and $[Ca^{2+}]_i$ rise in platelets were shown in Fig. 3A and Fig. 3B. EC exposed with RSV (5 μM to 40 μM) prior

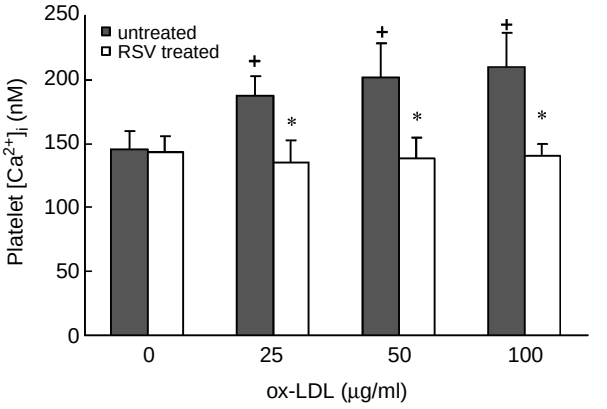


Fig. 2. RSV inhibits ox-LDL-induced elevation of the EC elicited $[Ca^{2+}]_i$ rise in platelets. EC were pre-exposed to ox-LDL or n-LDL for 1 h as indicated, with or without preexposure to RSV (20 μM, 30 min). $+P < 0.05$ for untreated *vs.* ox-LDL or n-LDL treated cells; $*P < 0.05$ for resveratrol treated *vs.* untreated cells (n = 4).

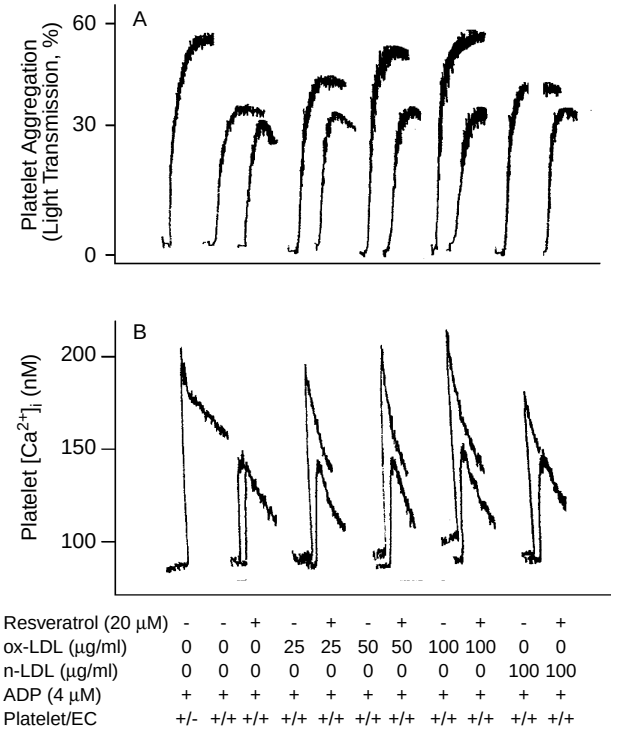


Fig. 3. Coincubation of platelets with EC effectively inhibited ADP-induced platelet aggregation (A) and $[Ca^{2+}]_i$ rise in platelets (B). The anti-platelet activation effect of EC was suppressed by preexposure of EC to ox-LDL in a dose dependent manner and was attenuated by a 30 min-exposure of EC to RSV prior to ox-LDL.

to ox-LDL effectively protected EC from the detrimental effects of ox-LDL. RSV ranging from 5 μM to 40 μM exerted similar effect on EC-mediated antiplatelet activity (Fig. 4), we therefore, chose a

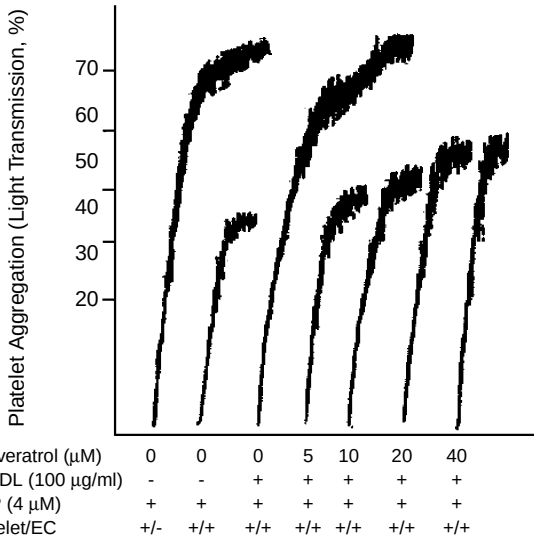


Fig. 4. Dose-dependent effect of RSV on ox-LDL-induced reduction of the anti-platelet aggregation activity of the endothelial cells. Cells were treated with RSV, ox-LDL, or n-LDL as indicated, and platelet aggregation was measured as described in the text.

