

Ganoderma lucidum extract stimulates glucose uptake in L6 rat skeletal muscle cells

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Received: 29 March, 2006; revised: 18 August, 2006; accepted: 08 September, 2006
available on-line: 10 September, 2006

The effect of *Ganoderma lucidum* extract on glucose uptake was studied in L6 rat skeletal muscle cells. *G. lucidum* extract increased glucose uptake about 2-fold compared to control. The extract stimulated the activity of phosphatidylinositol (PI) 3-kinase which is a major regulatory molecule in the glucose uptake pathway. About 7-fold increased activity of a PI 3-kinase was observed after treatment with *G. lucidum* extract, whereas PI 3-kinase inhibitor, LY294002, blocked the *G. lucidum* extract-stimulated PI 3-kinase activity in L6 skeletal muscle cells. Protein kinase B, a downstream mediator of PI 3-kinase, was also activated by *G. lucidum* extract. We then assessed the activity of AMP-activated protein kinase (AMPK), another regulatory molecule in the glucose uptake pathway. *G. lucidum* extract increased the phosphorylation level of both AMPK α 1 and α 2. Activity of p38 MAPK, a downstream mediator of AMPK, was also increased by *G. lucidum* extract. Taken together, these results suggest that *G. lucidum* extract may stimulate glucose uptake, through both PI 3-kinase and AMPK in L6 skeletal muscle cells thereby contributing to glucose homeostasis.

Keywords: *Ganoderma lucidum* extract, glucose uptake, phosphatidylinositol 3-kinase, AMP-activated protein kinase, skeletal muscle

INTRODUCTION

Ganoderma lucidum is an important medicinal fungus belonging to the Ganodermataceae family which has been popularly used for its health promoting properties (Stapleton *et al.*, 1996; Vavvas *et al.*, 1997; Thornton *et al.*, 1998; Wasser & Weis, 1999). It is consumed more for its pharmaceutical rather than nutritional value. The effects of *G. lucidum* extract on cancer, hypertension, hypercholesterolemia, and hepatitis have been demonstrated by several studies (Franz, 1989; Furusawa *et al.*, 1992; Wang *et al.*, 1997). In addition, it was reported that *G. lucidum* extract showed hypoglycemic activity by increasing plasma insulin and by affecting hepatic enzymes in alloxan-induced diabetic mice (Hikino *et al.*, 1985).

Zhang & Lin, (2004) reported that *G. lucidum* extract showed protective effect on alloxan-induced pancreatic islet damage.

Given that skeletal muscles account for more than 80% of insulin-stimulated glucose uptake, an impaired glucose uptake in skeletal muscle is responsible for the development of type II diabetes mellitus (Baron *et al.*, 1991). Two major regulatory molecules of glucose uptake, phosphatidylinositol (PI) 3-kinase and AMP-activated protein kinase (AMPK), are responsible for glucose uptake into the cytoplasm (Musi *et al.*, 2001; Xi *et al.*, 2001). PI 3-kinase is stimulated by insulin and mediates glucose uptake (Xi *et al.*, 2001). Activation of PI 3-kinase in impaired skeletal muscle showed a reduced effect on glucose uptake (Bjornholm *et al.*, 1997; Dohm *et*

al., 1998). Protein kinase B (PKB) is an enzyme that can be activated by insulin *via* a pathway involving a PI 3-kinase dependent mechanism and can promote glucose uptake (Chan *et al.*, 1999). AMPK mediates contraction-induced glucose uptake. AMPK α , the catalytic subunits of AMPK, exists as two isoforms, $\alpha 1$ and $\alpha 2$. AMPK $\alpha 1$ is ubiquitously expressed in various tissues such as skeletal muscle, liver, and pancreas, while AMPK $\alpha 2$ is more limitedly expressed in skeletal and cardiac muscles (Cheung *et al.*, 2000; Musi & Goodyear, 2002).

Although a hypoglycemic effect of *G. lucidum* extract has been reported, the exact mechanism of this effect has yet to be elucidated. Therefore, we investigated the effect of *G. lucidum* extract on glucose uptake in skeletal muscle cells by assessing the activities of PI 3-kinase and AMPK, two crucial mediators in the glucose uptake pathway.

MATERIALS AND METHODS

Preparation of extract. *G. lucidum* was purchased at Kyungdong market (Seoul, Korea) and was authenticated by the College of Oriental Medicine, Semyung University. Methanol extract of *G. lucidum* (yield: 19.7% of dry weight) was obtained by 48 h maceration at room temperature and was filtered through a 0.45 μm filter (Osmonics, Minnetonka, MN, USA), lyophilized, and kept at 4°C.

