

Role of the water extract from *Coccinia indica* stem on the stimulation of glucose transport in L8 myotubes

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Abstract

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Hypoglycemic effect of *Coccinia indica* used for treatment of diabetes in traditional remedies has known to relate with increased transport of glucose into peripheral tissues. However, the cellular mechanisms for this effect remain unclear. This present study reports that the water extract (WE) of *C. indica* stem exhibited a dose-dependent induction of 2-deoxyglucose (2-DG) uptake in rat L8 myotubes. Maximal uptake was observed with approximately 3-fold increase in 2-DG transport in 16 h treatment compared with the control. Effect of WE was stronger than that of 1 mM metformin. The effects of insulin and WE were additive. WE-induced glucose uptake was significantly inhibited by cycloheximide and partially reversed by SB203580. GLUT1 protein was markedly increased in response to WE. Conversely, WE had no effect on GLUT4 protein level. Redistribution of GLUT4 to the plasma membrane was demonstrated. Triterpenoids and carbohydrates were detected in WE. In conclusion, new GLUT1 protein synthesis is necessary for WE-stimulated glucose transport while p38-MAPK-dependent activation of transporter intrinsic activity partly contributes to WE action. These results may explain and support the use of *C. indica* for the prevention and treatment of diabetes.

Key words : *Coccinia indica*, glucose transport, GLUT1, GLUT4, L8 myotube

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บทคัดย่อ

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บทบาทของสารสกัดน้ำจากเถาตำลึงต่อการกระตุ้นการนำกลูโคสเข้าสู่เซลล์กล้ามเนื้อ
เพาะเลี้ยงชนิด L8

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มีการใช้ตำลึงในการรักษาโรคเบาหวานตามภูมิปัญญาพื้นบ้าน แต่กลไกการทำงานในระดับเซลล์ของตำลึงที่ทำให้น้ำตาลเข้าสู่เนื้อเยื่อทั่วร่างกายเพิ่มมากขึ้นยังไม่เป็นที่ทราบแน่ชัด การศึกษาครั้งนี้พบว่า สารสกัดน้ำจากเถาตำลึงมีฤทธิ์กระตุ้นการนำกลูโคสเข้าสู่เซลล์กล้ามเนื้อเพาะเลี้ยงชนิด L8 ของหนู โดยฤทธิ์ของสารสกัดแปรผันโดยตรงกับความเข้มข้นที่ใช้ทดสอบ ฤทธิ์การนำเข้ากลูโคสเมื่อกระตุ้นด้วยสารสกัดนานาน 16 ชั่วโมงสูงเป็น 3 เท่าของสภาวะควบคุมและสูงกว่าฤทธิ์ของ metformin (1 mM) ทั้งยังมีผลเสริมฤทธิ์ของอินซูลิน การนำกลูโคสเข้าเซลล์ที่เพิ่มขึ้นโดยสารสกัดน้ำกลูโคสยังได้ด้วย cycloheximide และเปลี่ยนแปลงลดลงเล็กน้อยด้วย SB203580 สารสกัดน้ำทำให้ปริมาณรวมของตัวพาหุกลูโคส GLUT1 เพิ่มขึ้นแต่ไม่มีผลต่อ GLUT4 และทำให้มีการเคลื่อนย้ายตัวพาหุกลูโคส GLUT4 ไปที่ผิวเซลล์ พบสารประกอบ triterpenoids และ carbohydrates ในสารสกัดน้ำ ดังนั้นจึงสรุปได้ว่าการสร้างตัวพาหุกลูโคส GLUT1 จำเป็นต่อการกระตุ้นการนำกลูโคสเข้าสู่เซลล์กล้ามเนื้อของสารสกัดน้ำจากเถาตำลึง ส่วนการเพิ่มเอนทิตีของตัวพาหุกลูโคสที่ขึ้นกับการกระตุ้น p38-MAPK มีผลต่อการทำงานของสารสกัดน้ำเพียงบางส่วน ผลการศึกษานี้จึงช่วยสนับสนุนการใช้ตำลึงเพื่อการป้องกันและรักษาโรคเบาหวานได้

