BASIC RESEARCH STUDIES

Ellagic acid inhibits oxidized LDL-mediated LOX-1 expression, ROS generation, and inflammation in human endothelial cells

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Background: **LOX-1, a lectin-like receptor on endothelial cells, facilitates the uptake of oxidized low-density lipoprotein (oxLDL). Expression of LOX-1 is involved in the pathobiological effects of oxLDL in endothelial cells, including reactive oxygen species (ROS) generation, suppression of endothelial nitric oxide synthase (eNOS) activity, and leukocytic adhesion. Moderate consumption of phenolic-enriched food may have a protective effect against the development of atherosclerosis via the antioxidant capacity of phenolic compounds at the endothelial level. In this study, we determined whether ellagic acid, a polyphenolic compound widely distributed in fruits and nuts, protects against oxLDL-induced endothelial dysfunction by modulating the LOX-1-mediated signaling pathway.**

Methods: **Human umbilical vein endothelial cells (HUVECs) were pretreated with ellagic acid at doses of 5, 10, 15, and 20 μM for 2 hours and then incubated with oxLDL (150 μg/mL) for an additional 24 hours.**

Results: **LOX-1 protein expression was markedly lower after exposure to oxLDL in HUVECs pretreated with ellagic acid or diphenyleneiodonium, a well-known inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, than in HUVECs exposed to oxLDL alone, suggesting that ellagic acid deactivates NADPH oxidase. We also found that oxLDL activated the membrane assembly of $p47^{phox}$, Rac1, gp91 and $p22^{phox}$, and the subsequent induction of ROS generation; however, ROS generation was markedly suppressed in cells pretreated with ellagic acid or anti-LOX-1 monoclonal antibody. In addition, oxLDL down-regulated eNOS and up-regulated inducible NO synthase (iNOS), thereby augmenting the formation of NO and protein nitrosylation. Furthermore, oxLDL induced the phosphorylation of p38 mitogen-activated protein kinase, activated the NF-κB-mediated inflammatory signaling molecules interleukin-(IL) 6 and IL-8 and the adhesion molecules intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin, and stimulated the adherence of THP-1 (a human acute monocytic leukemia cell line) to HUVECs. Pretreatment with ellagic acid, however, exerted significant cytoprotective effects in all events.**

Conclusion: **Findings from this study may provide insight into a possible molecular mechanism by which ellagic acid inhibits LOX-1-induced endothelial dysfunction. Our data indicate that ellagic acid exerts its protective effects by inhibiting NADPH oxidase-induced overproduction of superoxide, suppressing the release of NO by down-regulating iNOS, enhancing cellular antioxidant defenses, and attenuating oxLDL-induced LOX-1 up-regulation and eNOS down-regulation. (J Vasc Surg 2010;52:1290-300.)**

Clinical Relevance: **Increased oxidative stress is an important factor contributing to the development of atherosclerotic lesions. This study underscores the potential clinical benefits and application of ellagic acid in prevention of oxLDL-associated atherogenic diseases.**

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Atherosclerosis is a chronic inflammatory process. The initiation of atherosclerotic lesion formation is caused by sublethal changes in endothelial function, a process referred to as endothelial activation or dysfunction. Oxidized low-density lipoprotein (oxLDL) impairs endothelial function, giving rise to reactive oxygen species (ROS) generation, and reduced nitric oxide (NO) production. Adhesion molecules are then activated, which in turn mediates leukocyte recruitment and adherence to the endothelium.[1]

LOX-1 is a lectin-like receptor for oxLDL in endothelial cells, smooth muscle cells, and macrophages, the three most important cells involved in the development of atherosclerosis.[2] This receptor has been shown to be activated by shear stress, endothelin, angiotensin II, and oxLDL in atherosclreotic lesions in humans and experimental animal models. Specifically, uptake of oxLDL into the endothelium is mediated through interaction with the LOX-1 receptor. It is this interaction that mediates most of the toxic effects of oxLDL, including the release of inflammatory cytokines and the enhanced expression of adhesion molecules and pro-apoptotic proteins. The binding of oxLDL to LOX-1 activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on the cell membrane, which results in the quick increase of intracellular ROS, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Increased O_2^- not only reacts with intracellular NO, resulting in a decrease in intracellular NO, but also up-regulates LOX-1 expression, thereby contributing to further ROS generation.[3] ROS derived from NADPH oxidase is strongly associated with atherosclerosis,[4] and oxLDL-induced endothelial dysfuction is prevented by radical-scavenging agents.[3] Clinically, however, free radical scavengers such as recombinant human superoxide dismutase (SOD) and catalase enzyme have been shown to have limited effects, perhaps due to their short half-life and to their very low penetration in vascular tissues.[5]

There is increasing evidence regarding the link between the dietary intake of fruits and vegetables and the lower incidence of cardiovascular diseases. These beneficial effects are now suggested to be due to the antioxidant activity of constituent phenolic phytochemicals.[6] Phenolic phytochemicals possess phenolic rings and hydroxyl substituents with the ability to quench free radicals and prevent cellular damage, and, therefore, function as effective antioxidants. Polyphenolic compounds affect the development of atherosclerosis not only through antioxidant properties but also by modulation of serum lipids, thereby influencing the immune and inflammatory processes associated with the development of atherogenic diseases.

Ellagic acid is a natural polyphenolic compound present as ellagitannins in fruits and fruit juices, including grape juice (10.2 mg/100 g), grape wine (5.6 mg/100 g), blueberries (0.9 mg/100 g), blackberries (42.4 mL/100 g), raspberries (17.9 mg/100 g), and strawberries (19.8 mg/100 g).[7] Ellagic acid is released by the metabolism of ellagitannins by the microflora in the gut.[8] Several biological activities of ellagic acid have been investigated, including anti-oxidation and inhibition of lipid peroxidation,[9] anti-inflammation,[10] anti-proliferation and anti-angiogenesis,[11] and anti-carcinogenesis and chemoprevention.[12] Ellagic acid effectively lowers the levels of plasma lipids, reduces oxidative stress, and inhibits apoptosis in hyperlipidemic rabbits,[13] as well as scavenges oxygen and hydroxyl radicals, and inhibits lipid peroxidation and 8-OhdG formation in vitro and in vivo.[14] Previous studies have shown that ellagic acid inhibits oxLDL-induced aortic smooth muscle cell proliferation,[15] inhibits cytokine-induced ROS generation and expression of adhesion molecules,[16,17] and prevents oxLDL-induced apoptosis[18] in endothelial cells. The underlying mechanisms, however, are still largely unclear. Therefore, in this study, we used human umbilical vein endothelial cells (HUVECs) to test the hypothesis that ellagic acid protects against oxLDL-induced endothelial dysfunction by modulating the LOX-1-mediated signaling pathway. In so doing, we explored the effects of ellagic acid on oxLDL-induced ROS generation, antioxidant enzyme expression, NADPH oxidase activation, LOX-1 protein expression, and NF-κB-relative downstream inflammatory responses.

