Rubus coreanus Inhibits Oxidized-LDL Uptake by Macrophages Through Regulation of JNK Activation

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Abstract: Oxidized low-density lipoprotein (oxLDL) contributes to atherosclerosis in part by being taken up into macrophages via scavenger receptors and leading to foam cell formation. Herbal compounds that have been used to treat blood stasis (a counterpart of atherosclerosis) for centuries include extracts of medicinal plants in the Rosaceae and Leguminosae families. In this study, we investigated the effect of the unripe Rubus coreanus (Korean black raspberry) fruit extract on oxLDL uptake by murine macrophage cells. In the presence of Rubus coreanus extract (RCE), Dil-labeled oxLDL uptake was inhibited in a dose-dependent manner. SP600125, a specific JNK inhibitor, inhibited the uptake of Dil-oxLDL into macrophages. RCE also inhibited JNK phosphorylation in a time- and dose-dependent manner in macrophages treated with oxLDL. These results indicate that among the mitogen-activated protein kinases, JNK phosphorylation is inhibited by RCE, which is likely the mechanism underlying the RCE-induced inhibition of oxLDL uptake by macrophages.

Keywords: Rubus coreanus; oxLDL Uptake; Macrophage; Phospho-JNK; Atherosclerosis.
Introduction

Atherosclerosis is a major risk of coronary artery disease in humans. It is also the leading cause of death in many developing countries. Cascades of inflammatory processes involving monocytes, endothelial cells, vascular smooth muscle cells, and soluble mediators underlie atherosclerosis (Weber and Noels, 2011). Atherosclerotic lesions start as fatty streaks, which consist mainly of cholesterol-enriched macrophage foam cells underlying the endothelium or large arteries (Li and Glass, 2002; Libby, 2002). Recruitment of macrophages and their subsequent uptake of oxLDL due to activation of mitogen-activated protein kinases (MAPKs) and increases in levels of scavenging receptors are major cellular events that contribute to fatty streak formation (Moore and Freeman, 2006; Guan et al., 2010). The formation of oxLDL and oxidative stress are thought to play a critical role in early atherogenesis, eliciting endothelial dysfunction and macrophage activation (Stocker and Keaney, 2004). The uptake of oxLDL stimulates macrophages to accumulate cholesterol and to form foam cells, which make up fatty streaks, the hallmark of early atherosclerotic lesions; this is followed by the development of fibrous and atheromatous plaques (Ouimet and Marcel, 2012). Although atherosclerosis can affect arteries anywhere in the body, it is both preventable and treatable (Rader and Daugherty, 2008).

*Rubus coreanus*, the Korean black raspberry, is a member of the family *Rosaceae* and is distributed in South Eastern Asia, especially China, Japan, and the Korean peninsula. The fruits of this plant contain large amounts of flavonoids and polyphenols and have therefore been employed as a traditional medicine for centuries by people from these regions (Choi et al., 2006). The *Rubus coreanus* extract (RCE) has been reported to contain different pharmaceutically active natural components including flavonoids, tannins, triterpenoids, anthocyanin, and phenolic compounds (Yoon et al., 2003). Due to the presence of these active components, it has anti-inflammatory, anti-fatigue, anti-gastropathic, anti-rheumatic, and anti-oxidative properties; it is also used to treat spermatorrhea, enuresis, asthma, and allergic diseases (Shin et al., 2002; Choi et al., 2003; Lee and Choi, 2006; Nam et al., 2006). A recent study demonstrated that the antioxidant (total polyphenol, flavonoid, and anthocyanin) and oxygen radical scavenging capacity of *Rubus coreanus* fruits was higher than that of pear cactus and mulberry fruits (Lee et al., 2009). Polyphenols found in *Rubus coreanus*, including epicatechin, catechin, and proanthocyanin, have been reported to inhibit catecholamine release in rat adrenal medulla by increasing NO secretion, which might lead to the elevation of cGMP levels and prevention of cardiovascular disease (Kee and Lim, 2007). Among major potential targets identified for therapeutic intervention of atherosclerosis, inhibition of oxLDL uptake by macrophages, which would lower plasma LDL cholesterol, is a major target (Rader and Daugherty, 2008).

