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Insulin-Sensitizing and Beneficial Lipid-Metabolic Effects of the Water-Soluble Melanin Complex Extracted from *Inonotus obliquus*

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Inonotus obliquus **has been traditionally used for treatment of metabolic diseases; however, the mechanism remains to be elucidated. In this study, we found that the water-soluble melanin complex extracted from *I. obliquus* improved insulin sensitivity and reduced adiposity in high fat (HF)-fed obese mice. When the melanin complex was treated to 3T3-L1 adipocytes, insulin-stimulated glucose uptake was increased significantly, and its phosphoinositide 3-kinase-dependent action was proven with wortmannin treatment. Additionally, dose-dependent increases in Akt phosphorylation and glucose transporter 4 translocation into the plasma membrane were observed in melanin complex-treated cells. Adiponectin gene expression in 3T3-L1 cells incubated with melanin complex increased which was corroborated by increased AMP-activated protein kinase phosphorylation in HepG2 and C2C12 cells treated with conditioned media from the 3T3-L1 culture. Melanin complex-treated 3T3-L1 cells showed no significant change in expression of several lipogenic genes, whereas enhanced expressions of fatty acid oxidative genes were observed. Similarly, the epididymal adipose tissue of melanin complex-treated HF-fed mice had higher expression of fatty acid oxidative genes without significant change in lipogenic gene expression. Together, these results suggest that the water-soluble melanin complex of *I. obliquus* exerts antihyperglycemic and beneficial lipid-metabolic effects, making it a candidate for promising antidiabetic agent.**

Keywords: Inonotus obliquus; antidiabetic; high fat-fed mice; 3T3-L1 adipocytes; glucose uptake; PI 3-K; GLUT4; fatty acid oxidation.

INTRODUCTION

Due to the increasing sedentary behavior and increase in caloric intake, there is a prevalence of diabetes and impaired glucose tolerance which, together with subsequent metabolic disorder, is now one of the largest contributors to morbidity and mortality worldwide. Insulin resistance is an impairment of the biological response to insulin resulting in dysfunctional glucose uptake and an increased risk of development of type 2 diabetes (Kahn, 1998). Loss of insulin sensitivity in peripheral tissues such as muscle and adipose tissue has been attributed to hyperglycemia due to β-cell failure brought on by insulin hypersecretion. Normal glucose homeostasis is maintained by the action of insulin-regulated glucose transporter 4 (GLUT4) found in muscle and fat cells which facilitates glucose uptake (Bryant *et al.*, 2002). Induction of glucose uptake by acute insulin stimulation results in translocation of GLUT4 from intracellular vesicles to the plasma membrane (Pessin *et al.*, 1999) which is mediated by signaling proteins such as phosphoinositide 3-kinase (PI 3-K), 3-phosphoinositide-dependent protein kinase (PDK1), Akt, and atypical PKCζ/λ (Taniguchi *et al.*, 2006).

Due to the debilitating effect of insulin resistance, discovery of pharmacological agents both natural and artificial for enhancing insulin sensitivity has been a key focus for the treatment of type 2 diabetes and restoration of normal metabolic state (Muoio and Newgard, 2008).There exist several major insulin-sensitizing agents, including the thiazolidinediones (TZDs) and metformin, which are commonly used for the alleviation of insulin resistance. However, despite their widespread use, it has been shown that they have adverse effects such as gastrointestinal disturbances, liver damage, and, in cases of TZDs, increased weight gain (Moller, 2001). Due in large part to this, several recent findings showing the beneficial effects of AMP-activated protein kinase (AMPK) activation on modulation of insulin sensitivity and energy homeostasis have led to an intense interest in developing AMPK activators as potential therapies for type 2 diabetes and obesity (Zhang *et al.*, 2009). Adiponectin, an adipokine, is also known to stimulate fatty acid oxidation via activation of AMPK in liver and skeletal muscle and has been also found to stimulate glucose uptake in skeletal muscle (Wu *et al.*, 2003).

