Ellagic acid protects endothelial cells from oxidized low density lipoprotein-induced apoptosis by modulating the PI3K/Akt/eNOS pathway

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Keywords:
Ellagic acid
Endothelium
Apoptosis
ROS
oxLDL
Akt

Abstract
Endothelial apoptosis is a driving force in atherosclerosis development. Oxidized low-density lipoprotein (oxidized LDL) promotes inflammatory and thrombotic processes and is highly atherogenic, as it stimulates macrophage cholesterol accumulation and foam cell formation. Previous studies have shown that the phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase/nitric oxide (PI3K/Akt/eNOS/NO) pathway is involved in oxLDL-induced endothelial apoptosis. Ellagic acid, a natural polyphenol found in berries and nuts, has in recent years been the subject of intense research within the fields of cancer and inflammation. However, its protective effects against oxLDL-induced injury in vascular endothelial cells have not been clarified. In the present study, we investigated the anti-apoptotic effect of ellagic acid in human umbilical vein endothelial cells (HUVECs) exposed to oxLDL and explored the possible mechanisms. Our results showed that pretreatment with ellagic acid (5–20 μM) significantly attenuated oxLDL-induced cytotoxicity, apoptotic features, and generation of reactive oxygen species (ROS). In addition, the anti-apoptotic effect of ellagic acid was partially inhibited by a PI3K inhibitor (wortmannin) and a specific eNOS inhibitor (cavatatin) but not by an ERK inhibitor (PD98059). In exploring the underlying mechanisms of ellagic acid action, we found that oxLDL decreased Akt and eNOS phosphorylation, which in turn activated NF-κB and downstream pro-apoptotic signaling events including calcium accumulation, destabilization of mitochondrial permeability, and disruption of the balance between pro- and anti-apoptotic Bcl-2 proteins. Those alterations induced by oxLDL, however, were attenuated by pretreatment with ellagic acid. The inhibition of oxLDL-induced endothelial apoptosis by ellagic acid is due at least in part to its anti-oxidant activity and its ability to modulate the PI3K/Akt/eNOS signaling pathway.

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Introduction
Ellagic acid is a polyphenolic compound found in the form of ellagitannins in fruits such as pomegranate, blackberries, raspberries and strawberries. Ellagic acid is released by the metabolism of ellagitannins by the microflora in the gut (Larroso et al., 2006). Several studies have reported on the anti-oxidant, anti-inflammatory and anti-mutagenic activities of ellagic acid (Priyadarsini et al., 2002; Edderkaoui et al., 2008; Leelavinothan Pari, 2008; Papoutsi et al., 2008). Ellagic acid also has been shown to be a potent anti-carcinogenic agent (Zhang et al., 1993) and to be a better protector against oxidative stress than vitamin E (Hassoun et al., 1997). In addition, oral administration of ellagic acid has been reported to protect against alcohol toxicity by decreasing the activities of liver marker enzymes and lipid peroxidative markers as well as by increasing the anti-oxidant activity of pomegranate and blackberry extracts.
Atherosclerosis is considered to be a chronic inflammatory disease (Ross, 1999), and growing evidence indicates that chronic and acute overproduction of reactive oxidative species (ROS) under pathophysiological conditions is related to the development of cardiovascular diseases (Madamanchi et al., 2005). It has been reported that ROS generated by oxidized low-density lipoprotein (oxLDL) triggers endothelial apoptosis, a process that can accelerate the progression of endothelial dysfunction in atherosclerosis (Napoli, 2003). Several critical ROS-sensitive signaling events involved in oxLDL-induced endothelial apoptosis include activation of nuclear transcription factor kappa B (NF-kB), accumulation of intracellular calcium, disturbance of the balance of Bcl-2 family proteins, and reduction of mitochondrial transmembrane potential with concomitant release of mitochondrial protein cytochrome c and the subsequent activation of caspase-3 (Salvayre et al., 2002).