RSV concentration of 20 μM to perform the rest of the experiments. The exposure time required for the maximal effect of RSV appeared to be short a 30 min incubation and a 2 h incubation were similarly effective (data not shown).

It is well known that an elevated intracellular cGMP levels in platelets through EC-platelet interaction enhances the resistance of platelet to agonist-induced platelet activation (23). In our hands, the cGMP levels in ADP (4 μM) treated EC and platelets were $0.5 \pm 0.1$ pmol per $10^7$ and $3.7 \pm 0.1$ pmol per $10^9$ cells, respectively, when measured separately. Coincubation of platelets with EC in the presence of the same concentration of ADP raised the cGMP level to $27.2 \pm 0.1$ pmol per same numbers of both cell types when measured together. Treatment of EC with ox-LDL (100 μg/ml) for 1 h prior to coincubation with platelets reduced EC-elicited elevation of cGMP level by about 75% (Fig. 5). Again, a brief exposure of EC to RSV (20 μM) prior to ox-LDL preserved most of the capacity of EC to raise cGMP content in the EC-platelets coincubation system.

The antithrombogenic nature of EC has been shown to mediate the liberation of nitrogen oxide (NO) (35). Agonist-induced NO production by EC is a $Ca^{2+}$-calmodulin-dependent process involving the activation of NO synthase (NOS), which catalyzes the conversion of L-arginine to NO and L-citrulline (21). To examine the RSV effect before exposure of EC to ox-LDL, we examined the $[Ca^{2+}]_i$ in untreated

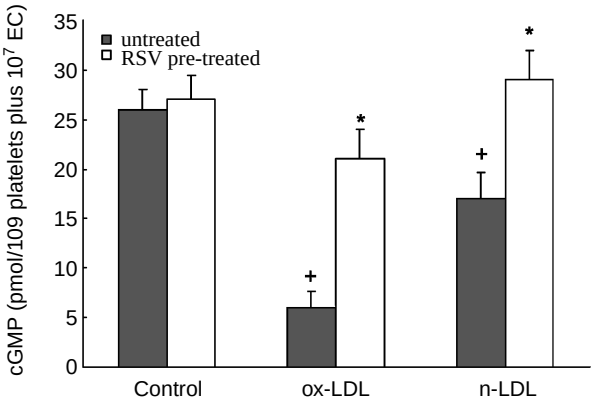


Fig. 5. RSV attenuates ox-LDL-induced reduction of EC-elicited cGMP rise in platelets. Endothelial cells were exposed to ox-LDL or n-LDL (100 μg/ml) for 1 h with or without RSV pretreatment (20 μM, 30 min) prior to coincubation with platelets. $+P < 0.05$ for control *vs.* ox-LDL or n-LDL treated cells; $*P < 0.05$ for untreated *vs.* RSV treated cells (n = 4).

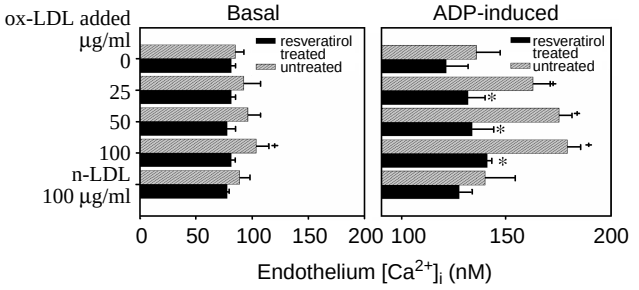


Fig. 6. Effect of RSV treatment (20 μM, 30 min) on basal (A) and ADP–evoked $[Ca^{2+}]_i$ levels (B) in EC with or without ox-LDL or n-LDL exposure. $+P < 0.05$ for control *vs.* ox-LDL or n-LDL treated cells; $*P < 0.05$ for untreated *vs.* RSV treated cells (n = 4).

