Anti-obesity and hypolipidemic effects of *Rheum undulatum* in high-fat diet-fed C57BL/6 mice through protein tyrosine phosphatase 1B inhibition

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Protein tyrosine phosphatase 1B (PTP1B) is important in the regulation of metabolic diseases and has emerged as a promising signaling target. Previously, we reported the PTP1B inhibitory activity of *Rheum undulatum* (RU). In the present study, we investigated the metabolic regulatory effects of RU in a high-fat diet (HFD) model. RU treatment significantly blocked body weight gain, which was accompanied by a reduction of feed efficiency. In addition, it led to a reduction of liver weight mediated by overexpression of PPARα and CPT1 in the liver, and an increase in the expression of adiponectin, aP2, and UCP3 in adipose tissue responsible for the reduction of total and LDL-cholesterol levels. Chrysophanol and physcion from RU significantly inhibited PTP1B activity and strongly enhanced insulin sensitivity. Altogether, our findings strongly suggest that 2 compounds are novel PTP1B inhibitors and might be considered as anti-obesity agents that are effective for suppressing body weight gain and improving lipid homeostasis. [BMB reports 2012; 45(3): 141-146]

INTRODUCTION
The increased incidence of type 2 diabetes mellitus and obesity in the population has fueled an intensified search for new therapeutic treatment options (1). The relationship between obesity and type 2 diabetes mellitus is associated with insulin resistance, which can be due to multiple defects in signal transduction (2). Insulin-mediated signal transduction is regulated by the balance between tyrosine phosphorylation and dephosphorylation at the earliest steps, namely the insulin receptor (IR) and its primary substrates - the insulin receptor substrate (IRS-1/2) proteins. Thus, protein tyrosine phosphatases (PTPs) are negative regulators in normal physiological states and play a role in some states of insulin resistance (3).

Protein tyrosine phosphatases (PTPs), have emerged as a new and promising class of signaling targets, since the discovery of PTP1B as a major drug target for diabetes and obesity (4). PTP1B knockout mice showed improved insulin sensitivity in glucose and insulin tolerance tests as a result of prolonged phosphorylation of the insulin receptor substrate proteins (5). In addition, increased leptin sensitivity in these knockout animals resulted in decreased adiposity and resistance to weight gain, and was associated with decreased leptin levels and an increased metabolic rate (6, 7). Further experiments with antisense oligonucleotides used to decrease PTP1B expression in adult animals resulted in improved insulin sensitivity in mice (9). Accordingly, inhibition of PTP1B is predicted to be an excellent and novel target to treat type 2 diabetes and obesity (3). In addition, only several of the recent inhibitors have been derived from natural products, which had been considered as a great diverse library for potential drugs (8).

*Rheum undulatum* L. (Polygonaceae, RU), a well known Chinese medicine, is a perennial herb that is distributed and cultivated mainly in South Korea (9). The rhizome of the species is one of the important herbal medicines that are used widely as laxatives, anti-inflammatory agents and anti-blood stagnation agents in East Asia (10, 11). Recently, it was found that RU and 3 compounds isolated from it (desoxyrhapontigenin, emodine, and chrysophanol) showed significant antidiabetic activity based on the oral glucose tolerance test (9). However, little is known about the metabolic disease regulating activity of RU and its anthraquinone derivatives at the molecular level. Previously, we showed PTP1B inhibitory activity of a hot water extract of RU (12). In the present study, we examined the effects of RU and its anthraquinone derivatives (chrysophanol and physcion) through the inhibition of PTP1B and not the activation of nuclear receptors responsible for metabolic disease. We then report the anti-obesity and hypolipidemic activities of RU in a high-fat diet-induced obese mouse model.
RESULTS

RU treatment significantly blocks body weight gain in HFD-fed mice
We examined the effects of RU on body weight, weight gain, and feed efficiency (Fig. 1). The values for body weight, weight gain, and feed efficiency in mice fed a HFD were significantly higher than the corresponding values for mice fed a standard semi-synthetic diet. Supplementation with RU for 8 weeks in HFD treated mice lowered body weights compared to the control HFD group (Fig. 1A). Weight gains were decreased by 52.6% and 23.6%, respectively, in metformin (MT) and RU treated groups (Fig. 1B). Especially, HFD + MT and HFD + RU groups showed significantly reduced feed efficiency (54.0% and 18.5%, respectively) compared to the control group (Fig. 1C). These results showed that RU can prevent the increase of body weight by modulating energy intake in HFD-fed mice.

