Differential Activation of Glucose Transport in Cultured Muscle Cells by Polyphenolic Compounds from *Canna indica* L. Root

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Effects of extracts of a plant, which has been used as a traditional medicine for treating diabetes on glucose transport activity was evaluated in cultured L8 muscle cells. The aqueous extract of *Canna indica* root (CI) at doses of 0.1—0.5 mg/ml, which contains total phenolic compounds equivalent to 6—30 μg of catechin caused a dose- and time-dependent induction of 2-deoxy-[3H]glucose (2-DG) uptake activity. The induced 2-DG uptake was significantly increased within 8 h and reached a maximum by 16 h. The CI extract increased the amount of glucose transporter isoforms 1 (GLUT1) and 4 (GLUT4) at the cell surface and enhanced expression of GLUT1 protein. Cycloheximide treatment almost completely reversed CI-induced 2-DG uptake to the basal level. Exposure of muscle cells to wortmannin and SB203580 diminished CI-mediated glucose uptake by 38 and 14%, respectively. The effect of CI and insulin was partially additive. Phytochemical analysis detected the presence of flavonoids and catechol in the CI. Taken together, these data provide evidence for differential effects of CI on regulated-glucose transport in muscle cells. Our findings suggest that GLUT1 protein synthesis and the activation of phosphatidylinositol 3-kinase (PI3K) are critical for the increase in glucose transporter activity at the plasma membrane and essential for the maximal induction of glucose transport by CI in L8 muscle cells.

Key words  *Canna indica*; polyphenol; glucose uptake; glucose transporter isoform 1 (GLUT1); L8 myotube

Peripheral resistance to insulin is a prominent feature of both insulin-dependent and non-insulin-dependent diabetes. One of the major factors regulating glucose uptake into muscle is the quantity of glucose transporter (GLUT) protein on the cell surface. In muscle and adipocytes, GLUT1 mediates basal or nonstimulated transport, whereas GLUT4, the insulin-responsive GLUT, facilitates increased glucose transport in the presence of insulin.13 Membrane GLUT4 is regulated by insulin via a PI3K-dependent process.1—3 However, increased GLUT4 content is insufficient to fully account for glucose transport activity observed under insulin-stimulated conditions. Specifically, transporter translocation accounts for approximately 30% of insulin-stimulated glucose uptake while activation of GLUTs is essential for maximal stimulation.4,5 Similarly, inhibition of p38 mitogen-activated protein kinase (p38 MAPK) activity prevents insulin-stimulated glucose transport but has no effect on glucose transporter translocation in adipocytes and skeletal muscle.6 Together, these evidence indicate that both PI3K and p38 MAPK activation are associated with basal and insulin-induced glucose transport.

Polyphenols present in plant-derived fruits and vegetables have been implicated in mediating glucose transport in *vitro* and *in vivo* studies.7—9 A tea polyphenol extract markedly increased basal and insulin-stimulated glucose uptake of adipocytes.10 In addition, several polyphenolic compounds were recently reported to inhibit sodium-dependent glucose transport in intestinal epithelial cells.11 Hence, polyphenols could play a role in controlling glucose uptake in the intestinal tract and peripheral tissues, and possibly contribute to blood glucose homeostasis.

A preliminary study of plants used in Thai folkloric medicine to treat diabetes determined a distinct effect of *Canna indica* L. (Cannaceae) watery extract on glucose uptake activity in a cell culture model, and, hence this extract was chosen for this present study. The aim of this work was to study the mechanism of action of *C. indica* on the stimulation of glucose uptake in L8 muscle cells.

MATERIALS AND METHODS

**Plant Extraction** The root of CI was collected in June 2004 from the medicinal plant garden of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Samples were dried at a 45 °C for 4 d and then ground to a powder. Five grams of grounded powder were extracted with 200 ml of distilled water at 70 °C for 30 min. The extract was filtered and then centrifuged at 5000 g for 20 min followed by freeze-drying (yield 72.5 mg/g dry weight). Varying concentrations in mg/ml were prepared from freeze-dry residue, and then used in further studies.

**Determination of Total Phenolics and Phytochemical Screening** The amount of total phenolic compounds was determined spectrophotometrically using Folin–Ciocalteu reagent as described by Lee et al.12 and was expressed in microgram of catechin equivalent (CE) based on a calibration curve for catechin. The content of total phenolics of CI root was 60.02±4.05 μg CE/mg freeze-dry residue. Phytochemical screening of CI root was performed using the methods previously described by Farnsworth13 and Harborne14 with slight modification. In brief, several reagents were prepared to test for the presence of flavonoids, coumarins, anthraquinones, cardiac glycosides, cyanogenic glycosides, coumarins, saponins and tannins. The results were compared with the positive standards of each test.

