Isolation of Intestinal Glucose Uptake Inhibitor from *Punica granatum* L.

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Abstract

Inhibition of intestinal glucose uptake is beneficial in reducing the blood glucose level for diabetes. To search for an effective intestinal glucose uptake inhibitor from natural sources, 70 native edible plants, fruits and vegetables were screened using Caco-2 cells and fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG). A compound that was able to inhibit glucose uptake was isolated from methanol extract of *Punica granatum* L. and called PG-1a. PG-1a appears to be a phthalic acid-diisononyl ester-like compound (PDE) with molecular weight of 418. The inhibitory effect of PG-1a on intestinal glucose uptake was dose-dependent with 89% inhibition at 100 µg/mL. Furthermore, the intestinal glucose uptake inhibitory effect of PG-1a was 1.2-fold higher than phlorizin, a well known glucose uptake inhibitor. This study suggests that PG-1a could play a role in controlling the dietary glucose absorption, and that PG-1a can effectively improve the diabetic condition, and may be used as an optional therapeutic and preventive agent.

Key words: *Punica granatum* L., glucose uptake, 2-NBDG, Caco-2 cell, brush border membrane vesicle

INTRODUCTION

Diabetes is a disease characterized by chronic hyperglycemia, which is not only a symptom of diabetes but also a pathogenic factor leading to a number of microvascular and macrovascular complications (1,2). Management of diabetes at present relies mainly on reduction of dietary carbohydrate intake and the use of hypoglycemic agents to lower the blood glucose levels. Control over the intestinal absorption of glucose would help to improve levels of glucose in the blood stream and reduce complications. One of the most effective ways to control the postprandial blood glucose level is to inhibit α -glucosidase or amylase, and therefore a number of inhibitors of these enzymes have been developed (3-5). However, inhibitors of these enzymes are not able to prevent glucose absorption when glucose itself has been ingested. Hence, it might be important to inhibit intestinal glucose absorption as well as glucosidase or amylase activity for the regulation of postprandial blood glucose levels. We have investigated the intestinal glucose uptake/transport inhibitor from various Korean edible plants, among which the methanol extract of pomegranate (*Punica granatum* L.) was selected for the isolation and purification of a glucose uptake inhibitor.

Punica granatum L. (PG) is an edible fruit cultivated

in many countries, including Afghanistan, India, China, Russia, and some parts of the United States. Pharmacological properties of PG extracts have been scrutinized, with anti-microbial (6), anti-parasitic (7), antiviral (8), anti-cancer (9) effects noted. An extract of the flowers lowers blood sugar in rodents (10), and the fresh juice inhibits LDL oxidation and atheromatous plaque formation in rodents and humans (11).

In this study, we used solvent partition, silica gel column chromatography, thin-layer chromatography (TLC), and high performance liquid chromatography (HPLC) to identify a potent inhibitor of intestinal glucose uptake from *Punica granatum* L. (PG). The isolated compound was then analyzed by EI-MS and ¹³C/¹H-NMR to elucidate its putative chemical structure. For the measurement of intestinal glucose uptake, we used a human intestinal epithelial cell line, Caco-2, and fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) as a tracer of intestinal glucose uptake. Caco-2, which is widely used to investigate the intestinal nutrient transport *in vitro*, is a human colon adenocarcinoma cell line that spontaneously exhibits various enterocytic characteristics such as brush border enzymes and glucose transporters (12). 2-NBDG is a new fluorescent derivative of glucose which is readily incorporated and metabolized into living cells, yield-

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ing a non-fluorescent derivative (13). Several studies suggested a potential use for the 2-NBDG in measurement of glucose uptake and viability in cell cultures using HepG2 human hepatocarcinoma cells, L6 rat skeletal cells, 3T3 adipocytes, erythrocytes, coronary endothelial cells, cardiomyocytes, human lung fibroblasts, and rabbit enterocytes (14-20). Therefore, the effects of intestinal glucose uptake inhibitor isolated from PG were investigated using Caco-2 cells and NBDG.