The extract of RU can improve glucose and lipid homeostasis in HFD-fed mice
We examined the effect of RU on fasting blood glucose levels. The blood glucose levels in HFD-fed mice were higher than levels in mice fed the standard semi-synthetic diet. The fasting blood glucose levels were suppressed in the HFD + MT and HFD + RU treated groups by 24.6% and 6.9%, respectively, compared to levels in the HFD group (Fig. 2A). We also examined the effects of MT and RU on plasma biomarkers (Fig. 2B, C). The HFD + MT and HFD + RU treated groups showed significant decreases of 26.5% and 13.6%, respectively in total cholesterol compared to the HFD group (Fig. 2B). Also, plasma LDL-cholesterol levels were significantly lower by 45.0% and 16.7% in the HFD + MT and HFD + RU treated groups, respectively, than in the HFD group (Fig. 2C). These results showed that some active compounds from RU may improve lipid homeostasis.

Fig. 1. The effects of *Rheum undulatum* extract on body weight, weight gain, and feed efficiency. MT (200 mg/kg) and RU (100 mg/kg) were administered to C57BL6 mice fed a HFD for 8 weeks. Body weight (A) was measured daily. At the end of the experimental period, weight gain (B) and feed efficiency [weight gain (g/8 wk)/food intake (g/8 wk)] (C) were measured. Data were expressed as mean ± S.E.M. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the HFD group.

Fig. 2. The effects of *Rheum undulatum* extract on blood bio-markers. Fasting blood glucose levels (A) were measured in blood taken from the retroorbital sinus after 8 weeks of MT (200 mg/kg) or RU (100 mg/kg) treatment. At the end of the experimental period, plasma samples were analyzed for total-cholesterol (B) and LDL-cholesterol (C). Data were expressed as mean ± S.E.M. (n = 5). *P < 0.05, **P < 0.001 as compared with the HFD group.
RU modulates expression level of genes for glucose and lipid metabolism in HFD-fed mice

We examined the effects of RU on liver and adipose tissue weights. Organ weight was expressed as its relative weight per body weight. The liver and adipose tissue weights in HFD-fed mice were higher than in mice fed the standard semi-synthetic diet. The HFD + MT and HFD + RU treated groups showed significant decreases in liver/body weight compared to the control HFD group (Fig. 3A). However, the HFD + MT and HFD + RU treated groups did not show any differences in epididymal white adipose tissue/body weight compared to the control HFD group (data not shown).

We then examined mRNA expression levels for genes related to glucose and lipid metabolism in liver and epididymal white adipose tissue via quantitative real-time PCR. The HFD + MT and HFD + RU treated groups had increased gene expressions for PPARα and CPT1 in liver compared to the control HFD group (Fig. 3B, C). The HFD + MT group showed significantly increased gene expressions for adiponectin, aP2, and UCP3 in adipose tissue. The HFD + RU group showed the same patterns as the HFD + MT group for adiponectin and aP2 expressions, but not for UCP3 mRNA expression, where expression levels showed a slight increase. (Fig. 3D-F).

Two anthraquinones, chrysophanol and physcion, from RU inhibit protein tyrosine phosphatase 1B (PTP1B)

We examined the inhibitory activities of chrysophanol and physcion from RU against PTP1B. PTP1B activities were measured using p-nitrophenyl phosphate (pNPP) as a substrate, and the known PTP1B inhibitor, ursolic acid (IC50 = 2.4 μM), was used as the positive control. The IC50 values resulting from this experiment were 3.8 μg/ml for RU, and 19.4 μM and 20.0 μM for chrysophanol and physcion, respectively. However, these compounds had no effect on hPPAR-γ/α/δ transactivation (data not shown). These results imply that chrysophanol and physcion were effective as PTP1B inhibitors.

