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α-Glucosidase Inhibition and Glucose-uptake Stimulation by Ethanol Extracts from Edible Mushroom *Hinmogi* (*Tremella fuciformis*)

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Abstract Ethanol extracts from the edible mushroom *hinmogi* (*Tremella fuciformis*, TF) were used in the investigation of effects on α-glucosidase *in vitro* and on glucose-uptake in 3T3-L1 mature adipocytes. Addition of the extract significantly inhibited α-glucosidase from small intestine of porcine and of rat (about 42 and 35% of control, respectively), and stimulated glucose uptake (about 100% of control), of which activity was higher than that of *maitake* (*Grifola frondosa*) X-fraction, a well known anti-diabetic substance. When the ethanol extracts were further partitioned successively by organic solvents and purified by silica gel chromatography, the non-polar layer (F-7) from hexane layer showed highest stimulatory activity of glucose-uptake among layers tested. The major components of the F-7 were 1-monooleoylglycerol and 1-monopalmitoylglycerol. Our report is the first description of TF with stimulatory activity of glucose-uptake. These results suggest that TF extracts may constitute a new source of glucose transport activator and could be employed as a potential anti-diabetic material for treatment and preventing diabetes.

Keywords: edible mushroom *Tremella fuciformis*, α-glucosidase, glucose-uptake, insulin, anti-diabetic

Introduction

Diabetes characterized as chronic hyperglycaemia has been classified into insulin dependent diabetes mellitus (IDDM, Type I diabetes) and non insulin dependent diabetes mellitus (NIDDM, Type II diabetes). An interaction between environmental factors and genetic susceptibility is regarded as the main cause of IDDM (1). NIDDM is associated with 3 major metabolic defects, (i) peripheral insulin resistance, (ii) enhanced hepatic glucose production and abnormal pancreatic insulin secretion, and (iii) aggravated symptoms by a variety of disorders including obesity, atherosclerosis, hyperlipidemia, and hypertension (2). At the molecular level, peripheral insulin resistance shown in fat- and muscle-cells from humans with non-insulin dependent diabetes mellitus has been associated with a defect in the transporter proteins transporting the glucose from an intracellular pool to the plasma membrane, and with decrement of insulin receptor number. According to the recent studies, insulin signaling might be overcome by exogenous insulin, sulfonylurea, and insulin secretagogues. However, several drawbacks have reported, such as a fatigue of the pancreatic β-cells, hypoglycemia, and parenteral exogenous insulin injections (3). Thus, new pharmacological approaches for the discovery of orally active molecules that mimic insulin's effects or make cells more sensitive to insulin action are likely to be beneficial. Vanadium showing the insulin-like activity and thiazolidinedione derivatives, insulin sensitizer, has been studied for more than 10 years (4). Colosolic acid from the leaves of *Lagerstroemia speciosa* used for the treatment of diabetes in Philippine folk medicine has been reported as a glucose transport activator (5). Some saponins from ginseng extracts were known to induce a stimulatory effect on glucose transport (6). Aqueous extracts of *Coriandrum sativam* promoted the insulin release and stimulated 2-deoxyglucose transport (7). Mushrooms valued as flavorful foods and medicinal substances for millennia are widely sold as nutritional supplements and touted as beneficial for health. Several mushrooms are claimed to exhibit biological actions such as antiviral, anticoagulant (8), anti-inflammatory (9), hypoglycemic (10), hypolipidemic (11), and hypotensive activities (12). Among them, the most thoroughly researched medicinal effect of mushrooms is their antitumor activity in mice as well as in humans. Recently we screened several edible mushrooms for novel anti-diabetic substance, of which the species *hinmogi* (Korean name, *Tremella fuciformis*; TF) showed potent anti-diabetic action. The body of this mushroom has long been considered a common food, and is widely used as a traditional drug in China. It was reported that extracts from TF showed cytotoxic effects on human colon adenocarcinoma (13). Prior to this report, however, systematic research on anti-diabetic activity of the extract from TF has not been performed. Some records of traditional Chinese anecdotes indicated that these organisms have for the treatment and prevention of diabetes. In order to clarify and provide the scientific basis for these popular beliefs, we extracted, fractionated, and partitioned active compound from TF, and obtained anti-diabetic substances inhibiting α-glucosidase action and elevating glucose uptake in 3T3-L1 cells.