MATERIALS AND METHODS

Samples

70 Korean plants were obtained from a local market (Gyeong-dong market, Seoul, Korea) or oriental medicine store in Seoul, Republic of Korea. Samples were identified by the Division of the Oriental Medicine, Semyung University, and the voucher of the specimens was preserved. Each sample was blanched for 5 min to inactivate enzymes, homogenized with Ultra-Turrax (IKA-Lab, Muhsamstrasse, Berlin, Germany), and centrifuged at $10,000\times g$ for 30 min. The samples were extracted with various solvents (hexane, acetone, methanol and hot water) for 2 hr and lyophilized.

Cell culture

Caco-2 cells obtained from American Type Culture Collection (Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA) and 1% antibiotic-antimycotic (Gibco Life Technologies). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and the medium was changed every other day.

Glucose uptake in Caco-2 cell

Cells were seeded on 24-well plate at a density of 6×10^4 cells/well and cultured for 13~14 days. Uptake experiments were performed using PBS containing 100 μ M 2-NBDG (Invitrogen, Eugene, OR, USA) and PG (0.1 mg/mL) at 37°C for 120 min. After incubation, the cells were washed with ice-cold PBS to stop the uptake. The 2-NBDG fluorescence intensity was measured with a spectrofluorometer (Excitation: 485 nm, Emission: 535 nm, TECAN Austria GmbH, Salzburg, Austria). The cell viability was determined with MTT assay and the glucose uptake inhibitory effect was adjusted as the viable cell mass.

Isolation of glucose uptake inhibitor, PG-1a

Pomegranates (*Punica granatum*, PG) were purchased from the Gyeong-dong market in Seoul, Korea. The dried fruits of PG (1 kg) were powdered and extracted

with methanol at room temperature for 5 days. Filtrate of the methanol extract (560 g) was evaporated at 40°C and was dissolved in deionized water and partitioned 4 times with hexane, chloroform, and ethyl acetate. The methanol residue fraction (20 g) with high inhibitory effect on intestinal glucose uptake was then subjected to a silica gel 60G column (5.5×45 cm) by gradient elution with a mixture of the solvents (CHCl₃-MeOH) at a flow rate of 2 mL/min, and resulted in 18 subfractions. The subfractions (3.5 g) that exerted the highest inhibitory effect on glucose uptake were rechromatographed on silica gel column (4×35 cm) with the same eluant at a flow rate of 0.5 mL/min, and the fractions that showed most inhibitory effect were purified by successive preparative thin-layer chromatography (TLC) on an ODS-gel plate (20×20 cm, Merk Co., Darmstadt, Germany) using methanol and water (4:1, v/v) as developing solvents. High-performance liquid chromatography (HPLC, Waters Co., Milford, MA, USA) equipped with analytical column of μ -BondapakTMC₁₈ type (reverse phase, 7.8×300 mm) was used for the further purification. The data were collected over the range 190~800 nm and detection was performed at 228 nm. Analytical separations were carried out at flow rate of 2.0 mL/min using a 65 min linear gradient of 90~100% methanol in distilled water.

Structural analysis of PG-1a

The putative chemical structure of the isolated glucose uptake inhibitor was analyzed by NMR analysis. ¹H and ¹³C-NMR spectra were measured on a Bruker model Avance-500 (Bruker, Rheinstetten, Germany) instrumented at 500 and 125 MHz, respectively. The sample (5 mg) was dissolved in methyl-d₃ alcohol-dl (MeOD), and analyzed with tetramethylsilane (δ_H 0.00; δ_C 0.00) as an international standard. Electron ionization mass (EI-MS, JEOL, Tokyo, Japan) was operated at 70 eV and 250°C in chamber, and sample (0.3 mg) dissolved in methanol was analyzed. The sample (0.3 mg) dissolved in meta nitrobenzyl alcohol (Meta MBA) was applied to fast atom bombardment mass (FAB-MS, JEOL) equipped with Xe gas and at room temperature.

Preparation of enterocytes

An approximately 3 cm long segment of the duodenum from ICR mice was excised and washed in ice-cold phosphate buffered saline (PBS) containing 0.1 mM dithiothreitol (DTT). The tissue was then incubated with Hank's salt solution supplemented with 1.5 mM EDTA and 0.5 mM DTT at 37°C for 20 min. The cells were collected, and incubation of this tissue was repeated to collect more cells. The cells were pelleted by low speed centrifuge ($700\times g$).