Akt, a downstream effector of PI3K, promotes cell survival in response to various death stimuli (Fulton et al., 1999). The activation of Akt involves its phosphorylation on threonine 308 and on serine 473 by PI3K (Datta et al., 1999). Besides mediating cell survival in endothelial cells, Akt activates endothelial nitric oxide synthase (eNOS), which leads to nitric oxide (NO) production (Fulton et al., 1999). Evidence suggests that the PI3K/Akt/eNOS pathway plays an important role in preventing ROS-induced endothelial damage (Chavakis et al., 2001) by scavenging superoxide anion, which in turn prevents superoxide anion from forming its dismutation product, hydrogen peroxide (Huie and Padmaja, 1993). Previous studies reported that oxLDL leads to dephosphorylation of Akt/eNOS in a dose- and time-dependent fashion in cultured umbilical vein endothelial cells (Chavakis et al., 2001), and our earlier study found that the anti-oxidants resveratrol inhibited the production of ROS and prevented oxLDL-induced endothelial apoptosis (Ou et al., 2006). We therefore aimed to determine whether treatment with ellagic acid attenuates oxLDL-induced apoptosis and, if so, whether the mechanisms underlying the process involve the PI3K/Akt/eNOS signaling pathway.

Materials and methods

Materials. Fetal bovine serum (FBS), medium 199 (M199) and trypsin–EDTA were obtained from Gibco (Grand Island, NY, USA); low serum growth supplement was obtained from Cascade Biologics (Portland, OR, USA); ellagic acid, wortmannin, PD98059, sodium nitroprusside (SNP), 4,6-diamidino-2-phenylindole (DAPI), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT); 1400 W, penicillin, and streptomycin were obtained from Sigma (St. Louis, MO, USA); cavitrin was obtained from Enzo (San Diego, USA); TUNEL (deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labeling) staining kit was obtained from Boehringer Mannheim (Mannheim, Germany); 2′,7′-dichlorofluorescein acetoxymethyl ester (DCF-AM, Molecular Probes, Eugene, Ore) as a probe for the presence of ROS. After preincubation for 2 h with the indicated concentrations of ellagic acid, HUVECs were incubated with DCF-AM for 1 h followed by incubation with oxLDL for 2 h. The fluorescence intensity was measured at 485-nm excitation and 535-nm emission (before and after exposure to oxLDL) using a fluorescence microplate reader (Labsystems, CA). The percentage increase in fluorescence per well was calculated by the formula [(Ft - F0) / F0] × 100, where F0 is the fluorescence at 2 h of oxLDL exposure and Ft is the fluorescence at 0 min of oxLDL exposure.

Measurement of ROS production. The effect of ellagic acid on ROS production in HUVECs was determined using a fluorometric assay using 2′,7′-dichlorofluorescein acetoxymethyl ester (DCF-AM, Molecular Probes, Eugene, Ore) as a probe for the presence of ROS. After preincubation for 2 h with the indicated concentrations of ellagic acid, HUVECs were incubated with DCF-AM for 1 h followed by incubation with oxLDL for 2 h. The fluorescence intensity was measured at 485-nm excitation and 535-nm emission (before and after exposure to oxLDL) using a fluorescence microplate reader (Labsystems, CA). The percentage increase in fluorescence per well was calculated by the formula [(Ft - F0) / F0] × 100, where F0 is the fluorescence at 2 h of oxLDL exposure and Ft is the fluorescence at 0 min of oxLDL exposure.

Cell cultures. HUVECs were isolated from human umbilical cords with collagenase and used at passages 2–3 as previously described (Kuo et al., 2009). 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.
NF-κB assay. To prepare nuclear extracts for the NF-κB assay, the cells were first resuspended in buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM PMSF, then vigorously vortexed for 15 s and allowed to stand at 4 °C.

Fig. 1. Effect of ellagic acid on oxLDL-induced endothelial cell death. HUVECs were incubated with oxLDL (150 μg/ml) in the absence (middle) or the presence (right) of 20 μM ellagic acid for 24 h. (A) Photomicrographs from phase-contrast microscopy. (B) Viability was determined via MTT assay. The values represent means±SEM from three separate experiments. *P<0.05 vs. untreated control; *P<0.05 vs. oxLDL treatment.

