



Original Article

The biological activity of *Coccinia indica* on glucose transporter 1 (GLUT1) promoter

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Abstract

Plant derivatives with purported hypoglycemic properties have been used in traditional medicine around the world. *Coccinia indica* (ivy gourd) is used in traditional medicine to treat diabetics in many countries. *C. indica* is able to cause a reduction in blood glucose level and has shown hypoglycemic activity *in vitro* and *in vivo*. However, the mechanism of this effect remains unknown. In this study, we generated the pGL3-glucose transporter 1 (GLUT1) promoter to elucidate the molecular mechanism of the regulation of GLUT1 gene expression in response to a water extract of *C. indica* stem (CI extract). A fragment of 2.1 kb of rat GLUT1 promoter, located at -2,106 to +134, was linked to firefly luciferase. The regulating transcription was analyzed in transient expression assay after transfection and exposure of L6 myocytes with the GLUT1 promoter system and CI extract, respectively. Under normal condition (5 mM glucose), promoter activity induced by 0.15 mg CI extract was markedly increased by 5.71 fold from the basal value. CI extract was more effective than 2 mM metformin. Surprisingly, promoter activity in hyperglycemic condition (15 mM glucose) induced by 0.50 mg CI was increased by 1.63 fold from the basal value. In addition, CI extract increased the 2-deoxyglucose (2-DG) uptake in L6 myocytes in a dose-dependent manner in both conditions, 5 mM and 15 mM glucose. GLUT1 protein was determined by Western blot analysis and the level also increased in a dose-dependent fashion. Interestingly, the activity of the -273 to +134 of GLUT1 promoter was increased by 2.12 fold from the basal value. This site is the transcription initiation site containing GC box and TATA box. These observations suggest that the hypoglycemic action of *C. indica* may regulate through the activation of GLUT1 promoter resulting in an increase of the GLUT1 protein expression.

Keywords: GLUT1 promoter, *Coccinia indica*, luciferase, L6 myocyte

1. Introduction

Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. The ethnobotanical information reports that about 800 plants may possess an anti-diabetic potential including *Coccinia indica* (synonym *Coccinia cordifolia*, ivy gourd) (Alarcon-Aguilara *et al.*, 1998). *C. indica* is used in Ayurveda and Unani systems of

medicine for the treatment of diabetes, skin eruptions, tongue sores, earaches, etc. in India (Chopra *et al.*, 1956; Chopra *et al.*, 1958). Several scientists reported that *C. indica* leaves, fruit and roots have a potential hypoglycemic action in normal animals (Mukherjee *et al.*, 1972; Chandrasekar *et al.*, 1989; Kumar *et al.*, 1993), diabetic animals (Singh *et al.*, 1985; Shbib *et al.*, 1993; Kar *et al.*, 2003) and type 2 diabetic patients (Kuriyan *et al.*, 2007). The hypoglycemic effect of *C. indica* is partly mediated through the depression of the key gluconeogenic enzyme glucose-6-phosphatase and fructose-1,6-bisphosphatase (Hossain *et al.*, 1992; Shbib *et al.*, 1993). However, further studies are needed to elucidate the mechanism of this action.

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The family of facilitative glucose transporters is GLUT proteins. These are widely distributed proteins present in the plasma membranes of almost all cells. At present, there are 13 isoforms that divide into 3 groups: Class I (GLUT1-GLUT4), Class II (GLUT5, 7, 9 and 11) and Class III (GLUT6, 8, 10, 12 and HMIT1) (Bryant *et al.*, 2002). GLUTs are encoded by distinct genes of the *SLC2A* family (Solute carrier family 2). The expression of the particular genes in this family, including the newest member, GLUT14, is specific to tissues and cells (Longo and Elsas, 1998; Joost and Thorens, 2001; Piatkiewicz *et al.*, 2007). GLUT1 is a ubiquitous glucose transporter isoform. It is widely expressed and facilitates as a basal glucose transporter in many cell types except liver and pancreatic beta cells (Bell *et al.*, 1990). The GLUT1 gene is rapidly activated by serum, growth factors and oncogenic transformation (Hiraki *et al.*, 1988). In addition, GLUT1 is considered to be a stress response protein. For example, oxidative stress increases GLUT1 expression both in protein and mRNA levels by the activation of AP-1 (transcription activation factor) binding to enhancer 1 of the *GLUT1* gene (Kozlovsky *et al.*, 1997).

