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Enhancement of Glucose Uptake in 3T3-L1 Adipocytes by *Toona sinensis* Leaf Extract

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The effects of substances extracted from *Toona sinensis* leaves, using 50% alcohol/water, on cellular [^{3}H]-2-deoxyglucose uptake in differentiated cultured 3T3-L1 adipocytes were investigated. Following treatment of cells with 0.001, 0.01, or 0.1 mg/mL extracts for 60 minutes, [^{3}H]-2-deoxyglucose uptake increased from a basal value of 0.23 nmol/min/mg protein to 0.30, 0.33, and 0.38 nmol/min/mg protein, respectively. In insulin-stimulated cells, cellular [^{3}H]-2-deoxyglucose uptake was enhanced by *Toona sinensis* leaf extract from a basal value of 0.35 nmol/min/mg protein to 0.41, 0.46, and 0.52 nmol/min/mg protein, respectively. Cellular glucose uptake was also enhanced by *Toona sinensis* leaf extract after incubation of cells with 20 mM glucose for 48 hours. Cellular glucose uptake with a combination of *Toona sinensis* leaf extract and insulin was significantly inhibited by pretreatment of cells with the protein synthesis inhibitor cycloheximide and the protein kinase C inhibitor calphostin C in normal-, medium- and high-glucose media. However, the glucose uptake-enhancing effect of *Toona sinensis* leaf extract was not diminished by cycloheximide and calphostin C in the absence of insulin. These results indicate that enhancement of cellular glucose uptake by *Toona sinensis* leaf extract in basal and insulin-stimulated 3T3-L1 adipocytes may be mediated by distinct mechanisms.

Key Words: 2-deoxyglucose uptake, 3T3-L1 adipocytes, *Toona sinensis* leaf
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Diabetes mellitus is a complex and chronic progressive disease that can eventually adversely affect the function of many organs. Most patients with type 2 diabetes mellitus exhibit hyperglycemia and peripheral insulin resistance. Current drug therapy for diabetes mellitus is aimed at improving glycemic control [1]. Despite the fact that oral hypoglycemic agents have been available for almost 30 years, their precise mode of action and role in the management of diabetes mellitus remain poorly defined and controversial [2]. The most commonly used class of oral hypoglycemics are sulfonylureas, whose mechanism of action probably involves insulin release from pancreatic β-cells [3–5]. The sulfonylureas also have extrapancreatic action that help with insulin resistance in type 2 diabetes [6]. They are valuable therapeutic agents, but nevertheless, they possess disadvantages such as a reduction in efficacy and the potential to induce hypoglycemia in the long term [7]. Thus, numerous other hypoglycemic compounds, including crude drugs, have been investigated as potential agents for treating type 2 diabetes.

Recently, it was reported that ginseng extract increased glucose uptake in rabbit brain [8] and sheep

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erythrocytes [9]. However, protopanaxatriol ginsenosides inhibit glucose uptake in cultured renal proximal tubular cells [10]. We have previously reported that many crude drugs, including *Radix asparagi* and *Radix ophiopogonis*, increase glucose uptake in basal and insulin-stimulated 3T3-L1 adipocytes [11].

Toona sinensis is an upland tree that is widely distributed throughout Southeast Asia. Its leaves are used as a vegetable in China and Malaysia, and the tender leaves have also been used as a carminative [12]. Although this plant has been used medicinally for a long time, its effects are not fully understood. Antiproliferative effects on human lung cancer cells have been reported recently [13]. In the present study, we evaluated the effects of substances extracted from *Toona sinensis* leaves, using 50% alcohol solution, on cellular glucose uptake by 3T3-L1 adipocytes. Our objective was to find new hypoglycemic compounds for potential use in patients with diabetes mellitus.

Materials and Methods

Materials

Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), 2-deoxyglucose, and bovine serum albumin (BSA, fatty acid-free) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). NaCl, KCl, $CaCl_2$, $MgSO_4$, sodium dodecyl sulfate (SDS), and disodium hydrogen phosphate (Na_2HPO_4) were obtained from E. Merck (Darmstadt, Germany). [^{3}H]-2-deoxyglucose (7.5 Ci/mmol; final concentration of 0.2 mM 2-deoxyglucose) was purchased from New England Nuclear Corp. (DuPont, Boston, MA, USA).

Cell culture

3T3-L1 cells, obtained from American Type Culture Collection, were grown in 6-well plates (Costa, Cambridge, MA, USA) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 3.8 mM L-glutamine, and 50 μg/mL gentamicin. The cultures were kept at 37°C in a humidified chamber with 10% CO_2. The medium was changed every 2 or 3 days.