MATERIALS AND METHODS

Cell cultures. HUVECs were isolated from human umbilical cords with collagenase and used at passages 2-3.[19] After dissociation, the cells were collected and cultured on gelatin-coated culture dishes in M199 with low serum growth supplement, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Subcultures were performed with trypsin-ethylenediaminetetraacetic acid (EDTA). Media were refreshed every second day. The identity of umbilical vein endothelial cells was confirmed by their cobblestone morphology and strong positive immunoreactivity to von Willebrand factor. THP-1, a human monocytic leukemia cell line, was obtained from ATCC (Rockville, Md) and cultured in RPMI 1640 medium with 10% fetal bovine serum at a density of 2 to 5×10^6 cells/mL, as suggested in the product specification sheet provided by the vendor.

Lipoprotein separation and oxidation. Native LDL was isolated from fresh normolipidemic human serum by the sequential ultracentrifugation method and then oxidatively modified.[20] LDL (ρ = 1.019-1.210 g/mL) in KBr solutions containing 30 mM EDTA was stored at 4°C in a sterile, dark environment and used within 3 days. Immediately before oxidation tests, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25 M gel (Pharmacia, St. Quentin, France) in 0.01M phosphate-buffered saline (PBS; 136.9 mM NaCl, 2.68 mM KCl, 4 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4). $CuSO_4$-oxidized LDL (1 mg protein/mL) was prepared by exposing LDL to 10 μM $CuSO_4$ for 16 hours at 37°C. Protein was measured by the Bradford method.[21]

Measurement of ROS production. The effect of ellagic acid on ROS production in HUVECs was determined by a fluorometric assay using dihydroethidium (DHE, Molecular Probes, Eugene, Ore) as a probe for the presence of superoxide. After preincubation for 2 hours with the indicated concentrations of ellagic acid, HUVECs were incubated with

DHE for 1 hour followed by incubation with oxLDL for 2 hours. The fluorescence intensity was measured at 540 nm excitation and 590 nm emission (before and after exposure to oxLDL) using a fluorescence microplate reader (Labsystems, San Jose, Calif). The percentage increase in fluorescence per well was calculated by the formula $[(Ft_2 - Ft_0)/Ft_0] \times 100$, where Ft_2 is the fluorescence at 2 hours of oxLDL exposure and Ft_0 is the fluorescence at 0 min of oxLDL exposure. OxLDL-induced superoxide formation, which occurs largely through activation of NADPH oxidase, but also through uncoupling of endothelial NO synthase, xanthine oxidase, peroxisomes, and through direct superoxide release, leads to endothelial dysfunction. In this regard, diphenyleneiodonium (DPI), a potent inhibitor of flavin-containing oxidase, has frequently been used to inhibit ROS production mediated by various flavoenzymes, including NADPH oxidase, quinine oxidoreductase, cytochrome P450 reductase, and nitric oxide synthase.[22] To evaluate the role of NADPH oxidase and LOX-1 in oxLDL-induced ROS generation, cells were preincubated with DPI (5 μM) or anti-LOX-1 mAb (40 μg/mL) for 2 hours before exposure to oxLDL.

Nitrite (NO_2^-) accumulation. NO_2^- accumulation was used as an indicator of NO production in the medium and was assayed by Gries reagent.[23] Briefly, 100 μL of Gries reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% H_3PO_4; Sigma, St. Louis, Mo) was added to 100 μL of each supernatant in triplicate wells of 96-well plates. The plates were read in a microplate reader (Molecular Devices, Palo Alto, Calif) at 550 nm against a standard curve of $NaNO_2$ in culture medium.

Immunoblotting. To determine whether ellagic acid could attenuate the expression of oxLDL-induced ROS-regulating proteins, HUVECs were grown to confluence, pretreated with ellagic acid for 2 hours, and then stimulated with oxLDL for indicated time periods. After treatment, cytosolic/membrane protein fractions of cells were extracted with a Mem-PER kit, and cytosolic/nuclear protein fractions of cells were extracted with a Cytoplasmic Extraction kit according to the manufacturer's instructions (Pierce, Rockford, Ill). Cytosolic SOD-1, SOD-2, nitrotyrosine, eNOS, iNOS, and IκBα membrane-bound gp91, $p22^{phox}$, $p47^{phox}$, and Rac-1, and nuclear NF-κBp65 were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot assay. The blots were incubated with blocking buffer (1x PBS and 5% nonfat dry milk) for 1 hour at room temperature and then probed with primary antibodies (1:1000 dilutions) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour. To control equal loading of total protein in all lanes, blots were stained with mouse anti β-actin antibody (1:50,000 dilution) for cytosolic fractions, mouse anti-flotillin-1 (1:1000) for plasma membrane fractions, and anti-proliferating cell nuclear antigen (1:1000) for nuclear fractions. The bound immunoproteins were detected by an enhanced chemiluminescent assay (ECL; Amersham, Berkshire, UK). The intensities were quantified by densitometric analysis (Digital Protein DNA Imagineware, Huntington Station, NY).

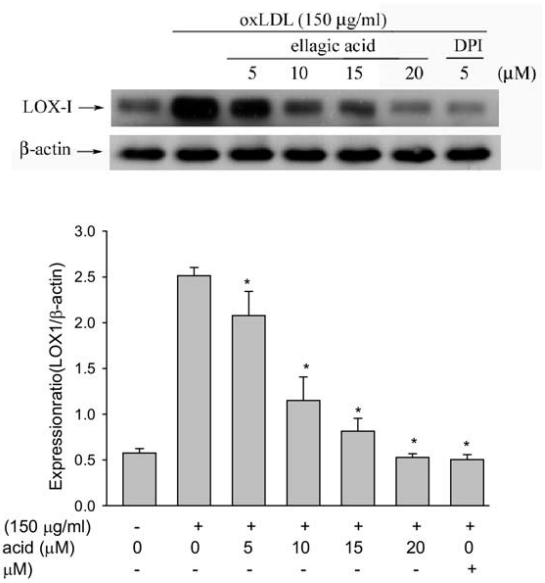

Fig 1. Inhibitory effect of ellagic acid on oxidized low-density lipoprotein (*oxLDL*)-induced endothelial LOX-1 protein expression. Human umbilical vein endothelial cells (*HUVECs*) were pretreated with ellagic acid (5-20 μM) or DPI (5 μM) for 2 hours followed by exposure to oxLDL (150 μg/mL) for a further 24-hour period. At the end of the incubation period, cells were lysed and proteins were analyzed by Western blot. Protein levels of LOX-1 were normalized to the level of β-actin. Data illustrated on the graph bars represent the mean ± SEM of three different experiments. $*P <$ .05 compared with oxLDL-stimulated HUVECs.

Assay for ET-1, interleukin-(IL) 6 and IL-8 secretion. Endothelial dysfunction caused by oxLDL has been attributed to reduced NO bioavailability and enhanced ET-1/cytokine release.[24] To determine the effects of ellagic acid on oxLDL-induced ET-1 and IL-8 secretion, HUVECs were seeded in 24-well plates at 0.5×10^5 cells. After 2 days, cells were pretreated with the indicated concentrations of ellagic acid for 2 hours followed by treatment with oxLDL (150 μg/mL) for 24 hours. At the end of the oxLDL incubation period, cell supernatants were removed and assayed for ET-1 and IL-8 concentration using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn). Data were expressed in ng/mL of duplicate samples.

