Anti-diabetic Properties of Chrysophanol and Its Glucoside from Rhubarb Rhizome

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An ethanol extract of rhubarb rhizome exhibited marked glucose transport activity in differentiated L6 rat myotubes. Activity-guided fractionation resulted in the isolation of two anthraquinones, chrysophanol-8-O-β-D-glucopyranoside (1) and chrysophanol (2). The anti-diabetic effect was examined by glucose transport activity, glucose transporter 4 (Glut4) expression in myotubes, and the level of insulin receptor (IR) tyrosine phosphorylation as influenced by tyrosine phosphatase 1B, each of which is a major target of diabetes treatment. Chrysophanol-8-O-β-D-glucopyranoside up to 25 μM dose-dependently activated glucose transport in insulin-stimulated myotubes. Increased tyrosine phosphorylation of IR due to tyrosine phosphate 1B inhibitory activity with an IC_{50} value of 18.34±0.29 μM and unchanged Glut4 mRNA levels was observed following chrysophanol-8-O-β-D-glucopyranoside treatment. Chrysophanol up to 100 μM exerted mild glucose transport activity and elevated the tyrosine phosphorylation of IR via tyrosine phosphate 1B inhibition (IC_{50}=79.86±0.12 μM); Glut4 mRNA expression was also significantly increased by 100 μM. The ED_{50} values of the two compounds were 59.38±0.66 and 79.69±0.03 μM, respectively. Therefore, these two anthraquinones from rhubarb rhizome, chrysophanol-8-O-β-D-glucopyranoside and chrysophanol, have mild cytotoxicity and anti-diabetic properties and could play metabolic roles in the insulin-stimulated glucose transport pathway.

Key words rhubarb; anthraquinone; diabetes; glucose transport

Type 2 diabetes mellitus is a heterogeneous metabolic disorder characterized by impaired insulin secretion from pancreatic beta cells and insulin resistance in peripheral tissues such as liver, adipose tissue, and skeletal muscle.1,2,3 Skeletal muscles account for approximately 75% of glucose absorption under insulin-stimulated conditions, and glucose transport is the rate-limiting step in primary glucose disposal and utilization especially in insulin-targeted skeletal muscle. Thus, its alteration is one of the major defects in type 2 diabetes both in vitro and in vivo.2—4) Increased glucose transport in skeletal muscle is reported to be mediated by glucose transporter 4 (Glut4), which translocates into the plasma membrane in response to insulin.5) The defect that causes type 2 diabetes has previously been shown to be a result of decreased intracellular Glut4 trafficking,6) and increased expression of Glut4 in skeletal muscle lowers blood glucose and enhances glucose transport and glucose utilization in skeletal muscles, among other changes.7—9) For these reasons, some targeted anti-diabetic drugs have been developed; for example, metformin (dimethylbiguanide), a commonly used glucose-lowering reagent, is thought to increase glucose uptake in skeletal muscle.10) Rosiglitazone and pioglitazone both act by reducing glucose production in the liver and increasing insulin-dependent glucose uptake in muscle cells.11) Therefore, we investigated the anti-diabetic therapeutic potential of several compounds to maximize glucose transport in L6 myotubes, which have been widely used to study the mechanism of insulin-stimulated glucose transport.

Several tyrosine phosphatases have been implicated in the regulation of insulin action, particularly tyrosine phosphatase 1B.12) Tyrosine phosphatase 1B is ubiquitously expressed and appears to be involved in the regulation of glucose homeostasis and energy expenditure by acting as a key negative regulator of insulin receptor (IR)- and insulin receptor substrate-1 (IRS-1)-mediated signaling pathways.13) The recent identification of tyrosine phosphatase 1B has raised the possibility that small molecule inhibitors of this enzyme could act as insulin sensitizers and anti-diabetic drugs by increasing glucose transport.14—16)

Rhubarb is the rhizome of Rheum undulatum L., R. palmatum, R. tanguticum Maxim., R. officinale Bail., and R. coreanum Nakai, which are distributed in Korea, Japan and China. The dried rhubarb rhizome is an important herbal medicine that is traditionally used for its purgation, antibacterial, anti-tumor, and anti-mutagenic properties and its ability to cure mental and renal disorders.17,18) It is known to contain bioactive components including anthraquinones, dianthrones, stilbenes, anthocyanins, flavonoids, tannins, organic acids, and chromones. The main bioactive constituents of rhubarb are anthraquinone derivatives that can inhibit cellular proliferation, induce apoptosis, and prevent metastasis.19,20) However, anti-diabetic properties of rhubarb are less well studied. In the course of screening natural medicinal plants that cause increased glucose transport activity in basal- and insulin-stimulated L6 myotubes, we selected Korean rhubarb rhizome and isolated its bioactive compounds.