Differentiation of 3T3-L1 cells to adipocytes

3T3-L1 pre-adipocytes were differentiated to adipocytes as described previously by Hong et al [11]. Briefly, 2 days after confluence (Day 0), the medium was removed and fresh medium containing 0.5 mM IBMX and 0.25 mM dexamethasone was added. After another 3 days, the medium was replaced with fresh culture medium. The cultures were then maintained as described above. By Day 8, more than 90% of the cells had differentiated into rounded cells with lipid droplets.

Measurement of glucose uptake activity

Substances were extracted from *Toona sinensis* leaves by boiling in 50% v/v alcohol/water for 3 hours. Following centrifugation at 2,000*g* for 10 minutes, the extracted substances were lyophilized. On days 8 to 12, differentiated adipocytes were incubated in serum-free medium with 1% BSA in the absence or presence of *Toona sinensis* leaf extract at concentrations of 0.001, 0.01, or 0.1 mg/mL at 37°C for 60 minutes. Glucose uptake was analyzed by measuring the uptake of [^{3}H]-2-deoxyglucose into cells as previously described [11]. Briefly, after treatment, cells were washed three times with Krebs-Ringer phosphate buffer containing 128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 10 mM Na_2HPO_4 at pH 7.4. [^{3}H]-2-deoxyglucose was added and the cells incubated for 10 minutes. The reaction was terminated by the addition of cold phosphate-buffered saline (PBS) containing 20 mM D-glucose. Cells were then washed three times with PBS, lyzed with 2% SDS, and counted for radioactivity. When measuring insulin-stimulated glucose uptake, 100 nM insulin was added 30 minutes before the addition of [^{3}H]-2-deoxyglucose. In the inhibitory experiment, 10^{-5} M cycloheximide and 10^{-7} M calphostin C were added 30 minutes before the addition of *Toona sinensis* leaf extract.

Statistical analysis

Overall significant differences between groups were determined using one-way ANOVA. The least significant difference was used to determine significant differences between individual samples. Values were considered to be significantly different from the control if p was less than 0.05.

Results

Basal glucose uptake was measured as 0.23 ± 0.01 nmol/min/mg protein. Cellular [^{3}H]-2-deoxyglucose

uptake in cultured 3T3-L1 adipocytes increased significantly to 0.30 ± 0.01 nmol/min/mg protein following incubation with 0.001 mg/mL *Toona sinensis* leaf extract for 60 minutes. With 0.01 mg/mL extract, this increased to 0.33 ± 0.01 nmol/min/mg protein, and with 0.1 mg/mL, to 0.38 ± 0.02 nmol/min/mg protein (Figure 1). The effects were dose-dependent, increasing 130%, 144%, and 165%, from the basal value. In the presence of insulin, cellular glucose uptake was significantly increased to 0.35 ± 0.02 nmol/min/mg protein (Figure 1), 152% of the basal value. With a combination of insulin and *Toona sinensis* leaf extract (0.001, 0.01, and 0.1 mg/mL), cellular glucose uptake increased to 0.41 ± 0.02, 0.46 ± 0.02, and 0.52 ± 0.02 nmol/min/mg protein, respectively (Figure 1). The effects were again dose-dependent, increasing 117%, 131%, and 148% compared to with insulin only.

Pretreatment of cells with 10^{-5} M cycloheximide for 30 minutes did not significantly inhibit the enhanced glucose uptake with 0.01 mg/mL *Toona sinensis* leaf extract (Figure 2). Similarly, 10^{-7} M calphostin C also failed to diminish the enhanced glucose uptake induced by 0.01 mg/mL *Toona sinensis* leaf extract (Figure 2). Following stimulation of glucose transport with insulin, cellular glucose uptake increased from the basal level of 0.23 ± 0.01 to 0.35 ± 0.02 nmol/min/mg protein (Figure 3A). Insulin-stimulated glucose uptake was significantly inhibited by pretreatment of cells with 10^{-5} M cycloheximide and 10^{-7} M calphostin C, to 0.29 ± 0.01 and 0.28 ± 0.01 nmol/min/mg protein, respectively (Figure 3A), which are 83% and 80% of the uninhibited insulin-stimulated value. In the presence of insulin and 0.01 mg/mL *Toona sinensis* leaf extract, cellular glucose uptake increased significantly to 0.53 ± 0.02 nmol/min/mg protein (Figure 3B). Pretreatment of cells with 10^{-5} M cycloheximide or 10^{-7} M calphostin C for 30 minutes significantly inhibited the enhancement of cellular glucose uptake by 0.01 mg/mL *Toona sinensis* leaf extract and insulin to 0.28 ± 0.02 and 0.28 ± 0.01, respectively (Figure 3B), 53% of the value without inhibition.