In a previous study, we demonstrated that a water extract of *C. indica* stem exhibited a dose-dependent induction of 2-deoxyglucose (2-DG) uptake and increased GLUT1 protein in rat L8 myotubes in a 16 h treatment compared with the control (Purintrapiban *et al.*, 2006). Since *C. indica* has a hypoglycemic activity *in vitro* and *in vivo*, we hypothesized that the action mechanism of the water extract from *C. indica* stem may control the transcription of GLUT1 promoter.

2. Materials and Methods

2.1 Materials

Rat L6 myoblast was obtained from the American Type Culture Collection (Rockville, MD). Tissue culture medium and supplements were obtained from Invitrogen (Carlsbad, CA). 2-Deoxy-D-[³H] glucose was supplied by Amersham Biosciences (Piscataway, NJ). pGL3-basic vector and Dual Luciferase assay system were procured from Promega (Madison, WI).

2.2 Cell culture

L6 myoblasts were grown in a monolayer and maintained in proliferation medium (Dulbecco's modified Eagle's medium (DMEM), low glucose with addition of 100 U/ml of penicillin, 100 µg/ml streptomycin, 2.5 g/l NaHCO₃ and 10% fetal bovine serum). Cells were incubated in a 5% CO₂ incubator at 37°C. Cells were grown to the myocyte stage, in either 100-mm dish for cell lysate preparation or a 48-well plate for luciferase and glucose uptake studies. In brief, 65,000 cells of L6 myoblasts were maintained in the proliferation medium for 1 day. Then, the medium was changed to a low-serum (2% horse serum, HS) containing medium for

2 days.

2.3 Preparation of *C. indica* water extract

The stems of *Coccinia indica* Wight & Arn (Cucurbitaceae) were collected from Songkhla province, Thailand. The plant specimen (voucher number CUC-001) has been identified and deposited in the herbarium of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Plant materials were cut, dried in a 42°C oven and ground into powder. One gram of ground sample was extracted in 20 ml phosphate-buffered saline (PBS) at room temperature for 30 min, and centrifuged at 12,000xg for 1 min. The supernatant (CI extract) was sterile-filtered and stored at 4°C. One microliter of CI extract is equally 0.05 mg of ground sample.

2.4 GLUT1 luciferase reporter constructs, transient transfection and luciferase reporter assays

The rat GLUT1 promoter region, located at -2106 to +134, was from Dr. M. Birnbaum (University of Pennsylvania, Philadelphia, PA). The different deletions of the 2,240 bp fragment of GLUT1 promoter were constructed into pGL3-basic luciferase reporter vector (Promega). The deletions were generated by cleaving with *Eco*RI and *Hind*III (-2106 to -1667), with *Kpn*I and *Xho*I (-979 to +134), with *Sma*I and *Xho*I (-207 to +134).

L6 myocytes were cotransfected using FuGENE6 (Roche, Indianapolis, IN) with 0.25 µg of pGL3-basic constructs expressing firefly luciferase containing GLUT1 promoter and with 0.001 µg of pRL-SL40 plasmid (expressing *Renilla* luciferase). After 30 h of transfection, cells were exposed to CI extract or 2 mM metformin in HEPES buffered saline, pH 7.4 (HBS) containing 2% HS with 5 or 15 mM glucose for 16 h. The cells were washed twice with PBS and then assayed for luciferase activity. Firefly and renilla luciferase assays were performed according to the manufacturer's protocol (Promega). In brief, cells were lysed with 65 µl of 1X PLB buffer and incubated at room temperature for 15 min. The differences in transfection efficiency between the experimental groups were normalized by calculating the ratio between the firefly and *Renilla* luciferase activities. Transfections and assays were performed in triplicate for each experiment group.

