JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Ellagic Acid Inhibits Oxidized Low-Density Lipoprotein (OxLDL)-Induced Metalloproteinase (MMP) Expression by Modulating the Protein Kinase C-α/Extracellular Signal-Regulated Kinase/Peroxisome Proliferator-Activated Receptor γ/Nuclear Factor-κB (PKC-α/ERK/PPAR-γ/NF-κB) Signaling Pathway in Endothelial Cells

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ABSTRACT: Previous studies have shown that vascular endothelium-derived matrix metalloproteinases (MMPs) contribute to the destabilization of atherosclerotic plaques, a key event triggering acute myocardial infarction. In addition, studies have reported that the PKC-MEK-PPARγ signaling pathway is involved in oxidized low-density lipoprotein (oxLDL)-induced expression of MMPs. Ellagic acid, a phenolic compound found in fruits and nuts, has potent antioxidant, anti-inflammatory, and anticancerous properties. However, the molecular mechanisms underlying its antiatherogenic effects remain to be clarified. This study aimed to assess whether the effects of ellagic acid on the fibrotic markers MMP-1 and MMP-3 are modulated by the PKC-ERK-PPAR-γ signaling pathway in human umbilical vein endothelial cells (HUVECs) that have been exposed to oxLDL. It was found that ellagic acid significantly inhibited oxLDL-induced expressions of MMP-1 and MMP-3. Pretreatment with ellagic acid and DPI, a well-known ROS inhibitor, attenuated the oxLDL-induced expression and activity of PKC-α. In addition, ellagic acid as well as pharmacological inhibitors of ROS, calcium, and PKC strongly suppressed the oxLDL-induced phosphorylation of extracellular signal-regulated kinase (ERK) and NF-κB activation. Moreover, ellagic acid ameliorated the oxLDL-induced suppression of PPAR-γ expression. In conclusion, the data suggest that ellagic acid elicits its protective effects by modulating the PKC-α/ERK/PPAR-γ/NF-κB pathway, resulting in the suppression of ROS generation and, ultimately, inhibition of MMP-1 and MMP-3 expression in HUVECs exposed to oxLDL.

KEYWORDS: ellagic acid, atherosclerotic plaques, matrix metalloproteinases

INTRODUCTION

Atherosclerosis is an inflammatory process that involves the participation of cytokines, growth factors, and modified lipoproteins. Clinical complications of atherosclerosis are often triggered by rupture of unstable plaques, whereas thinning of the atherosclerotic vessel wall due to elastin and collagen degradation and media necrosis might result in aneurysm formation and bleeding. The atherosclerotic sites vulnerable to rupture exhibit enhanced oxidant activity, which is manifested by deposition of oxidatively

Received: October 28, 2010
Revised: February 26, 2011
Accepted: April 1, 2011
Published: April 11, 2011

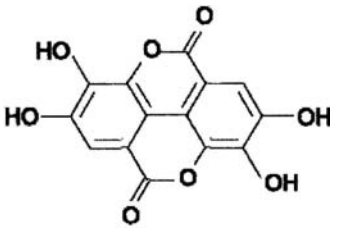

Figure 1. Chemical structure of ellagic acid.

modified low-density lipoproteins (oxLDL) and reduced levels of nitric oxide (NO) synthesis. Following the disruption of the endothelial lining, platelets adhere to the subendothelial surface where intense platelet aggregation and deposition of interspersed fibrin bands and inflammatory cells take place. Rupture of the atherosclerotic plaque is probably a result of intense inflammatory response and release of collagen-degrading metalloproteinases. Many studies have also demonstrated that matrix metalloproteinases (MMPs), a group of proteinases capable of degrading collagen and other matrix components, play an important role in the destabilization of atherosclerotic plaques. Vascular endothelial cells secrete MMPs through both the luminal and basolateral surfaces. MMP-1[1] and MMP-3,[2] when released from the basolateral surface, could be involved in the separation of endothelial cells from each other, a characteristic of endothelial dysfunction in the early stage of atherosclerosis. In addition, release of MMPs in the late stage of atherosclerosis could be a cause of the disruption of the basement membrane and subsequent rupture of the fibrous cap.

The protein kinase C (PKC) signaling pathway is a major regulator of cellular functions and is implicated in pathologies involving extracellular matrix remodeling. PKC-α is reported to be required for NF-κB activation in MMP expression of epithelial cells.[3] In other systems, PKC enhances MMP induction by oxLDL and is evoked via one or more mitogen-activated protein kinase (MAPK) pathways, including ERK, JNK, and P38 MAPK.[4] Therefore, PKC can influence multiple signaling pathways involved in the dysregulation of MMP expression.

Peroxisome proliferator-activated receptors (PPARs) comprise a superfamily of nuclear hormone receptor proteins that function as transcription factors. PPAR-γ regulates cellular proliferation and differentiation and is known to play an important role in obesity, diabetes, inflammation, and tumorigenesis. Several papers have indicated that activation of PPAR-γ inhibits the expression of MMPs in smooth muscle cells,[5] leukemia cells,[6] and chondrocytes.[7] Furthermore, the transcriptional activity of PPAR-γ has been shown to be inhibited by MAPK-mediated phosphorylation.[8]

It is well established that dietary polyphenolic compounds play significant roles in the prevention of atherosclerosis and cardiovascular diseases. Polyphenolic compounds affect the development of atherosclerosis not only through modulation of serum lipids but also by influencing the immune and inflammatory processes associated with the development of this disease. Ellagic acid (Figure 1), a phenolic compound present in berries and nuts, has been found to have antioxidative properties and to inhibit LDL oxidation.[9] Ellagic acid effectively lowers the levels of plasma lipids, reduces oxidative stress, and inhibits apoptosis in hyperlipidemic rabbits,[10] inhibits oxLDL-induced aortic smooth muscle cell proliferation,[11] and inhibits cytokine-induced adhesion molecule expression in endothelial cells.[12] In addition, we recently found that ellagic acid protects against oxLDL-induced endothelial dysfunction via modulation of LOX-1 expression and the PI3K/Akt/eNOS signaling pathway.[13,14] Losso et al.[15] suggested that inhibition of cancer cell proliferation by ellagic acid could be mediated by regulation of MMPs. Huang et al.[16] reported that ellagic acid exerts antiangiogenic effects by inhibiting the secretion of MMP protein in HUVECs, suggesting that ellagic acid may play a role in stabilizing atherosclerotic plaques. Ellagic acid has been shown to activate PPAR-γ.[17] Furthermore, activated MEK has been shown to suppress the transcriptional activity of PPAR-γ.[8] Therefore, we sought to investigate whether ellagic acid could suppress the oxLDL-induced expression of MMP-1 and MMP-3 in endothelial cells and whether the PKC/ERK/PPAR-γ/NF-κB signaling pathway is involved in mediating the process.