**Cell Culture and Incubations** L8 cells (ATCC, U.S.A.) were grown and differentiated into myotubes according to previous established method.15 Cells at the stage of myotubes were incubated with or without the CI extract or 1 mM metformin (positive control) in HEPES buffered saline, pH 7.4 (HBS) containing 15 mM glucose and 2% HS for the indicated period (time-dependent studies) or various doses (dose-
been shown to contain...been further maintained for 5 h without serum or insulin before the transport assay. Inhibitors were added into the culture medium at a certain time before the end of 16 h incubation (i.e. 2 μg/ml cycloheximide, 16 h; 100 nM wortmannin, 20 min; 10 μM SB203580, 30 min).

**Glucose Transport Assay** After the above incubations, cells were rinsed twice with HBS and incubated in HBS containing 10 μM 2-deoxy-[3H]glucose (2-DG, 1 μCi/ml) alone or with 100 nM insulin. At the end of incubation, the test media were collected and determined for lactate dehydrogenase (LDH) activity that was released upon cell lysis by the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI, U.S.A.). The 2-DG uptake was determined over a 10-min period at 37°C and terminated by three washes with 1 ml ice-cold 0.9% NaCl. The uptake of 2-DG into L8 myotubes was linear up to 15 min. Non-specific uptake, which was less than 10% of total uptake, was measured in the presence of 10 mM phloretin. The radioactivity associated with the cells was determined by cell lysis in 0.05 N NaOH, followed by liquid scintillation counting.

**Analysis of Glucose Transporters** Subcellular fractions and whole cell extracts were prepared as described by Yu et al. Briefly, cells were harvested and homogenized in homogenizing buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1 mM MgCl₂, protease inhibitors). Cell lysates were centrifuged at 200 g for 5 min. The supernatant (whole cell lysate) were collected for total protein analysis or further centrifuged at 16000 g for 15 min to obtain a plasma membrane (PM) pellet and a supernatant or soluble (S) fraction. The resulting pellet from this step has to obtain a plasma membrane (PM) pellet and a supernatant or soluble (S) fraction. The resulting pellet from this step has been shown to contain 95% of the α-subunit of Na⁺-K⁺-ATPase. Protein content was determined by the Bio-Rad Protein assay. Protein samples (150 μg) were separated in 10% SDS-PAGE and then transferred to PVDF. Western blots were performed as described earlier with antisera against GLUT4 (1:250, Biogenesis, Inc., U.S.A.) or GLUT1 (1:700, Santa Cruz Biotechnology, Inc., U.S.A.) and α-actin (1:700, Sigma-Aldrich, Inc., U.S.A.). α-Actin which expresses in differentiated myotubes was used as internal control.

**Statistical Analysis** Three to five independent experiments were conducted in all studies, and all assay conditions were performed in triplicate. Data were analyzed using ANOVA followed by Scheffe’s test. A level of significance of 5% was adopted for all comparisons.

**RESULTS AND DISCUSSION**

**Constituents in C. indica Root** Phytochemical screening yielded positive results for reactions with a 1% gelatin solution (gave precipitate) and a 1% ferric chloride solution (gave green-solution), suggesting the existence of condensed tannins catechol. The root extract also yielded positive results for Shinoda’s test by yielding a pink-red solution and for the test specific for chalcones and aurones and catechin suggesting the presence of several groups of flavonoids. The presence of high levels of phenolic compounds in parallel with elevated glucose uptake indicates that the active compounds of CI may act in concert in mediating glucose transport. These observations suggest that the potential activator(s) of glucose transport in CI root may contribute to polyphenolic compounds present in the extract.

**Effect on 2-DG Uptake** Figure 1 shows a dose- and time-dependent induction of glucose transport by CI in L8 myotubes. Prolonged (16 h) CI administration increased glucose uptake activity with maximal response (3.07 ± 0.17-fold, n = 5) at a dose of 0.4 mg/ml. Activation of 2-DG uptake was detected within 2 h, increased significantly at 8 h and reached a maximal level at 16 h of incubation. Metformin, the anti-diabetic drug that mainly mediates glucose transporter’s intrinsic activity and improved insulin action, elicited a smaller increase in 2-DG uptake in the time period studies. A slight toxicity (p > 0.05) was observed at maximal dose (0.4 mg/ml). CI became toxic to cells (p < 0.05) after attaining a peak value (Fig. 1A) or exposing the maximum dose longer than 16 h (Fig. 1B) in which either increased LDH activity or cell death were detected. To assess whether CI was capable of modulating insulin action, CI-pretreated cells were maintained in serum-free media for 5 h to enable the detection of insulin response. As shown in Fig. 2, insulin-stimulated glucose transport was significantly increased by 72% (+72.75 ± 30.29%) above that in the presence of insulin alone. These data suggest that CI can activate glucose transport independence of insulin and may exert its action partially through insulin-independent mechanisms since we detect the additive effect of insulin and CI.