Treatment with chrysophanol and physcion strongly enhances insulin signaling

Tyrrosine autophosphorylation of insulin receptors (IR) is one of the earliest cellular responses to insulin stimulation (16). Autophosphorylation begins with phosphorylation of Tyr1146 and either Tyr1150 or Tyr1151, while full kinase activation requires triple tyrosine phosphorylation (17). Akt Ser473 phosphorylation is an important node for signal transduction in the insulin pathway and for it's accepting a signal from upstream and passing it downstream. To confirm the hypothesis that chrysophanol and physcion enhanced the insulin signal path-

![Fig. 3. Measurements of liver weight and glucose/lipid metabolism regulating gene expression levels by qRT-PCR. Liver/body weight (A) was measured after 8 weeks of treatment with MT (200 mg/kg) or RU (100 mg/kg). Gene expression levels of PPARα (B) and CPT1 (C) in liver, and adiponectin (D), aP2 (E), and UCP3 (F) in epididymal white adipose tissue were quantified by quantitative real-time PCR. The mRNA levels were expressed as the fold increase relative to the HFD group after normalization by the GAPDH mRNA expression. Data were expressed as mean ± S.E.M. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the HFD group.](http://bmbreports.org)
way, we examined their effects on the phosphorylation of the insulin receptor (IR) and AKT Ser\textsuperscript{473} after treatment with insulin. RU (30 ug/ml), chrysophanol (30 μM), and physcion (30 μM) increased IRβ phosphorylation by 4.7, 5.2, and 4.7-fold, respectively, relative to the control (Fig. 4A, B). Also, RU (30 ug/ml), chrysophanol (30 μM), and physcion (30 μM) increased Akt Ser\textsuperscript{473} phosphorylation by 2.7, 2.7, and 2.9-fold, respectively, relative to the control. These results mean that RU, chrysophanol, and physcion enhance insulin signaling by inhibition of PTP1B.

DISCUSSION

Recently, we reported the PTP1B inhibitory activity of a hot water extract of RU (12), and in the present study, we isolated 2 anthraquinone derivatives, chrysophanol and physcion, from an extract of RU and identified their functional activity as novel PTP1B inhibitors. PTP1B is a key element in the negative regulation of the insulin signaling pathway and inhibition of this enzyme is predicted to be an excellent, novel therapeutic target for type 2 diabetes and obesity (4).

The blood-glucose lowering effect of RU and chrysophanol was previously reported after using an oral glucose tolerance test in non-diabetic ICR mice (9), however, that effect and its molecular mechanism were not explored in a high-fat fed mouse model. To check the glucose lowering effect of RU in a HFD model, we fed an RU extract to C57BL/6 HFD mice for 8 weeks. The results showed that RU has a weak fasting glucose lowering effect, but strong anti-obesity and hypolipidemic effects. RU treatment significantly blocked body weight gain in HFD mice, which was accompanied by a reduction of feed efficiency. This effect would not be due to the laxative activity of anthranoids contained in RU, because the laxative-effective dose of anthranoids is ≥25 mg (14), which is contained in 25 g of RU extract, and treatment dose was 100 mg/kg RU. In addition, RU treatment led to a reduction of liver weight, which might be mediated by PPARα and CPT1 overexpression in liver. Furthermore, RU treatment led to an increase in the expression of adiponectin, aP2, and UCP3 in adipose tissue, which might be responsible for the reduction of total cholesterol and LDL-cholesterol levels. Next, we isolated 2 compounds, chrysophanol and physcion, from RU and studied their mechanism of action regarding anti-obesity and hypolipidemic effects. These 2 compounds had no effect on the transcriptional activity of PPARs (data not shown). However, chrysophanol and physcion significantly inhibited PTP1B activity with IC\textsubscript{50} values of 19.4 and 20.0 μM, respectively. Furthermore, treatment with chrysophanol and physcion strongly enhanced insulin sensitivity, which was confirmed by an observation that treatment with these 2 compounds led to a dramatic increase in both tyrosine phosphorylation of the insulin receptor (IR) and Akt phosphorylation. Several stilbene derivatives showed potent PTP1B inhibitory activity (15). Because RU contains many kinds of anthranoids and stilbenes (9), its metabolic regulatory effects might be due to such compounds.

In summary, the present study demonstrated that chrysophanol and physcion from RU are novel PTP1B inhibitors and these compounds might be considered as anti-obesity agents that are effective for suppressing body weight gain and improving lipid homeostasis. In addition, their action of mechanism was not mediated by PPAR transactivation, which is a well known-target for glucose/lipid metabolism. Based on these results, we propose that PTP1B inhibition with RU is a powerful method to modulate metabolic diseases.