MATERIALS AND METHODS

Reagents. Fetal bovine serum, M199, and trypsin−EDTA were obtained from Gibco (Grand Island, NY); low serum growth supplement (Cascade, OR), ellagic acid, 2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxy)methyl ester (BAPTA-AM), diphenyleneiodonium chloride (DPI), Gö6976, PD98059, U0126, SB203580, wortmannin, Ly294002, penicillin, and streptomycin were obtained from Sigma (St. Louis, MO); anti-MMP-1 and anti-MMP-3 were obtained from R&D Systems (Minneapolis, MN); anti-PKCα, anti-NF-κBp65, and anti-IκBα were obtained from Cell Signaling (Beverly, MA), anti-PPAR-γ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PCNA was obtained from Transduction Laboratories (San Jose, CA); anti-ERK and anti-phospho-ERK were obtained from BD Biosciences (Franklin Lakes, NJ); and anti-flotilline-1 was obtained from Millipore (Bedford, MA). The PKC-α activity assay kit was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell Cultures. This experiment was approved by the Research Ethics Committee of the China Medical University Hospital. After receiving written consent from the parents, fresh human umbilical cords were obtained from normal full-term neonates shortly after birth and suspended in Hanks' balanced salt solution (HBSS) (Gibco) at 4 °C. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords with collagenase and used at passage 2−3 as previously described.[18] After dissociation, the cells were collected and cultured on gelatin-coated culture dishes in medium 199 with low serum growth supplement, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Subcultures were performed with trypsin−EDTA. Media were refreshed every 2 days. The identity of umbilical vein endothelial cells was confirmed by their cobblestone morphology and strong positive immunoreactivity to von Willebrand factor.

Lipoprotein Separation. Human plasma was obtained from the Taichung Blood Bank (Taichung, Taiwan), and LDL was isolated using sequential ultracentrifugation (p = 1.019−1.063 g/mL) in KBr solution containing 30 mM EDTA, stored at 4 °C in a sterile, dark environment, and used within 3 days as previously described.[14] Immediately before the 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) in 0.01 mol/L phosphate-buffered saline (136.9 mmol/L NaCl, 2.68 mmol/L KCl, 4 mmol/L Na_2HPO_4, 1.76 mmol/L KH_2PO_4) at pH 7.4. Copper-modified LDL (1 mg of protein/mL) was prepared by exposing LDL to 10 μM $CuSO_4$ for 16 h at 37 °C. After oxidation, the amount of thiobarbituric acid reactive substances (TBARS) in LDL ranged from 15 to 20 nmol/mg LDL.

Immunoblotting. HUVECs were grown to confluence, pretreated with ellagic acid for 2 h, and then stimulated with oxLDL for indicated

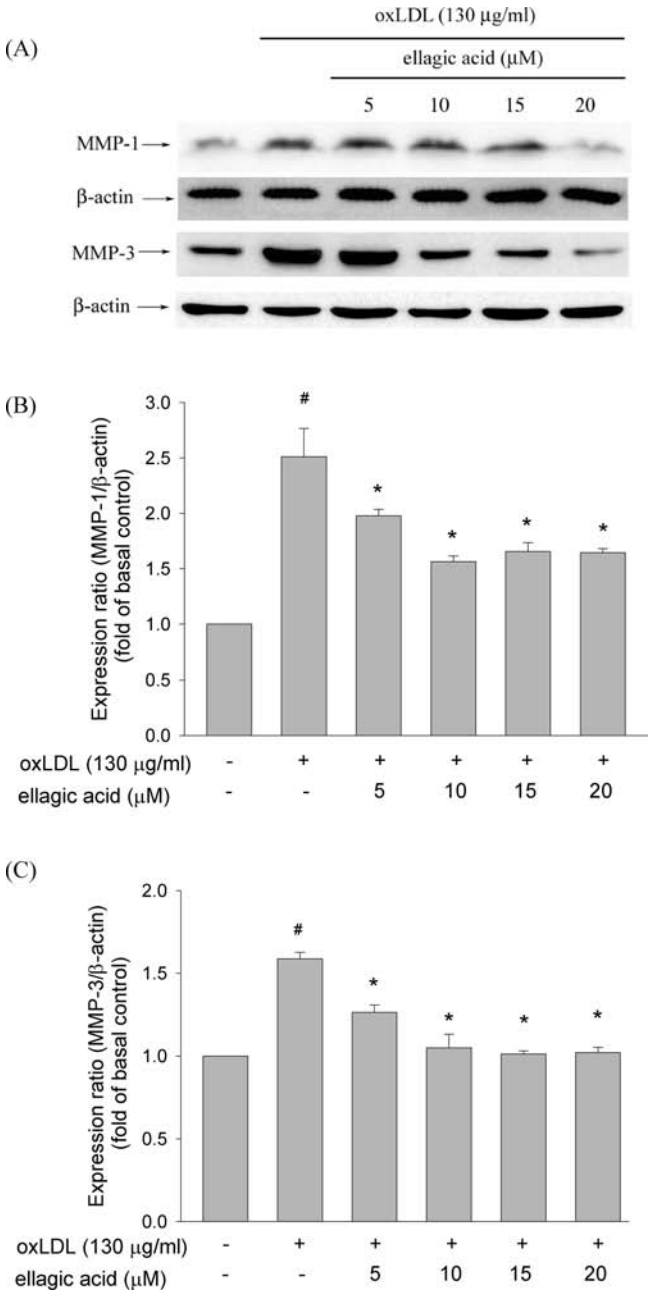

Figure 2. Effect of ellagic acid on oxLDL-induced MMP-1 and MMP-3 expressions. HUVECs were pretreated with ellagic acid (5−20 μM) for 2 h followed by exposure to oxLDL (130 μg/mL) for a further 24 h. At the end of the incubation period, cells were lysed and proteins were analyzed by Western blot. Protein levels of MMP-1 and MMP-3 were normalized to the level of β-actin. Data illustrated on the graph bars represent the mean ± SEM of three different experiments. #, $P < 0.05$ versus untreated control; ∗, $P < 0.05$ versus oxLDL treatment.

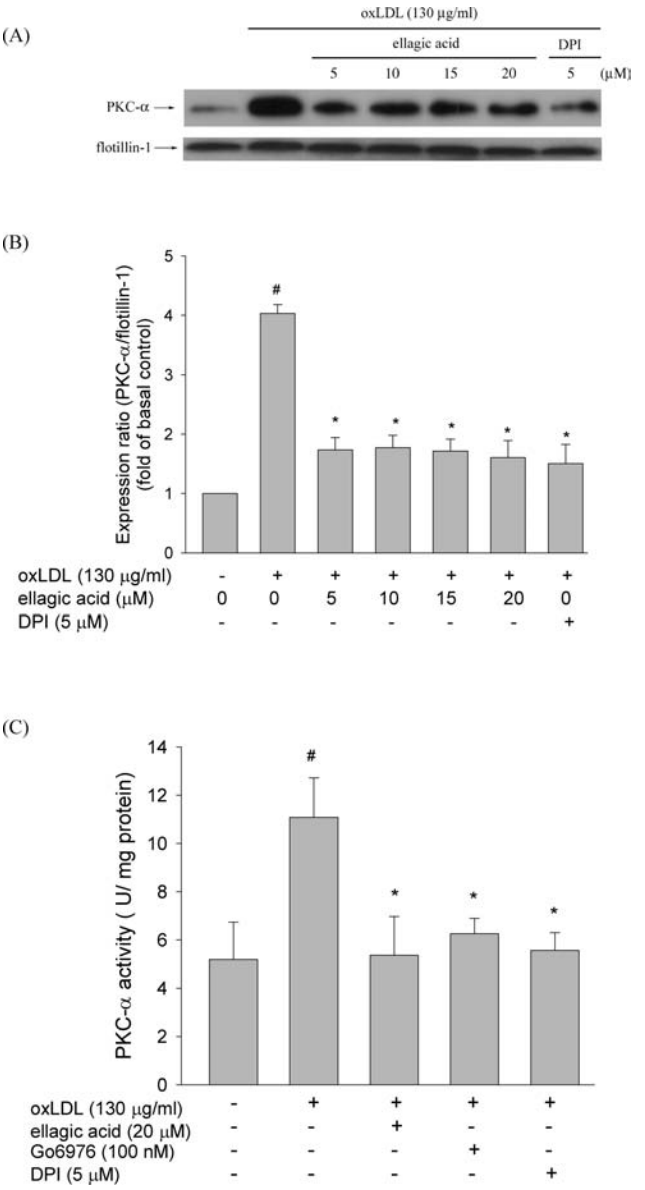

Figure 3. Effect of ellagic acid on oxLDL-induced PKC-α activation. HUVECs were pretreated with ellagic acid (5−20 μM) or DPI (5 μM) for 2 h followed by exposure to oxLDL (130 μg/mL) for a further 1 h. (A,B) Preparation of membrane and cytosolic proteins is described under Materials and Methods. The levels of membrane protein were normalized to the levels of flotillin-1. Representative Western blots and summary data show that ellagic acid protected against oxLDL-induced PKC-α translocation to the plasma membrane. (C) PKC-α activity in whole-cell lysates was measured by a fluorescein green assay kit. The values represent the mean ± SEM of three separate experiments. #, $P < 0.05$ versus untreated control; ∗, $P < 0.05$ versus oxLDL treatment.