Materials. All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) unless otherwise indicated. Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and other culture products were purchased from GIBCO BRL (San Diego, CA, USA). [γ - ^{32}P]ATP (6000 Ci/mmol) and 2- ^{3}H]deoxy-D-glucose (6.0 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA, USA). The anti-phospho specific antibodies that recognize a phospho-activated forms of AMPK $\alpha 1$, $\alpha 2$, p38 and PKB were from Upstate (Charlottesville, VA, USA) and Cell Signaling Technology (Beverly, MA, USA).

Cell culture. Monolayer of L6 skeletal muscle cells was maintained at subconfluent conditions in growth media containing DMEM with 0.045 g/ml glucose, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% fetal bovine serum. Cells were maintained in a humidified 37°C incubator with ambient oxygen and 5% CO_2 .

Glucose uptake assay. Glucose uptake was determined as previously described (Eichhorn *et al.*, 2001). Cells were cultured on 12-well culture plates, washed with Krebs-Ringer phosphate buffer (KRB) (pH 7.4, 25 mM Hepes, 118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.3 mM

MgSO_4 , 5 mM NaHCO_3), 0.07% bovine serum albumin, and 5.5 mM glucose and incubated in KRB for 60 min. Cells were treated with 100 nM insulin, or 20, 50, or 100 $\mu\text{g}/\text{ml}$ of *G. lucidum* extract for 30 min. Glucose uptake was measured by adding 20 μl glucose mixture (5 mM 2-deoxyglucose and 0.5 μCi 2- ^{3}H]deoxy-D-glucose in KRB) to 980 μl KRB followed by incubation for 20 min at 37°C. Non-specific glucose uptake was measured by parallel incubations in the presence of 10 μM cytochalasin B, which blocks transporter-mediated glucose uptake, and was subtracted from the total uptake in each assay. Uptake was terminated by washing the cells three times with 1 ml ice-cold phosphate-buffered saline (PBS). Cells were subsequently lysed with 0.5 ml of 0.5 M NaOH solution containing 0.1% SDS, and the solution was rotated for 15 min. Cell associated radioactivity was measured in a liquid scintillation counter (PerkinElmer Life and Analytical Sciences).

Immunoprecipitation. Immunoprecipitation was performed as previously described (Choi *et al.*, 2006). After washing twice with ice-cold PBS, treated with 50 $\mu\text{g}/\text{ml}$ *G. lucidum* extract were solubilized by incubation for 30 min in 1 ml of lysis buffer (pH 7.5, 50 mM Tris/HCl, 150 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 1 mM sodium orthovanadate, 100 mM NaF, 1 mM EGTA, 1% Triton X-100, 10% glycerol, leupeptin (5 $\mu\text{g}/\text{ml}$) and 1 mM phenylmethylsulfonyl fluoride) on ice. The cell lysates were then centrifuged at 15000 $\times g$ for 15 min at 4°C and supernatant was harvested. For immunoprecipitation, 20 μl of anti-phosphotyrosine antibody agarose beads was incubated with supernatant containing 500 μg of protein for 1 h at 4°C. Immunoprecipitates were washed three times with PBS containing 1% Nonidet P-40, three times with 100 mM Tris/HCl, pH 7.5, containing 500 mM LiCl_2 and finally twice with 25 mM Tris/HCl containing 100 mM NaCl and 1 mM EDTA.

PI 3-kinase assay. Crude PI 3-kinase was obtained by immunoprecipitation with antiphosphotyrosine antibody (Kim *et al.*, 2002). Cell lysates (500 μg protein) were incubated with 20 μl of antiphosphotyrosine antibody agarose beads. After washing, the immunoprecipitates with PI 3-kinase activity were resuspended in 100 μl of kinase assay buffer (20 mM Tris/HCl, 75 mM NaCl, 10 mM MgCl_2 , 200 $\mu\text{g}/\text{ml}$ phosphatidylinositol, 1 mM EGTA, 20 μM ATP, 10 μCi [γ - ^{32}P]ATP) and incubated for 30 min at room temperature with constant shaking. PI 3-kinase activity was measured by the phosphorylation of PI. The reaction was stopped by the addition of 100 μl 1 M HCl and the reaction products were extracted with 200 μl chloroform/methanol (1:1, v/v). The samples were centrifuged and the lower organic phase was harvested and applied to

a silica gel thin-layer chromatography (TLC) plate (Merck, Aichach, Germany) coated with 1% potassium oxalate. TLC plates were developed in chloroform/methanol/ammonium hydroxide/water (60:47:11.3:2, by vol.), dried, and visualized by autoradiography.