2.5 2-Deoxyglucose (2-DG) uptake assay and treatments

Cells were exposed to various agents (CI extract or 2 mM metformin) in HBS containing 2% HS with 5 or 15 mM glucose for 16 h. After the treatment period, cells were rinsed twice with HBS and incubated in HBS containing 23 nM 2-deoxy-[³H] glucose at 37 °C for 10 min. The glucose uptake reaction was terminated by washing the cells 3 times with an ice-cold saline solution. The cells were finally lysed in 0.15 ml of 0.05 N NaOH for 20 min. The 2-DG uptake of the cell

lysate was determined by liquid scintillation counting.

2.6 Cell lysate preparation and Western blot analysis

Lysate preparation was performed by the method described by Whiteman and Birnbaum (2003). Cells were washed three times with cold PBS and scraped in ice-cold high salt, non-denaturing lysis buffer (lysis buffer contains 20 mM tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% triton-X-100, 200 mM NaF, 5 mM EDTA, 1 mM EGTA and protease inhibitor cocktail). The cell suspension was placed on ice for 30 min and centrifuged at 10,000xg at 4°C for 15 min. The supernatant (total cell lysate) was used for protein determination by Bradford's method (1976) and determined for GLUT1 and α -actin protein by Western blot analysis as described by Purintrapiban *et al.* (2006).

2.7 Statistical analysis

Statistical significance was calculated using a paired Student's *t*-test. Results with a $p<0.05$ were considered significant for the mRNA expression experiments.

3. Results

3.1 Effect of CI water extract on GLUT1 promoter activity

To determine the effect of CI extract on a 2,240 bp fragment of GLUT1 promoter, we transfected cells with pGL3-GLUT1 followed by incubation with CI extract, metformin and insulin for 16 h. We also tested the effect of glucose under the treatment condition. The treatment solution contained 5 mM glucose or 15 mM glucose. In normal glucose, 5 mM, we found that CI extract concentration 0.15, 0.5, 2.0, 3.5 and 5.0 mg induced luciferase activity to 5.71, 5.8, 5.23, 3.16 and 4.05 fold from the basal value, respectively. In addition, 0.15, 0.5 and 2.0 mg of CI extract was more effective than metformin, an anti-diabetes drug (Figure 1A). In a hyperglycemic condition, 15 mM glucose, promoter activities were slightly increased by 1.35 and 1.63 fold from the basal value after treating cell with CI extract concentration 0.15 and 0.5 mg, respectively. Surprisingly, metformin did not affect the promoter activity (Figure 2A).

3.2 Effect of CI extract on 2-Deoxyglucose (2-DG) uptake assay

To test whether CI extract stimulates GLUT1 expression resulting in an increase of glucose uptake, L6 myocytes were treated with various CI extract concentration and incubated for 16 h. Under normal condition (5 mM glucose), the uptake of 2-DG was greatly enhanced in a dose-dependent manner with a maximal induction up to 1.96 fold above the basal value (Figure 1B). In addition, 3.5 mg of CI extract increased 2-DG uptake up to 2.31 fold above the basal value in the condition of 15 mM glucose (Figure 2B). Metformin

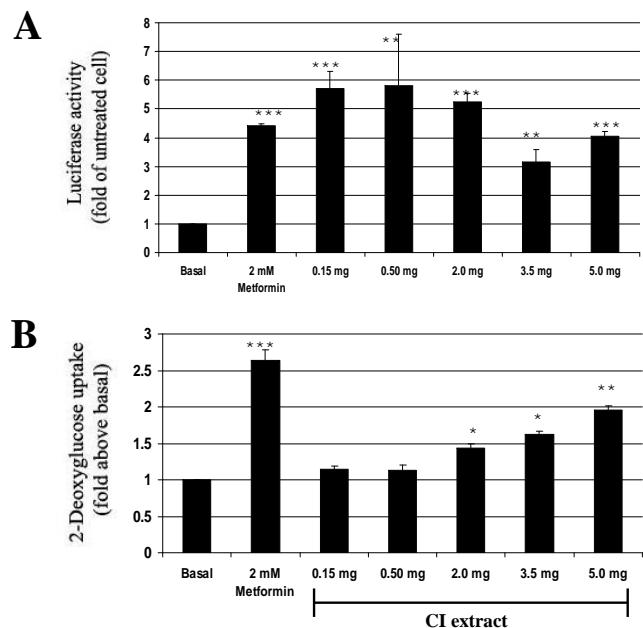


Figure 1. Effect of *C. indica* extract on GLUT1 promoter activity in normal glucose condition.

