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Original Article

Salacia oblonga extract increases glucose transporter 4-mediated glucose uptake in L6 rat myotubes: Role of mangiferin☆

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SUMMARY

Background and aims: To evaluate if the antidiabetic properties of *Salacia oblonga* extract are mediated not only by inhibiting intestinal α-glycosidases but also by enhancing glucose transport in muscle and adipose cells.

Methods: S. oblonga extract effects on 2-deoxy-D-glucose uptake were assayed in muscle L6-myotubes and 3T3-adipocytes. In L6-myotubes, the amount and translocation of glucose transporters were assayed. A fractionation of the extract was carried out to identify the active compounds. Furthermore, we analyzed the phosphorylation status of key components of signaling pathways that are involved in the molecular mechanisms regulating glucose uptake.

Results: S. oblonga extract increased 2-deoxy-D-glucose uptake by 50% in L6-myotubes and 3T3-adipocytes. In L6-myotubes, the extract increased up to a 100% the GLUT4 content, activating GLUT4 promoter transcription and its translocation to the plasma membrane. Mangiferin was identified as the bioactive compound. Furthermore, mangiferin effects were concomitant with the phosphorylation of 5′-AMP-activated protein kinase without the activation of PKB/Akt. The effect of mangiferin on 2-deoxy-D-glucose uptake was blocked by GW9662, an irreversible PPAR-γ antagonist.

Conclusions: S. oblonga extract and mangiferin may exert their antidiabetic effect by increasing GLUT4 expression and translocation in muscle cells. These effects are probably mediated through two independent pathways that are related to 5′-AMP-activated protein kinase and PPAR-γ.

1. Introduction

Effective blood glucose control is a key step in the management of type II diabetes to prevent related metabolic complications associated with this disease. Any pharmacological or nutritional intervention that reduces resistance to insulin or protects the β-cells should prevent or delay the progression of diabetes at best. In the prediabetic state of insulin resistance, glycemic control can be achieved by using oral agents that either interfere with the absorption of glucose (α-glycosidase and/or pancreatic α-amylase inhibitors) or facilitate glucose disposal in peripheral tissues (insulin-sensitizing agents).

In the Ayurvedic system of traditional Indian medicine, several plants from the *Salacia* genus (*oblonga*, *reticulata* and *prinoides*) have been extensively used for the prevention or remedy of type 2 diabetes and obesity.[1] Increasing evidence suggests regulatory effects of *Salacia* species on dietary nutrients, including inhibition of carbohydrate and lipid absorption.[2,3] Several authors[4,5] isolated and identified the active compounds (salacinol, kotalanol and mangiferin) of the roots and stems of *Salacia* and concluded that its mode of action is a competitive inhibition of the intestinal brush border α-glycosidase activity, similar to the antidiabetic drugs acarbose and miglitol. Results from animal[6] and

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AGIs, inhibitors of intestinal α-glycosidases; 5′-AMPK, 5′-AMP-activated protein kinase; 2-DG, 2-deoxy-[^{3}H]D-glucose; DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular signal-regulated kinases 1 and 2; FCS, fetal calf serum; HRP, horseradish peroxidase; PI3K, phosphoinositide-3 kinase; PPAR, peroxisome proliferator-activated receptors.

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randomized double-masked, human[7] studies have demonstrated that the water-soluble portion of the methanolic extract of *Salacia* reduces carbohydrate absorption and postprandial glycemia, providing an alternative to the conventional medical use of α-glycosidase inhibitors. Furthermore, toxicological, cytogenetic and safety studies of *Salacia oblonga* extract in rats support that at the doses used in human, no adverse health effects would be expected.[8]

Up to now, all the postprandial hypoglycemic effects of *S. oblonga* extract have been attributed to the reduction of intestinal carbohydrate absorption by the inhibition of α-glycosidase activity. However, the potential antidiabetic effects of *Salacia* species due to a direct regulation of glucose disposal in peripheral tissues remain unknown. In the present study, the main objective has been to assess the effects of *S. oblonga* extract on 2-deoxy-D-glucose uptake as well as the underlying mechanisms of action in differentiated L6-myotube cells. At the same time, we have identified the bioactive component of *S. oblonga*, *mangiferin*, responsible for this action and given some insights into the signaling pathways that are modulated by this compound. Furthermore, we also compared the antidiabetic activity of mangiferin with antidiabetic drugs used in clinical practice.

2. Materials and methods

2.1. Materials

A hot water *S.* extract (SOE) was obtained in a powder form from Tanabe USA, Inc. Mangiferin from *Mangifera indica* bark was purchased from Sigma (St. Louis, MO, USA; catalogue #M3547, lot. 055K1117). Chromium picolinate was obtained from Nutrition 21 (NY, USA). Cell culture media and supplements were from Sigma and Invitrogen (Carlsbad, CA, USA). Calf serum and fetal calf serum (FCS) were from Cultek (Madrid, Spain). GW9662 and HRP-conjugated secondary antibodies were from Sigma. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Phospho-p44/42 mitogen activated protein kinase E10 (Thr202/Tyr204), PKB/Akt and phospho-PKB/Akt (Ser473), AMPKα2 and phospho-AMPKα2 (Thr172) antibodies were from Cell Signaling (Beverly, MA, USA).

2.2. Cell culture

The L6.C11 rat skeletal muscle myoblast line (ECACC no. 92102119) and wild-type Chinese Hamster Ovary (CHO-k1; ATCC no. CCL-61) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS, 2 mM glutamine plus 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO, USA) in an atmosphere of air/CO_2 (95:5) and maintained at subconfluent densities in growth media. L6-myoblasts were differentiated into myotubes by exchanging the growth medium with a differentiation medium consisting of DMEM containing 2% (v/v) fetal bovine serum for 5–6 days (>50% fusion into multinucleated myotubes).

Mouse 3T3-L1 fibroblasts were maintained in DMEM supplemented with 10% calf serum. Differentiation of 3T3-L1 cells into adipocytes was induced by treatment of 48 h postconfluent cells with an adipogenic mixture consisting of 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine in the presence of 10% FCS. The differentiation medium was withdrawn 2 days later and replaced with medium supplemented with 10% FCS and 1 μg/ml insulin for 3 days, and then the cells were cultured in DMEM containing 10% FCS.

2.3. 2-Deoxy-[^{3}H]D-glucose uptake

Cells were grown in 48-well plates (Corning, NY, USA). They were differentiated into myotubes or adipocytes and then incubated in serum-free medium for 18–24 h. Treatments were performed in serum-free medium unless otherwise indicated. Triplicate measurements of 2-deoxy-[^{3}H]D-glucose (2-DG) uptake were taken after 10 min of incubation following the method described by Yonemitsu et al.[9]

2.4. α-Glycosidase inhibitory activity

α-Glycosidase inhibitory properties of each SOE fraction were assayed using intestinal mucosa isolated from obese Zucker rats, following Dahlqvist methodology.[10] Sucrase inhibition, IC_{50}, for each fraction was established.