The mushroom *Inonotus obliquus* is a parasitic fungus belonging to the Hymenochaetaceae family which grows on birch and other trees. Its use as a traditional medicine dates back to the 16th century where it has been used for gastrointestinal, cardiovascular diseases and diabetes in Eastern Europe and parts of Asia in the regions of Russia and Mongolia. Recent studies on the extracts of *I. obliquus* have shown it to have various biological activities including antihyperglycemic (Mizuno *et al.*, 1999), antioxidant (Cui *et al.*, 2005), and antitumor activities (Kim *et al.*, 2005; Song *et al.*, 2008). One study showed that *I. obliquus* has reducing effects

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on free fatty acid, triglyceride, and total and low density lipoprotein-cholesterol in the plasma of diabetic mice. This effect was complemented by increased plasma insulin, plasma high density lipoprotein-cholesterol, and hepatic glycogen. Additional histological morphology examination of alloxan-induced diabetic mice showed that *I. obliquus* extract restored damage in pancreatic tissues (Sun *et al*., 2008). *I. obliquus* or simply Chaga is consumed after water infusion as with most teas and herbal medicines and therefore necessitated analysis of the effect of the water-soluble extract of *I. obliquus*. Previous analysis of the constituent of the water extract of *I. obliquus* showed it to be mainly composed of melanin complex (Babitskaya *et al*., 2002; Mazurkiewicz, 2006), which has been found to have antioxidant and genoprotective effects (Babitskaya *et al*., 2000).

From the fact that the water extract of *I. obliquus* is being traditionally used for the purpose of preventing and treating diabetes, it is required to understand the action of melanin complex in *I. obliquus* with scientific precision. In the present study, we extracted melanin complex from *I. obliquus* and investigated its *in vitro* and *in vitro* effects on glucose and lipid metabolism. Our data demonstrate that the water-soluble melanin complex significantly improves insulin sensitivity with reducing fat accumulation in high fat (HF) diet-fed mice and also enhances insulin-stimulated glucose uptake in 3T3-L1 adipocytes, which is mediated through a PI 3-K pathway. It is also observed that treatment of melanin complex is associated with increases in fatty acid oxidation through enhanced expression of adiponectin and fatty acid oxidative genes in 3T3-L1 adipocytes. In addition, conditioning of hepatocytes and muscle cells with conditioned media from melanin complex-treated 3T3-L1 cells showed increased activation of AMPK.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Grand Islands, NY), and fetal bovine serum (FBS) and bovine calf serum were from Hyclone Laboratories Inc. (Logan, UT). Anti-GLUT4 and anti-AMPKα1 antibodies were from AbChem (Cambridge, MA) and Upstate (Lake Placid, NY), respectively. Antibodies against Akt, and phospho-Akt (Ser473), phospho-AMPKα (Thr172), β-actin, and horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG were from Cell Signaling Technology (Beverly, MA). Unless specifically mentioned, reagents including proteases were purchased from Sigma–Aldrich (St. Louis, MO).

Animals. Seven-week-old male C57BL/6J mice were purchased from Hyochang Bioscience (Daegu, South Korea) and fed a normal diet (AIN-76A) for a week to stabilize all metabolic conditions. Mice were maintained on a 12-h dark/light cycle at a constant temperature of 22 ± 1 °C and a humidity of 55 ± 10%. After stabilization, mice were divided into two diet groups, of which the chow diet group was fed a regular diet (20% kcal from protein, 64% kcal from carbohydrate, 16% kcal from fat), and the HF diet (HFD) group was fed a HFD (20% kcal from protein, 20% kcal from carbohydrate, 60% kcal from fat). Each group was divided into two subgroups, melanin complex-treated and control group, which received a daily dose of melanin complex (50 mg/kg/day) and PBS orally for 10 weeks, respectively. Mice were fasted for 4 h and sacrificed, and tissues of liver, epididymal fat, mesenteric fat, and quadriceps muscle were rapidly excised, snap-frozen in liquid nitrogen, and stored at −75 °C until processed for experiments.

Glucose tolerance test. After 10 weeks of melanin complex treatment, mice were fasted for 16 h and followed by intraperitoneal injection of glucose (2 g/kg). Blood samples were obtained by tail bleeding, and the blood glucose level was checked by Accu-Check Go (Roche Diagnostics GmbH, Germany) 0, 10, 20, 30, 60, 90, and 120 min after glucose injection.