Fig. 2. Effect of ellagic acid on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL (150 μg/ml) in the absence (middle) or the presence (right) of 20 μM ellagic acid for 24 h. Top: cells stained with DAPI. Middle: cells stained using TUNEL assay. Bottom: flow cytometric analysis (control: white; oxLDL: black; oxLDL+ellagic acid: gray).

Fig. 3. Inhibitory effects of ellagic acid on oxLDL-induced ROS production in HUVECs. After preincubation for 2 h with the indicated concentrations of ellagic acid (5-20 μg/ml), 150 μg/ml oxLDL was added to the medium for 2 h followed by a 1 h incubation with H₂O₂-sensitive fluorescent probe DCF-AM (10 μM). (A) Fluorescence images show the ROS level in control cells (left), in HUVECs stimulated with oxLDL (middle), and in HUVECs pretreated with 20 μM ellagic acid (right). (B) Fluorescence intensity of cells was measured with a fluorescence microplate reader. Fluorescence distribution of DCF-AM oxidation is expressed as a percentage of increased intensity. Data are expressed as the mean±SEM of 3 independent analyses. *P<0.05 vs. untreated control; *P<0.05 compared with oxLDL-stimulated HUVECs.
for 10 min. The samples were then centrifuged at 2000 rpm for 2 min. The pelleted nuclei were resuspended in 30 μL buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF and incubated for 20 min on ice. The nuclear lysates were then centrifuged at 15,000 rpm for 2 min. Supernatants containing the solubilized nuclear proteins were stored at −70 °C for subsequent NF-κB assay. The nuclear translocation of NF-κB was measured by an NF-κB p65 ActivELISA kit according to the manufacturer’s instructions. The absorbance at 405 nm was determined using a microplate reader (SpectraMax 340).

Measurement of [Ca²⁺]i. To determine the effect of ellagic acid on the rise of oxLDL-induced intracellular calcium, HUVECs were seeded onto 24-mm glass coverslips, pretreated with ellagic acid for 2 h, and then stimulated with oxLDL (150 μg/ml) for a further 24 h. The cells on the coverslips were loaded with 2 μM fura-2 AM (Molecular Probe) in M199 and allowed to stand for 30 min at 37 °C. After loading, the cells were washed with HEPES buffer (mM) (NaCl, 131; KCl, 5; CaCl₂, 1.3; Mg₂SO₄, 1.3; KH₂PO₄, 0.4; HEPES, 20; glucose 25, pH 7.4) to remove excess fluorescent dye. Then, the fluorescence of the cells on each coverslip was measured and recorded using an inverted Olympus IX-70 microscope. [Ca²⁺]i in endothelial cells was monitored at an emission wavelength of 510 nm with excitation wavelengths alternating between 340 nm and 380 nm with a Delta Scan System (Photon Technology International, Princeton, NJ, USA), and calculated using Grynkiewicz’s method (Grynkiewicz et al., 1985).

Measurement of mitochondrial membrane potential. The lipophilic cationic fluorochrome 5,58,6,68-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was used to explore the effect of ellagic acid on mitochondrial membrane potential (ΔΨm). JC-1 exists either as a green fluorescent monomer at depolarized membrane potentials or as a red fluorescent J-aggregate at hyperpolarized membrane potentials. ΔΨm exhibits potential-dependent accumulation in mitochondria, as indicated by the fluorescence emission shift from 530 nm to 590 nm. After cells had been treated with oxLDL (150 μg/ml) for 24 h in the presence or absence of various concentrations of ellagic acid, cells (5×10⁴ cells/24-well plates) were rinsed with M199, and JC-1 (5 μM) was loaded. After 20 min of incubation at 37 °C, cells were examined under a fluorescence microscope. Determination of the ΔΨm was carried out using a FACScan flow cytometer (Bedner et al., 1999).