MATERIALS AND METHODS

Cell culture

3T3-L1 (mouse embryonic fibroblast, CL-173) cells were purchased from the American Type Culture Collection, and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA) and 1% penicillin/
streptomycin (Invitrogen) at 37°C with 5% CO₂ in air.

**Plant materials**

*Rheum undulatum* (Korean rhubarb, Polygonaceae) rhizomes were purchased from an herb market in Seoul, Korea. The plant was authenticated and deposited at the KIST Gangneung Institute Herbarium, Gangneung, Korea (Voucher No. KIST-091).

**Extraction, isolation and identification of compounds**

RU (300 g) was extracted 3 times with ethanol and evaporated under vacuum at 40°C. The extract (105 g) was reconstituted in 0.5 L of water and re-extracted with 0.5 L of ethyl acetate (EtOAc). The EtOAc soluble fraction was evaporated under vacuum at 40°C. The EtOAc extract (10 g) was subjected to silica gel chromatography using an n-hexane-EtOAc gradient system (100 : 1 to 1% MeOH) to provide 9 fractions. Fraction 2 was separated into 3 subfractions (2-1 to 2-3) by silica gel chromatography using an n-hexane-EtOAc gradient system (100 : 1 to 100% EtOAc). Further separation of subfraction 2-2 by using an n-hexane-EtOAc gradient system (100 : 1) gave 2 subfractions. Further purification of subfractions 2-2-1 and 2-2-2 by using an n-hexane-EtOAc gradient system (200 : 1) resulted in the isolation of chrysophanol (93.7 mg) and physcion (7.7 mg). The structures of chrysophanol and physcion were confirmed by comparing the spectroscopic data (1H, 13C-NMR and MS) with those reported in the literature.

**Physcion**

\[\text{MS: } m/z 284 (M^+); \text{1H-NMR (CDCl}_3\text{)} \delta: 2.38 (3H, s, CH₃), 3.87 (3H, s, OCH₃), 6.61 (1H, s, H-7), 7.01 (1H, s, H-2), 7.29 (1H, s, H-5), 7.55 (1H, s, H-4), 12.05 (1H, s, -OH), 12.25 (1H, s, -OH); \text{13C-NMR (CDCl}_3\text{)} \delta: 22.2 (CH₂), 56.1 (OCH₃).\]

**Chrysophanol**

\[\text{MS: } m/z 254 (M^+); \text{1H-NMR (CDCl}_3\text{)} \delta: 2.47 (3H, s, CH₃), 7.09 (1H, s, H-2), 7.29 (1H, dd, /J = 1.0, 8.5 Hz, H-7), 7.64 (1H, d, /J = 1.5 Hz, H-4), 7.67 (1H, t, /J = 8.0 Hz, H-6), 7.82 (1H, dd, /J = 1.0, 7.5 Hz, H-5), 12.01 (1H, s, -OH), 12.12 (1H, s, -OH); \text{13C-NMR (CDCl}_3\text{)} \delta: 22.3 (CH₂), 113.7 (C-12), 115.9 (C-13), 119.9 (C-5), 121.4 (C-4), 124.4 (C-5), 124.6 (C-7), 133.4 (C-14), 133.6 (C-11), 136.9 (C-6), 149.3 (C-3), 162.4 (C-1), 162.7 (C-8), 182.0 (C-10), 192.5 (C-9).\]

**Animals and diets**

Male C57BL/6 mice (aged 7 weeks) were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan). Animal care and all experiments were conducted under the ethical guidelines of the Institutional Animal Care and Use Committee of the Korea Institute of Science and Technology. After adaptation for 1 week, mice were divided into 4 groups based on their body weight and plasma glucose levels and assigned to the normal diet (ND), high-fat diet (HFD), HFD + MT, or HFD + RU group. During the experimental period, the ND group mice were fed a normal diet and the other 3 groups of mice were fed a standard semi-synthetic diet (AIN-76A) containing 40% beef tallow. ND and HFD groups of mice were administered vehicle only (0.8% carboxymethyl cellulose, CMC), and the other 2 groups of mice were orally fed with MT (200 mg/kg) and RU (100 mg/kg) in 0.8% CMC for 8 weeks. During the experimental period, the mice had free access to food and water, and their body weight, food intake, and water intakes were measured daily. At the end of the experimental period, liver/body weight, and epididymal white adipose tissue/body weight were measured and blood samples were taken from the retro-orbital sinus to determine the plasma biomarkers.