Cell viability assay

The 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the toxicity of isolated glucose uptake inhibitor, PG-1a. Purified PG-1a (100, 200, and 500 $\mu\text{g/mL}$) was treated to isolated enterocytes and cultured for 24 hr. MTT (5.0 mg/mL) solution was added to each well and incubated for 4 hr at 37°C. Formazan formed by viable cells was dissolved with dimethyl sulfoxide (DMSO) and absorbance at 570 nm was determined by microplate reader (Bio-Tek, Winooski, VT, USA).

Statistical analysis

Results were presented as mean $\pm$ SD, and the data were analyzed by ANOVA followed by Turkey HSD's post-hoc test. A level of $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

To search for an effective intestinal glucose uptake inhibitor from natural sources, 70 native edible plants, fruits and vegetables were screened using Caco-2 cells, a well-accepted model of human intestinal absorption (Table 1). Among the screened extracts, the methanol extract of *Punica granatum* L. (PG) exhibited the highest inhibitory effect when compared with that of other extracts (Table 1).

Punica granatum L. (PG) fruit, commonly known as pomegranate, is widely consumed fresh and in beverage

Table 1. Inhibitory activity of various edible plants on intestinal glucose uptake

Plants used for screening	Glucose uptake inhibitory effect (%) ¹⁾		
	Fr. A	Fr. B	Fr. C
<i>Punica granatum</i>	35.3	38.0	29.0
<i>Rosa rugosa</i>	31.3	22.5	-6.2
<i>Citrus unshiu</i>	30.6	12.5	7.7
<i>Eucommia ulmoides</i> (leaf)	30.0	8.3	-9.4
<i>Eucommia ulmoides</i> (bark)	26.2	-4.4	-6.7
<i>Diospyrus kaki</i>	25.0	28.0	3.9
<i>Angelica gigas</i>	25.0	6.9	-2.8
Green tea	23.6	29.5	12.0
<i>Comus officinalis</i>	28.1	16.4	13.2
<i>Zizyphus jujube</i> var. intermis	22.3	23.2	3.8
<i>Rubus coreanus</i>	17.6	-4.8	6.1
<i>Raphnus sativus</i>	12.5	34.0	21.6
<i>Glycyrrhiza glabra</i>	14.9	13.9	0.7
<i>Raphanus sativus</i>	12.5	34.0	21.6
<i>Ginkgo biloba</i>	-8.0	12.0	3.5

¹⁾Glucose uptake inhibitory effect (%): The percentage of intestinal glucose uptake inhibition for each sample was calculated comparison with the control. The final concentration of each sample was 0.1 mg/mL. Fr. A: cold water extract, Fr. B: methanol extract, Fr. C: hot water extract.

forms. The edible part of PG is rich in polyphenolic compounds such as anthocyanins and hydrolysable tannins. There are several reports showing that some polyphenols reduce glucose uptake (21-23). We therefore attempted to isolate and purify the active compound from PG. The methanol extract of PG (CM) with an inhibitory effect on intestinal glucose uptake from the Caco-2 cells was subsequently refluxed 4 times with hexane (M-H), chloroform (M-C) and ethyl acetate (M-E) to fractionate each solvent-soluble compound. The final residue (M-M) was dried. The methanol residue fraction (M-M) that showed the highest inhibitory effect ($45 \pm 2\%$, Fig. 1) on glucose uptake in Caco-2 cells was chromatographed on the silica gel column yielding 18 sub-fractions. Among them, subfraction 4 (M-Ma) was found to inhibit glucose uptake by more than 40% ($43 \pm 2\%$, Fig. 2A), and was further chromatographed on second silica gel column to yield 26 sub-fractions. Fraction 12 (M-Mb) showing the highest inhibitory effect ($52 \pm 2\%$, Fig. 2B) on intestinal glucose uptake and was further chromatographed on preparative ODS TLC. Five bands (PG-1 ~ PG-5) were isolated and monitored for the glucose uptake inhibitory effect in Caco-2 cells. As depicted in Fig. 3, PG-1 with an R_f value of 0.43 exhibited a marked inhibitory effect ($75 \pm 3\%$), which was 1.4- and 2.0-fold of M-Mb (active fraction obtained by the 2nd silica gel column chromatography) and CM (methanol extract of PG), respectively.