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, IL). Cytosolic MMP-1, MMP-3, ERK, phospho-ERK, IκBα, membrane translocation of PKC-α, and nuclear translocation of NF-κBp65 were determined by SDS-PAGE and immunoblot assay. The blots were incubated with blocking buffer (1× PBS and 5% nonfat dry milk) for 1 h 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 h. To control equal loading of total protein in all lanes, blots were stained with mouse anti-β-actin antibody (1:50000 dilution) for cytosolic fractions, mouse anti-flotillin-1 (1:1000) for plasma membrane fractions, and anti-PCNA (1:1000) for nuclear fractions. The bound immunoproteins were detected by an enhanced chemiluminescent assay (ECL; Amersham, Berkshire, U.K.). The intensities were quantified by

densitometric analysis (Digital Protein DNA Imageware, Huntington Station, NY).

Protein Kinase C-α Assay. HUVECs were grown to confluence, pretreated with ellagic acid for 2 h, and then stimulated with oxLDL for an additional 1 h. At the end of the incubation period, cells were rinsed with ice-cold PBS and lysed by the addition of reaction buffer (50 mM HEPES, pH 7.2, 0.01% BSA, 10 mM $MgCl_2$, 1 mM DTT, and 1× lipid activator, provided in the kit). Protein kinase C-α activity in whole-cell lysate (10 μg) was measured with a PKC-α activity assay kit (nonradioactive) according to the manufacturer's instructions (Upstate Biotechnology).

Transfection with Small Interfering RNA (siRNA). On-target Plus SMART pool siRNAs for nontargeting control and PPAR-γ (NM_005037) were purchased from Dharmacon Research. Transient transfection was carried out using INTERFERin siRNA transfection reagent (Polyplus Transfection) according to the manufacturer's instructions. Two days after transfection, cells were treated with reagent as indicated for further experiments.

Statistical Analyses. Results are expressed as the mean ± SEM. Differences between groups were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. A P value of <0.05 was considered to represent statistical significance.

RESULTS

Ellagic Acid Inhibited the Expression of MMP-1 and MMP-3. In a recent study from our laboratory, we found that ellagic acid protects against oxLDL-induced endothelial dysfunction at concentrations ranging from 5 to 20 μM and that the phenolic compound is noncytotoxic at concentrations up to 100 μM.[14] In the present study, HUVECs were pretreated with indicated concentrations of ellagic acid (5−20 μM) for 2 h and then incubated with oxLDL (130 μg/mL) for a further 24 h. The protein levels of MMP-1 and MMP-3 were detected by Western blot. As shown in Figure 2, after a 24 h exposure to oxLDL, there was a 2.5-fold increase in MMP-1 expression and a 1.6-fold increase in MMP-3 expression; however, pretreatment of cells with ellagic acid at concentrations of >5 μM attenuated the expression of both metalloproteinases (all $P < 0.05$).

Ellagic Acid Inhibited the Expression of PKC-α Our most recent investigation demonstrated that ellagic acid protected against oxLDL-induced apoptosis by inhibiting the generation of reactive oxygen species (ROS), the earliest apoptotic signal in oxLDL-stimulated endothelial cells.[13] In addition, Wu et al. reported that ROS are capable of oxidizing molecules involved in the expression of MMP, such as PKC.[19] We, therefore, attempted to determine whether ellagic acid modulates PKC activation in HUVECs after exposure to oxLDL. As shown in Figure 3A,B, after a 1 h exposure to oxLDL, there was a 3.9-fold increase in the expression of PKC-α, but not in the expression of PKC-β, in the membrane fraction (data not shown); however, this increase in PKC-α expression was markedly reversed in HUVECs that had been pretreated with ellagic acid at concentrations of >5 μM (all $P < 0.05$). In addition, DPI, a well-known inhibitor of ROS, markedly antagonized the oxLDL-induced activation of PKC-α, suggesting that ellagic acid suppresses the oxLDL-induced activation of PCK-α by inhibiting the generation of ROS.

To confirm the effect of ellagic acid on oxLDL-induced PKC-α activation, HUVECs were pretreated with indicated concentrations of ellagic acid (5−20 μM) for 2 h and then incubated with oxLDL (130 μg/mL) for an additional 1 h. PKC-α activity was then measured in whole-cell lysates (Figure 3C). We found that oxLDL treatment resulted in a 2.1-fold increase in PKC-α activity relative to control cells ($n = 3$; $P < 0.05$). No increase in PKC-α activity, however, was seen in HUVECs that had been pretreated with ellagic acid, PKC-α/β inhibitor (Gö6976), or ROS inhibitor (DPI).

Ellagic Acid Inhibited OxLDL-Induced ERK/NF-κB Activation. Studies have shown that the expression of MMPs is regulated by MAPK[20] and PI3K/Akt,[21] that oxLDL increases the level of phosphorylation of MAPK in endothelial cells,[22] and that PKC-α activation, which occurs upstream of ERK activation, is involved in MMP expression.[23] Therefore, we investigated whether MAPK or PI3K activation is involved in oxLDL-induced expression of MMP-1 and MMP-3. After pretreatment with the ERK inhibitor PD98059, the P38 inhibitor SB203580, the MEK inhibitor U0126, the PI3K inhibitor wortmannin, and the Akt inhibitor LY294002 for 2 h, HUVECs were treated with oxLDL for another 24 h. We found that PD98059 as well as U0126, but not SB203580, and wortmannin as well as Ly294002 led to a marked reduction in levels of oxLDL-induced MMP-1 and MMP-3 expression, suggesting that oxLDL-induced MMP-1 and MMP-3 expression occurs via the MEK/ERK pathway and not via the P38 MAPK or PI3K/Akt signaling pathway (Figure 4A,B).

Next, we wanted to confirm whether ellagic acid inhibits oxLDL-induced expression of MMP-1 and MMP-3 by blocking ERK phosphorylation. Thus, HUVECs were pretreated with ellagic acid for 2 h followed by incubation with oxLDL for another 1 h. Phosphorylation of ERK in cytosolic fractions was determined by Western blot. As expected, we found that the level of oxLDL-induced ERK phosphorylation in cells exposed to 20 μM ellagic acid was similar to that seen in untreated control cells (Figure 4C,D).

