7-O-Methylaromadendrin Stimulates Glucose Uptake and Improves Insulin Resistance in Vitro

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The stimulation of glucose uptake into peripheral tissues is an important mechanism for the removal of glucose in blood and for the management of diabetes mellitus (DM). Since recent results have demonstrated the beneficial effects of flavonoids in relation to DM, this study was designed to examine the effects of 7-O-methylaromadendrin (7-O-MA), a flavonoid isolated from Inula viscosa, on glucose uptake into liver and fat tissue, and investigate the molecular mechanisms involved. 7-O-MA at 10 μM significantly stimulated insulin-induced glucose uptake measured by 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-a-glucose (2-NBDG) in both human hepatocellular liver carcinoma (HepG2) cells and differentiated 3T3-L1 adipocytes. Adipocyte-specific fatty acid binding protein (aP2) gene expression was increased by 7-O-MA in adipocytes, and both gene and protein level of peroxisome proliferator-activated receptor γ2 (PPARγ2) was also increased. Moreover, 7-O-MA stimulated the reactivation of insulin-mediated phosphorylation of phosphatidylinositol 3-kinase (PI3K)-linked protein kinase B (Akt/PKB) and adenosine 5’-monophosphate-activated protein kinase (AMPK) in high glucose-induced, insulin-resistant HepG2 cells, and this effect was blocked by either LY294002, a PI3K inhibitor, or compound C, an AMPK inhibitor. Therefore, these results suggest that 7-O-MA might stimulate glucose uptake via PPARγ2 activation and improve insulin resistance via PI3K and AMPK-dependent pathways, and be a potential candidate for the management of type 2 DM.

Key words 7-O-methylaromadendrin; glucose uptake; AMP-activated protein kinase; peroxisome proliferator-activated receptor γ2; phosphatidylinositol 3-kinase-linked protein kinase B

The much more common condition of diabetes mellitus (DM) is type 2 DM, which represents 90 to 95% of all diagnosed cases of diabetes.1,3 Insulin resistance, a specific feature of type 2 DM, is the condition whereby major organs such as muscle and liver become resistant to the action of the hormone, leading to increased glucose output from the liver and reduced uptake and metabolism of glucose by other organs.2 Studies on the molecular basis of insulin resistance have focused on the peroxisome proliferator-activated receptor γ (PPARγ). PPARγ plays an important role in the regulation of genes involved in adipocyte differentiation, lipid storage, glucose metabolism3 and insulin signal transduction.4,5 In this regard, PPARγ is a target for the treatment of insulin resistance as rosiglitazone, an anti-diabetic drug, does.

Insulin binding to its receptor induces activation of a complex network of downstream molecules, including phosphatidylinositol 3-kinase (PI3K) and the serine/threonine kinase PI3K-linked protein kinase B (Akt/PKB).6,7 Activation of Akt/PKB stimulates membrane translocation of the glucose transporter, GLUT4,7 leading to enhanced glucose uptake. Thus, Akt/PKB activation is a prerequisite for glucose uptake.

Adenosine 5’-monophosphate-activated protein kinase (AMPK) acts as an intracellular energy sensor. Increased recruitment of the AMPK signaling system may be effective in correcting insulin resistance.8 Activation of AMPK acutely stimulates glucose uptake via both GLUT1 and GLUT4, and enhances the long-term expression of GLUT4.9 Thus, AMPK is responsible for mediating the stimulation of glucose uptake.

The whole plant of Inula viscosa L. was traditionally used in folk medicine for treating DM.10,11 Previous investigations revealed the presence of a series of flavonoids in the whole plant including quercetin, apigenin, naringenin, saku ranetin, and aromadendrin.12,13 Some of the flavonoids were reported with somewhat conflict in regulating insulin-stimulated glucose uptake in adipocytes.15,16 For instance, sakuranetin was revealed to enhance insulin-stimulated glucose uptake in adipocytes,15 while apigenin suppressed.17 Quercetin was reported with somewhat conflict in regulating insulin-stimulated glucose uptake.17,18 7-O-Methylaromadendrin (7-O-MA) is also one of flavonoids isolated from I. viscosa. The preliminary data using our screening system indicated that 7-O-MA showed significant activities on stimulating insulin-induced glucose uptake into both HepG2 cells and differentiated 3T3-L1 adipocytes. Therefore, the specific aim of this study was to investigate the ability of 7-O-MA to improve diabetic conditions and the possible molecular basis. Our results observed that 7-O-MA increased expression of PPARγ2 and adipocyte-specific fatty acid binding protein.
(aP2) in adipocytes, and activated Akt/PKB and AMPK in high glucose-induced, insulin-resistant HepG2 cells. The findings suggested that 7-O-MA might have a significant ability to ameliorate insulin resistance and should be valuable for further study.

