Water Extracts of Immature *Rubus coreanus* Regulate Lipid Metabolism in Liver Cells

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Hyperlipidemia is a major contributor to atherosclerosis and hypolipidemic drugs such as statin are highly prescribed to treat elevated lipid level in plasma. *Rubus coreanus*, which is widely cultivated in south-east Asia, have been reported to show significant cholesterol lowering action in hyperlipidemic subjects. Our objective was to determine the cellular effect of *Rubus coreanus* extract (RCE) on cholesterol biosynthesis in human hepatic cells (HepG2) and to elucidate the molecular mechanism by which it causes change in cholesterol metabolism. RCE treatment lowered cholesterol biosynthesis as well as secretion from HepG2 cells. This effect was associated with lowering the release of apolipoproteins from hepatic cells. RCE treatment also showed an increase in phosphorylation of foam box protein 01 (FoXo-1) and 5-adenosine monophosphate-activated protein kinase (AMPK), thus lowering expression of phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase, which might be a major pathway for cholesterol biosynthesis inhibition. Apart from these, RCE also lowered sterol regulatory element-binding protein-1 (SREBP-1) expression in HepG2 cells, showing a long term regulation of cholesterol biosynthesis activity. These results indicate that one of the anti-hyperlipidemic actions of RCE is due to inhibition of cholesterol biosynthesis in hepatic cells and provides first documentation of a hypolipidemic bio-molecular action of *Rubus coreanus*.

**Key words** *Rubus coreanus*; cholesterol; apolipoprotein B; sterol regulatory element-binding protein-1; HepG2

A reduced life span is an outcome associated with many prevalent diseases, including diabetes, obesity and high blood pressure. Hyperlipidemia is one of the well known risk factors for atherosclerosis. Fibrates, statins and other various types of hypolipidimic drugs are widely used to treat patients with elevated levels of lipids in their plasma. Among all, statins are frequently prescribed by physicians. Statins inhibit lipid synthesis through activation of 5-adenosine monophosphate-activated protein kinase (AMPK). AMPK acts as negative regulator, thus inhibiting the activity of 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase, the major rate limiting enzyme during cholesterol biosynthesis. Treatment with statins also alters hepatic apolipoprotein B (apoB) secretion and cholesteregeneration. Most of the cholesterol synthesis takes place in liver. Thus inhibition of cholesterol biosynthesis and secretion is major target for prevention of hyperlipidemia.

Although there are proven benefits of commercial allopathic drugs on both primary and secondary prevention of heart disease, the high cost of statin treatment, in addition to possible side effects such as liver function abnormalities and rhabdomyolysis, may limit their widespread use. In seeking to prevent these diseases, many researchers have looked into potentially therapeutic benefits of naturally occurring functional food as an alternative for fibrates and statin treatment.

*Rubus coreanus*, belonging to the family Rosaceae, is widely growing in south-east Asian countries (Japan, China, and Korea), and is used as a Korean traditional medicine. Chemical constituents of *Rubus coreanus* extract (RCE) include flavonoids, tannins, triterpenoses, anthocyanin, and lots of polyphenolic compounds. It shows anti-inflammatory, anti-fatigue, anti-gastropathic, anti-rheumatic, treatment of spermatorrhea, enuresis, asthma, allergic disease, and antioxidant activity. Cholesterol lowering actions of RCE has been reported in normal rat as well as in healthy human. Similarly, anti-hyperglycemic with anti-hyperlipidemic effects of RCE in streptozotocin induced diabetic rats has also been reported. However, the molecular mechanism by which RCE shows lipid lowering effect has not been portrayed precisely. The objective of the present study was to investigate the effect of RCE on cholesterol biosynthesis in hepatocytes, and mechanism by which it acts.

**MATERIALS AND METHODS**

**Preparation of RCE** RCE was kindly provided by Gochang Black Raspberry Research Institute, Gochang Gun, Republic of Korea. The immature fruits of *Rubus coreanus* were collected from Gochang (Jeollabuk-Do) area in South Korea, and were kept at −20°C. In brief, fruits were extracted with 5 L of distilled water at 100°C using a reflux condenser. 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 fruit was approximately 5.1%.