Tung et al. reported that high levels of ROS play an essential role in the up-regulation of MMPs through the MAPK/NF-κB pathway.[24] To evaluate whether upstream regulators of NF-κB are involved in the protective effects of ellagic acid against oxLDL-induced expression of MMPs, we incubated HUVECs with pharmacologic inhibitors of ROS (DPI), calcium (BAPTA), and PKC-α/β (Gö6976). As shown in Figure 5, pretreatment of HUVECs with DPI, BAPTA, Gö6976, and ellagic acid significantly diminished the level of oxLDL-induced ERK phosphorylation and NF-κB activation, a downstream molecule of ERK phosphorylation. Collectively, these results strongly suggest that ROS generation and the calcium-dependent PKC-mediated ERK signaling pathway might be involved in the suppression of oxLDL-induced NF-κB activation by ellagic acid.

Effect of Ellagic Acid on PPAR-γ Expression. The MARK/ERK1/2 cascade plays a central role in intracellular signaling in response to extracellular stimuli. Studies have shown that one of the targets of the ERK cascade is PPAR-γ, a nuclear receptor that promotes differentiation and apoptosis[25] and down-regulates the expression of MMPs.[6] However, studies have also shown that activated MEK suppresses the transcriptional activity of PPAR-γ.[8] We, therefore, attempted to determine whether PPAR-γ is involved in the protective effects of ellagic acid against oxLDL-induced expression of MMP-1 and MMP-3. As shown in Figure 6A, B, treatment with oxLDL for 24 h led to a marked decline in protein expression of PPAR-γ. In contrast, pretreatment with ellagic acid resulted in a dose-dependent increase in the level of PPAR-γ (all $P < 0.05$).

Knockdown of PPAR-γ Using siRNA Antagonized the Suppression of OxLDL-Induced MMP-1 and MMP-3 Expressions by Ellagic Acid. To further investigate whether PPAR-γ is

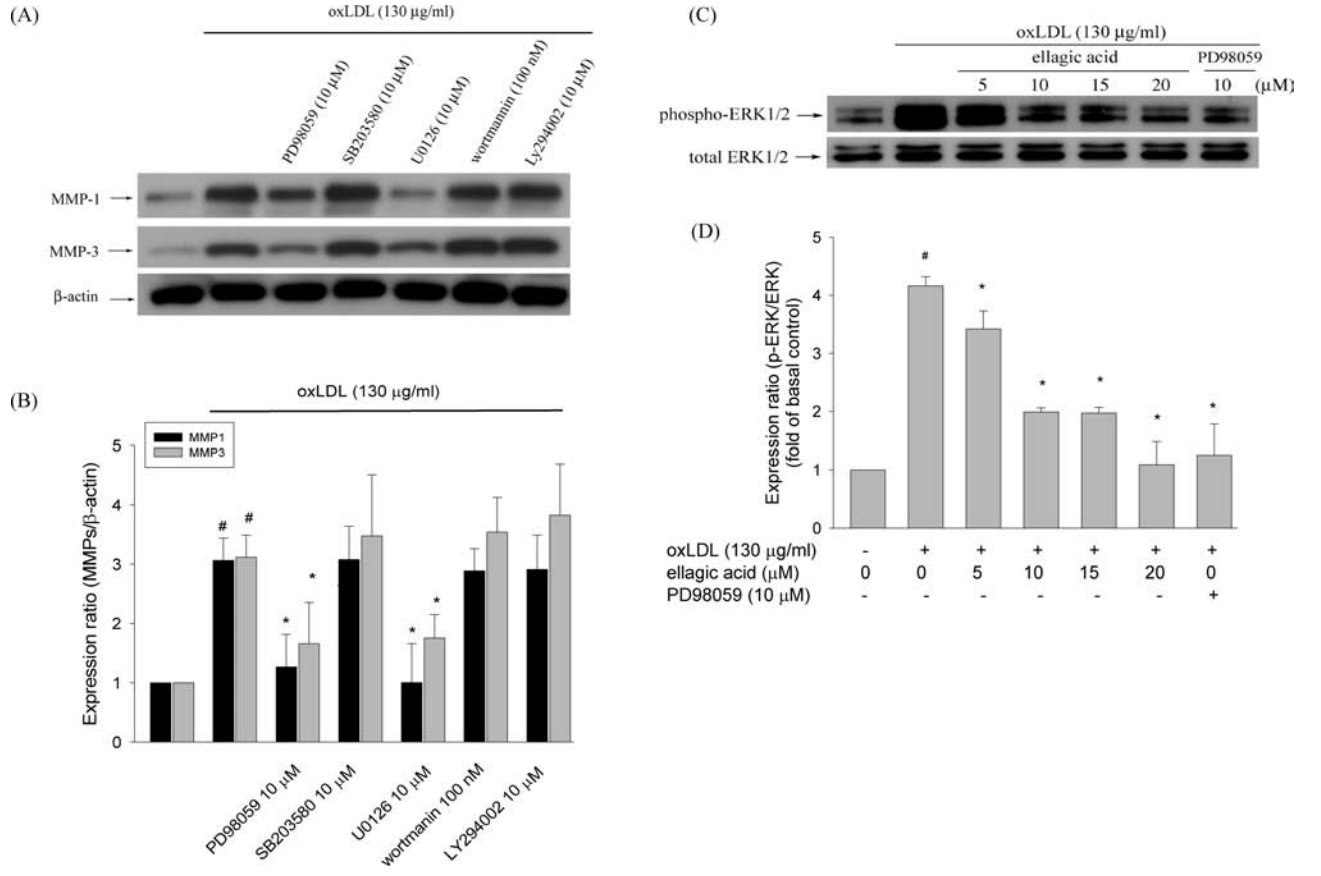

Figure 4. Activation of ERK by oxLDL in HUVECs. Treatment with PD98059 and U0126, but not SB203580, and wortmannin as well as Ly294002 attenuated the expressions of MMP-1 and MMP-3 in oxLDL-treated HUVECs. HUVECs were pretreated with each inhibitor 1 h prior to ellagic acid treatment followed by incubation with oxLDL (130 μg/mL) for 24 h (A,B) or 1 h (C). At the end of the incubation period, cells were lysed and proteins were analyzed by Western blot. Protein levels of MMP-1, MMP-3, and phosphor-ERK were normalized to the level of β-actin and total ERK, respectively. The data illustrated on the graph bars represent the mean $\pm$ SEM of three different experiments. #, $P < 0.05$ versus untreated control; $*$, $P < 0.05$ versus oxLDL treatment.

involved in the effects of ellagic acid on suppression of MMP-1 and MMP-3 expression in HUVECs exposed to oxLDL, we used a PPAR-γ siRNA and examined the changes in levels of MMP-1 and MMP-3 expression. Our results showed that PPAR-γ siRNA significantly antagonized the effect of ellagic acid on the suppression of MMP-1 and MMP-3 expression in HUVECs exposed to oxLDL (Figure 6C,D).

DISCUSSION

Several studies have reported that MMP-1 and MMP-3 play important roles in the destabilization of atherosclerotic plaques.[1] Our previous studies revealed that ellagic acid exerts its protective effects against oxLDL-induced endothelial dysfunction via modulating LOX-1[13] and the Akt/eNOS/NO signaling pathway.[14] However, the signaling pathways through which ellagic acid regulates the expression of MMP-1 and MMP-3 are poorly understood. In this study, we found that ellagic acid protects against oxLDL-induced MMP-1 and MMP-3 expression by modulating the ROS-mediated PKC-α/ERK/NF-κB signaling pathway. In addition, our finding that the suppressive effects of ellagic acid on oxLDL-induced expression of MMPs were abolished in cells exposed to PPAR-γ siRNA extend the understanding of the mechanisms involved in PPAR-γ activation. Furthermore, our finding that DPI attenuated PKC-α/ERK/NF-κB activation in HUVECs exposed to oxLDL might imply that inhibition of oxLDL-induced ROS generation plays a critical role in the protective effect of ellagic acid (Figure 7).