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Materials and Methods

Materials *Hinmogi* (*Tremella fuciformis*; TF) was purchased from a local market in China, and mucosa

material of porcine small intestine was obtained from a local market in Korea. Rat intestinal acetone powder, Trinder 100, insulin from bovine pancreas, dexamethasone, 3-isobutyl-1-methylxanthine, sucrose, and *p*-nitrophenyl-α-D-glucopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum, penicillin, streptomycin sulfate, and glutamine were purchased from Gibco (Rockville, MD, USA). Silica gel (70-230 meshes) and silica gel 60 F_{254} TLC plate (0.2 mm) were from Merck (Darmstadt, Germany). All solvents and reagents were from Fisher (Fair Lawn, NJ, USA) and Sigma, respectively, unless otherwise stated.

Extraction and fractionation The dried TF was ground using a food mixer (model DS2200; Daesung Electronics, Seoul, Korea). The dried powders (10 g) of TF were suspended in 1 L of 75% ethanol and extracted under reflux at 76°C for 1 hr. This procedure was repeated twice and thereafter the suspension was filtered on Whatman No. 2 paper (Maidstone, Kent, England). A clear supernatant was then concentrated in a rotary evaporator under reduced pressure. The resulting concentrated product was denominated as ethanol-soluble fraction (ESF) and used for assay of α-glucosidase inhibitory activity after dissolving in water. The ESF was further partitioned successively with hexane, chloroform, ethyl acetate, *n*-butanol, and water. Each layer was evaporated in a vacuum rotary evaporator at 50°C to remove the solvent and used for glucose-uptake assay in 3T3-L1 adipocytes. The hexane layer showing highest glucose-uptake activity was further fractionated using silica gel column chromatography (Merck; 70-230 meshes; 5×43 cm) with mixture of chloroform/methanol/water, 6 : 4 : 1 (v/v/v). Five-hundred mg of hexane layer was mixed with silica gel, loaded on a silica gel packed column using slurry packing, and eluted with a solution of *n*-hexane/chloroform/ethyl acetate/methanol (15 : 1 : 1 : 1, v/v/v/v). Each eluate with 4 mL fraction size was collected in tubes. The collected tubes were divided into 7 fractions (F-1 to F-7), dried under N_2 stream, monitored by thin-layer chromatography (TLC) on silica gel 60 F_{254} plate (Merck Kieselgel; layer thickness 0.2 mm), and used for glucose-uptake assay in 3T3-L1 cells.

Gas chromatography-mass spectrometry (GC-MS) analysis The subfraction of hexane layer, F-7, was developed on TLC (Merck Kieselgel 60 F_{254} plate, 0.5 mm thickness), scratched, and dissolved in petroleum ether/diethyl ether 9 : 1. After dry, the sample was subjected to GC/MS analysis as described previously (14,15). Briefly, the sample was converted to trimethylsilyl (TMS) ethers by adding Sylon BTZ [*N,O*-bis(trimethylsilyl)acetamide (BSA): chlorotrimethylsilane (TMCS): *N*-trimethylsilyli-midazole (TSIM), 3 : 2 : 3, Supelco Bellefonte, PA, USA] silylating reagent, following the procedure supplied with the reagent. Two-hundred μL of pyridine and 100 μL of sylon BTZ were added to the freeze-dried residue, which was allowed to react for 5 min at room temperature. Gas chromatography was performed using a Hewlett-Packard (HP) 6890 series equipped with split injector and mass spectrometer detection system (HP 5973). The column was an HP-5MS (60 m×0.25 mm i.d. with 0.25 μm film thickness); injection temperature was 290°C and sample injection volume was 1 μL with a split ratio of 50 : 1. The carrier gas was helium at a flow-rate of 0.8 mL/min. The following temperature gradient was used for eluting the products: 100°C for 5 min, to 150°C at a rate of 10°C/min and maintained for 15 min, to 250°C at 10°C/min and maintained for 15 min, finally to 300°C at 10°C/min and maintained for 60 min (total run time of 115 min). Mass spectra were collected by the scan mode and solvent delay time was 15 min. For qualitative analysis, the samples and authentic standard substances were verified by comparing the GC retention times and the total ion chromatogram (TIC). From overlapped peaks, all mass spectra were compared with those of a mass spectrum library (Wiley 275 database) or the spectra of the TMS derivatives of authentic standard compounds.