2.5. Sub-cellular fractionation

Membrane fractions from myotubes were prepared using the differential centrifugation modified method described by Yonemitsu et al.[9] Membranes were resuspended in homogenization buffer (20 mM $NaHCO_3$, 0.25 M sucrose, 5 mM Na_3N, 1 M leupeptin, 1 μM aprotinin A, 1 μM pepstatin, pH 7.0) to a final concentration of 1–3 mg protein/ml and stored at −80 °C. 5′-Nucleotidase and cytochrome *c* reductase activities (data not shown) were assayed as marker enzymes for plasma membranes and low-density microsomes, respectively, following Girón et al.[11] Protein concentration was measured using the Bio-Rad Protein Assay (Hercules, CA, USA).

2.6. GLUT1 and GLUT4 protein analyses

L6-myotube fraction proteins (30 μg for cell homogenates, 40 μg for plasma membranes and 50 μg for low-density microsomes) were electrophoresed in 10% SDS-polyacrylamide gels and the resolved proteins were then transferred onto nitrocellulose filter membranes (Millipore, Bedford, MA, USA). After transfer, the membranes were blocked by using 5% non-fat milk and incubated in a 1:600 dilution of a polyclonal anti-GLUT1 antibody (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) or 2.5 μg/ml of a monoclonal anti-GLUT4 antibody (Biogenesis Ltd, Poole, UK). The antigen–antibody complexes were detected with HRP-conjugated secondary antibodies using an enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Quantification was performed by densitometry after scanning the autoradiographs with the NIH image software.[12]

2.7. Reporters' analyses

The pGL3-ratGLUT4 construct was made by cloning a genomic DNA fragment encompassing positions −2212 to +164 of the rat GLUT4 gene from clone −2212/+147G4CAT into pGL3-Basic vector (Promega, Madrid, Spain), which codes for *firefly* luciferase. The DNA mixture used comprised the pGL3-ratGLUT4 reporter and the reference plasmid pRL-TK (ratio 1:5). The pRL-TK plasmid codes for *Renilla* luciferase under the control of the TK promoter and serves as an internal control to correct for differences in transfection efficiencies between plates within an experiment. For transfection experiments, cells were used at 80–90% confluence. Transfection and luciferase activity assays were performed according to Girón et al.[13] Before studying the basal/stimulated function of the rat GLUT4 promoter, transfected cells were washed and placed in a medium without FCS for 18 h prior to exposure to stimuli. Where indicated, stimuli (SOE 25 and 50 μg/ml or mangiferin 1 and 2 μg/ml) were added to the medium for 24 h and

luciferase activity was determined. All transfections were performed at least three times, in triplicate, using at least two preparations of plasmid DNA.

The expression plasmids pSV SPORT PPAR-γ1 and pSV SPORT PPAR-γ2 and the reporter plasmid PPRE X3-TK-Luc[14] were kindly provided by Dr. Bruce M. Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School, Addgene plasmids 8886, 8862 and 1015). CHO-k1 cells were transiently transfected with either pSV SPORT PPAR-γ1 or pSV SPORT PPAR-γ2, the PPAR responsive element-driven luciferase reporter vector PPRE X3-TK-Luc, and the control plasmid pRL-TK. After 24 h, the cells were treated with rosiglitazone (10 μM) or mangiferin (2 μM) and cultured for 24 h in DMEM containing 2% (v/v) charcoal/dextran stripped FBS (Invitrogen). The cells were then used for dual luciferase reporter gene assay.

2.8. SOE extraction and isolation

A fractionation protocol was carried out on a hot water extract from the dried roots and stems of *S. oblonga*. A scheme of the protocol and the recovery obtained is depicted in Fig. 3A. In brief, SOE (**a**) (2 g) was extracted with hot methanol for 3 h in a Soxhlet apparatus. Evaporation of the solvent under reduced pressure provided the methanol extract (**c**), and a part of it was sequentially partitioned in an ethyl acetate–H_2O (1:1) mixture for five times. Removal of the solvent under reduced pressure gave the ethyl acetate soluble fraction (**e**) and H_2O-soluble fraction (**d**). The methanol insoluble fraction (**b**) was dissolved in hot water and partitioned in *n*-butanol. The aqueous fraction termed **j** was lyophilized and the *n*-butanol fraction (**h**) was evaporated under reduced pressure. For some assays, fraction **h** was solubilized in water and re-extracted again with cold (−20 °C) *n*-butanol.

2.9. Identification of isolated active fractions

^{1}H NMR, ^{13}C NMR, DEPT, HMBC and HSQC spectra of SOE active fractions were recorded at the "Centro de Instrumentación Científica", University of Granada, using a Varian DirectDrive 500 MHz NMR apparatus (Palo Alto, CA, USA). Identity of mangiferin as the active fraction was carried out by comparison with the described NMR spectra data for mangiferin.[15] To further confirm the nature of the SOE active fractions, either commercial mangiferin from *M. indica* bark or mangiferin purified from *S. oblonga* extracts was separated by HPLC using a Water 600 controller, a Water 600 pump, a Water 717 plus autosampler and a Water 2996 Photodiode Array Detector. The column used was an XTerra RP18, 5 μm, 4.6 × 250 mm. Water and acetonitrile plus a 0.1% TFA were used as solvents.

2.10. Protein phosphorylation analysis

L6-myotubes were incubated in an FCS-free medium for 18–24 h and then treated with mangiferin (2 μM) or insulin (50 nM) in serum-free medium. After treatment, plates were processed as described by Girón et al.[13] Proteins were separated by SDS-polyacrylamide gels and immunoblotted with selected antibodies.

2.11. Western blots of 5′-AMP-activated protein kinase (5′-AMPK)

L6-myotubes were incubated in an FCS-free medium for 18–24 h and then treated in the absence or presence of mangiferin (2 μM) or AICAR (0.5 mM). Plates were flash frozen in liquid nitrogen and scraped with lysis buffer as described by Girón et al.[13] Samples were separated by SDS-PAGE and immunoblotted with anti-AMPKα2 or anti-phospho-AMPKα2 (Thr172) antibodies.

2.12. Statistical methods

Results are expressed as mean ± SEM from four to six independent experiments. Statistical significance was tested with ANOVA followed by Dunnett's multiple comparison test. A p value < 0.05 was considered significant. In experiments using X-ray films, different exposure times were used to ensure that bands were not saturated.

3. Results

3.1. Effect of *S.* oblonga extract on 2-DG uptake

The effect of SOE on basal and insulin-stimulated glucose uptake in L6-myotubes is shown in Fig. 1. When myotubes were incubated for 18 h in a serum-free medium with increasing amounts of SOE (0–75 μg/ml), 2-DG uptake was stimulated in a dose–response mode, with a semi-maximal activating concentration (EC_{50}) and a maximum relative increase in 2-DG uptake (B_{max}) of 9.4 ± 2.1 μg/ml and 38.4 ± 2.4%, respectively (Fig. 1A). To study if SOE shows any additive effect on insulin-stimulated glucose uptake, L6-myotubes were incubated with SOE (50 μg/ml), insulin (100 nM) or both compounds for 18 h. Results showed that treatment with the hormone or SOE alone for 18 h produced a significant increase in glucose uptake of 50% and 25%, respectively, whereas, incubation with both effectors exerted an additive increase in 2-DG uptake (Fig. 1B).