**Effect on Glucose Transporter Levels and Redistribution** It is now apparent that GLUT4 and GLUT1 are the principal isoforms that respond to various stimuli. The effect of CI on these isoforms was analyzed by Western blot. Figure 3A shows a significant increase of GLUT4 protein levels in the plasma membrane compared to those observed
GLUT1 protein levels upon prolonged incubation with CI may be attributed to altered GLUT1 gene expression, to enhanced translation of GLUT1 mRNA or to increased stability of GLUT1 protein. These possibilities need additional study.

**Effect of Inhibitors on CI-Stimulated 2-DG Uptake**

The effect of CI reflects its ability to recruit GLUT4 and a possibility of further regulation of subsequent intrinsic activation of GLUT4 at the plasma membrane that commonly occurs following insulin-receptor activation.28,29 In addition, accumulation of GLUT1 indicates that new synthesized protein is required. To explore whether the effect of CI depends on the action of PI3K and p38 MAPK, and an induction of the synthesis of proteins relevant for the stimulation of glucose transport, we utilized various inhibitors and determined their inhibitory effects on glucose uptake activity. SB203580 primarily causes a reduction of glucose uptake without an effect on GLUT4 translocation through the inhibition of p38 MAPK activity.6 Addition of 10 \( \mu \text{M} \) SB203580 to L8 cells 30 min before assay of glucose uptake activity slightly decreased CI-stimulated glucose uptake (−14.28 ± 2.36%, \( p>0.05 \)) and had no effect on the basal uptake (Fig. 4). Treatment with 100 \( \mu \text{M} \) wortmannin, a specific inhibitor of PI3K, caused a partial reduction (−38.20 ± 7.55%, \( p<0.01 \)) in the basal rate of uptake indicating a wortmannin-sensitive GLUT4 pool which is recruited to the cell surface via a PI3K-dependent signal. These data suggest a contribution of p38 MAPK-modulated GLUT4 activation in combination with a PI3K-mediated GLUT4 translocation in CI-mediated transport activity. Even though a SB203580 effect on GLUT1 may not be fully excluded, either no or a small effect of the inhibitor on induced-glucose uptake levels in GLUT1 predominantly expressed cells have been shown.30,31 Figure 4 also illustrates that a decrease in basal uptake of 45% was observed in the presence of 2 \( \mu \text{g/ml} \) cycloheximide. This implies that the synthesis of new transporter protein is important for maintaining the amount of transporter that normally regulates basal glucose transport. As expected, an increment of glucose transport due to CI induction (ca. 3-fold above basal values) was almost completely reversed by this inhibitor. These data indicate that protein synthesis is necessary for CI-stimulated glucose transport. Although cycloheximide blocked the synthesis of all GLUT isoforms, the inhibition may mainly associated with the expression of GLUT1 and minimally affected GLUT4 expression.

Insulin resistance is believed to cause by defects of insulin-post-receptor signals which affect translocation of GLUT4 to the plasma membrane.12,23 Several stimuli are
known to increase the amount of GLUT4 protein in the cell surface by different pathways, which are insulin-independent and wortmannin-insensitive. Furthermore, the normal response to these stimuli has been demonstrated in insulin-resistant muscle. \textsuperscript{31,33—36} These include stimulation of 5'-AMP-activated kinase-dependent mechanism by hypoxia and contraction/exercise and the mechanism involved Cbl-TC10, though the later is observed in adipocytes but not muscle cells.\textsuperscript{21,22} As shown in this study, CI offers a potential source of therapeutic agent(s) that may use to improve insulin signaling defects and/or activate insulin-independent pathways.

In conclusion, this study provides evidence of the stimulatory effect of glucose uptake and the modulation of insulin-responsive glucose transport by CI in cultured muscle cells. CI exerts its action mainly through GLUT4 translocation and up-regulation of GLUT1 protein expression, and partially via increased the activity of glucose transporter.

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REFERENCES