Adhesion assay. HUVECs at a density of 1×10^5 cells/mL were cultured in 96-well flat-bottom plates (0.1 mL/well) for 1 to 2 days. HUVECs were then pretreated with the indicated concentrations of ellagic acid for 2 hours, followed by stimulation with oxLDL (150 μg/mL) for 24 hours. Medium was removed and 0.1 mL/well of THP-1 cells (prelabeled with BCECF-AM 4 μM for 30 minutes in RPMI 1640 medium at 1×10^6 cell/mL density) were added to HUVECs. Cells were allowed to adhere at 37°C for 1 hour in a 5% CO_2 incubator. Non-adherent cells were gently aspirated. Plates were washed three times with M199. The number of adherent cells was estimated by microscopic examination and then the cells were lysed with 0.1 mL 0.25% Triton

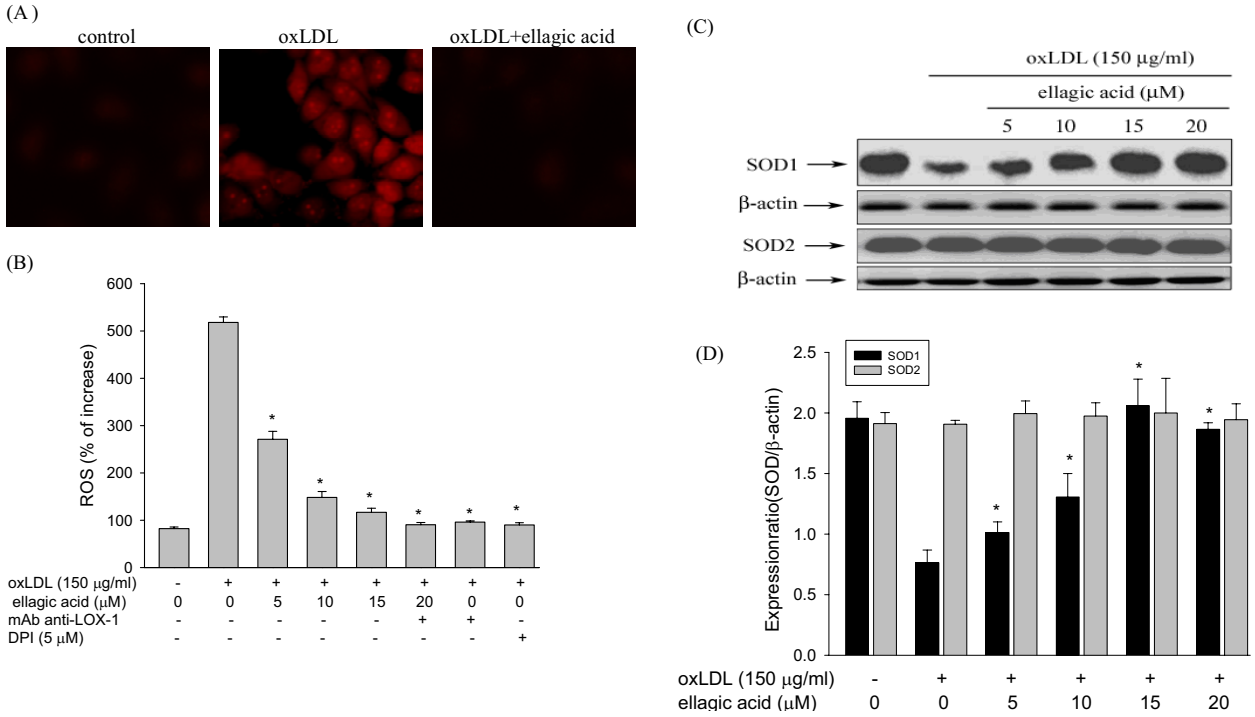

Fig 2. Inhibitory effects of ellagic acid on oxidized low-density lipoprotein (*oxLDL*)-induced reactive oxygen species (*ROS*) production in human umbilical vein endothelial cells (*HUVECs*). After pre-incubation for 2 hours with the indicated concentrations of ellagic acid (5-20 μM), cells were treated with 150 μg/mL oxLDL for 2 hours followed by a 1-hour incubation with superoxide-sensitive fluorescent probe DHE (10 μM). **(A)** Fluorescence images show the ROS level in control cells (left) and HUVECs stimulated with oxLDL alone (middle) and in the presence of 10 μM ellagic acid (right). **(B)** Fluorescence intensity of cells was measured with a fluorescence microplate reader. Fluorescence distribution of DHE oxidation is expressed as a percentage of increased intensity. **(C, D)** Representative Western blots of Cu, Zn-SOD (SOD-1), and Mn-SOD (SOD-2) protein levels in HUVECs pretreated with ellagic acid for 2 hours followed by stimulation with 150 μg/mL oxLDL for 24 hours. Data are expressed as the mean ± SEM of three independent analyses. *$P < .05$ compared with oxLDL-stimulated HUVECs.

X-100. The fluorescence intensity was measured at 485 nm excitation and 538 nm emission using a fluorescence microplate reader (Labsystems). The results of monocytes adhesion assays are reported as the fluorescence intensity per well of adherent THP-1 cells.

Adhesion molecule expression. To determine whether ellagic acid could modify oxLDL-induced adhesion molecule expression, HUVECs at a density of 1×10^5 cells/mL were grown to confluence and pretreated with 20 μM ellagic acid for 2 hours and stimulated with oxLDL (150 μg/mL) for 24 hours. At the end of stimulation, HUVECs were harvested and incubated with FITC-conjugated anti-intercellular adhesion molecule (ICAM)-1, anti-vascular cell adhesion molecule-1 (VCAM-1), and anti-E-selectin for 45 minutes at room temperature. After the cells had been washed three times, immunofluorescence intensity was analyzed using flow cytometry with a Becton Dickinson FACScan (Mountain View, Calif).

Statistical analyses. Results are expressed as mean ± SEM. Differences between the groups were analyzed using one-way analysis of variance followed by the Student's *t* test. A *P* value <0.05 was considered statistically significant.

RESULTS

Effect of ellagic acid on oxLDL-induced protein expression of LOX-1. First, we found that the endothelial viability was not affected by ellagic acid up to 50 μM. The ED50 (the half-maximal effective concentration) of ellagic acid was calculated to be 6.3 μM after exposure to 150 μg/mL oxLDL for 24 hours (data not shown). Therefore, 150 μg/mL of oxLDL and 5 to 20 μM of ellagic acid were used in the following experiments. Incubation of HUVECs with oxLDL enhanced LOX-1 protein expression. Pretreatment of HUVECs with ellagic acid for 2 hours at concentrations above 5 μM, followed by exposure to oxLDL for 24 hours resulted in suppression of LOX-1 protein expression. In addition, pretreatment with DPI, an inhibitor of ROS production, markedly inhibited oxLDL-induced LOX-1 up-regulation, strongly suggesting that ROS plays a critical role in the increased protein expression of LOX-1 (Fig 1).