Measurement of active caspase-3. To explore the effect of ellagic acid on oxLDL-induced caspase-3 activation, HUVECs were pretreated with ellagic acid for 2 h and then stimulated with oxLDL (150 μg/ml) for 24 h. The level of active caspase-3 was detected by flow cytometry using a commercial fluorescein active caspase kit (Mountain View, CA) (Telford et al., 2002) and a fluorescence microscope. The activity of caspase-3 was also measured using the EnzChek caspase-3 assay kit according to the manufacturer’s instructions (Molecular Probes, Mountain View, CA).

Fig. 4. The protective effect of ellagic acid depends on the PI3K/Akt/eNOS pathway. (A) HUVECs were pretreated with each inhibitor 1 h prior to ellagic acid treatment and then incubated for 24 h with oxLDL (150 μg/ml). Viability was determined via MTT assay. Treatment with wortmannin and cavtratin but not PD98059 attenuated the protective effect of ellagic acid in oxLDL-treated HUVECs. In addition, 1400 W and SNP partially reversed the cytotoxic effect of oxLDL. (B) HUVECs were pretreated for 2 h with the indicated concentrations of ellagic acid followed by oxLDL (150 μg/ml) for 1 h. Western blotting analysis for Akt, eNOS, phospho-Akt (p-Akt), and phospho-eNOS (p-eNOS). (C, D) Densitometric data are means±SEM from three separated experiments. *P<0.05 vs. untreated control; †P<0.05 vs. oxLDL treatment; ‡P<0.05 vs. oxLDL+ellagic acid treatment.
Eugene, OR). After being lysed by repeat freeze–thaw cycles, cells were incubated on ice for 15 min and centrifuged at 15,000 × g for 20 min. The protein concentrations of the supernatants were then determined. Equal amounts of protein (50 μg) were added to the reaction buffer containing 5 mM of caspase-3 substrate Z-DEVD-R110, and the mixture was incubated at room temperature for 30 min. The fluorescence generated from the cleavage of the substrate by caspase-3 was monitored with a fluorescence microplate reader (Labsystems, CA) at excitation and emission wavelengths of 496 nm and 520 nm, respectively.

Statistical analyses. Results are expressed as mean ± SEM. Differences between groups were analyzed using one-way ANOVA followed by Bonferroni’s post hoc test. A P-value < 0.05 was considered statistically significant.

Results

Ellagic acid protects against oxLDL-induced cytotoxicity

To examine toxicity of ellagic acid, HUVECs were treated with ellagic acid (5–100 μM) and incubated for 24 h. Ellagic acid did not influence cell viability in HUVECs (data not shown). HUVECs were then exposed to 150 μg/ml oxLDL alone for 24 h or pretreated with various concentrations of ellagic acid for 2 h followed by exposure to 150 μg/ml oxLDL for 24 h. Cell viability was assessed using MTT assay. As shown in Fig. 1, oxLDL significantly reduced the viability of HUVECs; however, ellagic acid significantly increased the viability in a concentration-dependent manner. The ED50 (the half-maximal effective concentration) of ellagic acid was calculated to be 6.3 μM.

Ellagic acid inhibited oxLDL-induced apoptosis

The induction of apoptosis in oxLDL-treated HUVECs was analyzed using TUNEL and DAPI staining assays and evaluated by microscopic observation and flow cytometry. As shown in Fig. 2, cells incubated with 150 μg/ml oxLDL for 24 h showed the typical features of apoptosis, including the formation of condensed and fragmented nuclei. Those apoptotic features were not observed, however, in HUVECs pretreated with 20 μM ellagic acid.

Ellagic acid inhibited oxLDL-induced ROS generation

A previous study demonstrated that oxLDL evokes a progressive rise in cellular ROS, which subsequently leads to the activation of apoptotic signaling (Schmitt et al., 1995). We, therefore, investigated the effects of ellagic acid on the generation of ROS, a potential factor related to oxLDL-induced endothelial cell injury, by using DCF-AM as a fluorescence probe. Pretreatment of HUVECs with ellagic acid (5–20 μM) for 2 h before exposure to 150 μg/ml oxLDL for 24 h. Therefore, 150 μg/ml of oxLDL and 5–20 μM of ellagic acid were used in the following experiments.