After incubation of cells with 20 mM glucose for 48 hours, glucose uptake was 0.15 ± 0.02 nmol/min/mg protein (Figure 4), or 65% of the basal value (0.23 ± 0.01). In these cells, 10^{-5} M cycloheximide and 10^{-7} M calphostin C failed to diminish the enhanced glucose uptake induced by *Toona sinensis* leaf extract (uninhibited cells, 0.29 ± 0.02 nmol/min/mg protein; cycloheximide, 0.28 ± 0.02 nmol/min/mg protein; calphostin C, 0.29 ± 0.01 nmol/min/mg protein) (Figure 4). When glucose-treated cells were stimulated with insulin, glucose uptake was significantly inhibited by pretreatment with cycloheximide and calphostin C, from 0.31 ± 0.02 to 0.25 ± 0.02 and 0.24 ± 0.01 nmol/min/mg

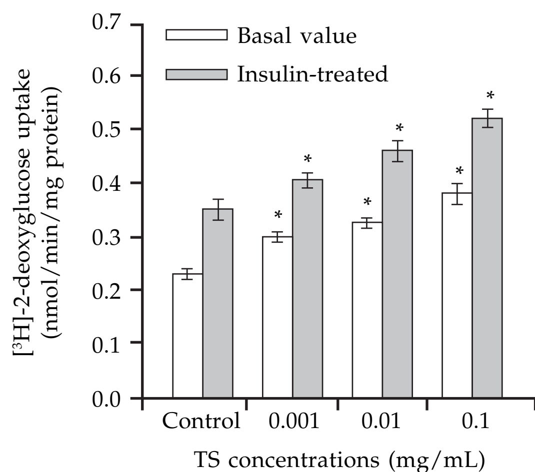

Figure 1. *Dose-dependent effects of* Toona sinensis *(TS) leaf extract on [³H]-2-deoxyglucose uptake in control and insulin-stimulated 3T3-L1 adipocytes. Values are mean ± standard error from three experiments with triplicate determination.* *$p < 0.05$ *compared with control* ($n = 9$).

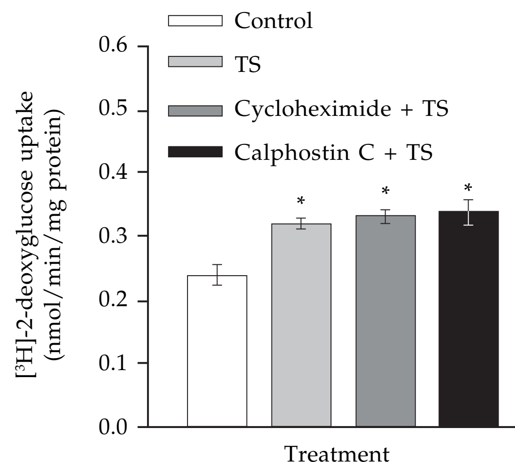

Figure 2. *Effects of cycloheximide and calphostin C on* Toona sinensis *(TS) leaf extract-stimulated [³H]-2-deoxyglucose uptake in normal medium. Differentiated 3T3-L1 adipocytes were pre-incubated with 10^{-5} M cycloheximide and 10^{-7} M calphostin C for 30 minutes following treatment of cells with 0.01 mg/mL TS leaf substances for 60 minutes. Values are mean ± standard error from three experiments with triplicate determination.* *$p < 0.05$ *compared with control* ($n = 9$).