**Chromatographic Conditions** High performance liquid
chromatography (HPLC) separation was performed on an Agilent 1100 system (Agilent, CA, U.S.A.) consisting of a quaternary pump and UV-Vis detector. Chromatography separation was performed using OptimaPak C18 column (250×4.6 mm i.d., 5 μm particle size). The mobile phase was solvent A (0.2 M ortho-phosphoric acid) and solvent B (20% 50 mM ammonium dihydrogen phosphate, pH 2.6 in 80% acetonitrile) in a gradient mode as follows: 0–10 min, 20% B; 10–15 min, 30% B; 15–20 min, 40% B; 20–25 min, 90% B; 25–30 min, 90% B; 30–32 min, 5% B; 32–40 min, 5% B. Rutin, myricetin, luteolin, queretin, kaempferol were detected at 360 nm. Similarly, caffeic acid, p-coumaric acid, ferulic acid, reserveratrol at 320 nm, gallic acid at 280 nm and ellagic acid at 260 nm wavelength. Chromatography was performed at 25°C with a flow rate of 1 mL/min, and the run time was 40 min. The injection volume was 10 μL.

Cells Human liver (HepG2) cells were cultured in RPMI 1640 medium (NY, U.S.A.) supplemented with 2 mM l-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Cells were maintained in a humidified atmosphere at 37°C, 5% CO2, and 20% O2. Cells were maintained at 70–80% confluence in serum-depleted conditions for 16 h prior to the experiments.

Western Blot Analysis Total lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyliden difluoride (PVDF) membranes. After blocking of nonspecific sites with 5% bovine serum albumin (BSA), the PVDF membranes were incubated with the indicated primary antibody, followed by a secondary antibody. Primary antibodies of phospho-foxhead box class 01 (p-FoXo-1), phosphoenolpyruvate carboxy-kinase (PEPCK), FoXo-1, and AMPK antibodies were purchased from Santa Cruz Biotechnology (NY, U.S.A.). The superoxide dismutase (SOD) activity kit (AD1-900-157) was obtained from Enzo Life Sciences (Lorrach, Germany). The cholesterol measurement kit (AM202-2) was purchased from Asan Pharm., South Korea. HPLC grade acetonitrile and ortho-phosphoric acid were purchased from Fischer Scientific (NJ, U.S.A.). Ammonium dihydrogen phosphate was obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system from Millipore (MA, U.S.A.). Standard of rutin, myricetin, luteolin, quercetin, kaempferol, caffeic acid, p-coumaric acid, ferulic acid, reserveratrol, gallic acid and ellagic acid were purchased from Sigma (MO, U.S.A.).

**RESULTS**

**Quantification of Phenolic and Flavonoid in Extract Sample by HPLC** We isolated and quantified phenolic and flavonoid compounds in RCE using HPLC method. HPLC conditions and HPLC solvent conditions for compounds analysis are listed in Supplementary Tables 1a, b. The results are shown in Table 1. Eight compounds were found and among them, ellagic acid was the most abundant, followed in descending order, by gallic acid > rutin > caffeic acid > quercetin > luteolin > kaempferol. Resveratrol, p-coumaric acid, myricetin and ferulic acid were not detected in the extract sample.

**RCE Lowers Intracellular and Extracellular Cholesterol Level in HepG2 Cells** Cells were incubated in the absence or presence of RCE for 24 h. The amount of intracellular cholesterol showed a dose-dependent decrease with 0.1–5 μg/mL of RCE (Fig. 1a). Highest cholesterol lowering effect was
shown by treatment dose of 5 μg/mL of RCE. Similarly, the amount of extracellular cholesterol released by HepG2 cells into the media were also lowered dose-dependently upon treatment with various dose from 0.1–5 μg/mL, however extracellular cholesterol lowering was most significant at 5 μg/mL of RCE (Fig. 1b). Potency of RCE for lowering cholesterol biosynthesis was compared with a cholesterol lowering drug, atorvastatin (1 μM and 10 μM). Intracellular and extracellular cholesterol was measured in higher dose of RCE, but most promising cholesterol biosynthesis inhibition effect was with 5 μg/mL of RCE. (Supplementary Figs. 1a, 1b). This result was similar to that with lipid lowering effect of curcumin and garlic, where low dose seems more effective then high dose for implementing cholesterol-lowering effects in human subjects.18,19