Thus, in this study, we sought to identify active compounds to improve glucose transport in myotubes and to evaluate their roles by measuring tyrosine phosphorylation of IR, tyrosine phosphate 1B activity, and Glut4 expression. Our data collectively suggest that isolated compounds from rhubarb rhizome are potential diabetes therapeutics.

MATERIALS AND METHODS

Plant Material Cultivated Korean rhubarb rhizome was purchased from Yuseong herbal drug market, Daejeon, Korea. A voucher specimen has been deposited in Chungnam National University.

Extraction and Isolation The dried and milled rhizome of rhubarb (5 kg) was extracted with ethanol, three times. The ethanol extract was combined and concentrated to yield...
a residue (650 g), which was suspended in water and then successively partitioned with hexane (30 g), EtOAc (300 g) and BuOH (100 g). The EtOAc-soluble fraction was diluted with acetone, and then filtrated through filter paper to give precipitated powder and acetone fraction (226.1 g). The acetone fraction was subjected to silica gel column chromatography using the mixtures of CHCl₃ and MeOH in increasing polarity (80:1 to 0:1) to separate into six fractions (#1—#6). The subfraction #3 was rechromatographed on silica gel column with a stepwise gradient of CHCl₃ and MeOH (4:1 to 0:1) to give chrysophanol-8-O-β-D-glucopyranoside (1) (600 mg). The hexane-soluble fraction was subjected to silica gel column chromatography, eluted with hexane–EtOAc (60:1 to 0:1) to give five fractions (H#1—H#5). The fraction H#1 was rechromatographed on silica gel column, hexane–EtOAc (40:1) as eluting solvent to obtain chrysophanol (2) (1.5 g).

**Cell Culture** L6 rat myoblasts (CRL-1458TM) were obtained from ATCC (Rockville, MD, U.S.A.), cultured in DMEM containing 10% FBS and 5% WEHI-conditioned medium to provide IL-3.20 After compound treatment for 1 h, cells were treated with 200 nM insulin for 20 min and added 1 mCi [3H]-glucose and 5 mM glucose as final concentrations for an hour. Glucose uptake (about 90% confluence), the medium was switched to DMEM with 2% horse serum and replaced every second day. Experiments were initiated on day 6 when myotube differentiation was completed.

**Glucose Transport Assay** Myotubes were cultured on 12-well plates, incubated with serum-free DMEM for 18 h, and washed with Krebs-Ringer Heps (KRH) buffer (NaCl 120 mM, KCl 5 mM, CaCl₂ 2 mM, MgSO₄ 1.5 mM, HEPES 20 mM). After compound treatment for 1 h, cells were treated with 200 nM insulin for 20 min and added 1 μCi 2-deoxy-O-[¹⁴C]-glucose and 5 mM glucose as final concentrations for an additional 15 min. The reaction was terminated by placing the plates on the ice, washing the cells three times with ice-cold PBS. The cells were lysed in 0.5 M NaOH with 0.1% SDS for 1—2 h at 37°C, and the radioactivity retained by cell lysates was determined by scintillation counter.21 Aliquots of 1 μg of total RNA from each sample were reverse transcribed to cDNA using an AccuPower® CycleScript RT PreMix (dT20) according to manufacturer’s instructions from Bioneer Inc. (Daejeon, Korea). PCR primers used in this study included; Glut4: 5'-CGG GAC GTG GAG CTG GCC GAG GAG-3’ and 5’-CCC CCT CAG CAG GTA GTG A-3’; Gapdh: 5’-TAG ACG GGA AGC TCA CTG GC-3’ and 5’-AGG TCC ACC ACC CTG TTG CT-3’.

**Cell Cytotoxicity** L6 myoblasts were seeded at 5×10⁵ cells/well in 96-well microplates and allowed to attach for 24 h. Compounds were added to the medium at various concentrations (0 to 100 μM). After 48 h treatment, cell proliferation and cytotoxicity were conducted by Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan). CCK-8 (10 μl) was added to each well and incubated for 3 h at 37°C, then assayed by measuring the absorbance at 450 nm using microplate reader (Dynatech MR700). Three replicated wells were used for each experimental condition.