ROS generated in endothelial cells include superoxide ($^{\bullet}O_2$), hydrogen peroxide (H_2O_2), peroxynitrite ($^{\bullet}ONOO$), NO, and hydroxyl ($^{\bullet}OH$) radicals. It is generally recognized that NADPH oxidase-derived superoxides are predominant sources of ROS in the vasculature. One recent investigation from our laboratory demonstrated that the membrane assembly of gp91, $p22^{phox}$, $p47^{phox}$, and Rac-1 after oxLDL exposure was reduced in cells pretreated with ellagic acid.[13] We, therefore, assume 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. ROS can act as a second messenger in the regulation of diverse cellular processes by oxidizing cysteine residues on critical target molecules including kinases, phosphatases, redox-sensitive transcription factors, cell cycle regulators, and cell membrane lipids. PKC, a member of the serine/threonine kinase family, regulates a variety of cell functions including proliferation, cell cycle, differentiation, cytoskeletal organization, cell migration, and apoptosis. PKC contains multiple cysteine residues that can be oxidized and activated by ROS. On the contrary, PKC activation is required for ROS generation, suggesting that ROS can be either upstream or downstream of PKC. Several studies

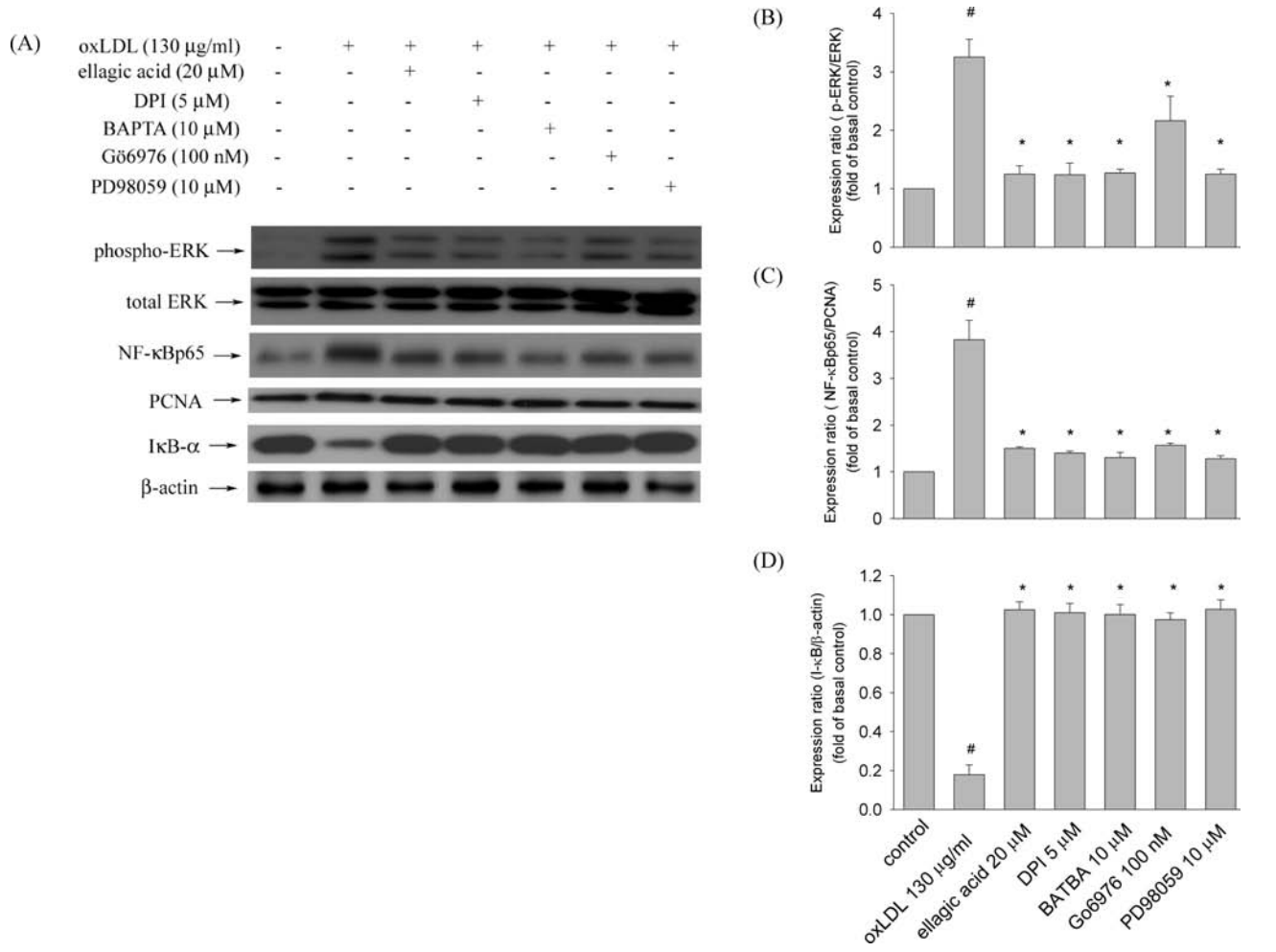

Figure 5. OxLDL-induced activation of ERK and NF-κB in the presence of pharmacologic inhibitors or ellagic acid. Pretreatment of HUVECs with indicated concentrations of DPI, BAPTA, Gö6976, PD98059, or ellagic acid attenuated ERK activation, IκB-α degradation, and NF-κB activation caused by oxLDL. HUVECs were pretreated with each inhibitor or ellagic acid for 2 h, followed by incubation with oxLDL (130 μg/mL) for 1 h. At the end of the incubation period, cells were lysed and proteins were analyzed by Western blot. Protein levels of phosphor-ERK, NF-κB, and IκB-α were normalized to the level of total ERK, PCNA, and β-actin, respectively. Data illustrated on the graph bars represent the mean $\pm$ SEM of three separate experiments. #, $P < 0.05$ versus untreated control; $*$, $P < 0.05$ versus oxLDL treatment.

have shown that ROS-mediated PKC activation might be an upstream event involved in MMP expression.[4] In this study, we hypothesized that ellagic acid exerts its antiatherogenic effect by blockading the ROS-mediated signaling pathway. Consistent with previous findings that the PKC-α signaling pathway is involved in the expression of MMP-1 and MMP-3,[26] we found that ellagic acid attenuated the oxLDL-induced expression of MMP-1 and MMP-3 by inhibiting the generation of ROS and the subsequent activation of PKC-α. This observation was further confirmed by the observations that pretreatment of HUVECs with DPI, a ROS inhibitor, attenuated the ox-LDL-induced activation of PKC-α.

MAPKs including ERK, JNK, and P38 are important downstream signaling molecules involved in the expression of MMPs. The MAPK signaling pathway is the major signaling cascade involved in cell migration in diverse systems. Previous studies have shown that the MAPK and PI3K/Akt signaling pathways are involved in MMP activation when endothelial cells are treated with visfatin,[27] fibronectin, or vitronectin.[28] However, we recently demonstrated that ellagic acid attenuated the dephosphorylation of Akt, a key signaling pathway involved in endothelial NO synthase expression, in cells exposed to oxLDL,[14] a finding consistent with that reported by Li and Renier.[28] Confirmatory evidence of the role played by ERK came from our experiments in which specific inhibitors of MEK and ERK (U0126 and PD98059, respectively) but not inhibitors of PI3K or Akt (wortmannin and LY294002, respectively) effectively suppressed the expression of MMPs in endothelial cells after exposure to oxLDL (Figure 4A,B). Thus, the signaling pathways involved in MMP expression seem to be cell type specific and depend on the type of stimuli to which cells are exposed. Our findings indicate that ellagic acid diminishes oxLDL-induced expression of MMPs by modulating the ERK but not the PI3K/Akt signaling pathway. We further demonstrated that the use of inhibitors of ROS (DPI), intracellular calcium (BAPTA), and PKC-α/β (Gö6976) inhibited the activation of NF-κB (Figure 5). These observations suggest that the reduction in MMP-1 and MMP-3 expression by ellagic acid may be associated with the inhibition of ROS-mediated calcium-dependent PKC activation and the subsequent phosphorylation of ERK and activation of NF-κB.