Immunoblotting. The cell lysates were centrifuged for 15 min at $12000 \times g$ at 4°C , and the supernatant was collected. Proteins were separated by SDS/PAGE and transferred onto nitrocellulose membrane (Schleicher & Schuell, Middlesex, UK). After transfer, the membrane was blocked in 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20 (TBS-T) and then was incubated for 1 h at room temperature with 1:1000 diluted antibody against phospho-AMPK $\alpha 1$, $\alpha 2$, p38 MAPK or β -actin (Cell Signaling Technology, Beverly, MA, USA). Next, the membrane was washed in TBS-T followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:2000). The immunoreactive bands were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia, Uppsala, Sweden).

Statistical analysis. Statistical analysis was performed using Student's *t*-test and one way analysis of variance (one way-ANOVA). The accepted level of significance was preset as *P* value < 0.05. Data are presented as means \pm S.E.M. All calculations were performed using SPSS software (SPSS, Inc., Chicago, IL, USA).

RESULTS

G. lucidum extract stimulates glucose uptake in skeletal muscle cells

We determined the effect of *G. lucidum* extract on glucose uptake using 2- ^{3}H deoxy-D-glucose in L6 skeletal muscle cells. Figure 1 shows that glucose uptake was increased by *G. lucidum* extract by about 85–90% compared to control (*P* < 0.01). The stimulatory effect of *G. lucidum* extract was dose-independent at dosages ranging from 20 to 100 $\mu\text{g}/\text{ml}$. At the doses of 20, 50, and 100 $\mu\text{g}/\text{ml}$ of the uptake *G. lucidum* extract was 183.4 ± 8.1 , 195.3 ± 6.8 , and $187.7 \pm 9.4\%$, respectively, compared to control. The stimulatory effect of *G. lucidum* extract on glucose uptake was as strong as that of 100 nM insulin ($180.4 \pm 8.6\%$) (Fig. 1), which was used as a positive control (Maleppillil *et al.*, 2005). These results indicate that *G. lucidum* extract stimulates glucose uptake in L6 skeletal muscle cells. Since the extract showed a dose-independent effect on glucose transport, the following experiments were set at 50 $\mu\text{g}/\text{ml}$ *G. lucidum* extract.

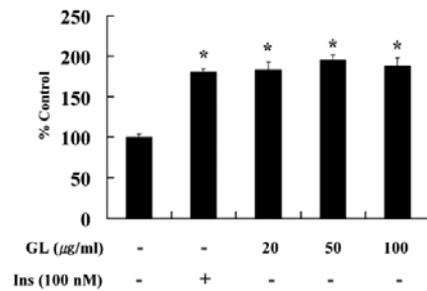


Figure 1. *G. lucidum* extract stimulates the rate of 2- ^{3}H deoxy-D-glucose uptake in L6 skeletal muscle cells.

Cells were treated with various doses of *G. lucidum* extract (20–100 $\mu\text{g}/\text{ml}$) for 30 min and 2- ^{3}H deoxy-D-glucose uptake was measured in a scintillation counter. Insulin (100 nM) was used as a positive control. Data represent the mean \pm standard error of five separate experiments, each performed in triplicate and presented as % control. Ins, insulin; GL, *G. lucidum* extract, **P* < 0.05 vs. control.

G. lucidum extract-stimulated glucose uptake is mediated by PI 3-Kinase

PI 3-kinase plays a critical role in insulin-stimulated glucose uptake. We performed PI 3-kinase assay using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. In Fig. 2A, autoradiogram of a TLC plate shows the incorporation of ^{32}P into the 3' position of PI. PI-3-phosphate (PI-3-P), which is formed by PI 3-kinase activity, was markedly increased by 100 nM insulin for 30 min (about 6-fold compared to control). Insulin was used as a positive control (Cedia *et al.*, 2005). The levels of PI-3-P were increased by *G. lucidum* extract at 10, 30 and 60 min compared to that of control. Densitometry analysis revealed that *G. lucidum* extract-stimulated PI 3-kinase activities at 10, 30, and 60 min were 5.8, 6.7, and 7.3-fold higher compared to those of the control, respectively. The *G. lucidum* extract-stimulated PI 3-kinase activity was attenuated by more than 90% by LY294002. The increase of PI 3-kinase activity by *G. lucidum* extract was significantly greater than that by 100 nM insulin (*P* < 0.05). As shown in Fig. 2B, the phosphorylation level of PKB, a downstream effector of PI 3-kinase, was increased by treatment with *G. lucidum* extract in a time-dependent manner. Treatment with LY294002 almost completely inhibited the *G. lucidum* extract-stimulated phosphorylation level of PKB.