To elucidate the role that *Rubus coreanus* plays on the generation of atherosclerosis, we investigated the effects of RCE on the uptake of oxLDL and the expression of various MAPK proteins in mouse peritoneal macrophages. We found that RCE significantly inhibited the uptake of oxLDL by macrophages. Furthermore, RCE inhibited JNK phosphorylation, which may be the major mechanism responsible for the RCE-induced inhibition of oxLDL uptake.
Materials and Methods

Preparation of Rubus coreanus Extract (RCE)

RCE was kindly provided by Gochang Black Raspberry Research Institute, Gochang-Gun, Korea.

The unripened fruits of Rubus coreanus Miq were collected from the Gochang (Jeollabuk-do) area in South Korea and kept at −20°C. In brief, unripened fruits were extracted with 5 L of distilled water at 100°C using a reflux condenser (Eyela, Japan). Then, the extracts were filtered through 0.26 mm filter paper (Advantech, No. 2). Aliquots from filtered samples were concentrated at 40°C using a 1 L rotary evaporator (Eyela, Japan). The concentrate was lyophilized in a freeze-dryer (Ilshin, Hong Kong) to generate powder, which was closed in an airtight container and stored at −20°C until used. The dried extract yield from the starting dried plant material was approximately 5.1%.

Materials

Except for phospho-JNK, all other primary and secondary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Phospho-JNK was purchased from Cell Signaling Technology Inc., (Danvers, MA), and RPMI medium 1640 was obtained from Gibco (Invitrogen, Grand Island, NY). Dil-labeled oxLDL (BT-920) and oxLDL (BT-910) were obtained from Biomedical Technologies Inc. (Stoughton, MA). All other chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO).

Mice

Peritoneal macrophages were harvested from pathogen-free 8- to 10-week-old male C57BL/6 mice. All mice were housed individually in cages under specific pathogen-free conditions, in an air-conditioned room at 22°C with a 12 h light/dark cycle and were fed chow ad libitum. Food was removed the night before the experiment. All animal experiments were approved by the Committee on Animal Care and Use of Chonbuk National University.

Preparation of Peritoneal Macrophages

C57BL/6 mice were injected with 3 ml of 3% thioglycolate (BD Bioscience) intraperitoneally (i.p.), and i.p. macrophages were harvested 72 h later as described previously (Ishida et al., 2008). The cells were suspended in RPMI 1640 medium containing 10% FBS and incubated at 37°C in 24-well plates or in 6-cm discs. Two hours later, non-adherent cells were removed, and the medium was replaced.

Uptake of Dil-Labeled Modified LDL

Dil-labeled oxLDL was used to monitor the uptake of modified LDL (Voyta et al., 1984). Macrophages were pretreated with reagents for 24 h in confocal dishes, and then
Dil-labeled oxLDL (10 μg/ml) was added and incubated for another 5 h. Fluorescent images were taken using a Nikon Eclipse E600 fluorescence microscope (Kawasaki, Kanagawa, Japan). The fluorescence intensity of each treatment group was measured using ImageJ software.

**Immunostaining**

Macrophage cells pretreated with various concentration of RCE and 1μM of SP600125, were fixed with 3.7% masked formaldehyde for 20 min. After washing fixed cells twice with PBS, blocking and permeabilized solution with 5% BSA and 0.2% Triton was added for an hour. As primary antibody p-JNK with dilution of 1:200 in 5% BSA was incubated in 4°C for overnight. For secondary antibody staining FITC conjugated secondary antibody with 1:100 dilution in 5% BSA was used. Finally, fluorescence image was recorded and quantified.

**Western Blot Analysis**

Total lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking with 5% BSA, the membranes were incubated with the indicated primary antibody, followed by the respective secondary antibody. Bands were detected using enhanced chemiluminescence reagents purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

**Protein Quantification**

The Bradford method was used to quantify the amount of protein present in the samples.

**Statistics**

All the results are presented as means ± SEM. The Microcal Origin8 software (Northampton, MA) was used for statistical calculations. p values were determined via ANOVA. p values less than 0.05 were considered significant.