OxLDL has been reported to up-regulate various MMPs in endothelial cells, such as membrane type 1-MMP (MT1-MMP),[29]

dx.doi.org/10.1021/jf1041867 |J. Agric. Food Chem. 2011, 59, 5100–5108

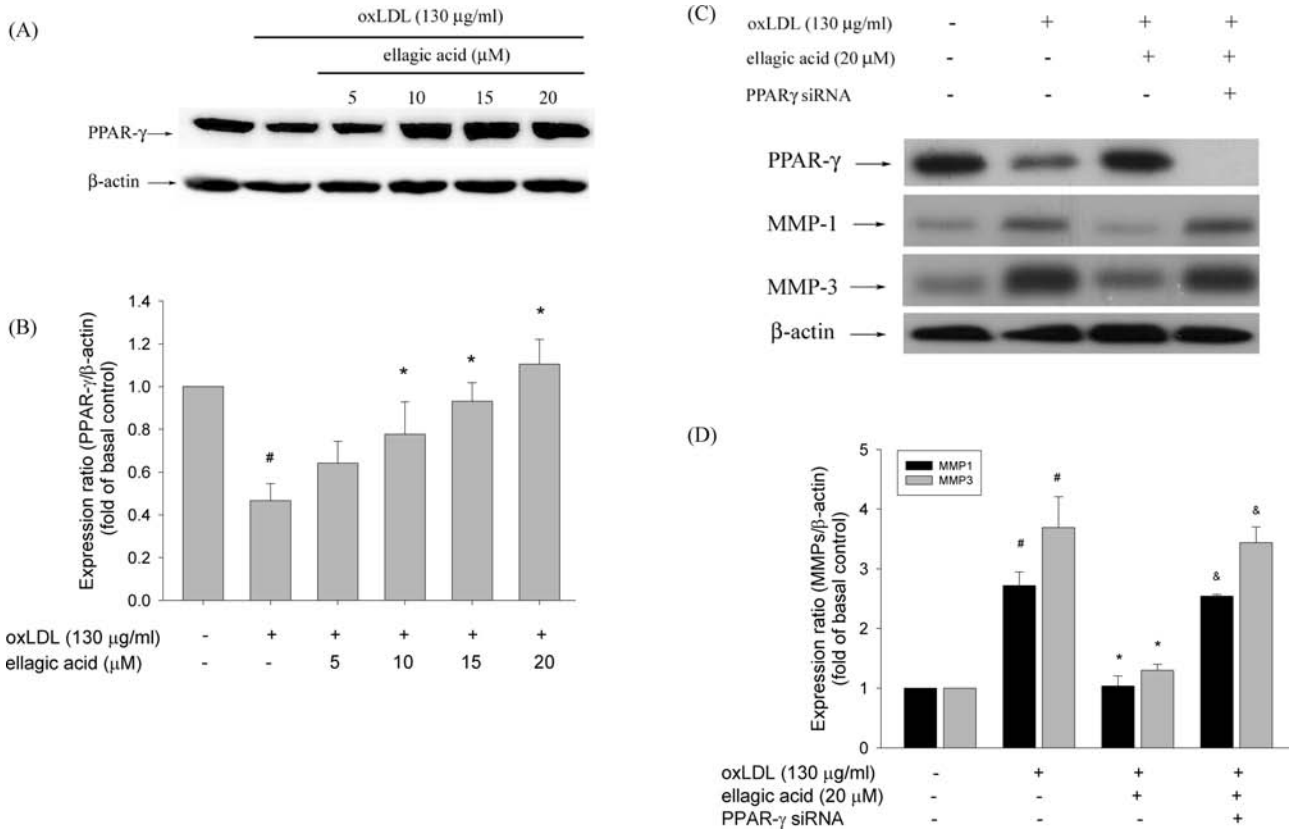

Figure 6. Ellagic acid reversed the oxLDL-diminished PPAR-γ expression, and inhibition of PPAR-γ with siRNA antagonized the effects of ellagic acid on oxLDL-induced MMP-1 and MMP-3 expressions. (A,B) Western blot showing PPAR-γ protein levels in HUVECs treated with oxLDL (130 μg/mL) for 24 h in the absence and presence of indicated concentrations of ellagic acid. (C) HUVECs transfected with PPAR-γ siRNA for 48 h and then treated with 20 μM ellagic acid for 1 h followed by exposure to 130 μg/mL oxLDL for 24 h. The cell lysates were analyzed by Western blot using anti-PPAR-γ, anti-MMP-1, MMP-3, or anti-β-actin antibody. Values represent the mean ± SEM from four separate experiments. #, $P < 0.05$ versus untreated control; ∗, $P < 0.05$ versus oxLDL treatment; &, $P < 0.05$ versus ellagic acid plus oxLDL treatment.

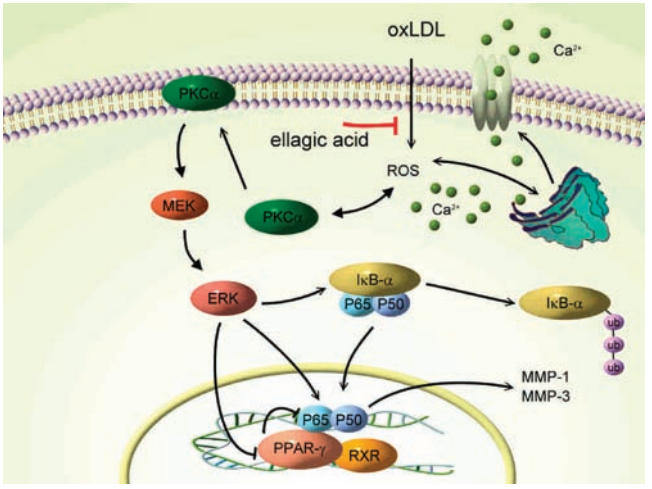

Figure 7. Schematic diagram showing the signaling cascades involved in the attenuation of MMP-1 and MMP-3 expressions in oxLDL-exposed cells treated with ellagic acid. As depicted, ellagic acid inhibited the signaling cascades initiated by oxLDL-generated ROS. → indicates activation or induction, and —| indicates inhibition or blockade.