**RESULTS AND DISCUSSION**

In the present study, we employed L6 myotubes to evaluate the effect of 56 medicinal plants on glucose transport; we ultimately selected Korean rhubarb rhizome. Plant medicines have a long history as treatments for diabetes, and numerous phytotherapies to manage risk factors such as hyperglycemia have been investigated in animal models and patients over the years.23 Muscular glucose transport activity-guided isolation of bioactive compounds from rhubarb rhizome yielded chrysophanol-8-O-β-D-glucopyranoside (1) from the EtOAc-soluble fraction and its aglycone chrysophanol (2) from the hexane-soluble fraction (Fig. 1). In studies of diabetes, chrysophanol-8-O-β-D-glucopyranoside has been reported to prevent hyperglycemia-associated diabetes through its mammalian intestinal α-glucosidase inhibitory activity, while chrysophanol inhibited postprandial hyperglycemia by 42.3%.23,24

To assess the anti-diabetic effect of the isolated compounds, we first conducted glucose transport assays with var-
Table 1. Inhibitory Effects of Compounds 1 and 2 on Tyrosine Phosphatase 1B

<table>
<thead>
<tr>
<th>Compound name</th>
<th>IC_{50} (µM)</th>
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<tbody>
<tr>
<td>Chrysophanol-8-O-glucopyranoside (1)</td>
<td>18.34±0.29</td>
</tr>
<tr>
<td>Chrysophanol (2)</td>
<td>79.86±0.12</td>
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a) The values represent the mean±S.D. of three experiments.

Fig. 2. Glucose Transport Activity by Compounds in Basal- and Insulin-Stimulated L6 Myotubes

(A) Differentiated myotubes were incubated in the presence of compound 1 (0—50 µM) for 1 h, and then glucose transport activity was investigated. (B) Cells were exposed with compound 2 (0—100 µM) for 1 h and conducted the glucose transport assay. Data are presented as means±S.D. of triplicate experiments.

Fig. 3. Effects of Compounds on Insulin Receptor Tyrosine Phosphorylation in 32D^{IR} Cells

(A) 32D^{IR} cells were incubated with compound 1 (0—50 µM) for 1 h followed by 5 min treatment with 200 nM insulin. The cell lysates (50 µg) were subjected to SDS-PAGE to measure P-Tyr and total IRβ. (B) Cells were incubated with compound 2 (0—100 µM), and conducted Western blotting using antibodies for P-Tyr and IRβ. The protein level was normalized by glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

In summary, two anthraquinones that affected glucose transport in myotubes with mild cytotoxicity were isolated from Korean rhubarb rhizome. Chrysophanol-8-O-β-d-glucopyranoside significantly enhanced insulin-stimulated glucose transport by IR activation, but did not influence Glut4 expression. Chrysophanol influenced insulin-responsive glucose transport by increasing IR phosphorylation and Glut4 mRNA expression. The results suggest that chrysophanol-8-

ious concentrations of the compounds. Incubation of L6 myotubes with compound 1 (0, 1, 5, 10, 25, 50 µM) for 1 h, significantly enhanced insulin-stimulated glucose transport to 139, 148, 231, 255 and 254%, respectively, compared with the negative control without insulin treatment (100%). Treatment with up to 10 µM compound 1 alone (basal level) did not influence the activity, while the activity was slightly increased to 132 and 131% at 25 and 50 µM, respectively (Fig. 2A). Compound 2 increased the activity to 141, 174, and 177% at doses of 10, 25 and 100 µM, respectively, in insulin-stimulated L6 myotubes, with no detectable changes in basal level (Fig. 2B). Our results suggest that compounds 1 and 2 affect insulin-stimulated muscular glucose transport and could be used as insulin sensitizers for diabetes treatment.

To investigate the role of compounds with IR-regulated glucose transport activity, we assessed tyrosine phosphatase 1B in vitro. As a critical negative regulator of the insulin signal transduction cascade, tyrosine phosphatase 1B acts to reverse tyrosine kinase action and is a key phosphatase for IR and IRS-1, major mediators of the glucose transport pathways.25,26) Tyrosine phosphatase 1B has also been implicated in insulin-dependent pathways and in the insulin insensitivity that is the most common pathology of type 2 diabetes and obesity.27) Therefore, we measured the tyrosine phosphatase 1B inhibitory activity of the isolated compounds. The results are shown in Table 1; compounds 1 and 2 inhibited this enzyme with IC_{50} values of 18.34±0.29 and 79.86±0.12 µM, respectively (Table 1). The anthraquinones from Saussurea lappa, emodin-8-O-β-d-glucopyranoside, rhein-8-O-β-d-glucopyranoside and chrysophanol, were reported to be moderate inhibitors against tyrosine phosphatase 1B in vitro.28) Similarly, compound 2 exhibited moderate inhibitory activity in this study, but compound 1 has not previously been reported to be a potent inhibitor of tyrosine phosphatase 1B. To further identify whether both compounds could inhibit tyrosine phosphatase 1B in cells, we tested the tyrosine phospho-
Cells were treated with compound lysis, total RNA was isolated and reverse transcribed to cDNA for RT-PCR analysis. (B) Myotubes were exposed to compound -D-glucopyranoside and chrysophanol could be considered as potential therapeutic reagents for diabetes due to their enhancing effects on insulin-dependent glucose transport. Further studies are needed to clarify their additional effects.

REFERENCES