MATERIALS AND METHODS

Materials 2-[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]-2-deoxy-d-glucose (2-NBDG) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Oil Red O, Accustain® Harris hematoxylin solution, and insulin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Rosiglitazone was purchased from Masung & Co., Ltd. (Seoul, Korea). LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Compound C ((6-[(4-(2-piperidin-1-yl)-etoxy)-phenyl]-3-pyridin-4-yl-pyrrazolo[1,5-a]pyrimidine) was purchased from Calbiochem (Darmstadt, Germany). Antibodies against PPARγ (D69, #2430, derived from rabbit) and phospho-AMPKα (Thr 172, #2531, derived from rabbit) were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #sc-25778, derived from rabbit), phospho-Akt1/2/3 (Ser 473)-R (#sc-7985R, derived from rabbit), and Akt/PKB (Akt1 (B-1), #sc-5298, derived from mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies (peroxidase anti-rabbit immunoglobulin (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies (peroxidase anti-rabbit immunoglobulin G (IgG) produced in horse, #PI-2000) to detect the primary antibodies were purchased from Vector Laboratories Inc. (Burlingame, CA, U.S.A.). The flavonoid 7-O-MA, isolated from I. viscosa, was kindly provided by Professor Yi-Nan Zheng of the Jilin Agricultural University in China.

Isolation of Compound The dried and powdered aerial parts of I. viscosa (2.4 kg) were extracted with hot MeOH (5 l×three times). After filtration and evaporation of the solvent in vacuo, the combined crude methanolic extract was suspended into H2O and consecutively partitioned with hexane, EtOAc, and BuOH. The EtOAc-soluble extract was recrystallized in CHCl3 to give 7-O-MA in DMEM containing 10% FCS and 1 μM insulin in the absence of 7-O-MA. After 12 d, the cells were stained, and lipid accumulation was measured as previously described with minor modifications. Briefly, the cells were fixed in 10% formalin for 1 h and then stained with Oil Red O solution (60% isopropanol, 40% water) for 2 h and Accustain® Harris hematoxylin solution for 15 min at room temperature. The cells were then washed three times with 60% isopropanol to remove unbound dye and photographed under a microscope. Subsequently, the Oil Red O stain was dissolved in isopropanol, and lipid accumulation was quantified by measuring the optical absorbance at 510 nm.

Determination of PPARγ2 and aP2 mRNA Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from differentiated 3T3-L1 cells using the TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.). The RNA (2 μg) was reverse transcribed into cDNA using Oligo dT primers and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. For the amplification of PPARγ2, 1 μl cDNA was used with an amplification cycle consisting of 30 s of denaturation at 94 °C, 40 s of annealing at 59 °C, and 60 s of extension at 72 °C for 30 cycles. For the amplification of aP2, annealing temperature is 54 °C. The sense and antisense primers for β-actin (NM_007393) were 5′-ATC TGG CAC CAC ACC TTC TAC-3′ and 5′-GAC AGC ACT GTG TTG GCA TAG-3′; for PPARγ2 (NM_011146) 5′-TCT GGG AGA TTC TCC TGC TGA-3′ and 5′-TGG TAC GGT TTC TTT CAT AAC ACA TTC CAC C-3′, respectively. After RT-PCR, electrophoresis on a 1.2% agarose gel was performed, and the DNA was visualized by ethidium bromide staining. The relative expression of PPARγ2 and aP2 was calculated with the β-actin gene as an endogenous control.