Ellagic acid-mediated protective action involves PI3K/Akt/eNOS activation

Activation of PI3K/Akt/eNOS and ERK1/2 is known to suppress apoptosis and promote cell survival (Amaravadi and Thompson, 2005). To investigate whether PI3K/Akt/eNOS or ERK1/2 signaling was involved in the action of ellagic acid, we pretreated cells with a PI3K inhibitor (wortmannin), a selective eNOS inhibitor (cavtratin), a selective iNOS inhibitor (1400 W), or an ERK1/2 inhibitor (PD98059) 1 h prior to ellagic acid treatment. As shown in Fig 4A, pretreatment with wortmannin as well as cavtratin but not PD98059 partially antagonized the protective action of ellagic acid against oxLDL-induced cell death. In addition, 1400 W and SNP (an exogenous NO donor) partially reversed the cytotoxic effects of oxLDL, indicating that PI3K/Akt/eNOS activation is involved in the protective action of ellagic acid. To confirm this, Western blot analysis was performed.
using phosphor-specific Akt (Ser473) and phosphor-eNOS antibody. As expected, ellagic acid significantly reversed the dephosphorylation of Akt and eNOS caused by oxLDL (Figs. 4B–D).

Ellagic acid prevented NF-κB activation and downstream apoptotic response

NF-κB, a redox-sensitive transcription factor, is involved in oxLDL-induced endothelial apoptosis (Ou et al., 2007). In addition, NO inhibits the cleavage of IκB and NF-κB activation (Marshall et al., 2000). We hypothesized that oxLDL induces NF-κB activation by reducing the bioavailability of NO and that oxLDL-induced NF-κB activation could be reversed by ellagic acid. To determine whether the effects of ellagic acid are associated with the NF-κB signaling pathway in oxLDL-stimulated HUVECs, we assayed NF-κBp65 nuclear translocation using ELISA and examined whether ellagic acid interferes with the expected NF-κB-mediated pro-apoptotic response. As shown in Fig. 5, ellagic acid inhibited the oxLDL-induced activation of NF-κB by nearly 100% at a concentration of 20 μM. As expected, wortmannin and cavtratin but not PD98059 partially antagonized the inhibitory effect of ellagic acid. Additionally, cells pretreated with 1400 W or SNP showed a marked reduction in the activation of NF-κB (all P<0.05).

Ellagic acid inhibited oxLDL-induced intracellular calcium ([Ca^{2+}]i) accumulation

Intracellular calcium rise is involved in oxLDL-induced endothelial apoptosis (Sanson et al., 2008). To determine the effect of ellagic acid on the increase in oxLDL-induced intracellular calcium concentration, HUVECs were seeded onto 24-mm glass coverslips, pretreated with ellagic acid for 2 h, and then stimulated with oxLDL (150 μg/mL) for an additional 24 h. As shown in Fig. 6, the basal [Ca^{2+}]i increased from 65 ± 7 nM to 356 ± 22 nM in oxLDL-treated cells; ellagic acid, however, significantly inhibited the oxLDL-induced rise in intracellular calcium (all P<0.05).

Ellagic acid modulates oxLDL-induced mitochondrial transmembrane permeability transition

Calcium is the most important signal for opening of the mitochondrial permeability transition pore (PTP), a mechanism that triggers apoptosis (Zoratti and Szabo, 1995). As a consequence of both the dysfunction of the electrochemical gradient caused by pore opening and the rupture of the outer mitochondrial membrane, the mitochondrial membrane potential (ΔΨm) generally collapses (Giovannini et al., 2002). When HUVECs were exposed to oxLDL (150 μg/mL), the ΔΨm became depolarized, as shown by the increase in green fluorescence (Fig. 7A, middle panel). Pretreatment with 20 μM ellagic acid reduced the change in ΔΨm as indicated by repression of green fluorescence and restoration of red fluorescence (Fig. 7A, right panel). The results of flow cytometry confirmed those findings (Fig. 7B). OxLDL caused a marked increase in JC-1 green fluorescence (middle panel) compared to control (left panel). Pretreatment with ellagic acid caused a marked inhibition of oxLDL-induced apoptosis (right panel).

