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Aqueous extract of unripe *Rubus coreanus* fruit attenuates atherosclerosis by improving blood lipid profile and inhibiting NF-κB activation via phase II gene expression

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ABSTRACT

Ethnopharmacological relevance: The fruit of *Rubus coreanus* has been used as a traditional herbal medicine for alleviation of inflammatory and vascular diseases in Asian countries.
Aim of the study: The anti-atherogenic effect of unripe *Rubus coreanus* fruit extract (URFE) and its underlying mechanism were analyzed in mice fed a high-fat diet (HFD) and in cell culture system.
Materials and methods: Mouse was freely given HFD alone or supplemented with URFE for 14 weeks, followed by analysis of atherosclerotic lesions and serum lipid levels. For in vitro assay, macrophages were pretreated with URFE, followed by stimulation with lipopolysaccharide (LPS). Expression levels of inflammatory genes (TNF-α, IL-1β, and iNOS) and phase II genes (heme oxygenase-1, glutamate cysteine lygase, and peroxiredoxine-1) as well as intracellular reactive oxygen species (ROS) level and NF-κB activation pathway were analyzed in cultured macrophages as well as mouse sera and aortic tissues.
Results: URFE supplementation reduced HFD-induced atherosclerotic lesion formation which was correlated with decreased levels of lipids, lipid peroxides, and inflammatory mediators (TNF-α, IL-1β, and nitric oxide) in sera as well as suppression of inflammatory gene in aortic tissues. In addition, pretreatment of macrophages with URFE also suppressed LPS-induced NF-κB activation, ROS production, and inflammatory and phase II gene expressions. Inhibition of phase II enzyme and protein activities attenuated the suppressive effects URFE on ROS production, NF-κB activation, and inflammatory gene expression.
Conclusion: These results suggest that URFE attenuates atherosclerosis by improving blood lipid profile and inhibiting NF-κB activation via phase II antioxidant gene expression.

1. Introduction

Hyperlipidemia plays an important role in the pathogenesis of atherosclerosis. Numerous studies have demonstrated that dyslipidemia, such as the increases in plasma concentrations of triglyceride, total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C), and a decrease in plasma concentration of high-density lipoprotein cholesterol (HDL-C), is an important risk factor for various cardiovascular diseases. Of these plasma lipids, LDL-C plays a crucial role in the development of atherosclerosis; however, the elevation of HDL-C prevents the initiation of atherosclerosis by blocking the atherogenic effects of LDL-C (Parthasarathy et al., 1990; Steinberg, 1997). These evidences indicate that improvement of blood lipid profile prevents

Abbreviations: URFE, unripe *Rubus coreanus* fruit extract; HFD, high-fat diet; LPS, lipopolysaccharide; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ROS, reactive oxygen species; NF-κB, nuclear factor-kappaB; IKK, IκB kinase; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; NOx, nitrite and nitrite plus nitrate; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; HO-1, heme oxygenase-1; GCL, glutamate cysteine ligase; Prx1, peroxiredoxin 1; ARE, antioxidant response element; Nrf2, nuclear factor E2-related protein 2; oxLDL, oxidized low-density lipoprotein; SnPP, tin protoporphyrin IX; BSO, buthionine sulfoximine; RT-PCR, reverse transcription-polymerase chain reaction; MDA, malondialdehyde

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blood vessel injury and the pathogenic development of atherosclerotic plaque formation leading to cardiovascular dysfunction.

Atherosclerosis is also regarded as a chronic inflammatory disease that results from the production of various cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), as well as soluble mediators, including inducible nitric oxide synthase (iNOS)-mediated NO production (Choi et al., 2003; Buttery et al., 1996). TNF-α and IL-1β promote the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells (Lee et al., 2006a) and the interaction between monocytes and endothelial cells, resulting in an increase in transmigration of circulating monocytes to the intima. The migrated monocytes mature to macrophages, take up lipids, and become foam cells, leading to inflammatory gene expression and atheroma plaque formation (Libby et al., 2009). The expression of the inflammatory genes is tightly regulated by activation of the transcription factor nuclear factor-κB (NF-κB), suggesting that NF-κB activation is a critical step in the pathogenesis of atherosclerosis.

Rubus species, including *Rubus coreanus* and *Rubus rigidus*, have been traditionally used as a therapeutic medicine in Asian and Western countries for inflammatory diseases, such as atherosclerosis (Ferreira et al., 2011; Guarrera, 2005; Patel et al., 2004; Yang et al., 2008). Recent studies demonstrate that the fruits of Rubus species significantly reduce the plasma levels of triglycerides, total cholesterol, and LDL-C in hypercholesterolemia animals and reduce the anti-inflammatory effects of immune-activated macrophages (Ferreira et al., 2011; Park et al., 2006). Of several compounds isolated from Rubus species, ellagic acid is a well-identified bioactive component, which regulates oxidized LDL (oxLDL) uptake and cholesterol efflux in murine macrophages (Park et al., 2011a,b) and inhibits NF-κB-mediated inflammation (Lee et al., 2010). Some herbal medicines or natural products prevent inflammatory diseases, including atherosclerosis, by inducing Nrf2-dependent phase II antioxidant enzymes and proteins, such as heme oxygenase (HO-1), glutamate cysteine ligase (GCL), and peroxiredoxin 1 (Prx1). The phase II gene products are critically involved in regulation of NF-κB activation, which is a key step in the pathogenesis of atherogenesis (Kim et al., 2008a, 2008b, 2012). Thus, the herbal medicines, which induce phase II genes, can be considered an effective anti-atherogenic strategy.