We also assayed the effects of SOE on 2-DG uptake in differentiated 3T3-adipocytes. As shown in Fig. 1C, the results obtained in these cells were similar to those from L6-differentiated myotubes.

3.2. Effects of *S.* oblonga extract on glucose transporters amount and translocation

We next studied whether the stimulation of 2-DG uptake was accompanied by an increase in the total amount of GLUT4 or GLUT1. After incubating myotubes with 10, 50 and 150 μg/ml SOE for 18 h, the amount of these two transporters in cell homogenates was quantified by Western blot. SOE-treated myotubes presented a significant increase in the protein content of GLUT4 when compared to control cells (Fig. 2A). In contrast, the amount of GLUT1 protein was not significantly modified by any treatment.

The next step was to examine the acute effect of SOE (50 μg/ml) on GLUT4 translocation, from low-density microsomes to the plasma membrane. Incubation with SOE for 2 h significantly increased the GLUT4 protein amount in plasma membranes with a concomitant decrease in GLUT4 concentration in the low-density microsome fraction (Fig. 2B). Activation of GLUT4 translocation by SOE was also associated with a 30% increase in 2-DG uptake (2 h, 150 ± 5 vs 200 ± 10 pmol min well^{-1}, $p < 0.05$).

3.3. SOE fractionation: identification of bioactive compounds

Since our results showed that SOE was able to stimulate 2-DG uptake through an increase in GLUT4 amount and translocation to the plasma membrane, a fractionation protocol was carried out to identify the compounds responsible for these effects. A scheme of the protocol used and the percentage of recovery is shown in Fig. 3. Each fraction was assayed for its capability either to modulate glucose uptake or to inhibit intestinal sucrase activity. Since the recovery of the fractionation process was known (see Fig. 3), the amount of each fraction used for the 2-DG uptake assay was adjusted to the corresponding amount in the original extract (50 μg/ml) to compare their effects (Table 1).

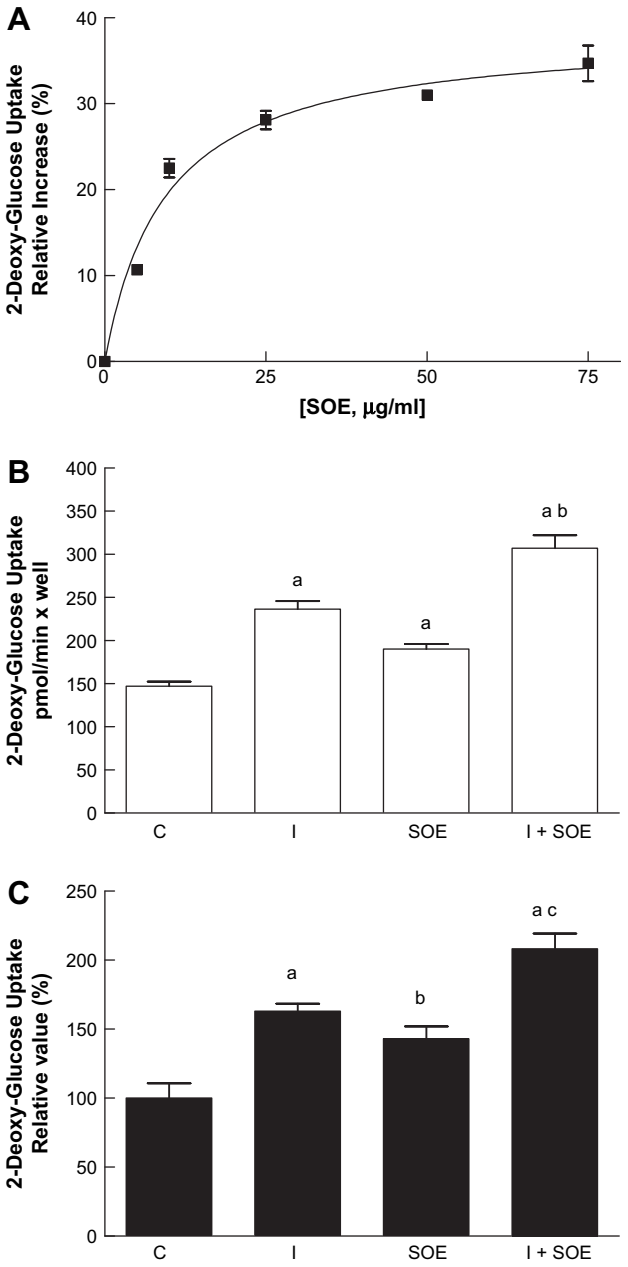

Fig. 1. *Effects of SOE on 2-DG uptake.* (**A**) L6-myotubes were deprived of FCS for 24 h and then incubated with increasing amounts of SOE (0–75 µg/ml) for 18 h. 2-DG uptake was then determined in quadruplicate as described in Section 2 and the relative increase of 2-DG uptake was plotted against SOE concentration. (**B**) L6-myotubes were deprived of FCS for 24 h and then incubated in the absence (C) or the presence of insulin (I: 100 nmol/l), SOE (SOE: 50 µg/ml) or both effectors for 18 h. Results are mean ± SEM of five independent experiments. [a] $p < 0.001$ compared with control cells; [b] $p < 0.01$ compared with cells treated with insulin. (**C**) Differentiated 3T3-adipocytes were deprived of FCS for 24 h and then incubated in the absence (C) or the presence of insulin (I: 100 nmol/l) or SOE (SOE: 50 µg/ml) or both effectors for 18 h. Results are mean ± SEM of five independent experiments. [a] $p < 0.001$ and [b] $p < 0.01$ compared with control cells; [c] $p < 0.005$ compared with cells treated with insulin.