Preparation of α-glucosidase from porcine and rat small intestine Porcine small intestinal α-glucosidase was prepared using a modification of the method described by Rhinechart *et al.* (16). The mucosa material of porcine small intestine was solubilized in Triton X-100/0.05 M potassium phosphate buffer (pH 6.8) and centrifuged (3,200×g, 4°C) for 20 min. The pellet was dissolved in 0.05 M potassium phosphate buffer (pH 6.8) and dialyzed in dialysis membrane (Cut-off Mw: 12,000-14,000) against the same buffer for 24 hr. The nondialyzed portion was centrifuged as described above to remove insoluble materials and the supernatant was lyophilized. This preparation was stored at −20°C until used for α-glucosidase activity assay. α-Glucosidase from rat small intestine was prepared from rat intestinal acetone powder by a modified method of Ohta *et al.* (17). One g of rat intestinal acetone powder was suspended in 100 mL of 0.05 M potassium phosphate buffer (pH 6.8) and centrifuged (3,200×g, 4°C) for 20 min. The supernatant was immediately used for α-glucosidase activity assay, as described below.

α-Glucosidase inhibitory assay α-Glucosidase inhibitory activity of mushroom extracts was determined using a slightly modified method described by Truscheit *et al.* (18). Briefly, the α-glucosidases prepared as described above and substrates (0.4 M sucrose and 2 mM *p*-nitrophenyl-α-D-glucopyranoside) were dissolved in 0.05 M potassium phosphate buffer, pH 6.8. Fifty μL of mushroom extracts (5 mg/mL) was pre-incubated with 100 μL of α-glucosidases obtained from porcine or rat for 10 min. One-hundred μL substrate solution (0.4 M sucrose) was then added to the reaction mixture and incubated for 20 min at 37°C. Subsequently, 0.8 mL Trinder 100 solution prepared according to the manufacture's specification was added, and absorption was measured at 505 nm after 20 min. The change of absorbance was proportioned to the enzyme activity remaining in the reaction. α-Glucosidase inhibitory activity is expressed as follows:

$$\text{Inhibitory activity (\% control)} = [(A-B)/A] \times 100\ (\%)$$

A is the absorbance in the absence of inhibitor, and B is that in the presence of inhibitor.

Cell culture and adipocytes differentiation 3T3-L1 Preadipocytes were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). 3T3-L1 Preadipocytes were grown in 12-well culture plates in

Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 units/mL penicillin, 50 μg/mL streptomycin sulfate, and 2 mM glutamine, which were maintained in 5% CO_2 humidified atmosphere at 37°C. The fresh medium was exchanged after every 48 hr. Differentiation of the confluent cells was induced by addition of same volume of fresh medium containing 5 μg/mL insulin, 0.75 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (19). After induction for 48 hr, the differentiated cells were maintained in the same media without 3-isobutyl-1-methylxanthine and dexamethasone for an additional 2 days. The fresh medium was exchanged after every 2 days. The cells between 10 and 14 days after induction of differentiation were used for assay of glucose uptake, at which time more than 95% of the cells expressed the adipocytes phenotype.

Glucose-uptake assay Glucose-uptake by 3T3-L1 adipocytes cultured as described in above was performed by previously described method (20). Briefly, 3T3-L1 adipocytes were incubated in Krebs Ringer phosphate (KRP) buffer (pH 7.4) containing 128 mM NaCl, 4.7 mM KCl, 1.65 mM $CaCl_2$, 2.5 mM $MgSO_4$, and 5 mM Na_2HPO_4 at 37°C for 30 min. The cells were then further incubated with or without insulin, and with or without mushroom extract for 1 hr at 37°C. The glucose-uptake reaction was initiated by adding 2-deoxy[^{3}H]glucose (PerkinElmer, Waltham, MA, USA) and stopped by twice rapid washing with 1 mL of ice-cold KRP. After suspending the cells in 500 μL of 0.1% sodium dodecyl sulfate (SDS), 2-deoxy [^{3}H]glucose uptake by the cells was counted following addition of 6 mL of Ultimagold (PerkinElmer). Measurements of 2-deoxy[^{3}H]glucose were made in duplicate, in which nonspecific uptake was determined in the presence of 10 μM of cytochalasin B without 2-deoxy[^{3}H]glucose.