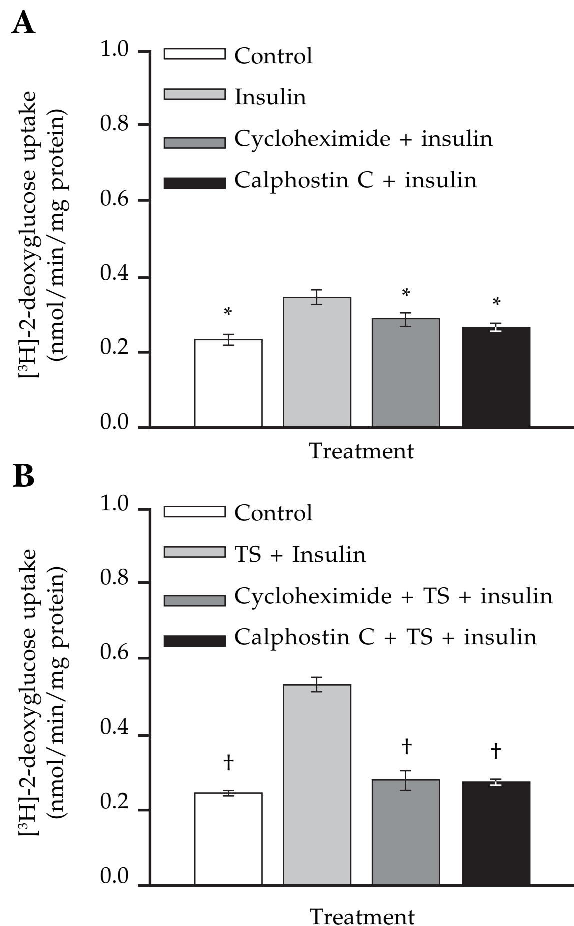

Figure 3. *Effects of cycloheximide and calphostin C on* Toona sinensis *(TS) leaf extract-stimulated [3H]-2-deoxyglucose uptake in insulin-stimulated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were pre-incubated with 10^{-5} M cycloheximide and 10^{-7} M calphostin C for 30 minutes following treatment of cells in the (A) absence or (B) presence of 0.01 mg/mL TS leaf substances for 60 minutes. Insulin was then added to cells for 30 minutes. Values are mean ± standard error from three experiments with triplicate determination.* *$p < 0.05$ *compared with cells treated with insulin only, and* †$p < 0.05$ *compared with cells treated with TS and insulin* (n = 9).

protein, respectively (Figure 5A), or 81% and 77% of the glucose uptake seen in insulin-stimulated cells. The addition of 0.01 mg/mL *Toona sinensis* leaf extract and insulin to glucose-treated cells significantly increased glucose uptake to 0.49 ± 0.02 nmol/min/mg protein (Figure 5B). However, pretreatment with 10^{-5} M cycloheximide or 10^{-7} M calphostin C significantly reduced the enhanced cellular glucose uptake to 0.24 ± 0.01 and 0.23 ± 0.02, respectively (Figure 5B), or 49% and 47% of the value without inhibition.

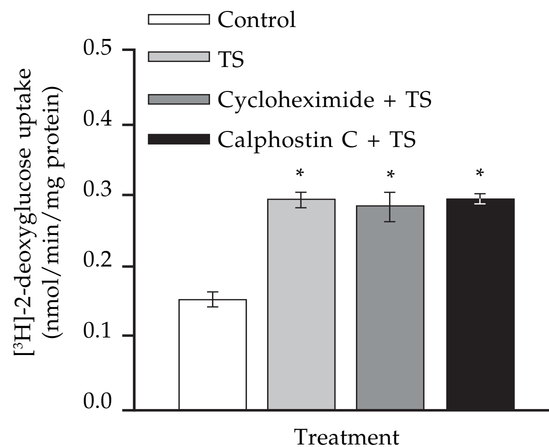

Figure 4. *Effects of cycloheximide and calphostin C on* Toona sinensis *(TS) leaf extract-stimulated [3H]-2-deoxyglucose uptake in 3T3-L1 adipocytes after incubation of cells with 20 mM glucose for 48 hours. Differentiated 3T3-L1 adipocytes were pre-incubated with 10^{-5} M cycloheximide and 10^{-7} M calphostin C for 30 minutes following treatment of cells with 0.01 mg/mL TS leaf substances for 60 minutes. Values are mean ± standard error from three experiments with triplicate determination.* *$p < 0.05$ *compared with control* (n = 9).

Discussion

The results of this study demonstrated that exposure to various concentrations of *Toona sinensis* leaf substances extracted with 50% alcohol enhanced both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes. There is currently little data on the effects of *Toona sinensis* leaf substances on glucose uptake in cultured adipocytes in vitro. The regulatory mechanism of these substances on cellular glucose uptake may be mediated by distinct mechanisms in the presence and absence of insulin, as shown by the fact that pretreatment with the protein synthesis inhibitor cycloheximide and the protein kinase C inhibitor calphostin C only inhibited enhancement of glucose uptake by *Toona sinensis* leaf extract in insulin-treated cells. Protein synthesis and protein kinase C may not be involved in *Toona sinensis* leaf extract-regulation of glucose uptake in the absence of insulin. However, the exact mechanism needs to be determined.