Ellagic acid modulates oxLDL-induced expression of pro-apoptotic proteins

A sustained rise in cytosolic calcium plays a central role in oxLDL-induced apoptosis and necrosis, possibly by activating the calcium-
dependent calpain proteases that mediate the cleavage of Bid and the release of cytochrome c (Vindis et al., 2005). In addition, following cellular stress, P53 is stabilized by phosphorylation via calcium-dependent protein kinase C (Guarda et al., 1993), allowing it to regulate the expression of numerous pro-apoptotic genes (Chipuk and Green, 2006). Evidence indicates that increased levels of phosphorylated P53 stimulate a conformational change in the pro-apoptotic protein Bax that favors its translocation to the mitochondria (Cheng et al., 2007). We, therefore, examined the effects of ellagic acid on P53-regulated intrinsic pro-apoptotic proteins. As shown in Fig. 8, incubation of HUVECs with oxLDL for 24 h resulted in an increase in phosphorylation of P53, although the total P53 protein level was unchanged. Moreover, oxLDL increased the mitochondrial translocation of Bax and caused a reduction in the expression of the anti-apoptotic protein Bcl-2, which resulted in the permeabilization of mitochondria and the release of cytochrome c. Pretreatment with ellagic acid abolished the P53-evoked pro-apoptotic signaling events in a dose-dependent manner (all \( P < 0.05 \)).

Ellagic acid reduced oxLDL-induced caspase-3 activation

Caspases are key mediators of cell death and caspase-3 is the primary executioner of apoptosis in HUVECs in response to oxLDL. We subsequently determined the active form of caspase-3 using flow cytometry. As shown in Fig. 9, active caspase-3 was significantly increased in cells treated with 150 μg/ml oxLDL for 24 h. In contrast, the activation of caspase-3 by oxLDL was suppressed in cells pretreated with 20 μM of ellagic acid. The activity of caspase-3 was confirmed using the EnzCaspase-3 assay kit. The results showed that oxLDL significantly upregulated caspase-3 activity by 4.5-fold, whereas pretreatment with ellagic acid effectively suppressed the activity of that apoptotic factor, implying that oxLDL has a stimulatory effect and that ellagic acid has an inhibitory effect on caspase-3 activation. Simultaneous treatment of HUVECs with ellagic acid and a PI3K inhibitor or eNOS inhibitor, but not and ERK inhibitor, partially abolished the inhibitory effects of ellagic acid on caspase-3 activity. Furthermore, the addition of 1400 W or SNP definitely inhibited oxLDL-induced activation of caspase-3.

Discussion

Oxidative stress induced by oxLDL in endothelial cells plays an important role in the pathogenesis of atherosclerosis. In our previous study, we demonstrated that oxLDL elicited vascular cell apoptosis by increasing intracellular ROS levels. The increased abundance of ROS subsequently activated NF-\( \kappa \)B which in turn activated a downstream signaling cascade resulting in apoptosis (Ou et al., 2007). The present study is to our knowledge the first report to show that ellagic acid elicits anti-apoptotic effects in oxLDL-stimulated endothelial cells by inhibiting ROS generation and modulating the PI3K/Akt/eNOS signaling pathway.

In recent years, there has been much interest in the anti-atherogenic effects of health foods and their active phytochemicals. The protective effects are, in part, attributed to their ability to inhibit excess ROS generation. The ROS generated by exposure to oxLDL in endothelial cells include superoxide (\( \cdot O_2^- \)), hydrogen peroxide (\( H_2O_2 \)), peroxynitrite (\( \cdot ONOO^- \)), NO, and hydroxyl (\( \cdot OH \)) radicals, molecules that activate diverse signaling pathways by oxidizing cysteine residues on specific target molecules including kinases, phosphatases, and redox-sensitive transcription factors. One of the most important ROS in the vasculature is superoxide, which is formed by the univalent reduction of oxygen.