G. lucidum extract-stimulated glucose uptake is mediated by AMPK, and p38 MAPK

To determine if the *G. lucidum* extract-stimulated glucose uptake was mediated by AMPK, we examined the phosphorylation levels of AMPK $\alpha 1$, $\alpha 2$ (catalytic subunits of AMPK) at Thr172 and p38 mitogen activated protein-kinase (p38 MAPK) proteins. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), a compound that activates AMPK, was then used as a positive control (500 μM) (Salt *et al.*, 2000).

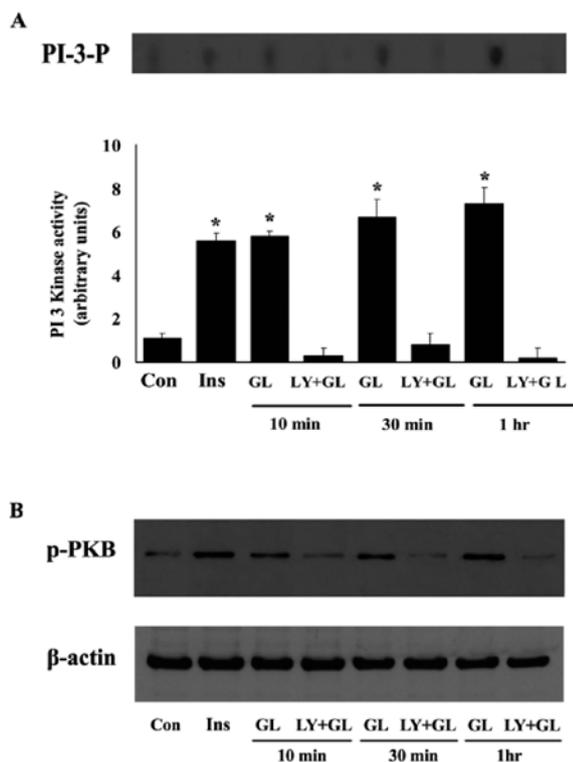


Figure 2. Effect of *G. lucidum* extract on phosphatidylinositol 3-kinase (PI 3-kinase) activity and phosphorylation of protein kinase B (PKB).

PI 3-kinase activity was measured in anti-phosphotyrosine immunocomplex. Cells were treated with 50 µg/ml *G. lucidum* extract and insulin (100 nM, for 30 min) was used as a positive control (A). The phosphorylation of PKB was analyzed by immunoblotting with antibodies specific to phosphorylation of PKB. Cells were treated with 50 µg/ml *G. lucidum* extract in the absence or presence of 20 µM LY294002 (PI 3-kinase inhibitor) and 100 nM insulin for the indicated time. Beta actin was used as an internal control (B). Con, control; Ins, insulin; GL, *G. lucidum* extract; PI-3-P, phosphatidylinositol-3-phosphate; LY, LY294002; p-PKB, phospho-protein kinase B. **P* < 0.05 vs. control.

As shown in Fig. 3A, the phosphorylation level of AMPK α1 was clearly increased by *G. lucidum* extract at 10, 30 and 60 min. The increased phosphorylation of AMPK α1 by *G. lucidum* extract was even greater than that by AICAR. Not as strong as for AMPK α1, a time-dependent increase in the phosphorylation level of AMPK α2 was also observed at 10, 30 and 60 min. p38 MAPK phosphorylation was accordingly increased at 10, 30 and 60 min (Fig. 3A). In an additional experiment, to determine whether AMPK and PI 3-kinase are component of the same signaling route, we investigated the phosphorylation level expression of PKB after treatment with AICAR in L6 skeletal muscle cells. The phosphorylation level of PKB was increased by insulin but not by AICAR (Fig. 3B). This result suggests that AMPK and PI 3-kinase may be component of different signaling route in L6 skeletal muscle cells. The intensity of β-actin, which was used as an internal control, was content.

DISCUSSION

G. lucidum extract has been used for long time in China to prevent and treat various human diseases such as hepatitis, hypertension, and hyperglycemic, tumorigenic and immunological diseases (Kabir *et al.*, 1988; Kim *et al.*, 1999; Cao *et al.*, 2005; Kimura, 2005). Although a hypoglycemic effect of *G. lucidum* extract has been reported, its mechanism is not fully understood.