and ADP-treated EC. Figure 6 shows that ox-LDL treatment increased $[Ca^{2+}]_i$ in both control and ADP-treated EC. However, pre-treatment with RSV attenuated this ox-LDL effect. In addition, treatment of EC with ox-LDL at 100 μg/ml for 1 h reduced NOS protein content by more than 50% and a 30-min exposure of EC to RSV prior to ox-LDL preserved the NOS protein (Fig. 7). Similar results were obtained when RSV pretreatment reduced ADP-induced NO production in exposure EC to ox-LDL. The NO metabolite release indicated as the level of nitrite plus nitrate. Figure 8 showed that ox-LDL obviously inhibited the level of of nitrite/nitrate production in EC, but this effect was prevented by preexposure of EC to RSV. EC coincubated with RSV and L-arginine (1 mM, a NO substrate) before oxLDL exposure was found to significantly increased endothelium-induced NO metabolite release (Fig. 8). These results

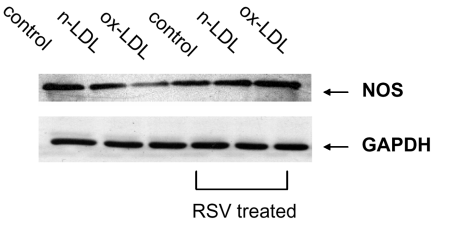


Fig. 7. RSV attenuates ox-LDL-induced reduction of the NOS protein in EC. Endothelial cells were exposed to ox-LDL or n-LDL (100 μg/ml) for 1 h with or without RSV pretreatment (20 μM, 30 min). Cell lysate were prepared as described. Aliquots equivalents to $6 \times 10^4$ cells were fractionated on 12% SDS-PAGE and immunoblotted with NOS antibody. After incubating with second antibody, the blot was visualized by autoradiography as described in the text.

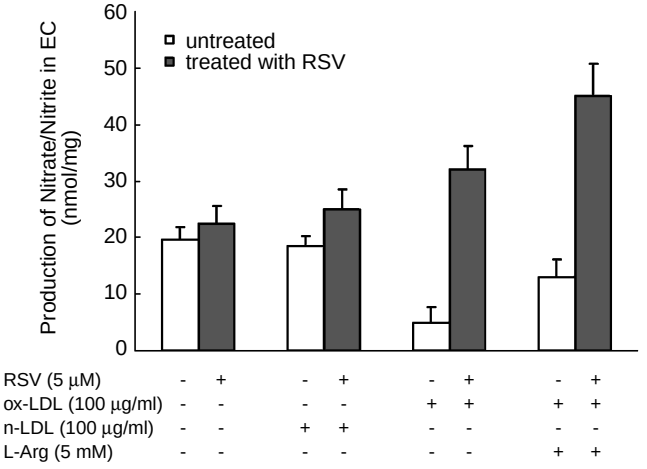


Fig. 8. Effect of RSV on ADP-induced nitrite plus nitrate level in oxLDL exposed EC. EC were treated with ox-LDL (100 μg/ml) or n-LDL (100 μg /ml) for 1 h with or without a 30 min-preexposure to RSV (20 μM) before they were coincubated with platelets and ADP (4 μM). Exposure to ox-LDL inhibited the release of NO metabolite to control cells significantly. Incubation of EC with a NO donor, L-arginine (1 mM for 15 min) did not reverse the effect of ox-LDL-inhibited release of NO metabolite. But preexposure RSV and L-arginine to EC before ox-LDL treatment increased the nitrate/nitrite release. $+P < 0.05$ for control *vs.* ox-LDL or n-LDL treated cells); $*P < 0.001$ for untreated *vs.* RSV treated cells (n = 4).

demonstrate that RSV pretreatment may provide a sustained increase in NO production in the vasculature *via* protection eNOS from ox-LDL damage.

## Discussion

The mechanism underlying the protective effect of RSV, a phenolic substance in red wine, on the anti-

platelet activation property of EC was examined after the EC were exposed to atherogenic levels of ox-LDL. We found that exposure of EC to ox-LDL reduced the anti-thrombogenic nature of EC, and the effect appeared to be mediated through the NOS-NO pathway. The prothrombongenic effects of ox- LDL on EC, including reduced anti-platelet aggregating activity, increased $[Ca^{2+}]_i$ and platelet cGMP levels, were all attenuated by RSV pretreatment.