MMP-1 and MMP-3,[30] and MMP-9.[31] Although previous studies have shown that ellagic acid inhibits MMP-2 activity in endothelial cells[16] and in ethanol-exposed hepatic cells,[32] this study is the first to demonstrate that ellagic acid inhibits oxLDL-stimulated MMP-1 and MMP-3 expression. Whether ellagic acid inhibits other types of MMPs in endothelial cells after exposure to oxLDL needs to be investigated. Recently, Hua et al. reported that aspirin inhibits the expression of MMPs in macrophages exposed to oxLDL through up-regulation of PPAR-γ, a nuclear receptor that promotes differentiation and anti-inflammation.[33] ERK has been reported to be involved in the suppression of transcriptional activity of PPAR-γ via phosphorylation in adipocytes.[8] In addition, Chung et al. have shown that PPAR-γ inhibits NF-κB-driven transcription by physically interacting with p50 and p65 proteins.[34] Moreover, Liu et al. demonstrated that PPAR-γ down-regulates the expression of MMP-9 and MMP-2.[6] Consistent with a recent finding that ellagic acid up-regulates PPAR-γ expression,[17] we found that the suppression of PPAR-γ caused by oxLDL was reversed in the presence of ellagic acid (Figure 6A,B). In an attempt to further illustrate whether PPAR-γ is involved in the effects of ellagic acid on inhibition of oxLDL-stimulated MMP-1 and MMP-3 expression in endothelial cells, knockdown of PPAR-γ via PPAR-γ siRNA was employed. We found that the suppressive effects of ellagic acid on oxLDL-induced expression of MMP-1 and MMP-3 were attenuated in cells transfected with PPAR-γ siRNA (Figure 6C,D). Our findings indicate that ellagic acid suppresses the oxLDL-induced expression of MMP-1 and MMP-3 by inhibiting the generation of ROS and subsequently

the activation of the calcium-dependent PKC-α and ERK/PPAR-γ/NF-κB signaling pathway.

Ellagic acid is found in fruits and fruit-derived beverages including blueberries (0.9 mg/100 g), blackberries (42.4 mg/100 g), raspberries (17.9 mg/100 g), strawberries (19.8 mg/100 g), grape juice (10.2 mg/100 g), and grape wine (5.6 mg/100 g).[35] The typical dietary intake of ellagic acid in humans is approximately 40−80 mg/day if 200 g of strawberries or blackberries is eaten.[35] Mertens-Talcott et al. reported that the peak plasma concentration of ellagic acid (C_{max}) 1 h after consumption of 800 mg of pomegranate extract, which contained 21.6 mg of ellagic acid, was 22.8 ng/mL, which is equivalent to 0.11 μM. This concentration could cause a significant increase of antioxidant capacity of plasma. The concentrations of ellagic acid used in our study (5−10 μM) are, therefore, far and above the minimum physiologically effective dose. In addition, the concentrations used in our study are similar to those that have been reported to inhibit lipopolysaccharide-induced expression of enzymes involved in the synthesis of prostaglandin E2 in human monocytes[36] and inflammation in endothelial cells.[12]

In conclusion, our data suggest that ellagic acid elicits its antiatherogenic effects by modulating the PKC-α/ERK/PPAR-γ/NF-κB pathway, resulting in the suppression of ROS generation and, ultimately, inhibition of MMP-1 and MMP-3 expression in HUVECs exposed to oxLDL. Our findings suggest that ellagic acid is a potential preventive agent against the development of cardiovascular diseases.

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Funding Sources

This study was supported by grants from the National Science Council (NSC 98-2320-B-039-020-MY3), China Medical University (CMU99-S-13), and Veterans General Hospitals University System of Taiwan Joint Research Program Tsou's Foundation (TCVGH 99G201), Taiwan, ROC.

ABBREVIATIONS USED

MMPs, metalloproteinases; oxLDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; NO, nitric oxide; PKC, protein kinase C; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; MAPK, mitogen-activated protein kinase; PPARs, peroxisome proliferator-activated receptors; LOX-1, lectin-like oxidized LDL receptor; EDTA, ethylenediaminetetraacetic acid; BAPTA-AM, 2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis-(acetoxymethyl) ester; DPI, diphenyleneiodonium chloride; PCNA, proliferating cell nuclear antigen; HBSS, Hanks' balanced salt solution; TBARS, thiobarbituric acid reactive substances; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ECL, chemiluminescent; siRNA, small interfering RNA; ROS, reactive oxygen species.

REFERENCES

(1) Libby, P. Molecular bases of the acute coronary syndromes. *Circulation* **1995**, *91* (11), 2844–2850.

(2) Henney, A. M.; Wakeley, P. R.; Davies, M. J.; Foster, K.; Hembry, R.; Murphy, G.; Humphries, S. Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88* (18), 8154–8158.

(3) Shin, Y.; Yoon, S. H.; Choe, E. Y.; Cho, S. H.; Woo, C. H.; Rho, J. Y.; Kim, J. H. PMA-induced up-regulation of MMP-9 is regulated by a PKCα-NF-κB cascade in human lung epithelial cells. *Exp. Mol. Med.* **2007**, *39* (1), 97–105.

(4) Wang, H. H.; Hsieh, H. L.; Wu, C. Y.; Yang, C. M. Oxidized low-density lipoprotein-induced matrix metalloproteinase-9 expression via PKC-δ/p42/p44 MAPK/Elk-1 cascade in brain astrocytes. *Neurotox. Res.* **2010**, *17* (1), 50–65.

(5) Marx, N.; Schonbeck, U.; Lazar, M. A.; Libby, P.; Plutzky, J. Peroxisome proliferator-activated receptor γ activators inhibit gene expression and migration in human vascular smooth muscle cells. *Circ. Res.* **1998**, *83* (11), 1097–1103.

(6) Liu, J.; Lu, H.; Huang, R.; Lin, D.; Wu, X.; Lin, Q.; Zheng, J.; Pan, X.; Peng, J.; Song, Y.; Zhang, M.; Hou, M.; Chen, F. Peroxisome proliferator activated receptor-γ ligands induced cell growth inhibition and its influence on matrix metalloproteinase activity in human myeloid leukemia cells. *Cancer Chemother. Pharmacol.* **2005**, *56* (4), 400–408.

(7) Francois, M.; Richette, P.; Tsagris, L.; Raymondjean, M.; Fulchignoni-Lataud, M. C.; Forest, C.; Savouret, J. F.; Corvol, M. T. Peroxisome proliferator-activated receptor-γ down-regulates chondrocyte matrix metalloproteinase-1 via a novel composite element. *J. Biol. Chem.* **2004**, *279* (27), 28411–28418.

(8) Hu, E.; Kim, J. B.; Sarraf, P.; Spiegelman, B. M. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. *Science* **1996**, *274* (5295), 2100–2103.

(9) Priyadarsini, K. I.; Khopde, S. M.; Kumar, S. S.; Mohan, H. Free radical studies of ellagic acid, a natural phenolic antioxidant. *J. Agric. Food Chem.* **2002**, *50* (7), 2200–2206.

(10) Yu, Y. M.; Chang, W. C.; Wu, C. H.; Chiang, S. Y. Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid. *J. Nutr. Biochem.* **2005**, *16* (11), 675–681.

(11) Chang, W. C.; Yu, Y. M.; Chiang, S. Y.; Tseng, C. Y. Ellagic acid suppresses oxidised low-density lipoprotein-induced aortic smooth muscle cell proliferation: studies on the activation of extracellular signal-regulated kinase 1/2 and proliferating cell nuclear antigen expression. *Br. J. Nutr.* **2008**, *99* (4), 709–714.

(12) Papoutsi, Z.; Kassi, E.; Chinou, I.; Halabalaki, M.; Skaltsounis, L. A.; Moutsatsou, P. Walnut extract (*Juglans regia* L.) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483. *Br. J. Nutr.* **2008**, *99* (4), 715–722.

(13) Lee, W. J.; Ou, H. C.; Hsu, W. C.; Chou, M. M.; Tseng, J. J.; Hsu, S. L.; Tsai, K. L.; Sheu, W. H. Ellagic acid inhibits oxidized LDL-mediated LOX-1 expression, ROS generation, and inflammation in human endothelial cells. *J. Vasc. Surg.* **2010**, *52* (5), 1290–1300.