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Type 2 diabetes mellitus, that accounts for approximately 90% of diabetic patients, has become a serious public health problem. Although the causes of non-insulin-dependent diabetes mellitus (NIDDM) are not completely known, obesity, hyperinsulinemia, hyperglycemia and insulin resistance are closely associated with NIDDM. Insulin lowers blood glucose levels by promoting glucose transport into insulin-sensitive tissues. The activation of phosphatidylinositol 3-kinase (PI3-kinase) in response to insulin leads to selective redistribution of glucose transporter 4 (GLUT 4) to the plasma membrane and subsequently increased glucose uptake in skeletal muscle and adipocytes (Okada *et al.*, 1994; Tsakiridis *et al.*, 1995). However, increased plasma membrane glucose transporter content is insufficient to fully account for the insulin-stimulated increase in glucose uptake. Association of GLUT4's activity with the p38 mitogen-activated protein kinase (p38 MAPK) activation (Konrad *et al.*, 2001) suggests that insulin and other agents that stimulate glucose uptake might regulate the

intrinsic activity of cell surface glucose transporters. Studies with specific PI3-kinase inhibitor wortmannin (Kaliman *et al.*, 1995), p38 MAPK inhibitor SB203580 (Sweeney *et al.*, 1999) and diabetic drugs such as α -lipoic acid (Konrad *et al.*, 2001) and troglitazone (Yonemitsu *et al.*, 2001) imply that both PI3-kinase and p38 MAPK activation are associated with basal and insulin-induced glucose transport. Thus, PI3-kinase- and p38 MAPK-dependent signaling pathways are logical targets for defects associated with insulin resistance and diabetes.

Coccinia indica (ivy gourd) or tum-lerng (Thai name) is a creeping plant that grows wildly in tropical countries. It is used in Ayurveda, a traditional East Indian healing system to treat "sugar urine" (Yeh *et al.*, 2003). Several studies showed evidence of the hypoglycemic effects of the *C. indica* watery extract in the experimental animals and diabetic patients which are insulin-mimetic properties (Azad Khan *et al.*, 1979; Kumar *et al.*, 1993; Shibib *et al.*, 1993; Kamble *et al.*, 1998). It was reported that *C. indica* modulated

activities of enzymes in glucose metabolism and lipolytic pathway, leading to an increase in peripheral glucose consumption (Kumar *et al.*, 1993; Shibib *et al.*, 1993; Kamble *et al.*, 1998). However, the mechanism of action of *C. indica* is not well understood. Due to the potential role for *C. indica* in the treatment of type 2 diabetes, further study was suggested (American Diabetes Association Guidelines Level I, A; Yeh *et al.*, 2003).

In this study, mechanisms of action of the water extract from *C. indica* stem were explored utilizing a cell culture model. We tested the effect of *C. indica* on glucose uptake activity and redistribution of glucose transporter in L8 muscle cells. The involvement of p38 MAPK and protein synthesis on *C. indica* action was assessed.

Materials and methods

Materials

Rat L8 myoblasts were purchased from American Type Culture Collection (Rockville, MD). Cell culture medium and supplements were from Life Technologies, Inc. (Gaithersburg, MD). Insulin, metformin, phloretin, SB203580, cycloheximide, protease inhibitor cocktail, anti-actin antibody and standard chemicals were from Sigma (St. Louis, MO). 2-Deoxy-D-[³H] glucose was purchased from Amersham Biosciences (Piscataway, NJ). CytoTox 96 non-radioactive cytotoxicity assay kit was from Promega (Madison, WI). Anti-GLUT1 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody to GLUT4 was from Biogenesis (Brentwood, NH). Electrophoresis and protein assay reagents were from Bio-Rad (Hercules, CA).

Preparation of *C. indica* water extract

The stems of *Coccinia indica* Wight & Arn. (Cucurbitaceae) were collected from the forest in Songkhla province, Thailand. Plant materials were chopped and dried in a 42°C oven. Five grams of ground sample was extracted in 200 ml water at 70°C for 30 min. The extract was filtered, and then centrifuged at 2,000xg for 20 min, followed by freeze-drying (yield 126 mg/g ground sample).