Results and Discussion

α-Glucosidase inhibitory activity *in vitro* α-Glucosidase inhibitor, retarding carbohydrate digestion, and reducing postprandial blood glucose, may be of particular interest as a means to aid diabetes treatment. The TF was extracted with 75% ethanol as described in the materials and methods. The inhibitory activities of the ethanol-soluble fraction (ESF) against α-glucosidases from porcine and rat small intestine were 42 and 35% of control, respectively (Fig. 1). It has been reported that in rats, oral administration of ESF and water-soluble-50% ethanol precipitate (X fraction) from *maitake* (*Grifola frondosa*) showed the blood glucose-lowering activity (21). The methanol extracts from fruits and seeds of balsam pear (*Momordica charantia*), one of the typical daily food materials in the prefecture of Okinawa in Japan, showed the α-glucosidase inhibitory activities of 50 and 78%, respectively (22). In addition, the methanol extract and ethyl acetate-soluble portion from the leaves of *Myrcia multiflora* were found to show inhibitory activities on α-glucosidase and on the increase of serum glucose level in sucrose-loaded rats and in alloxan-induced diabetic mice (23). Strong inhibitory activity of ESF from mushroom TF against α-glucosidase *in vitro* drove us to concentrate our studies on glucose-uptake in 3T3-L1 cells *in situ*.

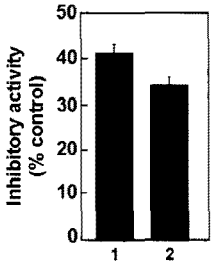

Fig. 1. The inhibitory activities of ethanol-soluble fraction (1 mg/mL) extracted from *Tremella fuciformis* on α-glucosidase against α-glucosidase from porcine small intestine (1) and from rat small intestine (2). Data shown are typical results from experiment repeated 3 times. Bars indicate standard variation.

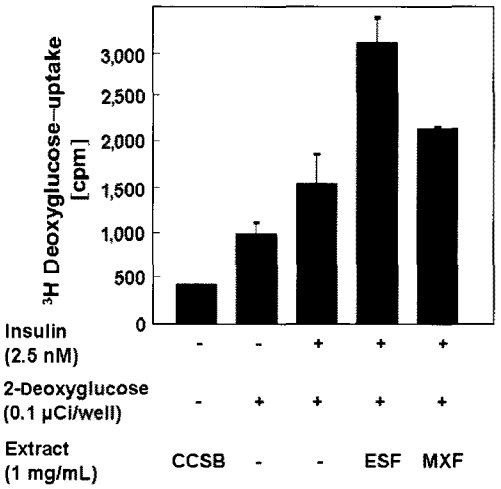

Fig. 2. The stimulatory effects of ethanol-soluble fraction from *Tremella fuciformis* on 2-deoxyglucose-uptake in 3T3-L1 adipocytes. CCSB, cytochalasin B; +, presence; -, absence. Data shown are typical results from experiment repeated 3 times. Bars indicate standard variation.

Stimulation of 2-deoxyglucose-uptake *in vivo* For preliminary experiments, 3T3-L1 adipocytes cultured for 10-14 days after induction of differentiation were incubated in Krebs Ringer phosphate (KRF) buffer with different concentrations of insulin, in which 50% stimulatory concentration in 2-deoxyglucose-uptake by insulin was 2.5 nM in concentration-dependent curve (data not shown). Therefore 2.5 nM of insulin was used for 2-deoxyglucose-uptake assay. To determine whether the α-glucosidase inhibitory activity of ESF from TF shown in Fig. 1 could enhance the insulin's 2-deoxyglucose-uptake activity, 2.5 nM 2-deoxy[^{3}H]glucose with/without 1 mg/mL of ESF was treated to 3T3 L1 adipocytes. The 3T3-L1 cells treated with insulin alone showed mild increase of 2-deoxyglucose-uptake, whereas the cells treated with mixture of insulin and ESF demonstrated the significant increase of 2-deoxyglucose-uptake compare to cells treated with insulin alone (Fig. 2). Furthermore ESF clearly showed a higher effect than maitake X-fraction (MXF), a well known anti-diabetic substance in 2-deoxyglucose-uptake. ESF from edible mushroom TF demonstrated significant inhibition of α-glucosidase *in vitro* (Fig. 1) as well as stimulatory

