Dietary Ellagic Acid Attenuates Oxidized LDL Uptake and Stimulates Cholesterol Efflux in Murine Macrophages1–3
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Abstract
Foam cell formation is the hallmark of early atherosclerosis. Lipid uptake by scavenger receptors (SR) in macrophages initiates chronic proinflammatory cascades linked to atherosclerosis. It has been reported that the upregulation of cholesterol efflux may be protective in the development of atherosclerosis. Ellagic acid, a polyphenolic compound mostly found in berries, walnuts, and pomegranates, possesses antioxidative, growth-inhibiting and apoptosis-promoting activities in cancer cells. However, the antiatherogenic actions of ellagic acid are not well defined. The current study elucidated oxidized LDL handling of ellagic acid in J774A1 murine macrophages. Noncytotoxic ellagic acid suppressed SR-B1 induction and foam cell formation within 6 h after the stimulation of macrophages with oxidized LDL, confirmed by Oil red O staining of macrophages. Ellagic acid at \( \leq 5 \) \( \mu \)mol/L upregulated PPAR\( \gamma \) and ATP binding cassette transporter-1 in lipid-laden macrophages, all responsible for cholesterol efflux. In addition, 5 \( \mu \)mol/L ellagic acid accelerated expression and transcription of the nuclear receptor of liver X receptor-\( \alpha \), highly implicated in the PPAR signaling. Furthermore, ellagic acid promoted cholesterol efflux in oxidized LDL-induced foam cells. These results provide new information that ellagic acid downregulated macrophage lipid uptake to block foam cell formation of macrophages and boosted cholesterol efflux in lipid-laden foam cells. Therefore, dietary and pharmacological interventions with berries rich in ellagic acid may be promising treatment strategies to interrupt the development of atherosclerosis. J. Nutr. 141: 1931–1937, 2011.

Introduction
Foam cell formation of macrophages is a hallmark and pivotal event in the pathogenesis of atherosclerosis (1). Because oxidized LDL is thought to act as an inflammatory factor in evoking atherosclerosis, therapeutic interventions targeting the arterial wall disorder focus on blocking LDL oxidation. Oxidized LDL particles are recognized by macrophage SR\( \alpha \), SR-A, SR-B CD36 antigen, and macrophage antigen CD68 (2,3). Macrophage SR-B1 is one of the membrane receptors responsible for the internalization of oxidized LDL that promotes cellular accumulation of cholesterol (4). Active components present in oxidized LDL as inflammatory factors include lipid hydroperoxides, oxysterols, and lysophosphatidylcholine, all formed by cellular oxidative mediators (5). LDL undergoes oxidation within lysosomes in macrophages and oxidized LDL cholesterol is degraded to oxysterols (6). It is now well accepted that oxysterols play important roles in the formation of atherosclerotic plaque (7). PPAR\( \gamma \) is expressed at high levels in macrophages as well as foam cells of atherosclerotic lesions (8). PPAR\( \gamma \) upregulates expression of the orphan receptor LXR identified as the nuclear receptor target of the cholesterol metabolites of oxysterols (9,10). Activation of the LXR pathway attenuates various mechanisms underlying atherosclerotic plaque development (9). Thus, transcriptional cascades of PPAR\( \gamma \) and LXRs pathways appear to be important in maintaining cellular cholesterol homeostasis in macrophages. In addition, PPAR\( \gamma \) elevates macrophage ABCA1 levels (11). ABCA1, a membrane transporter abundant in macrophages, mediates efflux of cholesterol and phospholipid to lipid-free apoA1 and apoE to then form nascent HDL (11). ABCA1 plays a crucial role in cholesterol homeostasis and reverse cholesterol transport (12). It is well established that the protective
effect of HDL against atherosclerosis is primarily attributed to its function in reverse cholesterol transports, a process by which excess cell cholesterol is taken up by HDL particles (13).