To screen for compounds present in the *C. indica* watery extract (WE) regarding to their chromatographic and spectroscopic behaviors, chromatographic analysis of WE was carried out on an Agilent Series 1100. Separation was performed on a RP-C18 column with sample injection volume of 10 µl (10 mg/ml). A gradient of 10 to 100% of methanol in water was used as mobile phase with a flow rate of 1 ml/min. Detection of eluates was employed by a photodiode array detector at different UV wavelengths (210, 254 and 366 nm).

Detection of active constituents

WE was subjected to phytochemical screening, using the previous methods described by Farnsworth (1966) and Harborne (1973) with slight modification. In brief, several reagents were prepared to test for the presence of flavonoids, coumarins, anthraquinones, iridoids, cardiac glycosides, saponins, alkaloids, tannins, carbohydrates, amino acids and peptides. The results were compared with the positive standards of each test.

2-Deoxyglucose uptake assay and treatments

Rat L8 myoblasts were maintained and differentiated into myotubes in Dulbecco's modified Eagle's medium (low glucose) as described previously by Purintrapiban and Ratanachaiyavong (2003). Cells were seeded at 35,000 cells/cm² in 24-well plates for transport experiments and 10-cm diameter dishes for protein preparations. Differentiation was initiated by culturing confluent myoblasts in the medium with 2% horse serum (HS). Exposure of myotubes to the experimental conditions was preceded by a 3 h incubation in culture medium supplemented with 15 mM glucose. To investigate dose- and time-dependent effects, cells were incubated with 0, 0.5, 1.0, 1.25, 1.5, 1.75 or 2.0 mg/ml WE in HEPES buffered saline (HBS), pH 7.4 containing 15 mM glucose and 2% HS for 16 h (standard protocol) and with or without 1.5 mg/ml WE for 2, 8, 16 or 24 h (time-dependent studies). In the combination studies with insulin, incubations were further maintained for 5 h without

serum or insulin to reduce the basal rate of glucose transport. In experiments where effects of inhibitors were assessed, cells were incubated with or without 10 μM SB203580 for the last 30 min or 2 $\mu\text{g/ml}$ cycloheximide for 16 h before being assayed for glucose uptake activity. After the above incubations, the test media were collected and used in the cytotoxic studies performed according to manufacturing instruction using the CytoTox 96 non-radioactive cytotoxicity assay. The cells were then rinsed with HBS and incubated in HBS containing either 10 μM 2-deoxy- ^3H glucose (2-DG; 1 $\mu\text{Ci/ml}$) alone or in the presence of 100 nM insulin. The 2-DG uptake was determined over a 10-min period at 37°C. Non-specific uptake was measured in the presence of 10 mM phloretin and was subtracted from the total uptake. The radioactivity associated with the cells was determined by cell lysis in 0.05 N NaOH, followed by liquid scintillation counting. An aliquot from each well was taken to determine the total cellular protein using the method of Bradford (1976).

Analysis of glucose transporter

Subcellular fractionation and whole cell lysate preparation were carried out as described by Yu *et al.* (1999) with modification. In brief, cells were treated with 20 strokes in homogenizing buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1 mM MgCl_2 , protease inhibitors). Cell lysates were centrifuged at 200xg for 5 min to remove cell nuclei. The supernatant (whole cell lysate) were collected for total GLUT1 and GLUT4 protein analysis or further centrifuged at 16,000xg for 15 min to pellet the plasma membrane (PM) from the supernatant or soluble (S) fraction containing intracellular pool of transporters. Protein samples (100-150 μg) were separated in 10% SDS-PAGE, and probed with antibodies against GLUT4 (1:250) or GLUT1 (1:700).

Statistical analyses

Three to five independent experiments were conducted in all studies, and all assay conditions were performed in triplicate. Data were analyzed using Student's t test, and differences were accepted

as significant at a p value < 0.05 .