The most commonly used oral hypoglycemic agents are the sulfonylureas, whose mechanism of action probably involves insulin release [3,4]. More recent studies have indicated that sulfonylurea may act, in part, by increasing insulin sensitivity in extrapancreatic tissues [14,15]. The hypoglycemic effect of drugs such

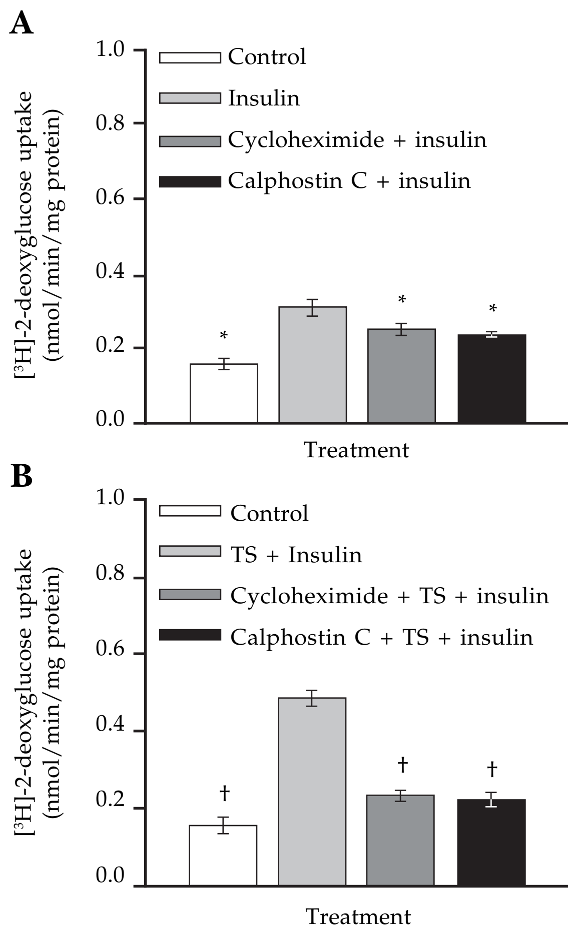

Figure 5. *Effects of cycloheximide and calphostin C on* Toona sinensis *(TS) leaf extract-stimulated [³H]-2-deoxyglucose uptake in insulin-stimulated 3T3-L1 adipocytes after incubation of cells with 20 mM glucose for 48 hours. Differentiated 3T3-L1 adipocytes were pre-incubated with 10^{-5} M cycloheximide and 10^{-7} M calphostin C for 30 minutes following treatment of cells in the (A) absence or (B) presence of 0.01 mg/mL TS leaf substances for 60 minutes. Insulin was then added to cells for 30 minutes. Values are mean ± standard error from three experiments with triplicate determination.* *$p < 0.05$ *compared with cells treated with insulin only, and* †$p < 0.05$ *compared with cells treated with TS and insulin* (n = 9).

as sulfonylureas is decreased gradually after long-term use. Thus, combination therapy with two or three different classes of oral antidiabetic agents is used. When maximum doses of oral antidiabetic agents do not adequately control glycemia, insulin therapy is necessary [16]. However, subcutaneous injection of insulin is inconvenient and painful for patients. It is thus important to find more convenient hypoglycemic compounds for type 2 diabetes patients. The present study shows that alcohol-extracted substances from *Toona sinensis* leaves possess significant glucose uptake-stimulating activity in 3T3-L1 adipocytes. Substances extracted using 50% alcohol can be easily taken orally, so are convenient for type 2 diabetes patients. Furthermore, *Toona sinensis* leaves are eaten as a vegetable in China and Malaysia. Although the mechanism of enhanced glucose uptake in adipocytes is uncertain, substances from *Toona sinensis* leaves may be used as an adjunctive antihyperglycemic agent.

3T3-L1 cells are an excellent experimental model to quickly screen the effects of crude drugs on glucose uptake. When 3T3-L1 fibroblasts grow exponentially, cells maintain the fibroblast phenotype, but when division ceases and the cells mature, as many as 20% to 40% of cells may undergo spontaneous differentiation to adipocytes over a period of 20 to 30 days [17]. The process of differentiation may be accelerated in a number of ways, for example, by increasing the amount of serum in the culture medium or by adding certain hormones or other chemical agents such as insulin, biotin, l-methyl-3-isobutylxanthine or dexamethasone to the medium. Fully differentiated 3T3-L1 adipocytes are particularly responsive to insulin, so sugar uptake can be acutely activated many fold by insulin [18]. In contrast to isolated adipocyte suspensions from animal tissues that lose cell viability rapidly, 3T3-L1 adipocytes can be studied in a stable cell monolayer that maintains cell viability and hormonal responsiveness for extended periods of time. Thus, 3T3-L1 cells are also a convenient experimental model to screen the effects of crude drugs on glucose uptake [11]. The results of this in vitro study in 3T3-L1 adipocytes may not reflect the in vivo situation. However, *Toona sinensis* leaves extracted with 50% alcohol solution may provide an alternative agent in the treatment of diabetes mellitus patients.

Acknowledgments

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