Effects of ellagic acid on oxLDL-induced ROS generation. Pretreatment of HUVECs with ellagic acid (5-20 μM) for 2 hours before exposure to 150 μg/mL oxLDL significantly decreased the level of ROS generation

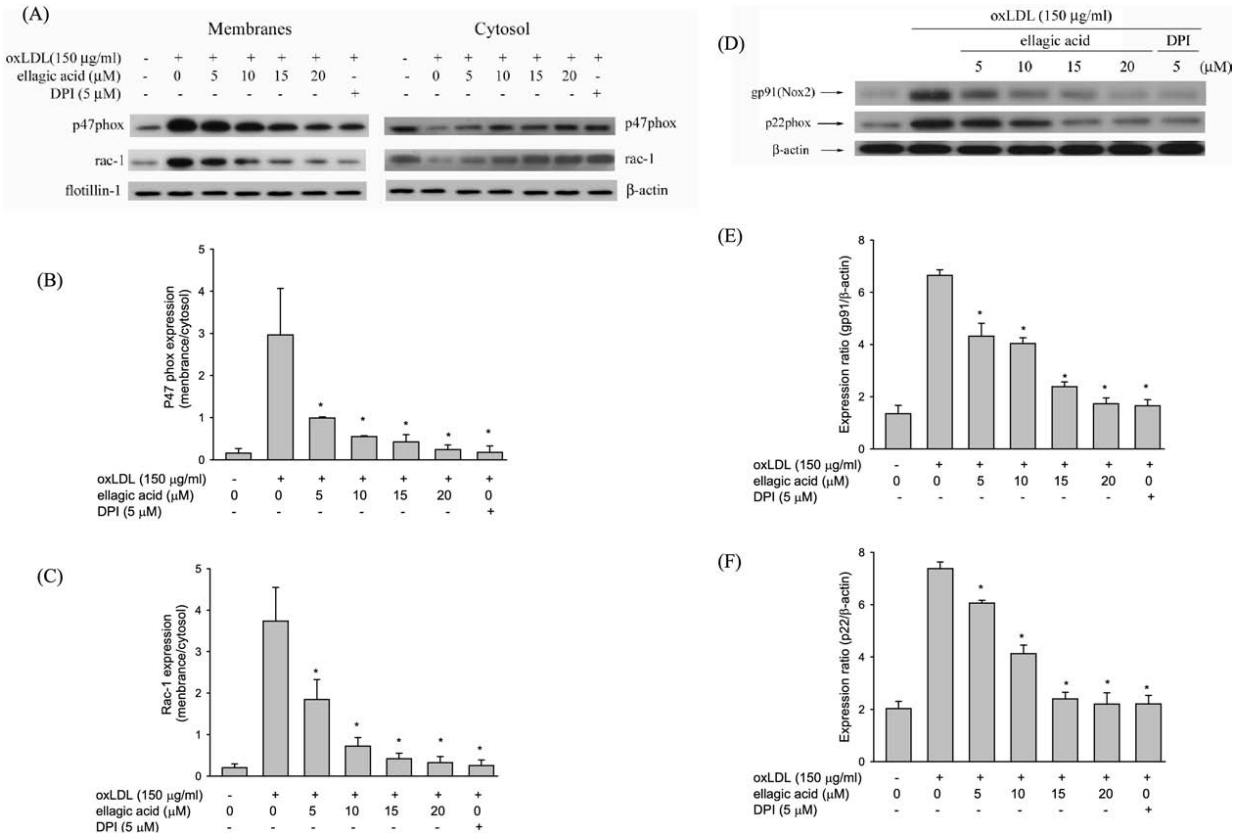

Fig 3. Ellagic acid attenuated the level of NADPH oxidase membrane assembly. Human umbilical vein endothelial cells (*HUVECs*) were pretreated for 2 hours with the indicated concentrations of ellagic acid followed by stimulation with oxidized low-density lipoprotein (*oxLDL*) (150 μg/mL) for 1 hour **(A-C)** or 24 hours **(D-F)**. Preparation of membrane and cytosolic proteins is described in the Material and Methods section. The levels of cytosolic protein and membrane protein were normalized to the levels of β-actin and flotillin-1, respectively. Representative Western blots and summary data showed that ellagic acid protected against oxLDL-induced $p47^{phox}$ and Rac-1 translocation to the plasma membrane, and gp91 as well as $p22^{phox}$ expression. The values represent means ± SEM from three separate experiments. $*P < .05$ vs oxLDL treatment.

in a dose-dependent manner (all $P < .05$; Fig 2A, 2B). In addition, oxLDL-induced ROS was abolished by pretreatment with monoclonal antibody of LOX-1 (mAb anti-LOX-1) or DPI (Fig 2B), suggesting that ROS generation, one of the earliest signals after oxLDL exposure, was largely dependent on the binding of oxLDL to LOX-1 and subsequent activation of NADPH oxidase.

Intracellular ROS levels are regulated by the balance between ROS generation and antioxidant enzymes. ROS are able to inactivate antioxidative enzymes, leading to oxidative stress. We next turned our attention to the expression of SOD isoforms in endothelial cells in response to oxLDL. Our results showed that Cu, Zn-SOD (SOD-1), but not Mn-SOD (SOD-2), expression was diminished after treatment with oxLDL for 24 hours; however, SOD-1 was expressed normally after pretreatment with ellagic acid at concentrations above 15 μM (Fig 2, *C* and *D*).

Effects of ellagic acid on oxLDL-induced membrane assembly of NADPH oxidase complex. The NOX family of NADPH oxidases is a major source of ROS in endothelial cells.[25] This enzyme complex is composed of two membrane components, Nox2 (also called $gp91^{phox}$) and $p22^{phox}$, and three cytoplasmic components, $p47^{phox}$, $p67^{phox}$, and the small GTPase rac-1. The process by which the NADPH oxidase enzyme complex is activated begins with the phosphorylation of $p47^{phox}$, which causes the translocation of the $p47^{phox}/p67^{phox}$ complex to the plasma membrane where $p47^{phox}$ interacts with $p22^{phox}$ and $p67^{phox}$ acts as the NOX activator through direct protein-protein interaction.[26] We, therefore, measured the effects of ellagic acid on NADPH oxidase activation after exposure to oxLDL. Membrane translocation assay showed that the levels of $p47^{phox}$ and Rac-1 in membrane fractions of HUVECs were three- to four-fold higher in cells treated with oxLDL for 1 hour than in untreated cells (Fig 3A-C). In addition, we found that the protein levels of gp91 and $p22^{phox}$ were increased significantly in HUVECs exposed to oxLDL for 24 hours. However, pretreatment of oxLDL-exposed cells with ellagic acid led to a dose-dependent reduction in gp91 and $p22^{phox}$ protein expression (5-20 μM, all $P < .05$; Fig 3D-F).