L6 myocytes were transfected with a 2,240 bp fragment of pGL3-GLUT1 promoter. Thirty hours later cells were treated with 2 mM metformin or 0.15, 0.5, 2.0, 3.5, 5.0 mg CI extract in 5 mM glucose for 16 h. Cells were then harvested and measured for luciferase activity (*Panel A*) and 2-Deoxyglucose uptake (*Panel B*). Firefly luciferase activity was normalized against Renilla luciferase activity to control for transfection efficiency. Results (in relative luciferase units) represent the average of three independent experiments performed in triplicate and are mean \pm S.D. transfections. * $p<0.05$, ** $p<0.01$, *** $p<0.005$ compared with basal.

showed the highest value, 2.64 and 3.55, in both conditions 5 mM and 15 mM glucose, respectively.

3.3 Effect of CI extract on GLUT1 protein

To make certain that upregulation of 2,240 bp fragment of GLUT 1 promoter leads to GLUT1 protein synthesis, L6 cells were treated with various concentrations of CI extract in 5 mM glucose for 16 h. Then the cell lysate samples were subjected to 10% SDS-PAGE and probed with antibodies against GLUT1 and α -actin. Here, we found that CI extract upregulated GLUT1 protein in a dose-dependent manner (Figure 3).

3.4 The TATA and GC box in rat GLUT1 promoter is necessary for the actions of CI extract

To test the binding site of the CI extract on the GLUT1 promoter, we generated three small fragments including -2106 to -1167, -979 to +134 and -207 to +134 and transfected into L6 myocytes. These transfected cells were treated

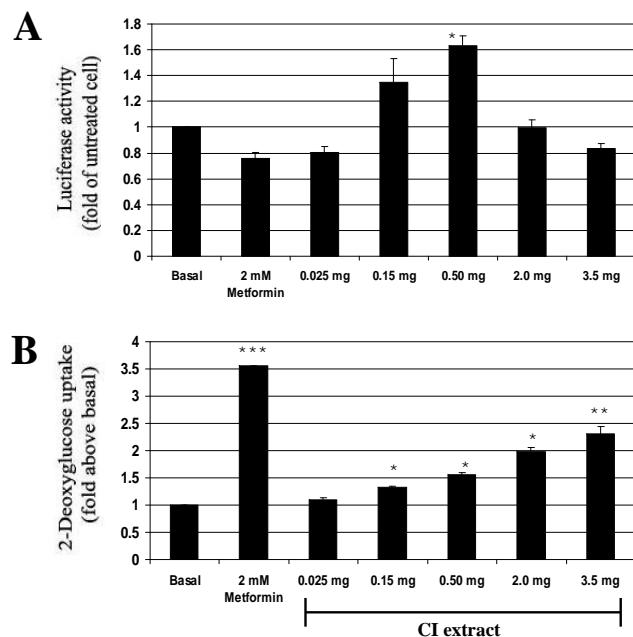


Figure 2. Effect of *C. indica* extract on GLUT1 promoter activity in high glucose condition.

L6 myocytes were transfected with a 2,240 bp fragment of pGL3-GLUT1 promoter. Thirty hours later cells were treated with 2 mM metformin or 0.025, 0.15, 0.5, 2.0, 3.5 mg CI extract in 15 mM glucose for 16 h. Cells were then harvested and measured for luciferase activity (Panel A) and 2-Deoxyglucose uptake (Panel B). Firefly luciferase activity was normalized against Renilla luciferase activity to control for transfection efficiency. Results (in relative luciferase units) represent the average of three independent experiments performed in triplicate and are mean \pm S.D. transfections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared with basal.

with 0.15 mg of CI extract. The luciferase activities increased by 1.62 and 2.12 fold from the basal value by treatment with -979 to +134 and -207 to +134 GLUT1 promoters, respectively (Figure 4).