Results

Detection of active constituents

The HPLC fingerprint chromatogram of WE at different UV wavelengths is shown in Figure 1. It showed that compounds processing a degree of unsaturation or chromophore that absorbed radiation at 254 nm were most prominent. Few compounds with less UV-absorbing property, which were detected at 210 nm, were also present. In order to determine the potential stimulators of glucose transport, WE was subjected to phytochemical screening. WE yielded positive results for Liebermann-Burchard test with red color solution. This is an indication of the presence of triterpenoid compounds. This fraction also yielded positive results for Molisch's test with a purple ring of the interphase between the concentrated sulfuric acid layer and the layer of a mixture of sample and α -naphthol solution suggesting the presence of free carbohydrates and/or bound sugars in the form of glycosidic compounds. We did not detect other compounds such as coumarins, anthraquinones, iridoids, cardiac glycosides, saponins, flavonoids, tannins, alkaloids, amino acids and peptides. It is evident that the active compounds in WE could be triterpenoids and carbohydrates.

Effects of WE on 2-DG uptake

In L8 myotubes, the uptake of 2-DG was greatly enhanced in a dose-dependent manner by 16 h incubation of WE with maximal induction up to 2.97 ± 0.37 folds above basal (Figure 2). A shorter incubation time (2 h) was insufficient to allow changes in basal rate of glucose transport to develop. The transport activity increased by 1.41 ± 0.08 folds after 8 h of incubation with WE and was maximal at 16 h (3.12 ± 0.33 folds, Figure 3). An antidiabetic drug metformin was chosen as a positive control because it has been shown to exhibit a long-term direct effect on glucose-mediated transport in cell culture about mM level (Hundal *et al.*, 1992; Wiernsperger, 1999). Interest-

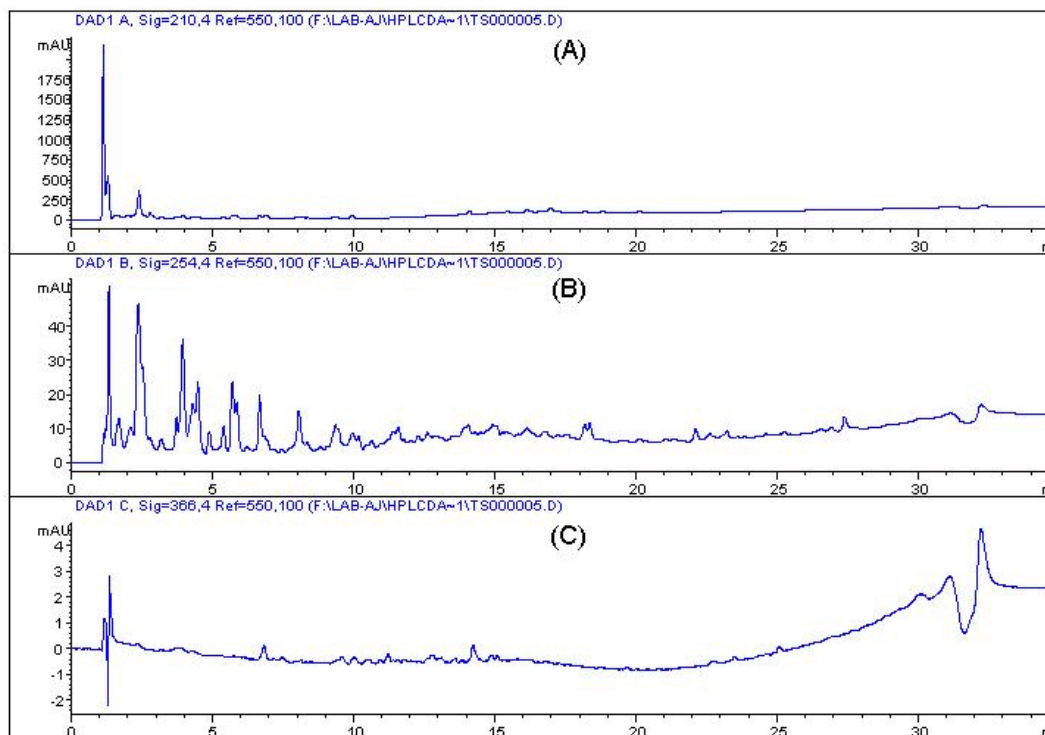


Figure 1. HPLC fingerprint chromatograms of three different UV wavelengths: 210 nm (A), 254 nm (B) and 310 nm (C) of *C. indica* extract. WE (10 μ l of 10 mg/ml) was analyzed with reversed-phase HPLC. The conditions were: RP-C18 column; solvent system, gradient of 10 to 100% of methanol in water; flow rate, 1 ml/min; detection, UV 210, 254 and 366 nm.