Ellagic acid is a polyphenol found in numerous plant foods, including berries, nuts, and pomegranates (14). In plants, ellagic acid is present in the form of ellagitannin, which is ellagic acid bound to a sugar molecule (15). As with other polyphenols, ellagic acid has chemoprotective activities, with growth-inhibiting and apoptosis-promoting properties in cancer cells (16,17). Our previous study showed that ellagic acid alleviated skin wrinkles and inflammation associated with chronic UV exposure leading to photoaging (18). In addition, ellagic acid prevented oxidized LDL-induced endothelial dysfunction by attenuating lectin-like oxidized LDL receptor-1–mediated signaling pathway (14). This compound reduced rat aortic smooth muscle cell proliferation induced by oxidized LDL via inactivation of the ERK pathway (19). Interestingly, pomegranate phenolics of punicalagin, punicalin, gallic acid, and ellagic acid diminished atherosclerotic lesions and raised cellular paraoxonase 2 activity in peritoneal macrophages of atherosclerotic apoE-deficient mice and J774A1 macrophages (20).

Considering that ellagic acid exerts antiatherogenic activity, it was hypothesized that ellagic acid improved macrophage cholesterol handling through enhancing the PPARy-LXRα-ABCA1 pathway for the clearance of excess cholesterol loaded by oxidized LDL. To test this hypothesis, this study elucidated that ellagic acid stimulated ABCA1-mediated cholesterol efflux in J774A1 murine macrophages. It was also examined that ellagic acid suppressed SR-B1 induction in the early stage of oxidized LDL uptake. In this study, the antiatherogenic activity of ellagic acid was tested in a lipid-laden macrophage model of arteriosclerosis.

Materials and Methods

Materials. Ellagic acid, DMEM chemicals, fatty acid-BSA, GW9662, pioglitazone hydrochloride, and Oil red O were obtained from Sigma-Aldrich Chemical, as were all other reagents, unless specifically stated elsewhere. FBS and penicillin-streptomycin were purchased from Lonza. SR-B1 antibody was purchased from Santa Cruz biotechnology. PPARy antibody was obtained from Cell Signaling Technology. Rabbit anti-LXRα antibody was obtained from ABR. ABCA1 antibody was purchased from Novus Biologicals. β-Actin antibody was obtained from Sigma Chemicals. HRP-conjugated goat anti-rabbit IgG and donkey anti-goat IgG were supplied from Jackson Immuno Research Laboratories. Ellagic acid was dissolved in DMSO for live culture with cells; its final culture concentration was ≤0.1%.

Preparation and oxidation of human plasma LDL. Human plasma LDL were prepared by a discontinuous density gradient ultra-centrifugation as previously described (21). A pooled human normolipidemic plasma LDL fraction was dialyzed and used within 4 wk. Protein concentration of the plasma LDL fraction was determined by the method (22) and the concentrations of TG and total cholesterol were measured using commercial diagnostic kits (ASAN SET TG-S and ASAN SET Total Cholesterol, Asan Pharmaceuticals).

Oxidized LDL were prepared by incubating with 10 μmol/L CuSO4 (Cu²⁺) in F-10 medium at 37°C for 24 h. The extent of LDL oxidative modification was regularly determined using TBARS measurements and the electrophoretic mobility assay (23).

Cell culture and cytotoxicity. Macrophage-like cell line J774A1 (mouse histiocytic lymphoma cells) were grown in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO₂ in air. Macrophages were pretreated with 1–10 μmol/L ellagic acid and exposed to 50 mg/L cholesterol-oxidized LDL for various times. For lipid uptake, cells were incubated in DMEM supplemented with 0.4% fatty acid-free BSA.

After incubation of J774A1 macrophages with Cu²⁺-oxidized LDL in the absence and presence of ellagic acid, 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium bromide (DUCHEFA Biochemie) assay was carried out to quantify cellular viability (21).

Lipid uptake. Oil red O staining was performed to identify foam cell formation of macrophages. Oil Red O is a fat-soluble diazo dye used for staining neutral TG and lipids and some lipoproteins (24). After culture of J774A1 cells with 50 mg/L oxidized LDL, cells were treated with 0.5% Oil Red O dissolved in 60% 2-propanol. After mounting, images were obtained by using an optical microscope. Oil Red O staining was quantified by incubating cells in 0.1 mol/L NaOH solution with 1% SDS.

Western-blot analysis. Western-blot analysis was performed using whole cell extracts from J774A1 macrophages as previously described (21). Equal protein amounts of cell lysates were electrophoresed on 6 or 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking nonspecific binding with 5% skim milk, membrane was incubated with polyclonal rabbit antibodies of SR-B1, PPARy, ABCA1, and LXRα. After washes, membrane was incubated for 1 h with a goat anti-rabbit IgG conjugated to HRP. The individual protein level was determined using Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotechnology) and Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore). Incubation with mouse β-actin antibody (Sigma-Aldrich Chemical) was also performed for comparative controls. Lamin B antibody was used as a nuclear control.