Fig. 8. Immunoblotting analysis of apoptosis-provoking proteins in response to oxLDL and ellagic acid. HUVECs were incubated with 150 μg/mL oxLDL in the absence or presence of indicated concentrations (5–20 μM) of ellagic acid for 24 h. Representative Western blots and summary data showing that oxLDL upregulated pro-apoptotic (phosphorylated P53, Bax) and downregulated anti-apoptotic (Bcl-2) proteins and increased the concentration of cytochrome c in the cytosolic fraction. Pretreatment with ellagic acid suppressed these apoptosis-provoking alterations. Results were subjected to densitometric analysis; the values are presented as means ± SEM of three separate experiments. *P < 0.05 vs. untreated control; #P < 0.05 vs. oxLDL treatment.
analyses. EnzCaspase-3 assay kit. Data are expressed as the mean±SEM of 3 independent oxLDL+ellagic acid treatment.

The nitrosylation of cys 62 on the p50 subunit (Peng et al., 1995). In normal physiological conditions. At these low levels, NO has been shown to have anti-inflammatory and protective effects by inhibiting NADPH oxidase-generated peroxynitrite leading to peroxynitrite formation and cell toxicity (Chandel et al., 2000). It has been shown that iNOS-derived overproduction of NO can lead to activation of NF-κB, which in turn leads to the up-regulation of several major pro-inflammatory mediators such as COX-2, iNOS, and the adhesion molecules VCAM-1 and ICAM-1. Our recent study demonstrated that ellagic acid exerts its protective effects by inhibiting NAPDH oxidase-generated overproduction of superoxide and down-regulating iNOS, thereby suppressing the overproduction of NO (Lee et al., in press). In the present study, we found that some of the adverse effects of oxLDL, namely inhibition of Akt and eNOS phosphorylation and activation of NF-κB, were abrogated by ellagic acid treatment. In addition, pretreatment with wortmannin or cavatatin partially antagonized the anti-apoptotic effect of ellagic acid, suggesting that the protective effect of ellagic acid is due, at least in part, to its ability to upregulate the PI3K/Akt/eNOS/NO signaling pathway.

Pathophysiological stimuli that induce endothelial activation via ROS generation and alteration of intracellular Ca^2+ ion homeostasis are now considered to be major contributors to the development of atherosclerotic coronary artery diseases (Madamanchi et al., 2005). An intense, delayed, and sustained calcium signal is a trigger of cell death but the initial targets and the subsequent sequence of events leading to cell death are only partly understood (Vindis et al., 2005). ROS generation may inhibit Ca^{2+}/ATPases, leading to sustained elevations of [Ca^{2+}]_i, which are associated with mitochondrial dysfunction via loss of membrane potential and release of cytochrome c. Previous studies have demonstrated that calcium channel blockers inhibit atherosclerosis in cholesterol-fed animals (Cristofori et al., 2000) and down-regulating the endothelial receptor for oxLDL (lectin-like oxidized low-density lipoprotein receptor-1; LOX-1), and inhibit CPP32-like protease activity (Sugano et al., 2002). It has also been shown that anti-oxidants prevent both oxLDL-induced ROS generation and Ca^{2+} elevation (Maziere et al., 2005). Our study found that ROS generation was the earliest apoptotic signal and that it usually occurred within 5 min after the addition of oxLDL (data not shown). Based on our findings, the anti-apoptotic effects of ellagic acid are due to its inhibition of ROS generation, which, in turn, represses the release of endothelial [Ca^{2+}]_i, stabilizes the mitochondrial membrane, and prevents the release of cytochrome c, a molecule required for the activation of caspase-3.