Induction of Insulin-Resistant HepG2 Cells and Immunoblotting Insulin-resistance was induced in HepG2 cells as previously described. The cells were seeded into 6-well plates at 3×104 cells/well for 24 h and serum-starved for the next 24 h. After 24 h of pretreatment with serum-free DMEM with normal (5.5 mM) or high (30 mM) concentration of D-glucose in the absence or presence of 7-O-MA, the response to insulin (100 nM for 10 min) was measured. For immunoblotting, protein samples (20—50 μg) obtained from either differentiated 3T3-L1 adipocytes or high-glucose induced, insulin resistant HepG2 cells were separated by...
10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF; Immobilon-P, Millipore, Burlington, MA, U.S.A.). The membrane was blocked and then incubated with primary antibody at room temperature for 3 h followed by incubation with secondary antibody for 1 h. Immunoreactivity of PPARγ2, GAPDH, Akt/PKB, phospho-Akt or phospho-AMPK was detected with the Enhanced Chemiluminescence (ECL) Western Blot Detection system (Western Blotting Luminol Reagent, Santa Cruz, CA, U.S.A.), visualized, and measured by densitometry using the “Chemidoc XRS” digital imaging system and “MultiAnalyst” software from Bio-Rad laboratories, Inc. (Hercules, CA, U.S.A.). The amount of PPARγ2 was expressed as percentages of that in the vehicle-treated control group, and the amount of phosphorylated Akt or AMPK was expressed as percentages of that in the insulin-treated normal concentration of α-glucose group. To confirm the Akt- or AMPK-mediated reactivation of insulin signaling, LY294002, a specific inhibitor of PI3K, or Compound C, a potent and highly selective inhibitor of AMPK, was pre-treated and the phosphorylation level of Akt or AMPK was analyzed by immunoblotting.

**Statistical Analysis** All data were expressed as the mean±the standard error of the mean (S.E.M.) from three independent experiments. Statistical significance was determined by one-way or two-way analysis of variance (ANOVA) for comparisons, followed by the Dunnett or least significant difference (LSD) post-hoc test using SPSS 13.0 software (SPSS, Chicago, IL, U.S.A.), and chosen as p<0.05.

**RESULTS**

**7-O-MA Enhanced Glucose Uptake in HepG2 and Differentiated 3T3-L1 Cells** 2-NBDG was used in glucose uptake assay system. 7-O-MA significantly increased the insulin-stimulated 2-NBDG uptake in both HepG2 cells (Fig. 2A, p<0.01) and 3T3-L1 adipocytes (Fig. 2B, p<0.01) at 10 μM. Note that 10 μM of 7-O-MA had greater insulin-mediated 2-NBDG uptake than that of rosiglitazone at the same concentration (p<0.01) in the case of HepG2 cells. Thus, 7-O-MA-stimulated insulin-induced glucose uptake.

**Effect of 7-O-MA on Adipogenesis, PPARγ2 and aP2 mRNA Expression and PPARγ2 Protein Level** To compare the adipogenesis of 7-O-MA with rosiglitazone, differentiated 3T3-L1 adipocytes were used to measure the intracellular lipid droplets that appeared during differentiation. Oil Red O staining, shown in Fig. 3A, illustrated that rosiglitazone and 7-O-MA at 10 μM led to obvious conversion to adipocytes with markedly enhanced lipid accumulation compared to the controls. These results, shown in Fig. 3B, indicated that 10 μM rosiglitazone and 7-O-MA increased lipid accumulation by about 265% and 175%, respectively. Note that 10 μM of 7-O-MA increased the lipid content in adipocytes to a lesser extent than rosiglitazone. To determine whether 7-O-MA increases PPARγ2 and aP2 mRNA expression, RT-PCR was performed. The administration of 10 μM rosiglitazone increased the mRNA expression of PPARγ2 by 88% compared to the control; 10 μM 7-O-MA also enhanced the mRNA expression of PPARγ2 by approximately 56% (Fig. 3C). In the similar manner, 10 μM 7-O-MA and rosiglitazone caused 54% and 84% increase in the mRNA expression of aP2, respectively (Fig. 3D). Moreover, the level of PPARγ2 protein was also slightly increased by 1 and 10 μM 7-O-MA (Fig. 3E).

**Fig. 2. Effect of 7-O-Methylaromadendrin on 2-NBDG Uptake in HepG2 Cells and 3T3-L1 Adipocytes** (A) Effect of 7-O-methylaromadendrin (7-O-MA) on insulin-stimulated 2-NBDG uptake in HepG2 cells. (B) Effect of 7-O-MA on insulin-stimulated 2-NBDG uptake in 3T3-L1 adipocytes. HepG2 cells or differentiated 3T3-L1 adipocytes were treated with 1 μM insulin in the absence or presence of 7-O-MA for 1 h, and insulin-stimulated 2-NBDG uptake was measured. The average of all fluorescence intensities recorded from 1000 cell events was regarded as the fluorescence intensity of each test. The relative fluorescence intensities minus the background levels were used for statistical analysis. The values are expressed as the mean±S.E.M. and were determined from the average of three independent experiments. Significant differences from the control (2-NBDG and insulin treated group) are marked by *p<0.05 or **p<0.01. Significant difference compared to 10 μM 7-O-MA with the same concentration of rosiglitazone (Ros) is indicated by #p<0.01.