In skeletal muscle, glucose transport can be activated by at least two regulatory molecules, PI 3-kinase which is stimulated by insulin, and AMPK which is induced through muscle contraction (Zierath *et al.*, 2000). PI 3-kinase activity has been suggested to be required for insulin-induced glucose uptake in skeletal muscle (Cheatham *et al.*, 1994) and PKB, a downstream mediator beyond PI 3-kinase,

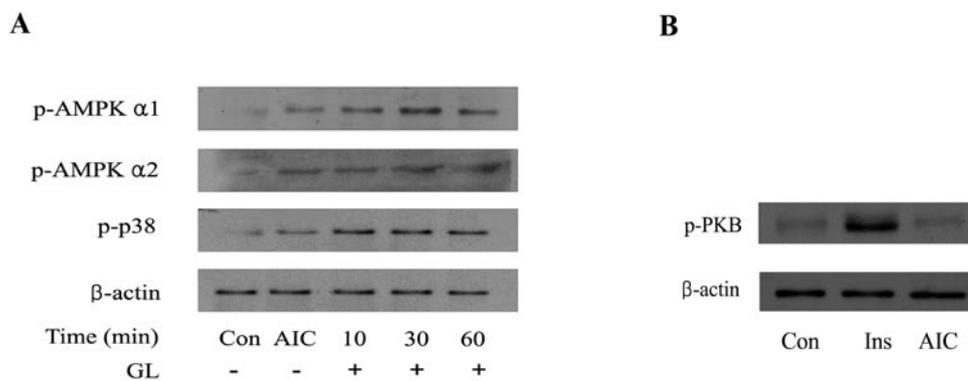


Figure 3. Effects of *G. lucidum* extract on the phosphorylation of AMP-activated protein kinase (AMPK) α1, α2 at Thr-172, p38 mitogen activated protein kinase (p-38 MAPK) and protein kinase B (PKB).

Cell lysates were analyzed by immunoblotting with antibodies specific to phospho-AMPK α1, α2 at Thr-172, phospho-p38 MAPK and phospho-PKB. Cells were treated with 50 µg/ml *G. lucidum* extract or 500 µM AICAR for the indicated time. β-actin was used as an internal control. GL, *G. lucidum* extract; AIC, 5-aminoimidazole-4-carboxamide ribonucleoside; p-AMPK α1, α2, phospho-AMP-activated protein kinase α1, α2; p-p38, phospho-p38 mitogen activated protein kinase; p-PKB, phospho-protein kinase B.

has been also implicated in insulin-stimulated glucose uptake (Brazil *et al.*, 2004). To identify the signaling regulatory molecules involved in *G. lucidum* extract-stimulated glucose uptake, we investigated PI 3-kinase and PKB activities. As shown in Fig. 2A, *G. lucidum* extract stimulated PI 3-kinase with increasing time (10, 30, and 60 min) and this-stimulated PI 3-kinase activity was decreased to basal line by LY294002. Moreover, PKB, a downstream mediator of PI 3-kinase, was also increased by *G. lucidum* extract. These data showing that PI 3-kinase is activated by *G. lucidum* extract treatment suggest that PI 3-kinase is involved in *G. lucidum* extract-stimulated glucose uptake in L6 skeletal muscle cells.

AMPK is another regulatory protein in the glucose uptake pathway and energy metabolism (Cortright & Dohm, 1997; Brozinick & Birnbaum, 1998). Activation of AMPK leads to the activation of p38 MAPK, implicating p38 MAPK as a downstream effector of AMPK (Xi *et al.*, 2001). We found that treatment of skeletal muscle cells with *G. lucidum* extract increased the phosphorylation levels of AMPK $\alpha 1$, $\alpha 2$ at Thr-172, and of p38 MAPK (Fig. 3A). Ha *et al.* (2006) reported that topiramate stimulated the rate of glucose transport in L6 skeletal muscle cells. From these results, we suggest that *G. lucidum* extract also increases glucose uptake by stimulating AMPK activity in skeletal muscle cells.

Recently, several reports have suggested that AMPK and PI 3-kinase are elements of one signaling route and cross talk to each other (Ouchi *et al.*, 2004; Zou *et al.*, 2004; Longnus *et al.*, 2005). Others, however, considered AMPK and PI 3-kinase to be on separate signaling pathways in skeletal muscle (Hayashi *et al.*, 1998; Zierath *et al.*, 2000; Mu *et al.*, 2001). In our experiments, it was found that PI 3-kinase and AMPK might be on different routes in mediating glucose uptake in L6 skeletal muscle cells (Fig. 3B).

In conclusion, we found that *G. lucidum* extract significantly stimulated glucose uptake in L6 skeletal muscle cells. An interesting result was that the increase of glucose uptake by *G. lucidum* extract might be mediated by both PI 3-kinase and AMPK.

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