Apolipoprotein Secretion from Liver Cells is Regulated by RCE

Apolipoproteins, which bound together with lipids especially very low density lipoprotein (VLDL), to secrete them into lymphatic and circulatory system, play an important role in lipid homeostasis in the body. In order to explain the mechanism for the lowering of cholesterol secretion by RCE, we examined the apolipoprotein secretion from HepG2 cells into media. Metabolic labeling and immunoprecipitation results revealed that RCE inhibited the secretion of apoA1 and apoB as seen in Figs. 2a–c. Newly synthesized apoB follows a sequence of steps that leads either to its degradation within the cell or bind together with lipid particles for the formation and secretion of a mature

<table>
<thead>
<tr>
<th>Compound</th>
<th>Contents (μg/mL)</th>
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<tbody>
<tr>
<td>Caffeic acid</td>
<td>8.6±0.35</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>425.1±2.34</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>181.2±3.05</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.4±0.11</td>
</tr>
<tr>
<td>Luteolin</td>
<td>0.7±0.24</td>
</tr>
<tr>
<td>Myricetin</td>
<td>ND</td>
</tr>
<tr>
<td>ρ-Coumaric acid</td>
<td>ND</td>
</tr>
<tr>
<td>Quercetin</td>
<td>4.1±0.67</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>ND</td>
</tr>
<tr>
<td>Rutin</td>
<td>31.6±0.72</td>
</tr>
</tbody>
</table>

Each value in the table represents mean±S.D. (n=3). ND, not detected.
Thus, we measured cholesterol biosynthesis (Figs. 3a, 3b) as well as apolipoprotein secretion into media by HepG2 cells with time sequence at 5 and 10 h after treatment with RCE (Fig. 3c). Results showed a time-dependent lowering of apoA and apoB (Figs. 3c–e). Coomassie brilliant blue (CBB) staining of gel is shown as a loading control. 

RCE Lowers Lipid Biosynthesis by Upregulating Phosphorylation of FoXo-1 and AMPK in HepG2 Cells

To further elucidate the mechanism by which RCE lowers secretion of cholesterol, apoA and apoB from hepatic cells, we determined the levels of p-FoXo-1, PEPCK, G6Pase, p-AMPK and AMPK by Western blotting (Fig. 4a). Treatment of HepG2 cells with RCE increased the expression of p-FoXo-1 and p-AMPK in a concentration-dependent manner (Figs. 4b, 4c). Similarly, expression of G6Pase and PEPCK was gradually decreased with increase in concentrations of RCE. Here, β-actin was used as a loading control. As RCE constituents measured by HPLC showed ellagic acid as the highest constituent. Therefore, we examined whether the RCE effect on expression of these molecules was due to ellagic acid. Treatment of HepG2 cells with ellagic acid (5 µM and 10 µM) showed similar effects to that of RCE (Figs. 4a–c).

**RCE Lowers Fatty Acid Biosynthesis by Regulatation of Sterol Regulatory Element-Binding Protein-1 (SREBP-1)**

SREBP-1 plays a critical role in long term regulation of cholesterol homeostasis in liver cells. To elucidate the mechanism by which RCE inhibits lipid biosynthesis, we further performed immunoblot experiment with SREBP-1 (Fig. 5a, b). Treatment with RCE at 5 µg/mL lowered the expression of SREBP-1 in a time-dependent manner. Equal loading was confirmed using a β-actin antibody.

**DISCUSSION**

*Rubus coreanus*, one of the traditional Korean medicinal plants, is well known for its various therapeutic benefits. In this study, we have found that RCE treatment lowers cholesterol biosynthesis lowering intracellular cholesterol as well...
as extracellular cholesterol (Figs. 1a, b). Major chemical constituents of RCE include ellagic acid, gallic acid, rutin, caffeic acid, quercetin, luteolin, and kaempferol (Table 1). Cholesterol biosynthesis lowering potency of RCE was compared with that of different doses of Atorvastatin. Here, RCE lowered release of apolipoproteins, increasing phosphorylation of FoXo-1, AMPK, thus decreasing expression of PEPCK, SREBP, and G6Pase dose dependently.

Hepatic secretion of apoB-containing lipoproteins (i.e., VLDL) plays an important role in lipid homeostasis in body. Overproduction of hepatic VLDL has been recognized to be linked to hyperlipidemia, because each VLDL particle contains one apoB molecule, the secretion of hepatic apoB directly reflects the production of VLDL by the liver. Inhibition of hepatic apolipoprotein secretion has been demonstrated to lower blood cholesterol and triglycerides in human. In this study, treatment of HepG2 cells with RCE lowered amounts of apolipoproteins secreted into media in a dose-dependent and time-dependent manner (Figs. 2a, b) showing that RCE exhibits apoB lowering effect.