ingly, the magnitude of induction by WE was clearly larger than that of metformin at 1 mM or 0.17 mg/ml. Cell toxicity was observed at high concentrations or with longer exposure (24 h) at concentration 1.5 mg/ml. WE and insulin relationship on 2-DG uptake stimulation was further examined as presented in Figure 4. In these experiments, combination studies were completed with WE at concentrations lower than its maximal induction level (1.25 mg/ml) to enable the detection of an additive effect. It is clear that WE was able to induce glucose transport per se, and was capable of further stimulation of glucose transport in the presence of insulin. The effects of both agents were additive. This suggested that the mechanism mediated by *C. indica* extract is, in part, likely to be independent from that mediated by insulin.

Effects of WE on glucose transporters

It is well established that insulin and other regulators of glucose transport promote glucose uptake by recruiting the insulin-sensitive glucose transporter GLUT4 to the plasma membrane. We studied effects of WE on GLUT4 translocation by Western blot analysis. As shown in Figure 5A, WE caused a slight increase (+35%) on GLUT4 redistribution in the PM fractions compared to control. These changes were accompanied by a corresponding decrease in an intracellular GLUT4 pool. Western blot analyses from four independent experiments revealed no notable change in the amount of GLUT4 total protein (0.95 ± 0.08 folds) in WE-treated cells (Figure 5B). In contrast, a significant increase (3.79 ± 0.74 folds) was detected in GLUT1 density in response to WE. Note that at

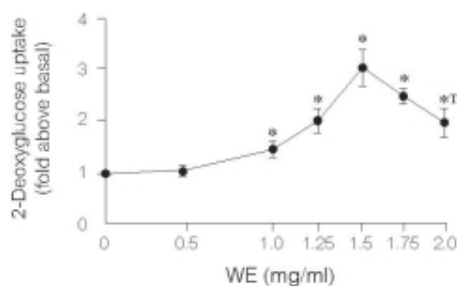


Figure 2. Concentration-dependent effect of *C. indica* extract on 2-deoxyglucose uptake. L8 myotubes were incubated with increasing doses of the water extract of *C. indica* (WE) for 16 h prior to 10 min determination of 2-DG uptake. Results represent means \pm SD from three independent experiments within which each point was assayed in triplicate. *, ($P < 0.05$) vs. basal uptake; T, toxicity.

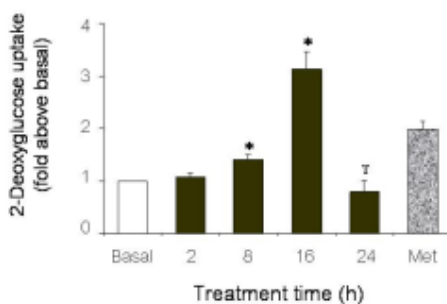


Figure 3. Time course of the stimulation of 2-deoxyglucose uptake by *C. indica* extract. L8 cells were incubated with or without 1 mM metformin (Met) for 16 h and 1.5 mg/ml *C. indica* extract (WE) for the time indicated prior to assess for glucose uptake activity. The results were normalized to untreated control in each time point. Results represent means \pm SD of three independent experiments within which each point was assayed in triplicate. *, $P < 0.05$ vs. basal uptake; T, toxicity.

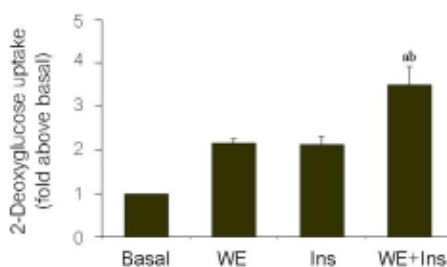


Figure 4. Combined effects of insulin and *C. indica* extract on 2-deoxyglucose uptake in L8 myotubes. Cells were incubated with 100 nM insulin (Ins) in the presence or absence of 1.25 mg/ml *C. indica* extract (WE), serum-deprived for 5 h, and then assayed for the glucose uptake activity in the presence or absence of insulin. Results represent means \pm SD from three independent experiments within which each point was assayed in triplicate. a, $P < 0.05$ vs. values with WE alone; b, $P < 0.05$ vs. values with insulin alone.