**Results**

**RCE Inhibits Modified LDL Uptake**

Mouse peritoneal macrophages were pretreated with RCE and then detected for their update of Dil-oxLDL. Fluorescence microscopy revealed a dose-dependent decrease in the amount of Dil-oxLDL in cells treated with RCE (Fig. 1A). A quantitative analysis of the fluorescence intensity from control and treated cells revealed that the presence of RCE inhibited the uptake of Dil-oxLDL into macrophages in a dose-dependent fashion (Fig. 1B).
Figure 1. Effect of RCE on the uptake of Dil-labeled oxLDL by mouse peritoneal macrophages. (A) Uptake of Dil-oxidized LDL as visualized by fluorescence microscopy. (B) Quantification of the fluorescence intensity of Dil-oxidized LDL uptake by macrophages. *p < 0.05 vs. OX-LDL group.
JNK Phosphorylation Inhibition Decreases oxLDL Uptake into Macrophages

To evaluate the mechanism of oxLDL uptake inhibition in macrophages, we investigated the effects of inhibiting several kinases of interest. We pretreated macrophages with PD98059, an extracellular signal-regulated kinase (ERK) inhibitor, SB203580, a p38 mitogen-activated protein kinase inhibitor, or SP600125, a JNK phosphorylation inhibitor. We found that the Dil-oxLDL uptake in the SP600125-pre-treated cells was significantly inhibited, whereas there was no decrease in Dil-oxLDL uptake in cells treated with PD98059 or SB203580 compared to the control group (Fig. 2A). The quantification data also showed that the Dil-florescence intensity and the ox-LDL uptake were significantly inhibited in the SP600125-treated cells compared to the PD98059- or SB203580-treated cells (Fig. 2B).

RCE Reduces JNK Phosphorylation to Inhibit Uptake of oxLDL by Murine Macrophages

To determine the mechanism by which RCE inhibits oxLDL uptake by macrophages, we assessed the effect RCE on JNK phosphorylation. To do this, we assayed for P-JNK by immunohistochemistry staining and Western blot analysis. Immunostaining demonstrated that RCE inhibited JNK phosphorylation in a dose dependent manner (Fig. 3A) and that

![Image](A)

Figure 2. Effect of various MAP kinase inhibitors on oxLDL uptake by murine peritoneal macrophages. (A) Uptake of Dil-oxidized LDL as visualized by florescence microscopy. (B) Quantification of the florescence intensity of Dil-oxidized LDL uptake by macrophages pretreated with 1 μM SP600125, 5 μM PD98059 or 5 μM SB203580. *p < 0.05 vs. OX-LDL group.
Figure 3. Effect of RCE on the JNK phosphorylation in mouse peritoneal macrophages. (A) Ten μg/ml ox-LDL was treated to macrophages with the indicated concentrations of RCE. One hour later, immunostaining was performed with anti-p-JNK1 and FITC-conjugated secondary antibody. (B) Ten μg/ml ox-LDL was treated to macrophages with or without either 10 μg/ml RCE or 1 μM SP600125. One hour later, immunostaining was performed with anti-p-JNK1 antibody and FITC-conjugated secondary antibody. (C) Expression of JNK phosphorylation at the indicated time periods in oxLDL-treated mouse peritoneal macrophages pretreated or untreated with RCE (10 μg/ml).
RCE inhibited JNK similarly to SP600125 (Fig. 3B). Western blot analysis results also showed that RCE pretreatment inhibited JNK phosphorylation (Fig. 3C). However, RCE had no effect on levels of phospho-p38 and phospho-ERK (data not shown). RCE alone had no effect on JNK activation, stress protein, GRP78 or other ROS scavenging proteins including Mn-SOD, CuZn-SOD and catalase (Fig. 4). These results indicate that RCE inhibits oxLDL uptake by macrophages by inhibiting JNK phosphorylation.