Haeusler et al. have recently demonstrated hepatic FoXo-1 deletion exacerbates lipid abnormalities and thus increases availability of substrates for hepatic cholesterol and triglyceride biosynthesis and VLDL secretion. Similarly, p-FoXo-1

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Fig. 4. Western Blot Expression of p-FoXo-1, G6Pase, PEPCK, p-AMPK and AMPK in HepG2 Cells Treated with RCE (0.1–5 µg/mL) and Ellagic Acid (1 and 10 µM) in Various Dose for 24 h

(a) Proteins (20 µg) of the total cell lysates were loaded in 8% and 10% SDS-PAGE. Immunoblot was performed as mentioned in Materials and Methods. β-Actin was used as loading control. (b), (c) represent fold induction bar graphs of p-FoXo-1 and p-AMPK expression after RCE treatment. Relative density of bands in (a) was quantified by densitometer. Values are mean±S.D. (n=3). *Significantly different from control cells; p<0.05.

Fig. 5. Protein Expression of SREBP-1 in HepG2 Cells Treated with RCE (5 µg/mL) in Different Duration of 0 h, 5 h, and 10 h

(a) Proteins (20 µg) of the total cell lysates were loaded in 8% SDS-PAGE. Immunoblot was performed as mentioned in Materials and Methods. β-Actin was used as loading control. Relative density of bands in (a) was quantified by densitometer. Values are mean±S.E.M. (n=4). *Significantly different from control cells; p<0.05.
tunes up lipolysis through its action on fibroblast growth factor (FGF21). In our study, RCE treatment upregulated the expression of p-Foxo-1 in a dose dependent manner, indicating lipolysis in HepG2 cells, thus lowering of cholesterol and triglyceride biosynthesis.

Polyphenols have been shown to exhibit beneficial effects on dislipidemia in patients with diabetic cardiovascular disease. Synthetic drug metformin and natural polyphenols including resveratrol, apigenin and theaflavins decrease lipid content in HepG2 cells, activating AMPK activity, thus increasing AMPK phosphorylation. The Thr-172 phosphorylation of the activation loop of AMPKα catalytic domain is essential for activation of both the α1 and α2 subunits of AMPK. AMPK phosphorylates and downregulates the expression of genes encoding the hepatic gluconeogenic enzymes G6Pase and PEPCK, which in turn downregulates lipid biosynthesis and upregulates fatty acid oxidation. AMPK phosphorylation inactivates a number of metabolic enzyme involved in ATP consuming cellular events including fatty acid, cholesterol and protein synthesis, involving acetyl-CoA enzyme A carboxylase (ACC) and HMG-CoA reductase, and also activates ATP-generating processes, including the uptake and oxidation of glucose and fatty acids. Thus AMPK activation is a logical therapeutic target for disease related to cellular proliferation, including atherosclerosis and cancer. In the present study, treatment of HepG2 cells with RCE lowered G6Pase and PEPCK expression in a dose-dependent manner. Similarly, p-AMPK was also increased. Thus, RCE might inhibit HMG CoA reductase enzyme activity. These results provide better understanding for the mechanism of lowering of cholesterol biosynthesis and depletion of apoB secretion from hepatic cells during treatment of RCE.

Transcriptional regulation of the genes involved in fatty acid metabolism is presently considered as the major long-term regulatory mechanism controlling lipid homeostasis. It is executed by a variety of transcriptional factors among sterol response element binding proteins (SREBPs) which are major transcription factors involved in fatty acid synthesis. Among SREBPs, SREBP-1c and SREBP-2 are the branching points of cholesterol and fatty acid metabolism. The main role of SREBP-2 is indeed geared towards cholesterol metabolism, whereas SREBP-1a and -1c link with both cholesterol and fatty acid homeostasis. In the present study, RCE treatment lowered expression of SREBP-1 in HepG2 cells treated with 5μg/mL (Fig. 4), which might result from inhibition of transcription of cholesterol and fatty acid.

In summary, RCE may be a suitable alternative to conventional lipid-lowering drugs in patients with elevated levels of cholesterol and at risk of atherosclerosis. While the present study has demonstrated that RCE decreases cholesterol levels in vitro, further in vivo study is warranted to explore role of individual active component(s).

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