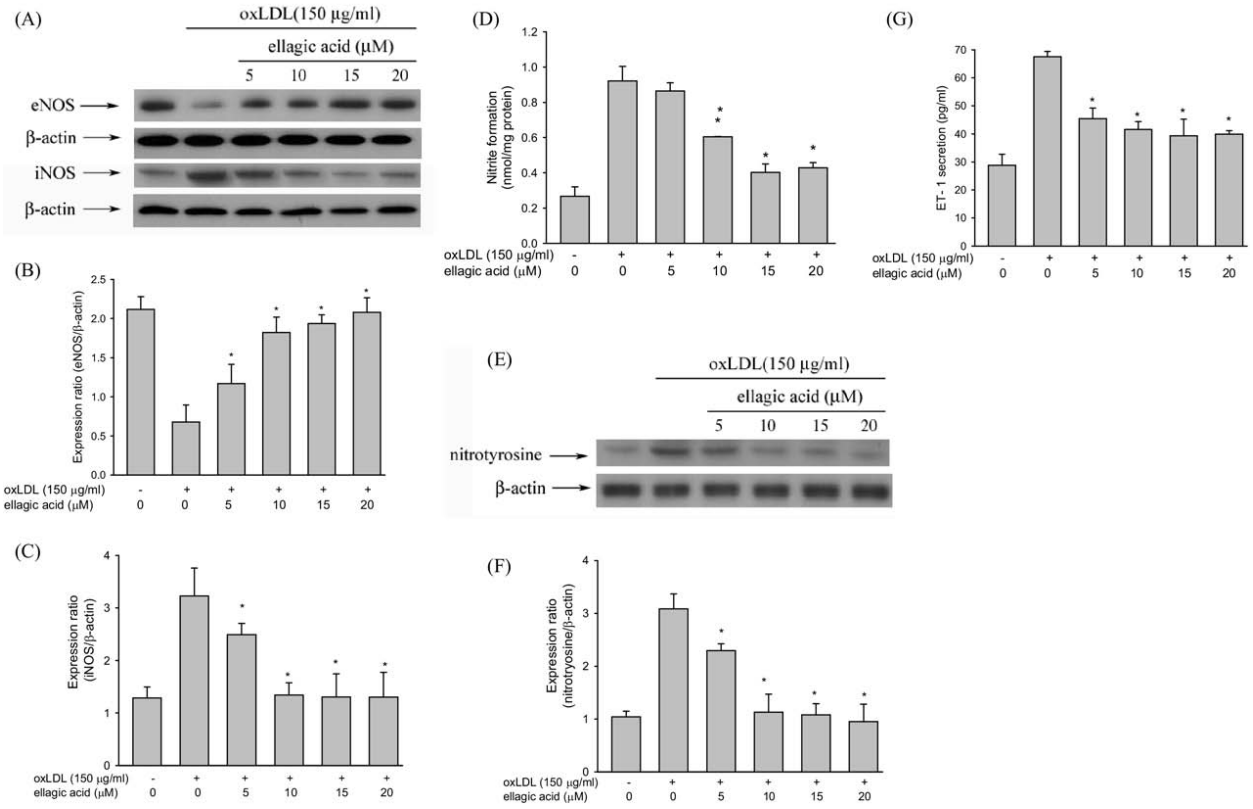

Fig 4. Ellagic acid ameliorated the oxidized low-density lipoprotein (*oxLDL*)-down-regulated eNOS and up-regulated iNOS protein expression **(A-C),** oxLDL-enhanced NO release **(D),** and nitration of tyrosine residues **(E, F)**. In addition, ellagic acid attenuated the oxLDL-enhanced secretion of ET-1 **(G)**. Human umbilical vein endothelial cells (*HUVECs*) were pretreated for 2 hours with the indicated concentrations of ellagic acid followed by stimulation with oxLDL (150 μg/mL) for 24 hours. At the end of the incubation period, level of eNOS, iNOS, and nitrotyrosine protein (normalized to the levels of β-actin) were determined by immunoblotting; content of NO was assayed using Gries reagent; ET-1 secretion was measured by ELISA. The values represent means ± SEM from three separate experiments. *$P < .05$ vs oxLDL treatment.

Ellagic acid ameliorated oxLDL-impaired NOS protein expression and enhanced ET-1. Nitric oxide (NO) is produced in endothelial cells by a constitutively expressed enzyme known as eNOS. Considerable evidence indicates that oxLDL-induced endothelial dysfunction is associated with down-regulation of eNOS and up-regulation of iNOS. ROS, especially superoxide, generated by oxLDL directly, reacts with NO to form peroxynitrite, a stable molecule that is toxic to endothelial cells. We, therefore, determined the effects of ellagic acid on protein expression of eNOS and iNOS as well as NO content and formation of nitrotyrosine, a stable final metabolite of peroxynitrate that can serve as a fingerprint for peroxynitrite-mediated damage of cellular proteins after exposure to oxLDL. As shown in Fig 4, oxLDL-diminished eNOS and enhanced iNOS protein expression levels returned to levels close to those seen in control cells when HUVECs were treated with 20 μM ellagic acid prior to stimulation with oxLDL. Furthermore, the oxLDL-enhanced NO release and nitrosylation of tyrosine residues were suppressed in HUVECs pretreated with ellagic acid.

It has been speculated that reduced NO release/bioavailability and enhanced release of endothelin-1 (ET-1) may contribute to oxLDL-induced endothelial dysfunction and the development of atherosclerosis.[27] We found that the oxLDL-enhanced ET-1 secretion that was observed in HUVECs after 24 hours of incubation with oxLDL was abolished in HUVECs pretreated with ellagic acid (Fig 4G).

Ellagic acid inhibited NF-kB activation via modulation of p38MAPK. It has been shown that oxLDL-induced ROS activates p38MAPK, which causes the activation and nuclear translocation of NF-κB and the subsequent regulation of pro-inflammatory gene expression.[28] We, therefore, determined whether the effects of ellagic acid are associated with the p38MAPK-NF-κB signaling pathway. Our data showed that oxLDL had no effect on the level of p38MAPK expression but that it markedly increased the level of phosphorylation of p38MAPK. In parallel experiments, HUVECs pretreated with ellagic acid showed very little p38MAPK phosphorylation and normal protein levels of that mitogen-activated protein kinase p38MAPK (Fig 5). NF-κB is a family of

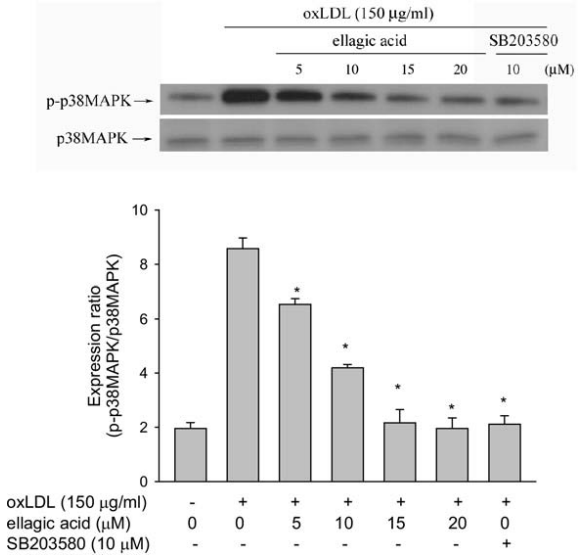

Fig 5. Ellagic acid inhibits oxidized low-density lipoprotein (*oxLDL*)-induced p38MAPK protein phosphorylation (p-p38MAPK). Human umbilical vein endothelial cells (*HUVECs*) were pretreated for 2 hours with the indicated concentrations of ellagic acid followed by stimulation with oxLDL (150 μg/mL) for 1 hour. Expression of p38MAPK was analyzed by Western blotting and quantified by densitometry. Treatment with 10 μM SB203580, a specific inhibitor of p38 MAPK, suppressed the oxLDL-induced phosphorylation of the p38MAPK protein. The values represent means ± SEM from three separate experiments. *$P < .05$ vs oxLDL treatment.