Figure 3. (Continued)

Figure 4. Effect of RCE alone on the expression of JNK, stress protein GRP78 and various ROS scavenging proteins at indicated time periods in mouse peritoneal macrophages.
Discussion

In this study, we showed that RCE inhibited the uptake of oxLDL by mouse peritoneal macrophages in a concentration-dependent manner. Furthermore, we showed that RCE regulated JNK phosphorylation, thereby inhibiting the uptake of oxLDL by macrophages. RCE treatment inhibited Dil-labeled oxLDL uptake by macrophages (Figs. 1A and 1B). The previously reported chemical constituents of *Rubus coreanus* include flavonoids, tannins, triterpenoids, and polyphenolic compounds (Kee and Lim, 2007). Among the *Rubus coreanus* components, compounds including tyrosol and resveratrol have been studied for their anti-atherosclerotic effects and have been demonstrated to lower oxLDL uptake by RAW 264.7 macrophage cells (Ou et al., 2006; Vivancos and Moreno, 2008). Resveratrol activated AMPK phosphorylation, leading to the regulation of gluco-lipid metabolism (Berrougui et al., 2009). Similar to RCE, other berry fruit extracts from pomegranates, grapes, and mulberries have also been shown to inhibit LDL oxidation thereby lowering macrophage-induced foam cell formation (Lui et al., 2008; Terra et al., 2009; Rosenblat et al., 2010).

RCE regulates ox-LDL uptake by inhibiting JNK activation (Figs. 3A to 3C). Multicellular organisms have three well-characterized subfamilies of mitogen-activated protein kinases (MAPKs) that control a vast array of physiological processes. These enzymes are regulated by a characteristic phosphorylation system in which protein kinase extracellular signal-regulated kinases (ERKs), c-jun amino terminal kinase (JNKs), and p38 phosphorylate and activate one another (Johnson and Lapadat, 2002). In this study, inhibition of neither ERK1/2 nor p38 MAPK diminished the uptake of oxLDL, while SP600125, a JNK phosphorylation inhibitor, significantly blocked the uptake of oxLDL by mouse peritoneal macrophages (Figs. 2A and 2B). These results are similar to those reported by Bennett et al., Sun and Chen (Bennett et al., 2001; Sun and Chen, 2011). Okutsu et al. also suggested that JNK was one of the main mechanisms for macrophage uptake of oxLDL (Okutsu et al., 2009). Okutsu and colleagues demonstrated Cilostazol that regulates JNK, is involved in oxLDL uptake. The anti-hyperlipidemic/anti-atherosclerotic effects of Cilostazol are explained by its ability to regulate JNK. Furthermore, JNK was also reported to be responsible for intracellular cholesterol accumulation leading atherosclerosis in THP-1 human monocytes in *Chlamydia pneumonia* infection (Liu et al., 2010). Oxidized LDL uptake and cholesterol accumulation in macrophages are thought to be enhanced by the involvement of scavenging receptors such as SR-A and CD36 (Fabbraio et al., 2000). Similarly, Ricci and colleagues showed that pharmacological inhibition of JNK activity significantly reduced plaque formation in vivo (Ricci et al., 2004). JNK-dependent phosphorylation of SR-A promotes the uptake of lipids into macrophages, thereby promoting foam cell formation. Similarly, macrophage-specific overexpression of SR-A has been shown to be sufficient for reducing atherosclerosis in ApoA-E deficient mice (Whitman et al., 2002). Our analysis to determine whether RCE has a regulatory effect on SR-A or CD36 in peritoneal macrophages resulted in inconclusive data (data not shown). The mechanism by which RCE inhibits JNK phosphorylation, thereby inhibiting oxLDL uptake by macrophages, requires further investigation.
In this study, there was no increase in the expression of various ROS scavenging proteins in RCE-treated macrophages (Fig. 4). However, this set of data alone may not be sufficient to conclude that RCE has inefficient antioxidant activities. There is a possibility that RCE might directly quench the free radicals, while not affecting the activity of the tested anti-oxidant proteins. Similar results have also been reported with other berries (Wakabayashi et al., 2003; Liu et al., 2008). Therefore, the free radical scavenging activity by RCE needs to be further investigated in the future.

In summary, RCE inhibited oxLDL uptake by macrophages by regulating JNK phosphorylation. RCE may therefore have a preventive or therapeutic effect against atherosclerosis, although more in vivo and human studies are required to confirm our findings.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

References