4. Discussion

GLUT1 is a ubiquitous glucose transporter that mediates the basal glucose transporter into cells. GLUT1 is highly expressed in conditions with a high demand for glucose, as in proliferating cells or in fetal tissue (Flier *et al.*, 1987). In adult life, GLUT1 levels are high in the endothelial cells of the blood-brain barrier, epithelial cells from the mammary glands, primate erythrocytes and cardiac myocytes from adult rats (Baldwin, 1993; Camps *et al.*, 1994; Fischer *et al.*, 1997; Vannucci *et al.*, 1997). GLUT1 may be involved in the survival of individual cells by providing them with basal glucose requirements. GLUT1 gene expression is regulated in different cell types by various stimuli including hypoglycemia, hypoxia, mitochondria inhibitors, prolonged insulin exposure, tumor necrosis factor alpha and iron chelators (Kozlovsky

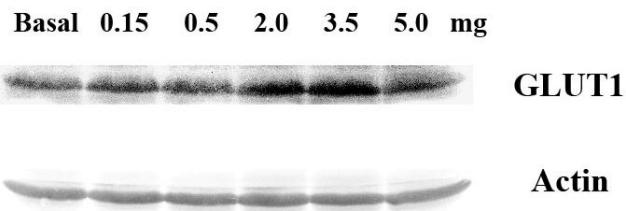


Figure 3. Effect of *C. indica* extract on GLUT1 protein.

L6 myocytes were treated with 0.15, 0.5, 2.0, 3.5 and 5.0 mg of CI extract or without CI extract (Basal) in 5 mM glucose and incubated for 16 h. Cell lysates were analyzed by 10% SDS-PAGE and detected with anti-GLUT1 and anti-actin antibodies.

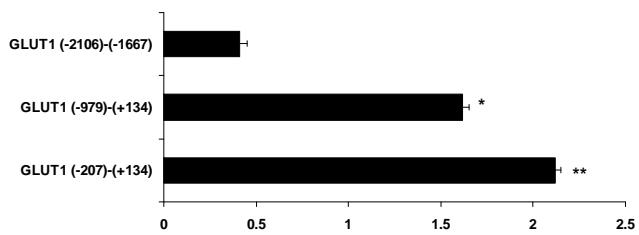


Figure 4. Analysis of CI extract on GLUT1 promoter region

Series of upstream region of the GLUT1 gene from (-2106) to (+134) relative to the transcription start site were generated. L6 myocytes were transfected with different site of GLUT1 promoter. Thirty hours later cells were treated with 0.15 mg CI extract in 5 mM glucose for 16 h. Cells were then harvested and measured for luciferase activity. Results (in relative luciferase units) represent the average of three independent experiments performed in triplicate and are mean \pm S.D. transfections. * $p < 0.05$ and ** $p < 0.01$ compared with basal.

et al., 1997). Insulin, stimuli and stress condition enhance glucose transport in a biphasic with the early phase mediated by posttranslational mechanisms and the late phase involved enhancement of GLUT1 gene expression (Rollins *et al.*, 1988; Shetty *et al.*, 1992; Barros *et al.*, 1997). In addition, activation of the GLUT1 gene by insulin deserves attention in blood glucose levels. Todaka *et al.* (1994) reported that enhancer-1 of GLUT1 gene (located at 2.7 kb upstream) is essential for rapid activation by insulin in 3T3-L1 adipocytes. Moreover, metformin affects the translocation of the GLUT1 and GLUT4. Previous results have shown that the incubation of L8 cell with 1-2 mM metformin increased the basal glucose uptake more than 2 fold from the basal value (Purintrapiban and Ratanachaiyavong, 2003). In the present work, we confirmed that metformin can directly activate GLUT1 promoter (Figure 1 and 2).