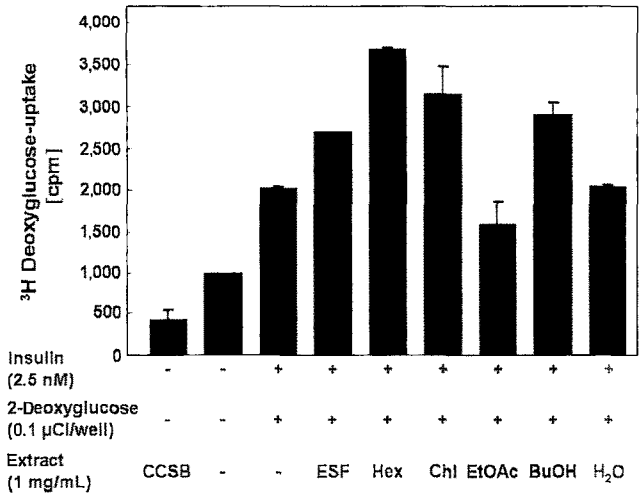

Fig. 3. The stimulatory effects of subfractions of ethanol-soluble fraction on 2-deoxyglucose-uptake in 3T3-L1 adipocytes. CCSB, cytochalasin B. Data shown are typical results from experiment repeated 3 times. Bars indicate standard deviation.

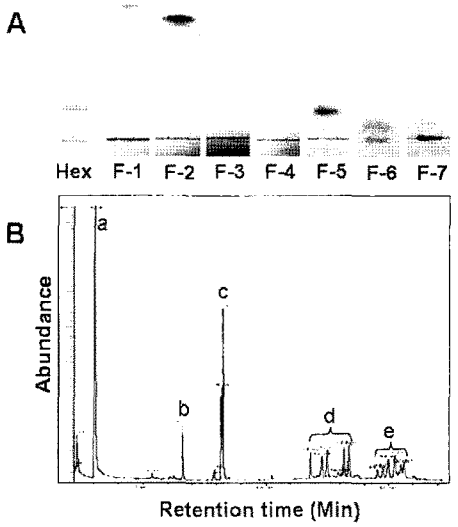

Fig. 4. TLC pattern of subfractions of hexane layer (A) and characterization of components of F-7 by GS-MS (B). a, TMS ethers; b, 1-monopalmitoylglycerol (retention time 6.61); c, 1-monoeleoylglycerol (retention time 8.26); d, cis-4b, 5, 9b, 10-tetrahydro-8-methoxy-5-methylindeno [1,2-b] indole, 2-(4-cyanophenyl)-5-dimethylaminomethylenaminopyrimidine (retention time 11.82); e, 3-methyl-4,5-dihydrobenz(h)imidazo [1,2-c] quinazolinium-1-olate (retention time 13.38).

activity of 2-deoxyglucose-uptake by insulin in 3T3-L1 cells in situ (Fig. 2). These results suggest that the anti-diabetic compounds would be present in TF. However, the experiments reported in Fig. 1 and 2 were performed with a crude preparation of ESF, without any further step of purification, which makes the nature of the active compounds questionable. Initially, we further partitioned the ESF as described in materials and methods. Hexane layer (Hex) showed the highest stimulatory activity of 2-deoxyglucose-uptake induced by insulin in 3T3-L1 cells among the layers tested, and chloroform- (Chl) and butanol-layer (BuOH) also showed relatively higher activities than ethyl acetate- (EtOAc) or water-layer (H_2O) (Fig. 3). These

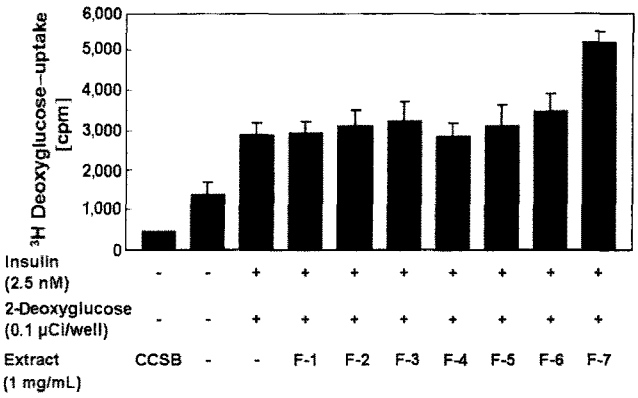

Fig. 5. The stimulatory effects of subfractions (F-1 to F-7) of hexane layer on 2-deoxyglucose-uptake in 3T3-L1 adipocytes. Data shown are typical results from experiment repeated 3 times. Bars indicate standard deviation.