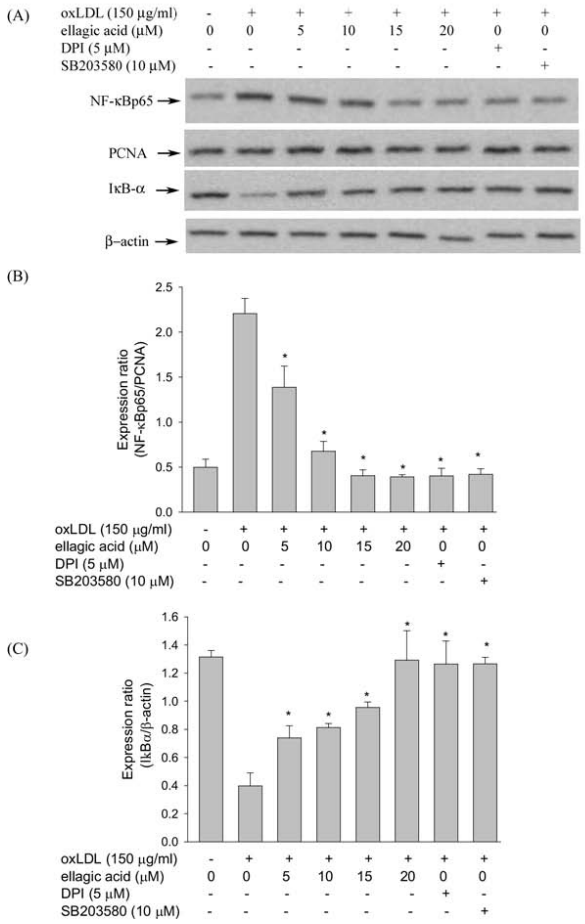

Fig 6. Effects of ellagic acid on oxidized low-density lipoprotein (*oxLDL*)-induced NF-κB activation. Human umbilical vein endothelial cells (*HUVECs*) were pretreated for 2 hours with the indicated concentrations of ellagic acid followed by stimulation with oxLDL (150 μg/mL) for 1 hour. Preparation of cytosolic and nuclear proteins is described in the Material and Methods section. The expression levels of the indicated proteins were analyzed by Western blotting and quantified by densitometry (**A**). The levels of cytosolic protein and nuclear protein were normalized to the levels of β-actin and proliferating cell nuclear antigen, respectively (**B, C**). The values represent means ± SEM from three separate experiments. *$P < .05$ vs oxLDL treatment.

dimers composed of members of the Rel/NF-κB family.[29] Its activation requires the dissociation of inhibitor factor, IκBα, and subsequent nuclear translocation, where NF-κB presents as a primary p65/p50 heterodimer and binds directly to its cognate DNA sequence.[30] As shown in Fig 6, after exposure to oxLDL, IκBα was degraded, thereby causing nuclear translocation of NF-κBp65. In contrast, in cells pretreated with various concentrations of ellagic acid or SB203580, a specific inhibitor of p38MAPK, NF-κB activation was markedly inhibited (all $P < .05$).

Ellagic acid inhibited NF-kB activated inflammatory response. Levels of inflammatory cytokines IL-6 and IL-8 were significantly higher in HUVECs that had been incubated for 24 hours with oxLDL at 150 μg/mL than in untreated control cells; however, ellagic acid significantly inhibited these NF-κB-mediated inflammatory responses in a dose-dependent manner (all $P < .05$; Fig 7, *A* and *B*). Flow cytometry revealed that the induction of adhesion molecules ICAM-1, VCAM-1, and E-selectin expression were attenuated by 20 μM ellagic acid (all $P < .05$; Fig 7C). To test the effect of ellagic acid on monocyte adhesion to HUVECs, confluent monolayers of HUVECs were pretreated with various concentrations of ellagic acid for 2 hours and then stimulated with oxLDL (150 μg/mL) for 24 hours, followed by incubation with THP-1 cells for 1 hour at 37°C. As shown in Fig 7D and E, oxLDL stimulated the adherence of THP-1 cells to HUVECs; however, ellagic acid treatment inhibited this adhesion in a dose-dependent manner (5-20 μM; all $P < .05$).

DISCUSSION

The present study is, to our knowledge, the first report to show the effects of ellagic acid on the protein expression of LOX-1 in oxLDL-evoked endothelial dysfunction. We first demonstrated that ellagic acid decreased the expression of LOX-1 in HUVECs that had been exposed to oxLDL. Second, we showed that ellagic acid inhibited superoxide generation, one of the earliest signals in oxLDL stimula-