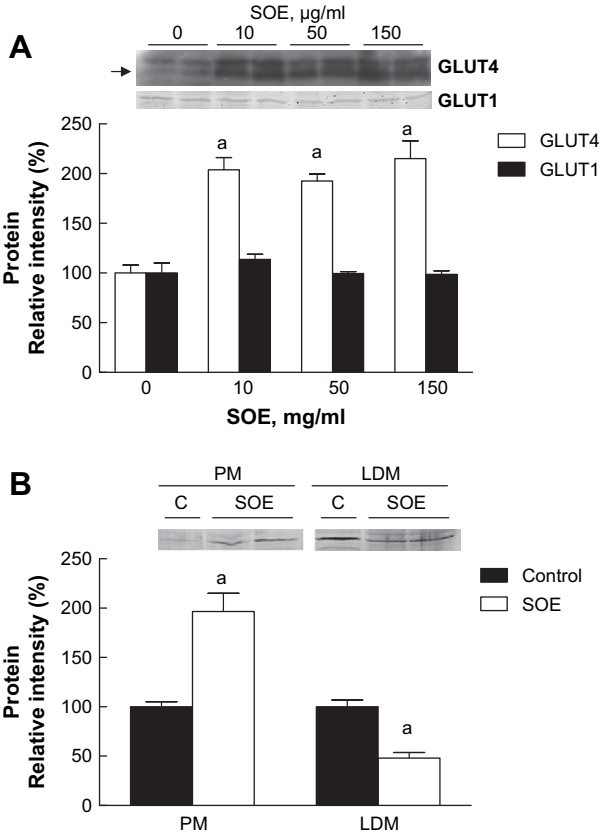

Fig. 2. *Effects of SOE on GLUT4 and GLUT1 protein levels in L6-myotubes.* (**A**) Cells were deprived of FCS for 24 h and incubated in the presence of increasing amounts of SOE (0–150 µg/ml) for 18 h. Representative Western blots showing GLUT4 and GLUT1 are shown. The densitometric quantification of GLUT4 and GLUT1 protein abundance using actin as a reference control (Western blot not shown) is shown. Signal densities from control cells were assigned a value of 100% for each transporter. Results are expressed as mean ± SEM for six samples per condition. [a] $p < 0.05$ compared with untreated cells. (**B**) Cells were deprived of FCS for 24 h and incubated in the presence of SOE (50 µg/ml) for 2 h. A sub-cellular fractionation was carried out as described in Section 2 and the GLUT4 protein amount was assayed by Western blot in the plasma membrane (PM) and low-density microsome (LDM) fractions. The densitometric quantification of GLUT4 protein in PM and LDM is shown. [a] $p < 0.05$ compared with untreated membrane fractions.

As it is shown in Table 1, the original *S. oblonga* extract (**a**) induced a significant increase in 2-DG uptake after 24 h of incubation. The methanol insoluble extract (fraction **b**) also exerted a stimulatory effect on 2-DG uptake, while no significant effects were obtained when the cells were incubated with the methanolic fractions **c**–**e**. The methanol insoluble fraction (**b**) was partitioned with water/*n*-butanol obtaining the **j** and **h** fractions, respectively. Aqueous fraction **j** was deprived of 2-DG uptake stimulating activity while the **h** *n*-butanol fraction contained most of the 2-DG uptake stimulating activity. Regarding the inhibitory capacity of different fractions of intestinal α-glycosidase activity, determined by the measurement of sucrase activity, results clearly indicated that the α-glycosidase inhibitory activity of each fraction was inversely associated with stimulatory action on 2-DG uptake in L6-myotubes. While fractions **c** and **d** showed the highest α-glycosidase inhibitory activities, they did not exert any significant capability to increase 2-DG uptake. On the contrary, fraction **h** showed the highest capacity to increase 2-DG uptake without any detectable sucrase inhibitory action.

Fraction **h** was identified as mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), a xanthone *C*-glycoside that was originally isolated from *M. indica* L. (Anacardiaceae). ^{1}H NMR, ^{13}C NMR, DEPT, HMBC and HSQC spectra of fraction **h** (see Supplementary material) matched those previously described for mangiferin.[15] Also, the mobility on TLC (data not shown) and the HPLC profiles of **h** were similar to those obtained with purified

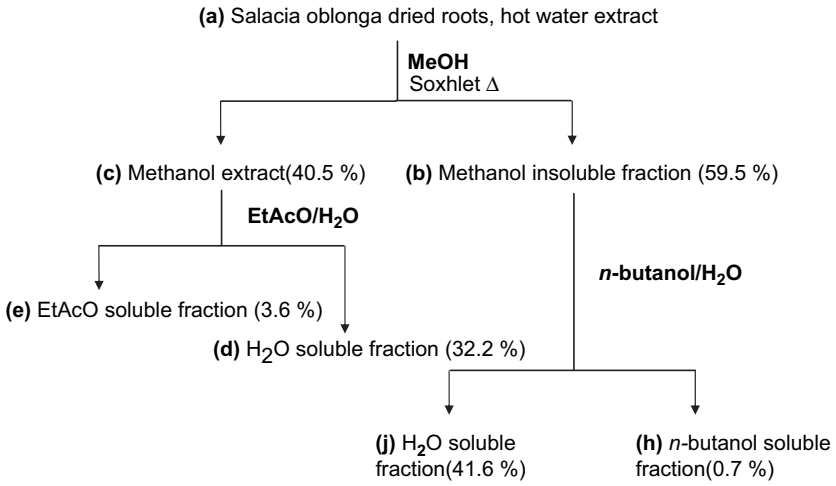

Fig. 3. *Salacia oblonga fractionation protocol.* The recovery for each fraction is shown.

mangiferin from *M. indica* (see Supplementary material). Finally, the peak separated by HPLC showed characteristic mangiferin absorbance peaks at 254, 317 and 364 nm.[16]

To further confirm the identity of fraction **h**, the capacity of this fraction to stimulate 2-DG uptake was compared with mangiferin from a commercial source. Incubation of L6-myotubes with commercial mangiferin from *M. indica* bark at 1 μM for 18 h was able to stimulate 2-DG uptake in a similar manner to that induced by **h** fraction (59.2 ± 1.7%, 65.1 ± 6.7%; relative increase with respect to basal condition), respectively.

3.4. 2-DG uptake, GLUT4 expression, translocation and signaling pathways modulated by mangiferin in L6-myotubes

Mangiferin, isolated from SOE, increased in a dose-dependent mode the 2-DG uptake after 18 h of incubation (Fig. 4A). Also, we assayed the capability of mangiferin (2 μM) to stimulate 2-DG uptake in differentiated 3T3-adipocytes upon 18 h incubation. Mangiferin significantly increased the relative 2-DG uptake in 3T3-L1 differentiated cells (100 ± 8.2 vs 147.2 ± 1.8, $p < 0.001$). Furthermore, in the adipocytes, the increase in glucose uptake mediated by mangiferin was additive to the effect of insulin (insulin 216.1 ± 2.6, mangiferin plus insulin 274.8 ± 2.8, $p < 0.001$ compared with insulin treated cells).

Table 1
2-DG uptake and sucrase inhibitory activities of SOE fractions.

Fraction	Recovery %	μg/ml in the assay	2-DG uptake relative value	Sucrase IC_{50} (μg/mg mucosa)
Control	–	–	100.0 ± 3.7	–
a	100.0	50.0	129.3 ± 4.2[a]	4.75
b	59.5	29.7	130.6 ± 4.3[a]	8.60
c	40.5	20.3	107.7 ± 10.0	3.24
d	32.2	16.1	115.5 ± 10.2	2.50
e	3.6	1.8	109.6 ± 5.8	ND
j	41.6	20.8	110.3 ± 2.3	14.17
h	0.7	0.3	145.6 ± 3.7[a]	ND

L6-myotubes were fasted for 24 h and then incubated with SOE fractions for 18 h. The 2-DG uptake was then determined in quadruplicate as described in Section 2. Results are expressed as mean ± SEM of five independent experiments. α-Glycosidase inhibitory properties of each SOE fraction were assayed using rat intestinal mucosa. Sucrase inhibition, IC_{50}, was established for each fraction. ND: not detected.

[a] $p < 0.05$ compared with control cells.

Since SOE effect on 2-DG uptake in L6-myotubes was due to an increase in GLUT4 amount in total cell homogenates, we assayed if purified mangiferin from *S. oblonga* was able to mimic these effects. Fig. 4B shows the effects of mangiferin on the total amount of GLUT transporters. An 18 h incubation of L6-myotubes in the presence of 2.5 μM mangiferin produced a significant increase in the total amount of GLUT4 transporter without any significant change in the GLUT1 (Fig. 4B).