**Reactivation of Akt and AMPK in Response to Insulin in High Glucose-Induced, Insulin-Resistant HepG2 Cells** To investigate the ability of 7-O-MA to reactivate Akt/PKB signaling via insulin in high glucose-induced, insulin-resistant HepG2 cells, Akt phosphorylation was measured. Under normal concentration of α-glucose condition, insulin stimulated an approximately 2.4-fold increase over the basal cellular level in the phosphorylation of Akt (Fig. 4A, lanes 1 and 2). However, under high concentration of α-glucose condition, insulin failed to phosphorylate Akt compared to normal concentration of α-glucose condition (Fig. 4A, lanes 2 and 4), suggesting an insulin-resistant state. At concentrations of 1 and 10 μM, rosiglitazone caused significant activation of Akt under high concentration of α-glucose condition (Fig. 4A, lanes 4, 5, and 6, p<0.01). Although not to the extent of rosiglitazone, 1 and 10 μM 7-O-MA significantly increased the insulin-mediated phosphorylation of Akt under high concentration of α-glucose condition (Fig. 4A, lanes 4, 7, and 8, p<0.01). To confirm whether the improvement of insulin resistance by 7-O-MA was PI3K-mediated, high glucose-induced, insulin resistant HepG2 cells were pre-treated with 20 μM LY294002 for 2 h followed by the addition of 10 μM 7-O-MA or rosiglitazone for 24 h. As expected, pretreatment of LY294002 caused further blockade of Akt phosphorylation.
cose-induced, insulin resistant HepG2 cells were treated with rosiglitazone or 7-O-MA for 24 h. Pretreatment of Compound C completely blocked the reaction of AMPK phosphorylation to insulin (Fig. 5B, lane 3), and this could not be reversed by either rosiglitazone or 7-O-MA (Fig. 5B, lanes 4 and 5).

DISCUSSION

Two major findings from this study are (1) 7-O-MA significantly increases insulin-mediated glucose uptake in both hepatocytes and adipocytes, and this stimulatory action may be associated with increased PPARγ2 expression, and (2) 7-O-MA significantly reactivates Akt and AMPK signaling in response to insulin in insulin resistant hepatocytes. Although further research is needed, this study is the first to link 7-O-MA, a flavonoid isolated from I. viscosa, with stimulation of glucose uptake and improvement of insulin resistance, and provide a valuable data for further developing the potential small molecule applying to the management of type 2 DM.

Recently, the beneficial effects of flavonoids, in relation to DM, have been partly ascribed to stimulate glucose uptake in peripheral tissues. Sakuranetin, one of the flavonoids from I. viscosa, had been revealed to significantly increase insulin-stimulated glucose uptake (1.5-fold at 100 µM in differentiated 3T3-L1 adipocytes), compared with control group. In our study, the activity of 7-O-MA on 2-NBDG uptake was assessed in HepG2 cells and 3T3-L1 adipocytes by flow cytometry. At the concentration of 10 µM, 7-O-MA significantly enhanced insulin-stimulated 2-NBDG uptake in HepG2 cells (ca. 5.0-fold) and 3T3-L1 adipocytes (ca. 2.0-fold), and showed stronger effects than those of rosiglitazone. In addition, sukarunetin, a 3-dehydroxylated 7-O-MA,
shows somewhat weaker activities on increasing insulin-stimulated glucose uptake in HepG2 cells (ca. 4.0-fold at 10 μM) and differentiated 3T3-L1 adipocytes (ca. 1.4-fold at 10 μM) compared with 7-O-MA. Therefore, the investigation of molecular mechanisms involved in the stimulation of glucose uptake by 7-O-MA was preferentially chosen.

As shown in Figs. 3A, B and D, 7-O-MA increased lipid accumulation and aP2 mRNA expression in adipocytes, suggesting that 7-O-MA induced differentiation of 3T3-L1 pre-adipocytes. Further measurement of gene and protein expression level of PP AR shows somewhat weaker activities on increasing insulin-stimulated glucose uptake in HepG2 cells (ca. 4.0-fold at 10 μM) and differentiated 3T3-L1 adipocytes (ca. 1.4-fold at 10 μM) compared with 7-O-MA. Therefore, the investigation of molecular mechanisms involved in the stimulation of glucose uptake by 7-O-MA was preferentially chosen.