To purify the active component of the intestinal glucose uptake inhibitor, the selected fraction from the TLC plate (PG-1) was subjected to μ -BondapakTMC₁₈ reverse phase column with an HPLC system. The final isolated compound, PG1-a, showed a significant single peak and

Fig. 1. Inhibitory effects of *Punica granatum* extract fractionated by solvent partitioning on intestinal glucose uptake in Caco-2 cell. The glucose uptake inhibitory activity for each fraction was calculated as the % of the control activity (100%). Final concentration of sample was 0.1 mg/mL. Data represent the mean ($n = 3$) $\pm$ SD. M-H: hexane extract, M-C: chloroform extract, M-E: ethyl acetate extract, M-M: methanol extract.

Fig. 2. Inhibitory effect of (A) M-M and (B) M-Ma subfractions separated by the silica gel open column chromatography on intestinal glucose uptake in Caco-2 cell. M-M: methanol extract, M-Ma: subfraction 4 of (A). A: Eluants were mixtures of CHCl_3 and MeOH (100:0 (No. 1, 2), 80:20 (No. 3~5), 70:30 (No. 6, 7), 60:40 (No. 8, 9), 50:50 (No. 10, 11), 40:60 (No. 12, 13), 30:70 (No. 14, 15), 20:80 (No. 16, 17), 0:100 (No. 18); v/v). B: Eluants were mixtures of CHCl_3 and MeOH (100:0 (No. 1), 85:15 (No. 2), 83:17 (No. 3~8), 80:20 (No. 9~14), 77:23 (No. 15~20), 75:25 (No. 21~23), 70:30 (No. 24, 25), 0:100 (No. 26); v/v). Sample concentration was 0.1 mg/mL. Data represent the mean ($n=3$) $\pm$ SD.

Fig. 3. Inhibitory effects of *Punica granatum* extract subfractions separated by the preparative TLC on intestinal glucose uptake in Caco-2 cell. TLC was carried out on ODS gel plate with methanol and water (4:1) solvent system and detected with UV lamp (256 nm). The glucose uptake inhibitory activity for each fraction was as the % of the control activity (100%). PG-1~PG-5: Fractions separated by the TLC, M-Mb: active fraction obtained by the second silica gel open column chromatography, CM: methanol extract of *Punica granatum*. The concentration of sample was 0.1 mg/mL. Data represent the mean ($n=3$) $\pm$ SD.

Fig. 4. HPLC (A) and thin-layer chromatography analysis (B) of the PG-1a, intestinal glucose uptake inhibitor isolated from *Punica granatum*. The HPLC analysis was performed with a Waters 2690 system equipped with a UV detector (at 228 nm). The column (7.8 $\times$ 300 mm) was eluted with the linear gradient of 90~100% methanol in distilled water at a flow rate of 2.0 mL/min. Injection volume was 60 μ L. For TLC analysis, 1 μ L (1/10 dilution in MeOH) sample was spotted on the ODS gel plate and developed with the solvent of methanol and water (4:1, v/v). PG-1a was visualized with 5% H_2SO_4 in EtOH (v/v).

spot on HPLC (Fig. 4A) and TLC (Fig. 4B), respectively. To identify the structure of this compound, the $^{13}\text{C}/^1\text{H}$ -NMR, FAB-MS, and EI-MS assays were performed. Based on the combination with spectral data obtained

by NMR and mass spectrometry, PG-1a is speculated to be a phthalic acid diester compound. In the FAB-MS and EI-MS, two peaks corresponding to $[\text{M}+\text{H}]^+$ 419 m/z and $[\text{M}+\text{Na}]^+$ 441 m/z were observed, suggesting the

Fig. 5. EI-MS spectrum (A) and FAB-MS spectrum (B) of PG-1a isolated from *Punica granatum*. The spectrum was recorded on positive ion EI mass spectrometer and on positive ion FAB mass spectrometer.

molecular weight of PG-1a to be 418 (Fig. 5). The base peak (149 m/z) of EI-MS suggested the presence of phthalic acid residue (Fig. 5A). The ^1H -NMR spectrum (Fig. 6A) exhibited characteristic peaks, including aromatic proton signals (7.7 and 7.6 ppm) and many substituted aliphatic protons (0.8~1.7 ppm). The ^{13}C -NMR spectrum (Fig. 6B) showed several informative signals comprising ester carbonyl carbon (169.4 ppm) and hydroxylated aliphatic carbon (67.3, 67.0 ppm). The compound PG-1a did not exhibit TLC-color reactions with FeCl_3 , ninhydrin and bromocresol green, which is in good agreement with the results of the NMR and mass spectrometer data. In the current study, we speculated that PG-1a is a phthalic acid-diisononyl ester-like compound. PG-1a showed no cytotoxicity on rat enterocytes at concentrations of 100, 200, and 500 $\mu\text{g/mL}$ (data not shown).