To validate if the 18 h incubation increase of 2-DG uptake and GLUT4 amount produced by SOE and mangiferin were related to GLUT4 gene expression the activity of GLUT4 promoter was analyzed (Fig. 4C). SOE treatment for 18 h produced a moderate but significant stimulation of the GLUT4 promoter activity. GLUT4 promoter activity was also significantly induced by mangiferin treatment.

Next, we assayed the short-term effect (1 h) of increasing concentrations of mangiferin (0–5 μM) on 2-DG uptake in L6-myotubes (Fig. 5A). Under this short-term incubation, only GLUT4 translocation can be responsible for the increase in 2-DG uptake. Mangiferin clearly produced a dose-related increase in the 2-DG uptake in 1 h. When the amount of transporters was assayed in the plasma membrane of L6-myotubes incubated with 2.5 μM mangiferin for 1 h, there was a significant increase of GLUT4 transporter without changes in the amount of the GLUT1 (Fig. 5B).

GLUT4 translocation to the plasma membrane is the rate-limiting step for glucose uptake in muscle.[17] Taking into account that mangiferin increased the 2-DG uptake in 1 h as well as elicited a higher translocation of GLUT4, the next step was to analyze the molecular mechanisms regulating glucose uptake mediated by mangiferin.

Phosphorylation status of protein kinase B (PKB/Akt) is a pivotal downstream element of the phospho-inositol-3 kinase (PI3K) cascade.[17] Our results showed that while insulin increased the phosphorylation of PKB/Akt, mangiferin treatment did not modify it (Fig. 5C). Next, since the effects of some oral antidiabetics have been related to the mitogen activated protein kinases,[13] we measured the total and phosphorylated ERK1/2 by Western blot. Mangiferin did not modify either the total or the phosphorylated ERK1/2 (Fig. 5C).

Stimulation of the 5′-AMPK signaling pathway is clearly involved in the control of GLUT4 translocation.[18] Therefore, we assayed whether mangiferin-stimulated glucose uptake was dependent on the activation of 5′-AMPK (Fig. 5D). As a positive

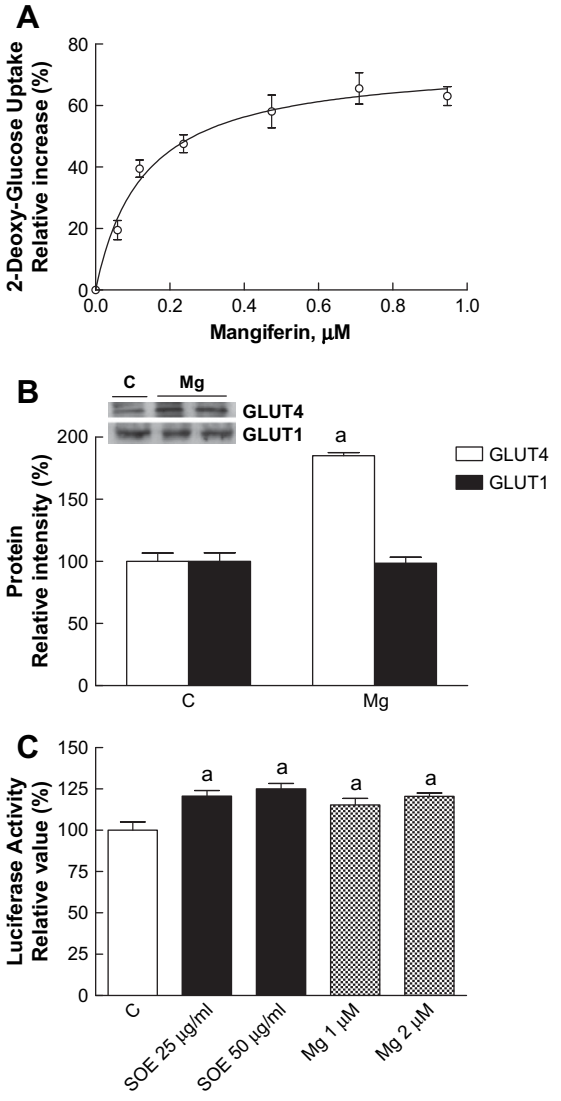

Fig. 4. *Long term effects (18 h) of mangiferin on glucose uptake and glucose transporters expression in L6-myotubes.* (**A**) Cells were deprived of FCS for 24 h and then incubated for 18 h with increasing concentrations (0–1 μM) of purified mangiferin from SOE. 2-DG uptake was then determined in quadruplicate as described in Section 2 and the relative increase of 2-DG uptake was plotted against mangiferin concentration. (**B**) Cells were deprived of FCS for 24 h and then incubated for 18 h in the absence (C) and presence of 2.5 μM mangiferin (Mg). Representative Western blots showing GLUT4 and GLUT1 in total cell homogenates are depicted. The densitometric quantification of GLUT4 and GLUT1 protein abundance using actin as a reference control (Western blot not shown) is shown. Signal densities from control cells were assigned a value of 100% for each transporter. Results are expressed as mean ± SEM for six samples per condition. $^{a}p < 0.05$ compared with untreated cells. (**C**) Effects of SOE and mangiferin on GLUT4 promoter activity. pGL3-ratGLUT4 construct was transfected in L6 cells. Differentiated cells were deprived of FCS for 24 h and then incubated in the absence (C), or in the presence of SOE or mangiferin for 24 h. At the end of the treatments, firefly luciferase activity was measured as described in Section 2.1. The luciferase activity from non-differentiated control cells was assigned a value of 100. Values are mean ± SEM ($n = 6$). $^{a}p < 0.05$ compared with control cells.

control, we used AICAR, a 5′-AMPK activator. As expected, AICAR increased 5′-AMPK phosphorylation (1.4-fold) and mangiferin produced an increase of 1.5-fold in the phosphorylated 5′-AMPK.

Taking into account that some effects of mangiferin on lipid metabolism have been related to PPAR stimulation,[5] we addressed the effects of mangiferin on PPAR-γ mediated signaling. L6-myotubes were pre-incubated for 30 min with GW9662 (0–1 μM), an inhibitor of PPAR-γ[19] and then treated with mangiferin (2 μM) for