Real time-PCR and RT-PCR. Following culture protocols, total RNA was isolated from J774A1 using a commercially available Trizol reagent kit (Molecular Research Center) (24). The levels of mRNA transcripts of SR-B1, ABCA1, and RXRα were quantified by real-time PCR using a SYBR Green PCR commercial kit (Qiagen). Primers and relative sizes of amplification products for PCR are shown in Supplemental Table 1. RT-PCR analysis was also performed for semiquantifying the levels of mRNA transcripts of ABCA1 and RXRα. The PCR condition for SR-B1 was 95°C for 10 min and 40 cycles at 95°C (10 s), 60°C (15 s), and 72°C (20 s), followed by melting curve analysis. The PCR condition for ABCA1 was 95°C for 10 min and 35 cycles at 94°C (30 s), 60°C (45 s), and 72°C (45 s), and the condition for RXRα was 95°C for 10 min and 35 cycles at 94°C (30 s), 60°C (45 s), and 72°C (45 s). The housekeeping gene GAPDH was used for an internal normalization for the coamplification.

Preparation of nuclear protein extract. Nuclear protein extracts from J774A1 cells were prepared using a detergent lysis procedure (21). Proteins were extracted from nuclear pellets by incubating with a high-salt buffer with vigorous shaking. The nuclear debris was pelleted by centrifugation at 2000 × g for 30 min to collect supernatants. For the measurement of protein levels of LXRα, Western-blot analysis was conducted with nuclear protein extracts using mouse LXRα primary antibody.

Cholesterol efflux assay. J774A1 macrophages were treated with 1–5 μmol/L ellagic acid for 24 h then equilibrated with 1 mg/L 3-dodecanoyl-NBD-labeled cholesterol (Cayman Chemical) for an additional 4.5 h. Cells exposed to NBD-labeled cholesterol were washed with PBS and incubated in DMEM for 4.5 h. Fluorescence-labeled cholesterol released from cells into medium for 6 h was detected by using a fluorometer at wavelengths of from λ = 485 to 538 nm. Cholesterol efflux was expressed as percent fluorescence in medium relative to total fluorescence.

Data analysis. The results are presented as means ± SEM. Statistical analyses were conducted using the SAS software package version 6.12 (SAS Institute). One-way ANOVA was used to determine inhibitory effects of ellagic acid on cellular effects of oxidized LDL in macrophages. Differences among treatment groups were analyzed with Duncan’s multiple-range test and considered significant at P ≤ 0.05.
Results

**Inhibitory effects of ellagic acid on lipid accumulation.** During 24-h incubation of J774A1 macrophages with ≤10 μmol/L ellagic acid, cell viability was not influenced (data not shown). In addition, ellagic acid per se did not cause lipid peroxidation.

This study investigated intracellular lipid accumulation in macrophages, as measured by Oil red O staining. There was heavy reddish staining in macrophages exposed to 50 mg/L oxidized LDL for 18 h (Fig. 1A). However, lipid accumulation declined significantly in macrophages exposed to 50 mg/L oxidized LDL for 24 h compared to those exposed for 18 h. This decline indicates lipid efflux from macrophages. When 5 μmol/L ellagic acid was applied to macrophages treating oxidized LDL for 18 h, the reddish lipid droplets diminished in a dose-dependent manner (Fig. 1B). These data show that 5 μmol/L ellagic acid retarded oxidized LDL uptake and foam cell formation in macrophages.

**Inhibition of SR-B1 induction by ellagic acid.** It has been shown that macrophage uptake of oxidized LDL entails SR-B1 induction (2,3). Oxidized LDL stimulated SR-B1 expression rapidly within 2 h, which was sustained up to 8 h (Fig. 2A). It should be noted that SR-B1 expression was slightly elevated at 18 h after the addition of oxidized LDL. When oxidized LDL were treated for 6 h, the SR-B1 expression declined due to the presence of ellagic acid (Fig. 2A). The inhibition of SR-B1 induction was accomplished via a direct modulation at its transcriptional level, as determined by using real-time-PCR. Treatment of macrophages with oxidized LDL enhanced the SR-B1 mRNA level, which was downmodulated by 10 μmol/L ellagic acid (Fig. 2B).