Oxidative modification of LDL has been implicated in the early stages of atherogenesis (13, 38) Exposure of isolated blood vessels to ox-LDL inhibited endothelium-dependent vessel relaxation, including to acetylcholine in precontracted rabbit aorta (3), and to 5-hydroxytryptamine, thrombin, and aggregating platelets in precontracted pig coronary artery (17, 30). Several studies have demonstrated that dietary antioxidants may help prevent the development and progression of atherosclerosis (6, 7). Consistent with these findings, we also showed that the ox-LDL- promoted vascular EC pinocytosis is effectively blocked by pretreatment of cells with antioxidants (4).

Considerable evidence indicates that atherogenic levels of ox-LDL could cause endothelial-dependent vasomotor abnormalites through inhibition of endothelium-derived NO production (26, 33). NO has been suggested to be the most important factor in the regulation of basal vascular tone and vasodilation, stimulated by acetylcholine and shear stress in humans *in vivo* (11, 28). Endothelial cell-derived NO could stimulate the activity of soluble guanylate cyclase and increase the level of cyclic GMP in the smooth muscle cells, resulting in vessel relaxation (22). NO generated by the endothelium could also inhibit platelet aggregation and adhesion to the blood vessel walls (22, 35). Our results showed that ox-LDL preexposure reduces the ability of EC to inhibit ADP-induced platelet aggregating and $[Ca^{2+}]_i$ rise, and to increase platelet cGMP content. We also showed that pretreatment of EC with RSV prior to ox-LDL attenuated the detrimental effects of ox-LDL on their nonthrombogenic property.

NO is generated in EC by the oxidation of L-arginine in a reaction that is catalyzed by the NOS (21, 22, 29). Recent studies have shown that oxidized, but not native LDL decreases cytokine-inducible NOS activity in murine macrophages without affecting its steady-state mRNA or protein expression (12, 18). Liao *et al*. demonstrated that ox-LDL reduces NOS expression in EC at both the transcriptional and post-transcriptional levels (18). Blair *et al*. reported that the caveolae protein, caveolin, directly binds eNOS, thereby regulating the generation of NO. They showed that an acute treatment of EC with oxLDL (only 1 h) inhibits eNOS activation by disrupting the association of eNOS with caveolae (1). These findings are consistent with our result, showing that ox-LDL exposed EC exhibits decreased NOS activity. We further showed that the ox-LDL effect on NOS is attenuated by pretreatment with RSV, indicating that RSV pretreatment preserved the antiplatelet activation property of EC *via* protection of NOS expression. RSV, when taken as food supplement or in beverages may provide a sustained increase in NO production in the vasculature. The attained NO concentrations may be sufficient to tip the redox balance from prooxidation to antioxidation state, providing a safeguard against EC damage due to LDL oxidation in the arterial walls. Under physiological conditions, the vascular endothelium could release vasoactive substances in response to various stimuli to regulate vascular tone. $PGI_2$ and NO are two of such substances that relax vascular smooth muscle cells and inhibit platelet activation (22, 35). In the current study, we show that ox-LDL exposure suppresses NOS expression in EC, and in contrast, the protein contents of $PLA_2$ and $COX_2$ are not affected (data not shown). We thus postulate that ox-LDL may act by suppressing the NOS-NO pathway to attenuate the antithrombogenic nature of the endothelial cells.

The oxidation of LDL in the arterial walls is believed to be one of the major culprit in the initiation of atherosclerosis. In this study, n-LDL had a modest effect but ox-LDL markedly reduces the ability of EC to inhibit ADP-induced platelet aggregation and $[Ca^{2+}]_i$ and cGMP rise. N-LDL was shown with a TBAR value of around 0.4 nmol/mg and ox-LDL was shown with a TBAR of 3.6- 4.9 nmol/mg (see the Materials and Methods). Therefore, this n-LDL effect may be caused by some oxidation of n-LDL during the incubation period (36). Our results seem to suggest that a reduction or retardation of the oxidation of LDL in the arterial walls and/or an increased resistance of vascular cells to the atherogenic effect of ox-LDL are/is beneficial in reducing the risk of CAD. By defending vascular cells against the oxidative stress exerted by ox-LDL, RSV might have the preventive values against CAD when taken as a food supplement or from foods rich in polyphenolic compounds.

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