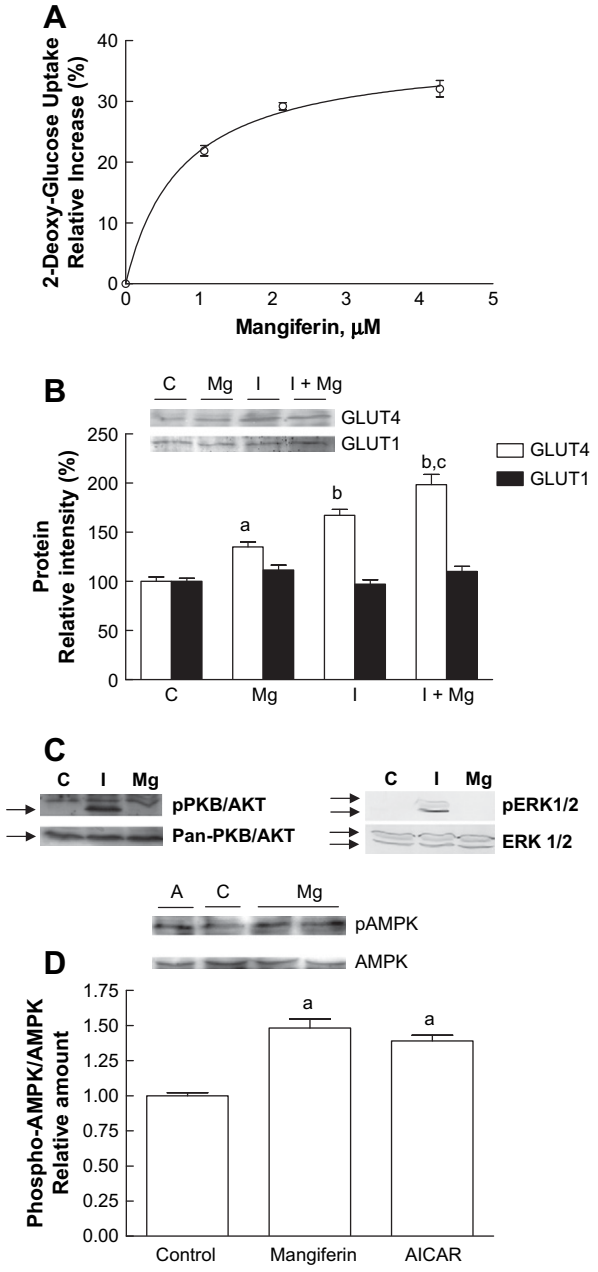

Fig. 5. *Short-term effects (1 h) of mangiferin on glucose uptake and glucose transporters expression and cell signaling in L6-myotubes.* (**A**) Cells were deprived of FCS for 24 h and then incubated for 1 h with increasing concentrations of mangiferin (0–5 μM). 2-DG uptake was then determined in quadruplicate as described in Section 2 and the relative increase of 2-DG uptake was plotted against mangiferin concentration. (**B**) Cells were deprived of FCS for 24 h and incubated in the presence of 2.5 μM mangiferin (Mg), 100 nM insulin (I) or both (I + Mg) for 1 h. A sub-cellular fractionation was carried out as described in Section 2 and the GLUT1 and GLUT4 protein amounts were assayed by Western blot in the plasma membrane fraction. The densitometric quantification is shown. $^{a}p < 0.05$ and $^{b}p < 0.001$ compared with untreated membrane fractions. $^{c}p < 0.05$ compared with insulin treated membrane fractions. (**C**) L6-myotubes were deprived of FCS for 24 h and then incubated in the absence (C) or presence of insulin (I, 50 nM) or mangiferin (Mg, 2 μM) for 15 min. Cells were lysed and total protein was subjected to SDS-PAGE and immunoblotted with specific antibodies. The results are a representative blot of three independent experiments. (**D**) L6-myotubes were deprived of FCS for 24 h and then incubated in the absence (C) or presence of AICAR (0.5 mM) or mangiferin (2 μM) for 15 min. Cells were lysed and total protein was subjected to SDS-PAGE and immunoblotted with anti-AMPKα2 or anti phospho-AMPKα2 (Thr172) antibodies. The results are a representative blot of three independent experiments.

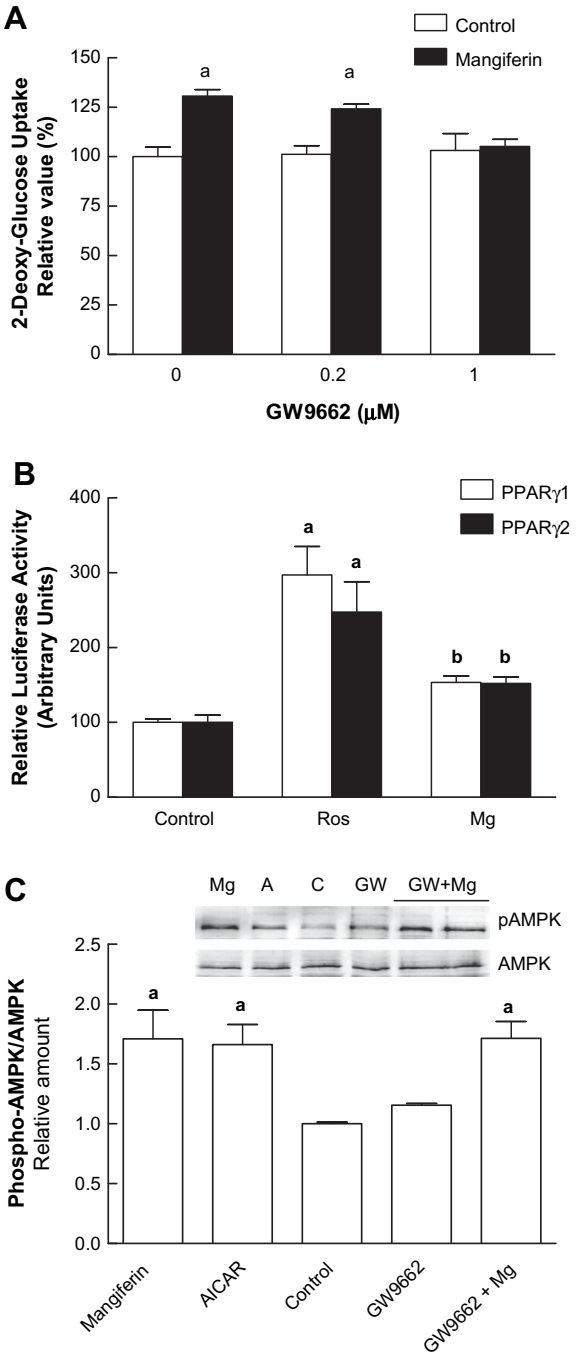

Fig. 6. *Analysis of mangiferin effects on the PPAR-γ signaling pathway.* (**A**) L6-myotubes were pre-incubated with increasing concentrations of GW9662 (0–1 μM) and then incubated for 2 h in the absence or presence of mangiferin (2 μM). GW9662 was added 30 min before the incubation with mangiferin and was maintained during the treatments. 2-DG uptake was then determined in quadruplicate as described in Section 2. Results are expressed as mean ± SEM of four independent experiments. [a] $p < 0.05$ compared with control cells. (**B**) Effects of mangiferin on PPAR responsive element-driven luciferase activity. CHO-k1 cells were transfected with plasmids pSV SPORT PPAR-γ1 and pSV SPORT PPAR-γ2 overexpressing PPAR-γ1 or PPAR-γ2 proteins in addition to the reporter plasmid PPRE X3-TK-Luc and control plasmid pRL-TK. Cells were treated with rosiglitazone (Ros, 10 μM) or mangiferin (Mg, 2 μM) and cultured for 24 h in DMEM containing 2% (v/v) charcoal/dextran stripped FBS. At the end of the treatments, firefly luciferase activity was measured as described in Section 2.1. The luciferase activity from non-treated transfected cells was assigned a value of 100. Values are mean ± SEM ($n = 4$). [a] $p < 0.01$ and [b] $p < 0.05$ compared with untreated cells. (**C**) L6-myotubes were deprived of FCS for 24 h and then incubated in the absence (C) or presence of mangiferin (Mg, 2 μM) or AICAR (A, 0.5 mM) for 15 min. To assay the effects of a PPAR-γ inhibitors, cells were pre-incubated with GW9662 (GW, 1 μM) and then incubated for 15 min in the absence or presence of mangiferin (2 μM). Cells were lysed and total protein was subjected to SDS-PAGE and immunoblotted with anti-AMPKα2 or anti phospho-AMPKα2 (Thr172) antibodies. Results are expressed as mean ± SEM of four independent experiments. [a] $p < 0.05$ compared with control cells.