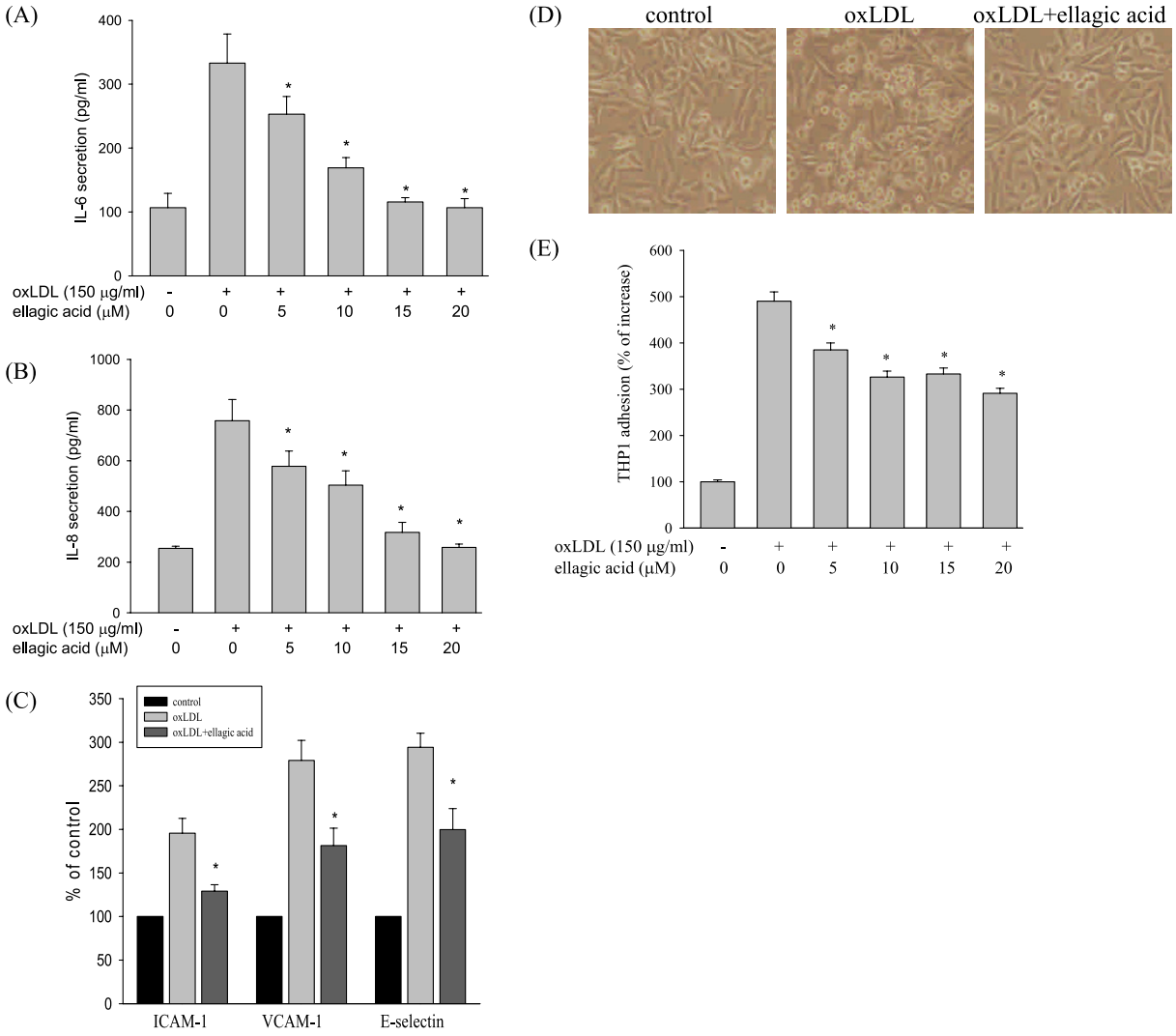

Fig 7. Effects of ellagic acid on oxidized low-density lipoprotein (*oxLDL*)-induced release of inflammatory cytokines, expression of adhesion molecules, and adhesiveness of THP-1 monocytic cells to human umbilical vein endothelial cells (HUVECs). Cells were incubated with indicated concentrations of ellagic acid for 2 hours and then incubated with ox-LDL for an additional 24 hours. ELISA measurements showing IL-6 **(A)** and IL-8 **(B)** protein levels in HUVECs treated with oxLDL in the absence or presence of indicated concentrations of ellagic acid. **(C)** HUVECs were incubated with oxLDL (150 μg/mL) in the absence (*light gray*) or presence (*dark gray*) of 20 μM ellagic acid for 24 hours. The histograms of cell surface expression of ICAM-1, VCAM-1, and E-selectin were generated by flow cytometry. **(D)** Representative fields of monocytes adhering to HUVECs with representative treatments as indicated. **(E)** Dose-dependent effect of ellagic acid (5-20 μM) on oxLDL (150 μg/mL)-induced adhesiveness of THP-1 to HUVECs was measured as described in the Materials and Methods section. The values represent mean ± SEM of three independent analyses. *$P < .05$ vs. oxLDL treatment.

tion, thereby blockading the LOX-1-mediated signaling pathway and the generation of ROS. Because regulation of LOX-1 involves NF-κB, inhibition of LOX-1 may result in a reduction in the oxidative stress-dependent activation of NF-κB.[28] Our finding that ellagic acid concomitantly decreases ROS production, NF-κB activation, and LOX-1 protein expression supports this possibility.

Increasing evidence indicates that ROS derived from NADPH oxidase act as a potent causal factor that initiates and accelerates the development of atherosclerosis.[31] Hence, compounds that suppress NADPH oxidase activation may ameliorate those complications. Previous studies have demonstrated that LOX-1 activation induces oxidative stress, and oxidative stress, in turn, stimulates LOX-1 expression, suggesting a positive loop between oxidative stress and LOX-1 expression.[32] LOX-1 activation has also been shown to activate NADPH oxidase and subsequent redox signals involving p38MAPK and NF-κB, the latter of which controls

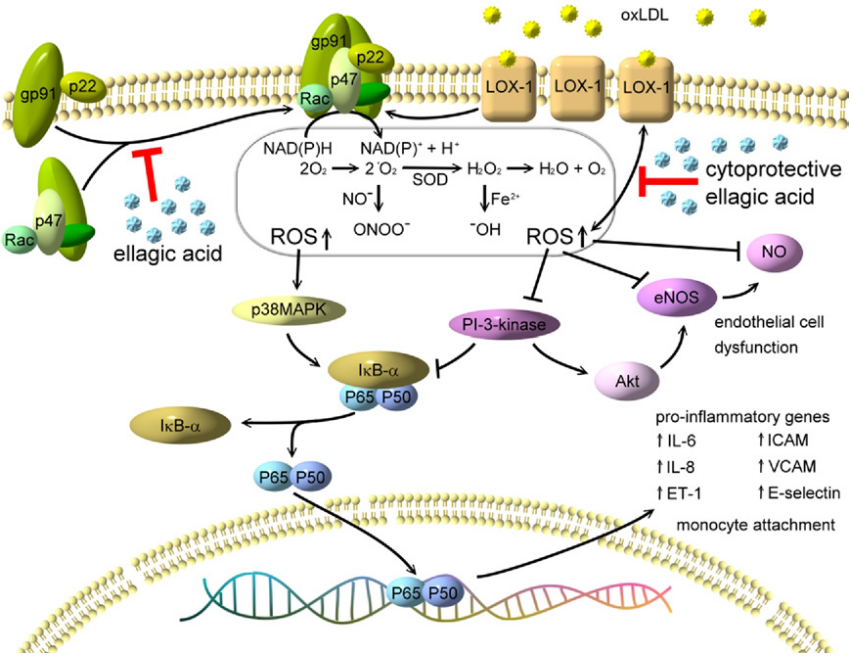

Fig 8. Schematic diagram showing cytoprotective signaling of ellagic acid in oxidized low-density lipoprotein (*oxLDL*)-induced endothelial dysfunction. As depicted, ellagic acid inhibits the LOX-1-mediated signaling cascades initiated by oxLDL-generated reactive oxygen species (*ROS*). The → indicates activation or induction, and ⊣ indicates inhibition or blockade.

the expression of a number of pro-inflammatory molecules, including cytokines, chemokines, inflammatory enzymes, and adhesion molecules in endothelial cells.[33] Cytoplasmic NF-κB is activated by the cleavage of IκB from the p50-65 heterodimer, which is then translocated to the nucleus. Cleavage of IκB requires an oxidizing milieu and appears to be one of the mechanisms through which ROS activates NF-κB.[34] The LOX-1-mediated signaling pathway involves the binding of oxLDL to LOX-1, which results in the activation of the NADPH oxidase subunits $p47^{phox}$ and Rac-1 on the cell membrane. Activation of those subunits induces a rapid increase in intracellular ROS and the subsequent activation of NF-κB-mediated downstream inflammatory response via phosphorylation of p38MAPK. In this study, our hypothesis was that ellagic acid acts as an antiatherogenic agent by blockading the LOX-1-mediated signaling pathway (Fig 8).