(14) Ou, H. C.; Lee, W. J.; Lee, S. D.; Huang, C. Y.; Chiu, T. H.; Tsai, K. L.; Hsu, W. C.; Sheu, W. H. Ellagic acid protects endothelial cells from oxidized low-density lipoprotein-induced apoptosis by modulating the PI3K/Akt/eNOS pathway. *Toxicol. Appl. Pharmacol.* **2010**, *248* (2), 134–143.

(15) Losso, J. N.; Bansode, R. R.; Trappey, A., 2nd; Bawadi, H. A.; Truax, R. In vitro anti-proliferative activities of ellagic acid. *J. Nutr. Biochem.* **2004**, *15* (11), 672–678.

(16) Huang, S. T.; Wang, C. Y.; Yang, R. C.; Wu, H. T.; Yang, S. H.; Cheng, Y. C.; Pang, J. H. S. Ellagic acid, the active compound of phyllanthus urinaria, exerts in vivo anti-angiogenic effect and inhibits MMP-2 activity. *eCAM* **2011**, DOI: 10.1093/ecam/nep207.

(17) Khateeb, J.; Gantman, A.; Kreitenberg, A. J.; Aviram, M.; Fuhrman, B. Paraoxonase 1 (PON1) expression in hepatocytes is upregulated by

pomegranate polyphenols: a role for PPAR-γ pathway. *Atherosclerosis* **2010**, *208* (1), 119–125.

(18) Kuo, W. W.; Huang, C. Y.; Chung, J. G.; Yang, S. F.; Tsai, K. L.; Chiu, T. H.; Lee, S. D.; Ou, H. C. Crude extracts of *Solanum lyratum* protect endothelial cells against oxidized low-density lipoprotein-induced injury by direct antioxidant action. *J. Vasc. Surg.* **2009**, *50* (4), 849–860.

(19) Wu, W. S. The signaling mechanism of ROS in tumor progression. *Cancer Metastasis Rev.* **2006**, *25* (4), 695–705.

(20) Kang, J. H.; Kim, J. K.; Park, W. H.; Park, K. K.; Lee, T. S.; Magae, J.; Nakajima, H.; Kim, C. H.; Chang, Y. C. Ascochlorin suppresses oxLDL-induced MMP-9 expression by inhibiting the MEK/ERK signaling pathway in human THP-1 macrophages. *J. Cell Biochem.* **2007**, *102* (2), 506–514.

(21) Wang, H. H.; Hsieh, H. L.; Wu, C. Y.; Sun, C. C.; Yang, C. M. Oxidized low-density lipoprotein induces matrix metalloproteinase-9 expression via a p42/p44 and JNK-dependent AP-1 pathway in brain astrocytes. *Glia* **2009**, *57* (1), 24–38.

(22) Apostolov, E. O.; Basnakian, A. G.; Yin, X.; Ok, E.; Shah, S. V. Modified LDLs induce proliferation-mediated death of human vascular endothelial cells through MAPK pathway. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *292* (4), H1836–H1846.

(23) Lin, C. W.; Shen, S. C.; Chien, C. C.; Yang, L. Y.; Shia, L. T.; Chen, Y. C. 12-*O*-Tetradecanoylphorbol-13-acetate-induced invasion/migration of glioblastoma cells through activating PKCα/ERK/NF-κB-dependent MMP-9 expression. *J. Cell Physiol.* **2010**, *225* (2), 472–481.

(24) Tung, W. H.; Tsai, H. W.; Lee, I. T.; Hsieh, H. L.; Chen, W. J.; Chen, Y. L.; Yang, C. M. Japanese encephalitis virus induces matrix metalloproteinase-9 in rat brain astrocytes via NF-κB signalling dependent on MAPKs and reactive oxygen species. *Br. J. Pharmacol.* **2010**, *161* (7), 1566–1583.

(25) Burgermeister, E.; Chuderland, D.; Hanoch, T.; Meyer, M.; Liscovitch, M.; Seger, R. Interaction with MEK causes nuclear export and downregulation of peroxisome proliferator-activated receptor γ. *Mol. Cell. Biol.* **2007**, *27* (3), 803–817.

(26) Reuben, P. M.; Brogley, M. A.; Sun, Y.; Cheung, H. S. Molecular mechanism of the induction of metalloproteinases 1 and 3 in human fibroblasts by basic calcium phosphate crystals. Role of calcium-dependent protein kinase Cα. *J. Biol. Chem.* **2002**, *277* (17), 15190–15198.

(27) Adya, R.; Tan, B. K.; Punn, A.; Chen, J.; Randeva, H. S. Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. *Cardiovasc. Res.* **2008**, *78* (2), 356–365.

(28) Jin, Y. J.; Park, I.; Hong, I. K.; Byun, H. J.; Choi, J.; Kim, Y. M.; Lee, H. Fibronectin and vitronectin induce AP-1-mediated matrix metalloproteinase-9 expression through integrin $\alpha5\beta1$/$\alpha v\beta3$-dependent Akt, ERK and JNK signaling pathways in human umbilical vein endothelial cells. *Cell. Signal.* **2011**, *23* (1), 125–134.

(29) Rajavashisth, T. B.; Liao, J. K.; Galis, Z. S.; Tripathi, S.; Laufs, U.; Tripathi, J.; Chai, N. N.; Xu, X. P.; Jovinge, S.; Shah, P. K.; Libby, P. Inflammatory cytokines and oxidized low density lipoproteins increase endothelial cell expression of membrane type 1-matrix metalloproteinase. *J. Biol. Chem.* **1999**, *274* (17), 11924–11929.

(30) Li, D.; Liu, L.; Chen, H.; Sawamura, T.; Ranganathan, S.; Mehta, J. L. LOX-1 mediates oxidized low-density lipoprotein-induced expression of matrix metalloproteinases in human coronary artery endothelial cells. *Circulation* **2003**, *107* (4), 612–617.

(31) Li, L.; Renier, G. The oral anti-diabetic agent, gliclazide, inhibits oxidized LDL-mediated LOX-1 expression, metalloproteinase-9 secretion and apoptosis in human aortic endothelial cells. *Atherosclerosis* **2009**, *204* (1), 40–46.

(32) Devipriya, N.; Sudheer, A. R.; Srinivasan, M.; Menon, V. P. Effect of ellagic acid, a plant polyphenol, on fibrotic markers (MMPs and TIMPs) during alcohol-induced hepatotoxicity. *Toxicol. Mech. Methods* **2007**, *17* (6), 349–356.

(33) Hua, Y.; Xue, J.; Sun, F.; Zhu, L.; Xie, M. Aspirin inhibits MMP-2 and MMP-9 expressions and activities through upregulation of PPARα/γ and TIMP gene expressions in ox-LDL-stimulated macrophages derived from human monocytes. *Pharmacology* **2009**, *83* (1), 18–25.

(34) Chung, S. W.; Kang, B. Y.; Kim, S. H.; Pak, Y. K.; Cho, D.; Trinchieri, G.; Kim, T. S. Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-γ and nuclear factor-κB. *J. Biol. Chem.* **2000**, *275* (42), 32681–32687.

(35) Mertens-Talcott, S. U.; Talcott, S. T.; Percival, S. S. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *J. Nutr.* **2003**, *133* (8), 2669–2674.

(36) Karlsson, S.; Nanberg, E.; Fjaeraa, C.; Wijkander, J. Ellagic acid inhibits lipopolysaccharide-induced expression of enzymes involved in the synthesis of prostaglandin E2 in human monocytes. *Br. J. Nutr.* **2010**, *103* (8), 1102–1109.