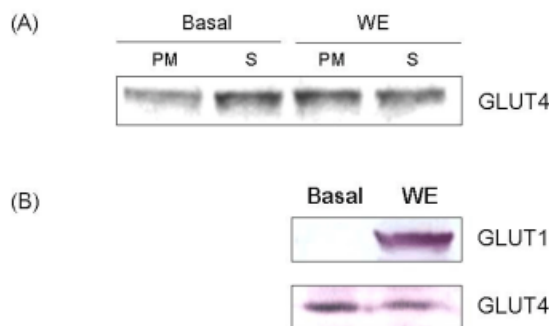


Figure 5. Effects of *C. indica* extract on GLUT4 redistribution (A) and total GLUT1 and GLUT4 protein (B) in L8 myotubes. Cells were incubated with and without 1.5 mg/ml *C. indica* extract (WE) for 16 h. Cell homogenates, plasma membrane (PM) and soluble (S) fraction were analyzed by SDS-PAGE and immunoblotting as described in Materials and Methods. Data represents one of the three independent experiments.

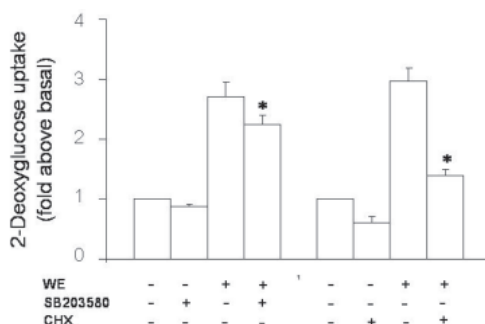


Figure 6. Effects of SB203580 and cycloheximide on the stimulation of 2-deoxyglucose uptake by *C. indica* extract. L8 cells were pre-incubated with 1.5 mg/ml *C. indica* extract (WE) for 16 h. SB203580 was added to a final concentration of 10 μ M at the last 30 min of WE pre-incubation period. Cycloheximide was given to a final concentration of 2 μ g/ml at the beginning and remained present throughout the WE treatment. Results represent means \pm SD of three independent experiments within which each point was assayed in triplicate. *, P<0.05 vs. values with WE alone.

the basal stage, GLUT1 density was barely detected. The results imply that WE promoted glucose transport by increasing the amount of transporters in the cell surface via GLUT4 recruitment and enhanced GLUT1 protein synthesis.

Effects of SB203580 and cycloheximide on WE-induced glucose transport

To examine whether p38 MAPK was involved in WE-stimulated 2-DG uptake, we treated

cells with p38 MAPK inhibitor. Figure 6 showed that SB203580, which interacted with the ATP-binding domain of p38 MAPK (Young *et al.*,1997), caused a slight reduction of the basal uptake, and partially prevented WE-stimulated 2-DG uptake (-17.24 \pm 1.92%). SB203580 at higher concentrations (20 μ M) did not cause further significant reduction. Cycloheximide, a protein synthesis inhibitor, did not have any effect in 1 h treatment (data not shown). In contrast, a powerful inhibitory

effect ($-53.15 \pm 4.13\%$) was demonstrated after prolonged exposure to WE-treated cells.

Discussion

Skeletal muscle is a major target of insulin-dependent glucose disposal. A number of studies have demonstrated that insulin and the antidiabetic drugs exert their action on blood glucose by facilitating glucose utilization in muscle cells (Klip *et al.*, 1990; Yonemitsu *et al.*, 2001). In addition, glucose transport is a rate-limiting step of muscle glucose disposal. The aim of this study was to determine the effects and mechanism by which the water extract of *C. indica* mediates a hypoglycemic response in experimental animals. To accomplish this, we used a cell culture model in which we studied glucose transport in L8 myotubes. We hypothesized that the underlying mechanism of lowering blood glucose level observed in animals treated with the test sample was caused by changes in glucose transporter availability at the cell surface. In earlier studies, the sensitivity of a L8 muscle cell model on glucose transport in response to insulin and metformin stimulation was documented (Purintrapiban and Ratanachaiyavong, 2003).