ROS generated in endothelial cells include superoxide ($\cdot O_2$), hydrogen peroxide (H_2O_2), peroxynitrite ($\cdot ONOO$), NO, and hydroxyl ($\cdot OH$) radicals. SOD protects against superoxide-mediated cytotoxicity by catalyzing $\cdot O_2$ to form H_2O_2. Cellular levels of superoxide are, therefore, normally low; however, Cu, Zn-SOD (SOD-1), but not Mn-SOD (SOD-2), is inactivated by H_2O_2 formed by dismutation of the superoxide anion. In this study, we found that ellagic acid significantly reduced superoxide generation caused by oxLDL, which subsequently ameliorated H_2O_2-attenuated SOD-1 expression (Fig 2C). We assume that ellagic acid protects against oxLDL-induced endothelial dysfunction by inhibiting LOX-1-mediated ROS generation, thereby preserving H_2O_2-inactivated SOD-1. However, whether the effect of ellagic acid is solely due to its antioxidant activity has not been clearly elucidated. Our finding that the membrane assembly of gp91, $p22^{phox}$, $p47^{phox}$, and Rac-1 after oxLDL exposure was reduced in cells pretreated with ellagic acid suggests that the beneficial effects of ellagic acid might be due, at least in part, to suppression of the membrane assembly of the NADPH oxidase complex (Fig 3).

Endothelial NO synthase (eNOS), a key regulator of vascular wall homeostasis, produces NO under normal physiological conditions. In contrast, the level of NO produced by inducible NO synthase (iNOS) is several orders of magnitude higher than that produced by eNOS in atherosclerotic lesions. In addition, the increase in NADPH oxidase activity leads to eNOS uncoupling, which results in the generation of superoxide rather than NO. Many studies have suggested that the interaction of NO with superoxide leads to peroxynitrite formation. This radical species is capable of nitrating tyrosine residues on key regulatory enzymes that are required for vascular tone.[35] Our data that nitrite formation was largely parallel to the expression level of iNOS (Fig 4, *A* and *C*) suggest that generation of NO as a precursor of nitrite in oxLDL-treated cells is due mainly to iNOS. However, pretreatment of HUVECs with ellagic acid suppressed the oxLDL-induced up-reguation of iNOS, thereby leading to a reduction in NO production (Fig 4D) and protein nitrosylation (Fig 4, *E* and *F*).

Decreased eNOS activity and enhanced release of endothelin-1 (ET-1) by oxLDL has been proposed to lead to an increase in oxidative stress, which would serve as a critical toxic mechanism for oxLDL in endothelial cells and is partially reversed by antioxidant treatment.[24] OxLDL antagonizes the vasodilatory effect of NO, and ROS and NO antagonize each other at the level of intracellular signaling; for example, NO released from eNOS inhibits cleavage of IκB and NF-κB activation, which is enhanced by ROS.[36] Consistent with a previous study that revealed that ellagic acid inhibits cytokine-induced adhesion molecule expression,[17] our data showed that the antiathrogenic effects of ellagic acid are due, at least in part, to the inhibition of adhesion molecules and subsequent monocytic adherence by moderating the reduction in eNOS expression caused by oxLDL.

The concentrations of ellagic acid (5-20 μM) used in our experiments were similar to those that have been reported to suppress aortic smooth muscle cell proliferation, anti-inflammation in human aorta endothelial cells, and G1 arrest and apoptosis in cervical carcinoma (CaSki) cells.[15,16,37] In animals, the peak plasma concentration after oral administration of an ellagic acid-phospholipid complex (equivalent to 80 mg/kg) was 2 μM. That concentration of ellagic acid was sufficient to restore the levels of antioxidant enzymes that had been impaired by administration of carbon tetrachloride.[38] The typical dietary intake of ellagic acid in humans, based on the consumption of 200 g of strawberries or blackberries, is approximately 40 to 80 mg/d.[39] The dose of ellagic acid used in the present study is equivalent to the dietary intake of approximately 200 g of berries. In humans, ellagic acid was detected in plasma at a maximum concentration of 31.9 ng/mL after 1 hour post-ingestion of 180 mL of pomegranate juice containing 25 mg of ellagic acid and 318 mg of ellagitannins.[40] Thus, the concentrations of ellagic acid used in the present study are comparable to those achievable physiologically. It is, however, unclear whether prolonged use of ellagic acid would lead to chronic accumulation in different tissues.

CONCLUSIONS

Ellagic acid possesses antioxidative, anticarcinogenic, and anti-inflammatory properties, making it a potential candidate agent for the prevention and treatment of several types of cancers and cardiovascular diseases. More research into the molecular mechanisms by which ellagic acid exerts its effects is needed. Our findings provide pharmacological evidence that ellagic acid helps prevent against the development of atherosclerosis.

AUTHOR CONTRIBUTIONS

Conception and design: LW, OH,
Analysis and interpretation: LW, OH, HW, CM, TJ, TK
Data collection: LW, OH, HW, CM, TJ, HS, TK
Writing the article: LW, OH
Critical revision of the article: HS, SW
Final approval of the article: HS, SW
Statistical analysis: HW, CM, TJ, TK
Obtained funding: LW, OH, SW
Overall responsibility: SW
WJL and HCO contributed equally to this work.

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INVITED COMMENTARY

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According to the findings of the National Vital Statistics Report and the Morbidity and Mortality Weekly report of the Centers for Disease Control and Prevention (CDC), cardiovascular disease continues to rank high among the leading causes of morbidity and mortality in adults in the United States. It is well known that most Americans are overfed and under-exercised, but what is much less well-known is how bad nutrition is causing an alarming increase in the frequency of obesity, heart disease, stroke, cancer, diabetes, and even dementia. Several epidemiological studies suggest that dietary patterns characterized by relatively high intake of fruits and vegetables are significantly associated with reduced risks of coronary heart disease and stroke. These foods have phytochemicals including carotenoids, and polyphenols like flavonoids, resveratrol and ellagitannins, iosthiocyanates and organosulfur compounds, each of which has been shown to be associated with lower risk of cardiovascular disease. Phytochemicals are potent antioxidants and anti-inflammatory agents, thereby counteracting oxidative damage and inflammation, which underlie the pathogenesis of cardiovascular disease. The authors present more data showing the importance of a diet high in polyphenols such as ellagic acid (ellagitannins). The authors show for the first time that pretreatment of endothelial cells with ellagic acid protected against ox-LDL endothelial cell dysfunction specifically through a LOX-1-mediated signaling pathway. The proatherosclerotic role of LOX-1 was made clear by Mehta and Sawamura when they reported that a LOX-1 knockout mouse model had reduced formation of atherosclerotic lesions in a proatherogenic genetic background under a high-cholesterol diet. Proatherosclerotic and pro-oxidative signaling and inflammatory response were measured as vascular NF-κB, CD68 expression, and p38 MAPK activation. Endothelial cell function was preserved, therefore providing proof of concept that LOX-1 was proatherosclerotic. There is evidence of LOX-1-increased expression in atherosclerotic plaques from human samples. There are also interesting studies showing that patients receiving ACE inhibitors and statins (Endothelial Protection, AT_1 blockage and Cholesterol-Dependent Oxidative Stress [EPAS] trial) independently and in combination had improved endothelial expression quotient of anti- and proatherosclerotic genes such as LOX-1. Further of interest is a small randomized controlled trial involving 19 patients with carotid artery stenosis found that pomegranate juice, high in ellagic acid, appeared to rescue blood pressure and carotid wall thickness. Inhibition of LOX-1 might be an interesting and novel therapeutic strategy in the treatment of atherosclerosis. One might even postulate from the authors' work that a diet high in polyphenols, especially ellagic acid, could lead to prevention of atherosclerotic disease or even regression.