Many genes have been reported to be linked with the regulation of programmed cell death under physiological and pathological conditions. The Bcl-2 and Bax genes have been shown to play a major role in determining cell survival or death after apoptotic stimuli (Choy et al., 2001). Cheng et al. showed that oxLDL-induced generation of ROS in endothelial cells leads to the activation of P33, which subsequently induces a conformational change in Bax that enables the mitochondrial translocation of that pro-apoptotic protein. In the same study they also showed that the anti-oxidants N-acetyl-cysteine (NAC) and superoxide dismutase (SOD) scavenge ROS and prevent oxLDL-induced expression of P33, Bax activation, and eventual apoptosis of endothelial cells (Cheng et al., 2007). Our results show that ellagic acid significantly reduced the activation of P33 and expression of Bax and significantly increased the expression of the anti-apoptotic protein Bcl-2 in a concentration-dependent manner. Our findings indicate that ellagic acid might protect against oxLDL-induced apoptosis by upregulating the Bcl-2/Bax ratio or by activating the PI3K/Akt/eNOS pathway.

The caspase pathway is an important effector of apoptosis and a well-identified downstream target for PI3K/Akt/eNOS. One mechanism by which PI3K/Akt/eNOS regulates cell survival involves the S-nitrosylation of cysteine 163 in the active center of the catalytic subunit p17 of caspase-3, which results in attenuation of its activity (Mannick et al., 1999). Investigators have linked NO to the inhibition of
caspase activation and prevention of endothelial apoptosis caused by oxLDL (Hoffmann et al., 2001). Our findings that ellagic acid reduced the activity of caspase-3 in oxLDL-treated HUVECs and that inhibitors of PI3K and eNOS blocked this effect clearly identify the PI3K/Akt/eNOS pathway as the major pathway that is modulated by ellagic acid.

Ellagic acid is found in fruits including grape juice (10.2 mg/100 g), grape wine (5.6 mg/100 g), blueberries (0.9 mg/100 g), blackberries (42.4 mg/100 g), raspberries (17.9 mg/100 g) and strawberries (19.8 mg/100 g) (Mertens-Talcott et al., 2003). The typical dietary intake of ellagic acid in humans is approximately 40–80 mg/day if 200 g of strawberries or blackberries is eaten (Mertens-Talcott et al., 2003). The concentrations used in our study range from 5 to 20 μM and are similar to previous report that demonstrated that ellagic acid (at 2.5–20 μM) inhibited the IL-1β–induced endothelial activation and expression of adhesion molecules (Yu et al., 2007), but seem to be relatively high compared with the physiologically achievable in vivo. The maximum concentration in plasma rarely exceeds 1 μM after the consumption of 10–100 mg of a single phenolic compound. The maintenance of a high concentration in plasma thus requires a repeated ingestion of the polyphenols over time (Scalbert and Williamson, 2000). It also is unknown whether prolong use of ellagic acid would lead to chronic accumulation in different tissues. In human, the 22.8 ng/ml (0.11 μM) of Cmax (peak level in the blood) in plasma after the consumption of 21.6 mg of ellagic acid has been reported that could cause a significant increase of anti-oxidant capacity of plasma (Mertens-Talcott et al., 2006). Recently, Murugan et al. (2009) developed a novel formulation with phospholipid that enhanced the bioavailability of ellagic acid. In addition, measurement of the plasma anti-oxidant capacity suggests that more phenolic compounds are present, largely in the form of unknown metabolites, produced either in our tissues or by the colonic microflora (Scalbert and Williamson, 2000). It will be important to learn more about these metabolites, particularly because of their potent biological activity.

Taken together, we propose a possible cytoprotective effect of ellagic acid (Fig. 10). Ellagic acid protects against atherogenic signaling triggered by oxLDL through inhibiting oxLDL-induced ROS generation and subsequent NF-κB activation, which in turn activated downstream pro-apoptotic signaling events, through the PI3K/Akt/eNOS pathway. Our findings suggest that ellagic acid might be a candidate agent for further development in the prevention of cardiovascular diseases.

**Acknowledgments**

This study was supported by grants from the Taichung Veterans General Hospital (TCVGH-993001C); the Taichung Veterans General Hospital and Tungs’ Taichung MetroHarbor Hospital (TCVGH-TTMH958502); and the National Science Council, Taiwan, ROC (NSC 98-2320-B-039-020-MY3 and NSC 98-2314-B-075A-002-MY3).

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