**Fasting blood glucose levels and plasma biomarker analyses**

After 8 weeks of treatment, the mice were fasted 16 hours and fasting blood glucose levels were measured in blood taken from the retro-orbital sinus by Accu-Chek (Roche, Mannheim, Germany). Then, the blood was collected in tubes containing 0.18 M EDTA and centrifuged at 5,000 rpm for 5 min at 4°C. After centrifugation, plasma was separated for estimation of total cholesterol, HDL-cholesterol, and triglycerides. Total cholesterol levels were measured by enzymatic methods using SICDIA L-T-CHO reagents (Eiken Chemical, Tokyo, Japan), and HDL-cholesterol levels were determined by enzymatic methods using L-Type HDL-C (Wako Pure Chemical, Osaka, Japan). Triglyceride levels were measured by GPO-HMPPS using SICDIA L TG reagent (Eiken). LDL-cholesterol levels were calculated from total cholesterol, HDL-cholesterol, and triglyceride levels.

**Total RNA isolation and gene expression analysis**

Liver and epididymal white adipose tissues from sacrificed mice were collected in liquid nitrogen and stored at -80°C until use. Total RNA was isolated from the tissues using the QiAgen Rneasy kit and the QiAgen Lipid Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. cDNA synthesis was performed with 1 µg of total RNA in 20 µl using random primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Oligonucleotide primers were designed for PPARα (GenBank Accession No. NM_011144.6), CPT1 (NM_009948.2), aP2 (NM_024406), UCP3 (AF053352), adiponectin (NM_009605), and GAPDH (NM_008084) using Primer3 program (http://web.bioneer.co.kr/cgi-bin/primer/primer3.cgi). Reactions were performed in a 25 µl volume containing 12.5 µl of SYBR Green reaction buffer, 1 µl of cDNA and 5 pmol of each primer using the 7,500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). After an initial incubation for 2 min at 50°C, the cDNA was denatured at 95°C for 10 min, followed by 40 cycles of PCR (95°C, 15 s, 60°C, 60 s). Data analyses were performed on a 7,500 System SDS software version 1.3.1 (Applied Biosystems). All samples were normalized by the corresponding expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Protein tyrosine phosphatase 1B inhibitory activity**

The enzyme activity was measured using para-nitrophenyl phosphate (p-NPP) as a substrate. p-NPP (20 mM) (New England
Biolabs, Inc., Beverly, MA, USA) and PTP1B (0.05 μg) (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA) were added to each well of a 96-well plate (final volume: 100 μl) in a reaction buffer containing 50 mM citrate buffer (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM DTT with or without natural extracts, and incubated at 37°C for 30 min. The contents of each well were then quenched by addition of 10 μl of 10 N NaOH. The amount of p-nitrophenol produced was estimated by measuring the absorbance at 405 nm. The nonenzymatic hydrolysis of p-nitrophenol produced was estimated by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.

**Immunoblot analysis**

3T3-L1 cells were cultured in DMEM containing 10% FBS and 1% antibiotics. At 80% confluence, the cells were incubated for 5 h in SF-DMEM (DMEM without FBS), and the medium was then replaced with SF-DMEM containing RU, chrysophansol, or physcion. One hour later, the cells were incubated for 0.5 h in SF-DMEM containing 10 μg/ml insulin. After stimulation, the cells were washed twice with PBS and harvested. Cell lysates were boiled in Laemmli sample buffer for 10 min, and 30 μg of each protein sample was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes, and the membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder. The membranes were then incubated overnight with primary antibodies for Akt, phospho-Ser473 Akt, IR β, and phospho-Tyr1131 IGF-I Rβ/Tyr1146 IGF-Rβ, all purchased from Cell Signaling (Beverly, MA, USA). Next, the membranes were washed with 0.1% Tween 20 in Tris-buffered saline and incubated for 2 h with a secondary antibody. The blots were developed using a chemiluminescence detection kit (Amersham Biosciences, Little Chalfont, UK).

**Statistical analysis**

Data are expressed as mean ± S.E.M. Significant differences compared with the HFD group were analyzed by one-way analysis of variance (ANOVA). Differences of P < 0.05 were considered significant.

**Acknowledgements**

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ008092), Rural Development Administration, Republic of Korea.

**REFERENCES**