**Upregulation of ABCA1 expression by ellagic acid.** When 50 mg/L oxidized LDL was added to macrophages, ABCA1 was induced from 8 h after treatment (Fig. 3A). The 18-h treatment with ellagic acid further upregulated oxidized LDL-elevated ABCA1 protein levels (Fig. 3A), indicating that ellagic acid may promote cholesterol efflux in lipid-laden foam cells. In addition, the transcriptional levels of ABCA1 were enhanced within 2 h by adding oxidized LDL, as determined by real-time PCR (Fig. 3B). The transcription of ABCA1 was also further upregulated by ≥1 μmol/L ellagic acid (Fig. 3B). In addition, RT-PCR data showed the upregulation of ABCA1 transcription by ellagic acid in a similar fashion (data not shown).

**PPARγ induction by ellagic acid.** Our investigation attempted to confirm that nuclear induction of PPARγ was responsible for ABCA1 upregulation. PPARγ expression was enhanced in 50 mg/L oxidized LDL-stimulated macrophages within 1 h and was sustained up to 18 h (Fig. 4A). When oxidized LDL were added for 18 h, the PPARγ induction was accelerated by ≥1 μmol/L ellagic acid (Fig. 4A). As expected, the upmodulated PPARγ was abolished by adding 10 μmol/L GW9662 (PPARγ inhibitor) to oxidized LDL-experienced macrophages cells (Fig. 4B). It was also investigated whether ABCA1 induction elicited by oxidized LDL involved PPARγ activation in lipid-laden macrophages. The ellagic acid augmentation of ABCA1 expression was abrogated in the presence of GW9662, indicating that nuclear PPARγ was a key modulator influencing macrophage cholesterol efflux (Fig. 4C). However, ellagic acid elevated ABCA1 expression in the presence of GW9662, suggesting that ellagic acid may promote ABCA1 expression independent of PPARγ signaling.

**Modulation of LXRα and RXRα by ellagic acid.** It has been reported that PPARγ-RXRα-LXRα signaling triggers ABCA1 induction in macrophages (9,11). The cellular level of LXRα was upregulated by PPARγ mediation, evidenced by using 10 μmol/L pioglitazone, a PPARγ agonist (Fig. 5A). Oxidized LDL increased total LXRα protein levels in macrophages, which was augmented by adding 1 μmol/L ellagic acid (Fig. 5B). Similar results were observed in the protein levels of nuclear LXRα. Accordingly, it is deemed that ellagic acid boosted ABCA1 transcription via a direct modulation of LXRα. We proposed a hypothesis that RXRα was highly invigorated by ellagic acid in the PPARγ-ABCA1 signaling. Interestingly, the present study revealed that RXRα transcription rather declined in lipid-laden macrophages treated with ellagic acid, as determined by RT-PCR (Fig. 5C) and real-time PCR assays (Fig. 5D).

FIGURE 1 Time course response of intracellular lipid accumulation (A) and inhibitory effects of ellagic acid on foam cell formation (B) of J774A1 murine macrophages. Microphotographs were obtained using an optical microscope at ×200 magnification. Values are mean ± SEM, n = 3. Means without a common letter differ, P < 0.05.
Promotion of cholesterol efflux by ellagic acid. Ellagic acid at $1 \text{m mol/L}$ promoted cholesterol efflux outside macrophages exposed to oxidized LDL (Fig. 6A), as performed by using NBD-labeled cholesterol. This was most likely due to ellagic acid-triggered ABCA1 induction (Fig. 3A). The cellular membrane transporter SR-B1 facilitates active efflux of cholesterol as well as internalization of oxidized LDL (2,3). In the present study, SR-B1 in the late stage of oxidized LDL handling (~12–18 h) was not involved in the cholesterol efflux promoted by ellagic acid (Fig. 6B). SR-B1 was induced in a temporal manner (Fig. 2A), with substantial expression during the early stage (4–6 h) and no notable induction during the late stage of the event.