Previous results have demonstrated that CI extract stimulates glucose transport and additively enhances the effect of insulin in L8 myotubes. This current study, we have shown that GLUT1 promoter activity was significantly increased in response to CI extract in L6 myocytes in both

conditions, 5 mM and 15 mM glucose. CI extract was capable of inducing GLUT1 promoter activity at a low concentration (0.15 mg) and the induction was reduced when treated with 3.5 mg CI extract. In contrast, 2-DG uptake and protein levels were increased in a dose-dependent manner after challenging with 0.15 to 5.0 mg CI extract (Figure 1 and 3). Taken together, these results indicated that *C. indica* upregulates the GLUT1 promoter leading to a hypoglycemic effect. However, only one CI extract concentration (0.50 mg) was capable of increasing GLUT1 promoter activity in a hyperglycemic condition (15 mM glucose). Surprisingly, the 2-DG uptake in this condition was increased in a dose-dependent manner, starting from treatment with 0.15 to 3.5 mg of CI extract and the results showed the same value as under a normal condition (5 mM glucose) (Figure 2). It is possible that GLUT1 expression is stimulated in response to high glucose concentration. Previous researchers reported that GLUT1-mRNA, GLUT1-protein and glucose transport increased after exposure of Clone 9 cells to hyperosmolarity, 600 mosmol/l (mannitol) (Hwang and Ismail-Beigi, 2001). Moreover, the expression of GLUT1 mRNA and protein of osteoblast in hyperglycemia (25 mM glucose) were 51% and 35% higher than in the normal glucose (5.5 mM) after 3 days of incubation (Fang *et al.*, 2006). Huang *et al.* (2002) also reported that high glucose (25 mM) increased GLUT1 protein by 2.5 fold in L6-GLUT4myc myotubes. Nevertheless, high glucose did not elevate the basal level of glucose uptake in L6-GLUT1myc myotubes. These results indicated that increased GLUT1 mRNA and protein by high glucose level may give rise to a high background of the promoter activity assays in hyperglycemia condition in this study. Thus, only one effective dose of CI extract could show the significant result (Figure 2).

The GLUT1 expression is controlled by the activity of the core promoter and two distinct enhancers. The core promoter of rat GLUT1 gene locates at the -99 to -33 region. This promoter drives the transcriptional activity of GLUT1 in a variety of non-muscle and muscle cells (Fandos *et al.*, 1999). The 44 bp GC-rich element located at -104 to -61, is necessary for the basal transcription of the GLUT1 gene. This site responds to hyperosmolarity (Hwang and Ismail-Beigi, 2001). Santalucia *et al.* (2005) described that the activity of the -99 to +134 of GLUT1 promoter is increased by phenylephrine. This region is relate to the transcription initiation site and acts as a minimal promoter, essential for the high level expression of GLUT1 in skeletal muscle cell lines and neonatal ventricular cardiac myocytes (NCMs), and contains binding sites for transcription factors (Santalucia *et al.*, 1999). The GLUT1 promoter contains a TATA box at -31 to -24 which could act as a binding site for TFIID, a multiprotein complex required in the initiation of transcription (Williams and Birnbaum, 1988). Several scientists have identified the GC box located at -91 to -86 in the GLUT1 core promoter that binds Sp1 and Sp3. Sp1 acts as a transcriptional activator (Hwang and Ismail-Beigi, 2007) and Sp3 as a repressor (Vinals *et al.*, 1997; Fandos *et al.*, 1999;

Santalucia *et al.*, 1999). A novel element located at -46 to -37 (MGIE-Muscle-Specific GLUT1 Element) has been reported to bind muscle-specific nuclear factors. This element could also bind Sp1, cAMP and serum resulting in activation of GLUT1 transcription (Sanchez-Feutrie *et al.*, 2004).

Finally, to confirm the binding activation and identify the binding target of the CI extract, we generated three deletions of the GLUT1 promoter. As the promoter activity obtained at a high glucose concentration restricted the effect of GLUT1 mRNA and protein, in this experiment cells were treated in normal glucose to eliminate the effect of high glucose. The results showed that CI extract regulates the GLUT1 promoter at the transcription initiation site (-207 to +134) (Figure 4). This site is also activated by many factors as described above. Since the GLUT1 promoter activity is upregulated in normal glucose condition and without additional supplement (insulin or other stimuli) or stress condition, we conclude that CI extract acts directly through the stimulation of the GLUT1 promoter at the transcription initiation site (GC and TATA box).

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