Table 2
Effects of mangiferin in comparison to chromium picolinate and oral antidiabetic agents on 2-DG uptake in L6-myotubes.

Compound	Basal conditions		Insulin-stimulated conditions	
	EC_{50}	B_{max}	EC_{50}	B_{max}
Mangiferin	129.0 ± 20.9 nM	74.5 ± 3.3%	153.4 ± 27.0 nM	78.9 ± 4.1%
Acarbose	ND	ND	–	–
Chromium picolinate	ND	ND	27.0 ± 5.1 μM	209.3 ± 11.9%
Metformin	77.5 ± 7.2 μM	87.0 ± 9.7%	–	–
Rosiglitazone	630.5 ± 91.2 nM	58.4 ± 2.1%	844.6 ± 170.0 nM	57.2 ± 15.2%

Cells were deprived of FCS for 24 h and then incubated with increasing concentrations of agents for 18 h in the presence or absence of 10 nM insulin. 2-DG uptake was then determined in quadruplicate as described in Section 2 and relative increase in 2-DG uptake was plotted against concentration and adjusted to a hyperbola. Values of semi-maximal stimulating concentration (EC_{50}) and maximal increase in 2-DG uptake (B_{max}, % of the basal transport) are shown. ND = not detected; – not assayed.

2 h and 2-DG uptake was measured (Fig. 6A). Our results showed that GW9662 at 1 μM significantly blocked the increase in 2-DG uptake induced by mangiferin.

To confirm these results, we assayed the effects of mangiferin on the stimulation of transcription from a reporter gene containing three copies of the PPAR-γ response element.[14] These assays were carried out in CHO-k1 cells transfected with plasmids over-expressing PPAR-γ1 or PPAR-γ2 proteins. Our results showed (Fig. 6B) that mangiferin increased PPAR-mediated transcriptional activity, but these effects were lower than those induced by rosiglitazone. In addition, we assayed if the 5′-AMPK phosphorylation mediated by mangiferin was dependent of PPAR-γ activation process. 5′-AMPK phosphorylation was not inhibited by GW9662 in L6-myotubes incubated with mangiferin (Fig. 6C).

3.5. Relative effects of mangiferin on 2-DG uptake compared to other oral antidiabetic compounds

Finally, we compared mangiferin purified from SOE, to chromium picolinate, a well-recognized insulin sensitizer ingredient, and to several currently used antidiabetic agents, in their ability to stimulate glucose uptake in basal and insulin-stimulated conditions (Table 2). The effects of incubation of L6-myotubes either with mangiferin or with oral antidiabetic agents (chromium picolinate, acarbose, metformin and rosiglitazone) for 18 h on glucose uptake were compared in terms of semi-maximal stimulation concentration (EC_{50}) and maximal increase with respect to untreated cells (B_{max}, %). At basal conditions, half-maximal stimulation was achieved with mangiferin at much smaller concentrations as compared to either metformin (600-fold higher) or rosiglitazone (5-fold higher). In addition, under these conditions, maximal stimulation induced by mangiferin was significantly higher as compared to rosiglitazone ($p < 0.05$) and similar to that promoted by metformin. Neither chromium picolinate nor acarbose per se caused any increase in basal glucose uptake. Furthermore, in insulin-stimulated conditions, maximal stimulation induced by mangiferin was also higher as compared to chromium picolinate and rosiglitazone. In contrast, acarbose was the only agent that was able to inhibit the intestinal α-glycosidase activity (sucrase IC_{50} 4.75 μmol glucose/mg intestinal mucosa).

4. Discussion

Inhibitors of intestinal α-glycosidases (AGIs) (i.e., acarbose) are used in the treatment of non-insulin-dependent diabetes mellitus and represent a huge percentage of the antidiabetic drug market.[20] AGIs work by reducing the rate of breakdown of complex carbohydrates in the gut, thus reducing the availability of absorbable monosaccharide, such as glucose. However, due to the fact that AGIs treatment is less effective in reducing hemoglobin A1c compared to other oral antihyperglycemic medications,[21] and due to the gastrointestinal side effects (bloating, flatulence, nausea and diarrhea) frequently associated with oral administration of AGIs, the use of oral antidiabetic agents with direct systemic effects (i.e., metformin and rosiglitazone) has become a cornerstone in medical management of diabetes.

Studies in animals and humans have documented that extracts from *Salacia* genus have the capability to reduce glycemia.[6] As of today, the antidiabetic effects of *S. oblonga* extracts have been mainly attributed to the intestinal inhibition of the glucose uptake, due to its α-glycosidase inhibitory activity. Moreover, Krishnakumar et al.[22] also suggested that the glucose lowering activity of *S. oblonga* extract might be also exerted through stimulation of the β-cells of the pancreas islets because insulin levels were higher in diabetic rats treated with SOE compared to control. However, insulin-sensitizing effects have not been previously demonstrated for this family of natural oral antidiabetic agents.

In this article, we have studied the effects of SOE on glucose uptake in cell cultures of differentiated L6-myotubes. In these cells, the insulin-sensitizing action, the 2-DG uptake and the levels of GLUT transporters were measured. L6 muscle cell cultures were chosen since differentiated rat L6-myotubes are a validated model to study glucose uptake by muscle in response to insulin-sensitizing compounds.[9,23] Furthermore, the use of a cell culture system allowed us to dissociate the intestinal α-glycosidase inhibition mediated by SOE from its systemic effects on glucose uptake by muscle cells. This way, any possible interference due to the intestinal α-glycosidase activities in animal models that could indirectly alter glucose uptake by peripheral tissues[24] was prevented.

In both L6-myotubes and differentiated 3T3-adipocytes, we have found that incubation with SOE for 18 h produced a significant increase in 2-DG uptake (Fig. 1). These effects were dose-dependent and additive to the increase in 2-DG uptake mediated by insulin. The effects of SOE in L6-myotubes were mediated by a raise in the total amount of GLUT4 transporter without major changes in GLUT1 protein. GLUT4 represents the isoform that is most likely to be regulated by acute physiological stimuli (e.g., insulin, IGF-1, and exercise). Also, the quantity of GLUT4 in the plasma membrane was higher after SOE treatment, pointing to a double effect of the compound: an increase in the total synthesis and in the translocation of the GLUT4 transporter in myotubes. A number of studies have demonstrated that translocation of the glucose transporter GLUT4 to the plasma membrane is a prerequisite for the stimulation of glucose uptake by insulin in muscle and fat tissues.[23] In addition, a decrease in GLUT4 translocation from light microsomes to the plasma membranes has been implicated as a possible cause of insulin resistance.[25]

Since SOE was able to stimulate glucose uptake, we investigated the identification of the bioactive compounds of SOE that had insulin-sensitizing properties. It has been described that SOE contains three active components: salacinol, kotalanol, and mangiferin with α-glycosidase activity.[2–5] Salacinol is so far the best-characterized active compound contained in this extract, and exhibits the highest inhibitory effect on intestinal α-glycosidase activities.[26] To address this question we carried out a fractionation of SOE, by using a simple organic solvents procedure, and the capability of each fraction to modulate glucose transport in the L6 cell culture system was assayed. As far as we know, the fractionating protocol used in this study is the easier procedure described for the characterization of *Salacia* extracts,[4] because no chromatographic procedures were used.