To confirm the inhibitory effect of PG-1a on intestinal glucose uptake, we examined the 2-NBDG uptake in Caco-2 cells. PG-1a showed dose dependent inhibitory effect with $89 \pm 5\%$ inhibition of glucose uptake at 100 $\mu\text{g/mL}$ concentration as compared to control (Fig. 7). The

effect of PG-1a was 2.3-fold higher than methanol extract of PG (CM). Furthermore, PG-1a showed a 1.2-fold higher inhibitory effect than phlorizin, which is known as a classical inhibitor of glucose uptake at the same concentration. The yields and IC_{50} values are listed in Table 2. The IC_{50} values of PG-1a and phlorizin on intestinal glucose uptake in Caco-2 cell assay system were 56.2 $\mu\text{g/mL}$ and 66.7 $\mu\text{g/mL}$, respectively.

Inhibition of intestinal glucose uptake would be beneficial for glucose homeostasis. Currently, a number of strategies are used to treat diabetes, such as α -glucosidase inhibitors and/or inhibition of glucose uptake in the intestine (24). α -Glucosidase inhibitors, such as acarbose and miglitol, which inhibit the breakdown of complex carbohydrates into simple sugars, work only with a meal containing complex carbohydrates (5,25). On the other hand, PG-1a isolated in our study was found to inhibit the intestinal absorption, which could compensate for the limitations of current α -glucosidase inhibitors.

We propose that the PG-1a might affect the sodium-dependent glucose co-transporter (SGLT)-1 gene expression. We have previously reported that PG treat-

Fig. 6. ^1H -NMR (A) and ^{13}C -NMR (B) spectrum of intestinal glucose uptake inhibitor, PG-1a, isolated from *Punica granatum*. PG-1a was dissolved in MeOD and recorded on high resolution NMR spectrometer operating at 125 and 500 MHz, and 25°C .

Fig. 7. Inhibitory effect of intestinal glucose uptake inhibitor, PG-1a, isolated from *Punica granatum* in Caco-2 cell. The glucose uptake inhibitory activity for each fraction was calculated as the % of the control activity (100%). CM: methanol extract of *Punica granatum*, Phlorizin: Classical inhibitor of SGLT1. The concentrations of PG-1a were 20, 50, and 100 $\mu\text{g/mL}$. CM and phlorizin were 100 $\mu\text{g/mL}$. Data represent the mean ($n=3$) $\pm$ SD. * $p<0.05$ vs. CM, # $p<0.05$ vs. phlorizin.

ment reduced the SGLT1 protein expression in Caco-2 cells and SGLT1 activity in brush border membrane vesicles obtained from the mice small intestine after PG treatment (26). In terms of glucose transporter inhibitors, phlorizin and its analogs have been developed for use in diabetes and show potential for clinical application (27), and phthalic acid-diisononylester exhibited more potent effect than phlorizin.

Table 2. Yield and IC_{50} of intestinal glucose uptake inhibitor, PG-1a, isolated from *Punica granatum*

	Yield (%)	IC_{50} ($\mu\text{g/mL}$) ¹⁾
CM (methanol extract)	60	131.6
M-M (fractionated methanol extract)	45	111.1
M-Ma (first silica gel)	17.8	116.3
M-Mb (second silica gel)	7	96.2
PG-1 (preparative TLC)	2	68.7
PG-1a (preparative HPLC)	0.3	56.2

¹⁾ IC_{50} = The concentration required for 50% inhibition of intestinal glucose uptake.

In conclusion, our results suggest that phthalic acid-diisononyl ester-like compound isolated from *Punica granatum* L. might perform a potent inhibitory activity in intestinal glucose uptake, indicating the need to further elucidate the mechanism underlying the action of phthalic acid-diisononylester.

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