As shown in Figs. 3A, B and D, 7-O-MA increased lipid accumulation and aP2 mRNA expression in adipocytes, suggesting that 7-O-MA induced differentiation of 3T3-L1 pre-adipocytes. Further measurement of gene and protein expression level of PP AR, indicating that 7-O-MA induced differentiation of 3T3-L1 pre-adipocytes. Since PP AR and PP AR shares similar structure to sakuranetin and shows similar activities on stimulating insulin-mediated glucose uptake and improving PPARγ mRNA expression, but fail to exhibit PPARγ ligand activity even at high doses (100 μM) in luciferase reporter assay. This observation suggested that sakuranetin modulates other mechanism that leads to enhanced expression of PPARγ but not activates PPARγ. In our preliminary study, sakuranetin which isolated together with 7-O-MA from I. viscosa was also examined to be consistent with those of reported data in affecting glucose uptake and PP AR expression in 3T3-L1 cells. It is possible that 7-O-MA regulates the expression of PPARγ by the similar mechanism to sakuranetin, which might not act as a PPARγ activator. However, it needs much further study to elucidate the mechanisms for both sakuranetin and 7-O-MA.
in future. Nevertheless, the present findings indicate that 7-O-MA stimulates glucose uptake partly ascribed to increase in PPARγ2 expression.

When pre-adipocytes differentiate into adipocytes, the morphological alterations are induced by the presence of oil droplets in the cytoplasm, thus, too much lipid accumulation may facilitate obesity. Rosiglitazone is a potent PPARγ-activator and this can cause adipocyte differentiation, leading to simultaneously increase in both glucose uptake and lipid accumulation. This action may provide weight gain, a well-known side-effect of rosiglitazone. In our study, we found that the lipid droplet content induced by 10 μM 7-O-MA was much lower than with 10 μM rosiglitazone treatment (Figs. 3A, B). In accordance with these results, 7-O-MA has less ability to stimulate PPARγ2 and aP2 mRNA expression than rosiglitazone (Figs. 3C, D). These results suggest that at the same concentration showing maximal efficacies in this study, 7-O-MA may have less chance to elicit weight gain due to adipogenesis as compared with rosiglitazone.

In insulin signaling pathway, Akt phosphorylation at Serine 473 by PI3K is important for downstream signal transduction and related physiological responses such as glucose uptake and glycogen accumulation.1,5,6,28 When HepG2 cells were treated with high concentration of glucose, insulin signaling measured by Akt phosphorylation in response to insulin was shut off and this state was insulin-resistant.21 In our study, as rosiglitazone did, 7-O-MA significantly increased Akt phosphorylation by about 24% in high glucose-induced, insulin-resistant HepG2 cells (Fig. 4A), suggesting that 7-O-MA has an ability to improve insulin resistance. This effect was blocked by LY294002, a specific inhibitor of PI3K (Fig. 4B), indicating that the effect of 7-O-MA on the improvement of insulin resistance is PI3K-dependent.

Insulin stimulated the phosphorylation of AMPK and this activation was shut off in insulin resistant state of cells (Fig. 5A). 7-O-MA significantly prevented from the inhibition of insulin-stimulated AMPK activation in high glucose-induced insulin-resistant HepG2 cells and this effect was blocked by compound C, a specific inhibitor of AMPK (Fig. 5B), indicating that 7-O-MA could re activate insulin-stimulated AMPK phosphorylation in insulin resistant state of cells. AMPK activation could cause insulin sensitization, which is benefit for the treatment of type 2 DM and its associated disorders.30 Therefore, our results suggest that 7-O-MA has an ability to improve insulin resistance.

In conclusion, insulin-mediated glucose uptake was stimulated by 7-O-MA, an active compound in I. viscosa in both hepatocyte and adipocyte, and this effect might be via the up-regulation of gene and protein expression of PPARγ2. As compared with rosiglitazone, 7-O-MA has lower effect on lipid accumulation in adipocyte. Furthermore, 7-O-MA activated insulin-stimulated Akt/PKB and AMPK phosphorylation in high glucose-induced, insulin resistant HepG2 cells. Although the possible involvement of other mechanisms of 7-O-MA to improve diabetic conditions should be studied, our findings suggest that 7-O-MA may be a potential candidate for the management of type 2 DM.

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