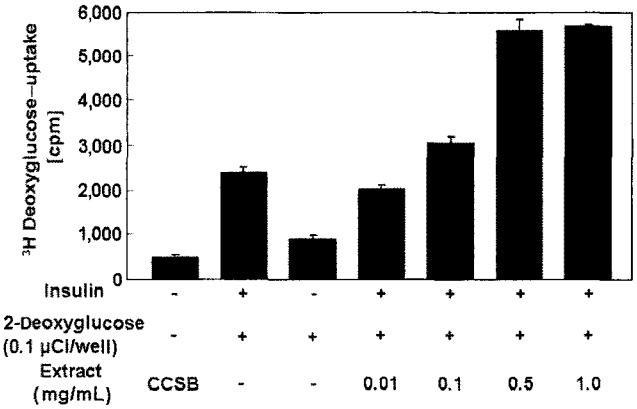

Fig. 6. The concentration-dependent stimulatory effects of F-7 on 2-deoxyglucose uptake in 3T3-L1 adipocytes. Data shown are typical results from experiment repeated 3 times. Bars indicate standard deviation.

results suggest that non-polar compounds might stimulate the 2-deoxyglucose-uptake even though the single active compound and mechanism were not clarified in this study. The Hex, which showed highest stimulatory activity of 2-deoxyglucose-uptake was further fractionated on a silica gel column chromatography and divided into 7 fractions (F-1 to F-7). For characterization of components, each fraction was developed on TLC and major bands were extracted followed by GS-MS analysis as described in the materials and methods. Each fraction was clearly well separated by silica gel column chromatography, showed the different mobility, and appeared to contain 2 or more compounds with similarities in solubility and in polarity on TLC (Fig. 4A). GC-MS analysis of F-7 showed that the major components were 1-monopalmitoylglycerol (peak b in Fig. 4B) and 1-monoeleoylglycerol (peak c in Fig. 4B), and the minor components were cis-4b, 5, 9b, 10-tetrahydro-8-methoxy-5-methylindeno [1,2-b] indole, 2-(4-cyanophenyl)-5-dimethylaminomethylenaminopyrimidine (peak d in Fig. 4B), and 3-methyl-4,5-dihydrobenz(h)imidazo [1,2-c] quinazolinium-1-olate (peak e in Fig. 4B). Among the 7 fractions, F-7 possessed the highest activity on 2-deoxy-glucose-uptake (Fig. 5). Other fractions (F-1 to F-6) showed nearly the same degree of insulin in 2-deoxyglucose-

uptake, which means that those fractions have no activity. These results clearly suggest that major compounds with 2-deoxyglucose-uptake stimulatory activity were partitioned to F-7. In addition, F-7 significantly stimulated 2-deoxyglucose-uptake in 3T3-L1 adipocytes at 0.5 and 1.0 mg/mL (Fig. 6). The banaba extract using hot water and banaba methanol eluent stimulated glucose uptake in 3T3-L1 adipocytes with an induction time and a dose-dependent response similar to those of insulin (24). Chalcones, originally isolated from natural plant sources, have been reported to possess a variety of biological properties. A chalcone derivative, 3-nitro-2'-benzeyloxychalcone (compound 1) stimulated glucose uptake and potentiated insulin-stimulated glucose uptake in a concentration-dependent manner in 3T3-L1 adipocytes (25). In this study, we demonstrated that extracts from an edible mushroom TF stimulated 2-deoxyglucose uptake in 3T3-L1 cells, which are consistent with previous observations that TF might lower blood glucose levels in diabetic mice and reduce the glycogen content and the plasma cholesterol in streptozotocin-induced diabetic mice (26). Our results suggest a new source of glucose transport activator, which requires further studies on characterization of the major active compound and on elucidation whether hexane layer of TF stimulates 2-deoxyglucose-uptake via either an insulin receptor or another signal transduction pathway, or whether it translocates glucose tranporter proteins from an intracellular pool to the plasma membrane. An understanding of the mechanism of TF action will be valuable for the study on prevention and treatment of obesity, and on insulin resistance and Type II diabetes.

Acknowledgments

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