Discussion

Five major findings were observed from this study: 1) noncytotoxic ellagic acid at $\leq 5 \text{ µmol/L}$ attenuated oxidized LDL uptake and cellular lipid accumulation in J774A1 murine macrophages; 2) oxidized LDL markedly induced the membrane receptor SR-B1 within 2 h, which was dose-dependently reversed by 5 µmol/L ellagic acid; 3) ellagic acid promoted oxidized LDL-induced expression of ABCA1 responsible for cholesterol efflux; 4) oxidized LDL-induced upregulation of PPARγ and LXRα was further accelerated by ellagic acid, indicating its direct modulation of ABCA1 transcription. Unexpectedly, the RXRα transcription declined in ellagic acid-treated, lipid-laden macrophages; and 5) ellagic acid fostered cholesterol efflux from lipid-laden foam cells. These observations demonstrate that noncytotoxic ellagic acid blocked oxidized LDL internalization through disturbing early induction of membrane SR-B1. The results also suggest that the ability of ellagic acid to enhance the ABCA1-mediated cholesterol efflux from lipid-laden foam cells may be mediated through transcriptional mechanisms pertaining to PPARγ-LXRα signaling. However, it was found that ellagic acid could be involved in ABCA1 activation independent of PPARγ signaling.

LDL oxidation is thought to be a crucial early event evoked in the pathogenesis of atherosclerosis (1). Under pathophysiological conditions such as chronic hyperlipidemia, lipoproteins aggregate within the intima of blood vessels and become oxidized.

**FIGURE 3** Time course responses of ABCA1 induction to oxidized LDL and its upregulation by ellagic acid (A), and ABCA1 mRNA levels in the absence and presence of ellagic acid, as measured by real time-PCR (B). Bands are representative of 3 independent experiments. β-Actin was used for comparative controls. Data are expressed relative to GADPH. Values are mean ± SEM, n = 3. Means without a common letter differ, $P < 0.05$. ABCA1, ATP-binding cassette transporter-A1.
by the action of oxygen free radicals generated by resident cells in the vessel wall. Macrophages taking up oxidized LDL are transformed to lipid-laden foam cells, forming part of atherosclerotic plaques in the fatty streak lesions (1). Several membrane receptors involved in internalizing oxidized LDL have been identified as SR. Unlike LDL receptors, these receptors are not negatively regulated by high levels of intracellular cholesterol. Macrophage SR include SR-A, SR-B CD36 antigen, and macrophage antigen CD68 (2,3), where SR-A is denoted as acetylated LDL receptor mainly expressed on macrophages (3). Oxidized LDL as the natural ligand for SR-A partially competes for the binding of acetylated LDL to macrophages (25). SR-B recognizes oxidized LDL but not acetylated LDL as receptors expressed on macrophages (2). Our previous study revealed that oxidized LDL induced SR-A and SR-B1 rapidly within 4 h (24).

In addition, SR-B is upregulated by cytokines present in atheromatous lesions (26). This study attempted to examine whether the natural compound ellagic acid modulated membrane SR-B1 induction elicited by oxidized LDL in murine macrophages.

Ellagic acid is a polyphenol found in numerous plant foods, including berries, nuts, and pomegranates (14). Ellagic acid is present in the natural form of ellagitannin bound to a sugar molecule (15). This compound has chemoprevention properties in cancer cells (16,17) and potentiates anticarcinogenic effects attributed to polyphenols such as quercetin in fruits (27). We have previously shown that ellagic acid suppressed skin wrinkles and inflammation resulted from chronic UV exposure (18). Pomegranate phenolic compounds including ellagic acid reduced atherosclerotic lesions in peritoneal macrophage of atheroscle-
The current report demonstrated that ellagic acid reduced oxidized LDL uptake and cholesterol influx in J774A1 murine macrophages by reducing membrane expression of SR-B1. In addition, ellagic acid promoted cholesterol efflux in lipid-loaded macrophages by inducing ABCA1 expression. The ABCA1 induction by ellagic acid was mediated by redirecting PPARγ-responsive cellular later signaling, thus transferring effluxed cholesterol onto lipid-poor apolipoproteins, initiating the formation of HDL particles. The SR-B1 inhibition by ellagic acid might be mediated by counteracting PPARγ-responsive early signaling. Accordingly, ellagic acid works as a PPARγ modulator and is an antiatherogenic agent blocking foam cell formation or enhancing cholesterol efflux pertaining to reverse cholesterol transport. Although ellagic acid may serve as a modulator against atherogenesis in vitro, its dietary in vivo role as an antiatherogenic agent remains unclear.

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Literature Cited