The quantity of bioactive components in plants used for herbal medicine is dependent on agronomical factors, weather condition, and the degree of fruit ripening. Indeed, unripe *Rubus coreanus* fruits elicit a strong inhibitory effect on the production of inflammatory mediators, whereas its ripe fruit extract had a weak inhibitory effect (Yang et al., 2008). The ethanol extract of the unripe fruits has been shown to contain about 4.1 mg/g extract of ellagic acid, a major bioactive compound, which is much higher than in its ripe fruit extract (Yang et al., 2008). Based on these evidences, we hypothesized that unripe *Rubus coreanus* fruit extract (URFE) can prevent the pathogenesis of atherosclerosis by improving blood lipid profile and suppressing inflammatory processes. We here found that URFE decreased high fat diet-induced atherosclerosis by lowering blood lipid level and inhibiting NF-κB-mediated inflammatory gene expression via induction of Nrf2-dependent phase II genes.

2. Material and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Life Technology Inc. (Rockville, MD). Antibodies for p-IκBα (sc-7977), IκBα, NF-κB p65, iNOS, TNF-α, and IL-1β were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for HO-1, Prx1, and p-IKKαβ, and Nrf2 were obtained from Cell Signaling Technology (Beverly, MA). Prx1 siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). U0126, Wortmannin, and PD98059 were purchased from Calbiochem (La Jolla, CA). URFE was obtained from Gochang Black Raspberry Research Institute (Jeollabuk-do, Korea). The unripe fruits of *Rubus coreanus* Miquel were collected in the Gochang area of Jeollabuk-do (Korea) and used for extraction as previously described (Bhandary et al., 2012). URFE was prepared by pulverizing and extracting the unripe fruits (100 g) with 1 l of distilled water at 100 °C using a reflux condenser. Then, the extract was filtered and concentrated. The concentrate was lyophilized in a freeze-dryer and stored at −20 °C until use. The yield of dried extract from starting dried fruits was approximately 5.1%, and its phytochemical compound profile has been previously reported (Bhandary et al., 2012). Other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

The immortalized murine macrophage RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin) containing 5% fetal bovine serum (Hyclone Labs, Logan, UT) at 37 °C in 5% CO_2/95% air. Cells were pretreated with URFE for 4 h and stimulated with LPS (100 ng/ml). For assaying the role of phase II genes in anti-inflammatory effect, cells were transfected with 80 nM Prx1 siRNA for 24 h using Lipofectamine reagent (Invitrogen) and treated with 20 μM tin protoporphyrin IX (SnPP), 20 μM buthionine sulfoximine (BSO), and triple combination (TC) of Prx1 siRNA, SnPP, and BSO for 4 h before stimulation with 100 ng/ml LPS. Peritoneal macrophages were collected from the peritoneal cavity of 7-week-old male C57BL/6 J mice (Jackson Laboratory) given an i.p. injection of 1.5 ml of 4% thioglycollate broth 7 days before harvest. Cells were cultured in 96-well plates (4×10^5 cells/well) at 37 °C in 5% CO_2/95% air overnight.

2.3. Western blot analysis

Whole-cell lysates as well as cytosolic and nuclear fractions were prepared as previously described (Lee et al., 2006b). Protein (40 μg) were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat-dried milk and sequentially hybridized with primary and secondary antibodies against target proteins. Protein bands were visualized by incubating membranes with chemiluminescent solution for 2 min and exposing to X-ray film.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were obtained from macrophages and tissues using Trizol reagents (Life Technology Inc., USA). RT-PCR was performed as described in a previous method (Lee et al., 2006a). After cDNA was synthesized from 5 μg mRNA using reverse transcriptase, PCR was performed in 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 μM each target primer. Amplification conditions were as follows: denaturation at 94 °C for 5 min for the first cycle and for 45 s starting from the second cycle, annealing of HO-1, GCL-catalytic subunit (GCLC), GCL-modifier subunit (GCLM), and actin at 56 °C for 30 s, annealing of iNOS at 65 °C for 1 min, and annealing of Prx1, IL-1β, and TNF-α at 51 °C for 45 s, and extension at 72 °C for 45 s for 30 cycles. Final extension was performed at 72 °C for 10 min. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The primers used were designed as previously described (Kim et al., 2012) and as follows: 5′-GGCCCTGGAAGAGGAGATA-3′ (sense) and