In cultured muscle cells, elevation of glucose levels such as observed in type II diabetes results in a reduction of glucose transport activity (Klip *et al.*, 1992; Purintrapiban and Ratanachaiyavong, 2003). Our observations that WE increases glucose uptake into muscle cells in high glucose condition (15 mM) implied that this plant extract directly induces up-regulation of glucose transport activity. The effective dose around 1-2 mg/ml suggested a potentially interesting in yielding active product for beneficial use in the treatment of type 2 diabetes mellitus. WE's ability to cause redistribution of GLUT4 reflects a specific action toward glucose utilization in muscle cells, and may involve insulin-mediated signaling. Despite the fact that insulin and *C. indica* extract can induce similar increase in glucose transport activity as demonstrated in the co-incubation study, we have shown that the combined effect was beyond that elicited by each agent alone. This suggests an in tandem operation

of insulin-dependent and independent pathways.

Studies already contain several suggestions indicating different mechanisms in mediating muscle glucose transport, i.e. 1) the existence of separate mechanisms for contraction/hypoxia and insulin with the end result of increased GLUT4 content in the plasma membrane (Douen *et al.*, 1990; Mu *et al.*, 2001; Chen *et al.*, 2002), and 2) proposed mechanisms involved in regulation of GLUT1 expression in response to insulin (PI3-kinase- and p70 S6K-dependent) may differ from those observed in response to oxidative stress (activation of AP-1/enhancer 1 binding of the GLUT1 gene), endothelin-1 (MAPK-dependent) and dexamethasone (PI3-kinase- and possibly mTOR/PHAS-I-dependent) (Kozlovsky *et al.*, 1997; Ewart *et al.*, 1998; Somwar *et al.*, 1998; Ishibashi *et al.*, 2000). Moreover, it is known that insulin can use p38 MAPK for its signaling functions and subsequent activation of transporter intrinsic activity (Rudich and Klip, 2003). In this study, SB203580 had a slight effect on *C. indica*-induced glucose uptake. This suggested that the p38 MAPK-dependent signaling is not a major contribution to the stimulated transport. Although an increased glucose transport profoundly depends on transporter translocation and activation at the plasma membrane, it may not explain the marked effect of *C. indica* extract either alone or in combination with insulin. In addition, the onset of glucose transport induced by this plant was slow (8-16 h) suggesting that the effect requires the synthesis of new proteins. Interestingly, the total amount of GLUT4 isoform did not change upon WE exposure indicating that treatment may increase the transporter susceptibility to degradation, and this process may occur at a corresponding rate to a GLUT4 protein synthesis during 16 h treatment. In contrast to GLUT4 content, GLUT1 protein expression is highly upregulated in response to WE, which can be diminished nearly to the basal level by cycloheximide. A similar finding has been observed in adipocytes and muscle cells following chronic exposure to insulin that insulin markedly decreased GLUT4 gene expression but elevated GLUT1 mRNA and protein levels (Koivisto *et al.*,

1991; Sargeant and Paquet, 1993). The reason that WE treatment could further elevate the transport action beyond a magnitude of the changes maximally observed in response to insulin is likely due to its potent effect on GLUT1 protein expression. The mechanism(s) by which the plant extract may act which are either direct stimulation of GLUT1 promoter or alteration of GLUT1 transcript/protein stability remain to be established. However, it is apparent that the GLUT1 system plays an important role in *C. indica*-induced transport activity.

In summary, these results provide evidence for a previously uncharacterized mechanism of *C. indica* in its antidiabetic activity. Our data demonstrate that *C. indica* stimulates glucose transport and additively enhances the effect of insulin in L8 myotubes. The mechanisms of action are mainly dependent on the upregulation of GLUT1 protein expression with a concomitant subcellular redistribution of GLUT1 and GLUT4 proteins and the increased transporter content in the plasma membrane. Signaling via p38 MAPK leading to transporter activation is at least partially involved.

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