Fraction **h**, obtained after *n*-butanol partition of the methanol insoluble fraction of the original extract, displayed the highest stimulating effect on basal glucose uptake. NMR spectra of fraction **h** as well as the solubility of this fraction preclude the presence of salacinol and therefore points out to another compound as responsible for the insulin-sensitizing properties of SOE. ^{1}H NMR, ^{13}C NMR, DEPT, HMBC and HSQC spectra of fraction **h** matched those previously described for mangiferin.[15] Also, the mobility on TLC and the HPLC profiles of **h** were similar to those obtained with purified mangiferin from *M. indica* bark. Furthermore, the identity of fraction **h** was confirmed by comparing the effect of this fraction with commercially purified mangiferin. Results showed that commercial mangiferin was able to stimulate basal glucose uptake in a manner similar to fraction **h**.

Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), is a xanthone *C*-glycoside that was originally isolated from *M. indica* L. (Anacardiaceae). Mangiferin is distributed among at least 16 plant families including Anacardiaceae, Iridaceae and Gentianaceae. Studies in rodents have suggested that mangiferin might exhibit antidiabetic properties, by ameliorating post-prandial glycemic, mainly due to its ability to inhibit the intestinal α-glycosidase activities.[27]

Our present results demonstrated that mangiferin stimulates the basal glucose uptake in L6 skeletal muscle cells. Furthermore, the compound was also able to significantly increase 2-DG uptake in 3T3-adipocytes supporting the hypothesis of a systemic action of SOE and mangiferin by increasing whole peripheral glucose uptake. Even, mangiferin exhibited a higher capacity to stimulate basal glucose uptake in L6-myotube culture compared to currently available antidiabetic agents. In our experiments, the rank order of potency for glucose uptake in basal conditions was as follows: mangiferin > rosiglitazone > metformin. These effects were not mimicked by acarbose or by chromium picolinate, well-known AGI and insulin sensitizer agents. This strong action mediated by mangiferin may be explained not only due to an increased translocation of GLUT4 to the plasma membrane, as shown in Fig. 5B but also might be mediated through a direct stimulation of GLUT4 protein expression (Fig. 4C).

In our study SOE and its bioactive compound mangiferin increased glucose transport in L6-myotubes in the absence of insulin and showed an additive effect in insulin-stimulating conditions. In skeletal muscle, glucose uptake can be modulated by at least two separate signaling pathways, one stimulated by insulin and one activated during exercise.[18] Our results may suggest an insulin-independent signaling mechanism of SOE in skeletal muscle.

The use of an L6 muscle cell culture system has facilitated the analysis of signaling pathways that are involved in the increased 2-DG uptake mediated by mangiferin. First, we studied the phosphorylation status of PKB/Akt, a downstream target of PI3K.[17] Our results (Fig. 5C) showed that, while insulin clearly increased PKB/Akt phosphorylation, mangiferin had no effects on the phosphorylation status of the protein. Since the PKB/Akt is a key element of the insulin signaling pathway, this result suggests that the mangiferin effects on 2-DG uptake could be independent of the insulin signaling pathway.

5′-AMPK is a sensor that responds to changes in cellular energy balance (AMP/ATP ratio).[28] Activation of 5′-AMPK translates in a stimulation of catabolism and an increase in glucose

uptake in peripheral tissues through a stimulation of GLUT4 translocation to the plasma membrane.[18] Therefore, we have assayed the effects of mangiferin on 5′-AMPK phosphorylation. Our results (Fig. 5D) show that mangiferin is able to stimulate 5′-AMPK phosphorylation. This increase (1.5-fold) is similar to the increase obtained using a well-known 5′-AMPK activator, AICAR.

Some systemic effects of *Salacia* genus extracts on lipid metabolism have been related with PPAR stimulation.[5] These authors suggested the involvement of mangiferin in the regulation of the lipid metabolism. Therefore, we have assayed the effects of a PPAR inhibitor on the mangiferin-mediated glucose uptake in L6-myotubes. Mangiferin stimulation of 2-DG uptake was clearly inhibited by GW9662 (Fig. 6A). This inhibitor specifically inhibits PPAR-γ acting as a tight binding irreversible inhibitor of the protein.[19] Furthermore, mangiferin was able to stimulate transcription in a well-known reporter system designed to measure PPAR-γ activity,[14] albeit these effects were lower than those induced by rosiglitazone. In addition, the 5′-AMPK phosphorylation mediated by mangiferin was independent of PPAR-γ activation process (Fig. 6C).

It is not surprising that mangiferin is able to act through two signaling pathways: a PPAR-γ activation and an increase in 5′-AMPK phosphorylation. A number of PPAR-γ agonists, such as rosiglitazone, can exert their antidiabetic properties, increasing the ADP/ATP ratio and activating 5′-AMPK,[29] which lead to increase in glucose uptake.

Finally, we measured the effects of mangiferin on ERK1/2 phosphorylation and its possible influence on 2-DG uptake. Some antidiabetics have been proposed to act through ERK1/2 signaling pathway.[13] Also, the phosphorylation by ERK1/2 and other kinases has been proposed to modulate the PPAR signaling pathway.[30] Mangiferin induced 2-DG uptake is independent of the phosphorylation of ERK1/2, since no changes in the phosphorylation status of ERK1/2 were detected upon incubation with mangiferin (Fig. 5C).

Taking together all our results, we can conclude that SOE has a direct effect in stimulating 2-DG uptake in muscle cell lines in culture. This increased uptake is due to a double effect, an increase in the GLUT4 transporter total amount together with a higher presence of the transporter in the plasma membrane. These effects of SOE were mediated by its bioactive compound mangiferin. Moreover, mangiferin is able to increase glucose uptake by activating 5′-AMPK and PPAR-γ in rat L6 skeletal muscle cells. In conclusion, *S. oblonga* extracts and mangiferin, through their effects in increasing glucose uptake, may be considered as a useful therapy to ameliorate the metabolic perturbation in insulin resistance and also under conditions of insulin deficiency.

Conflict of interest

None declared.

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JML-P conceived the study, carried out the data analyses and drafted the manuscript. RR participated in the conception of the study. RS designed the experiment, carried out the data analyses and drafted the manuscript. MDG collected the data, carried out the data analyses and drafted the manuscript. NS, AH, MM and MLJ collected the data. All authors read and approved the final manuscript.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.clnu.2009.04.018.

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