Cell culture. 3T3-L1 fibroblasts were grown and maintained in high-glucose DMEM containing 10% FBS at 37 °C in a 5% CO_2 environment. To differentiate 3T3-L1 preadipocytes into adipocytes, postconfluent (2 days) cells were incubated in the DMEM–FBS medium supplemented with 0.5 mM isobutylmethyl xanthine, 1 μM dexamethasone, and 1 μg/mL insulin (day 0). After 2 days, the medium was replaced with DMEM–FBS containing 1 μg/mL insulin only (day 2). From day 4, the medium was replaced with DMEM–FBS every 2 days for the next 4 days. Insulin-mediated glucose uptake was induced by acute exposure to insulin (100 nM) for 10 min after being treated with or without melanin complex. Prior to glucose transport assay and subcellular fractionation, cells were washed with 3.0-mL Krebs Ringer phosphate buffer (KRP). 3T3-L1 adipocytes were treated or not with melanin complex at specific concentrations (0.1–3 mg/mL) for the times noted in the figure legends. C2C12 myotubes, a skeletal muscle cell line, were maintained in high-glucose DMEM containing 10% FBS at 37 °C in 5% CO_2. To induce differentiation, the medium was replaced with high-glucose DMEM supplemented with 2% horse serum at confluence and repeatedly replaced every 2 days for the next 4 days. HepG2 hepatoma cells were grown and maintained in 10% FBS at 37 °C in 5% CO_2; cells were grown to semi-confluence prior to melanin complex treatment.

Extraction of water-soluble melanin complex. *I. obliquus* was purchased from an oriental medicinal herb store in Ulaanbaatar, Mongolia. To extract the water-soluble melanin complex, 10 g of the well-dried chopped sample was mixed with 100 mL of distilled water, and the mixture was sit overnight at room temperature and filtered (Mazurkiewicz, 2006). Repeated 3 times, the collected filtrate solvent was evaporated by a rotary evaporator. Melanin complex stocks for experimental use were dissolved in DMEM.

Glucose transport assay. Cytochalasin B-inhibitable glucose transport was assayed as previously described by

Thomson *et al.* (1997) with some modifications. Briefly, 3T3-L1 adipocytes were exposed to melanin complex in DMEM containing 10% FBS and 1% penicillin/streptomycin for 24 h. Afterwards, KRP wash was performed at pH 7.4, and cell cultures were incubated with or without 20-μM cytochalasin B for 10 min at 37 °C. Insulin (100 nM) was added or not for 10 min, followed by the addition of [^{3}H]2-deoxyglucose (0.2 mM, 0.2 μCi) for another 10 min. Termination of glucose uptake was done with three washes of ice-cold PBS. Cells were dissolved in 0.1% sodium dodecyl sulfate (SDS) and counted for radioactivity.

Western blot analysis. For protein detection of Akt, phospho-Akt, AMPK, phospho-AMPK, and GLUT4, Western blotting procedure was used as described previously (Kim *et al.*, 2009). Briefly, whole-cell lysates were centrifuged at 4 °C for 15 min to remove the insoluble materials. The proteins in the supernatants were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes or PVDF membranes. Membranes were blocked with TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) containing 5% skim milk for 1 h at room temperature and incubated with the appropriate antibodies. The proteins were visualized by enhanced chemiluminescence using HRP-conjugated anti-rabbit or mouse IgG. Densitometric analysis of the bands was performed using AlphaImager 2200 (Alpha Innotech Inc., San Leandro, CA) and analyzed by AlphaEase FC software (Alpha Innotech Inc.).

Subcellular fractionation. Total membranes were obtained and prepared as described by Kitzman *et al.* (1993) followed by subcellular membrane isolation as described by Fisher and Frost (1996). Proteins were separated by SDS-PAGE and transferred to nitrocellulose for Western blot analysis.

Real-time quantitative PCR. Total RNA extraction, reverse transcription, and quantitative PCR were performed as described previously (Kim *et al.*, 2010). Briefly, total RNA from 3T3-L1 adipocytes, HepG2 hepatocytes, and C2C12 myotubes were extracted with TRI reagent (Molecular Research Center Inc., Cincinnati, OH). cDNA was obtained by reverse transcription of total RNA (0.5–1 μg) with Multiscribe (Applied Biosystems, Foster City, CA). Quantitative PCR analysis was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific forward and reverse primers on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Quantification of gene transcripts for acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), peroxisome proliferator-activated receptor gamma, sterol regulatory element-binding protein 1-c, acyl CoA oxidase (AOX), carnitine palmitoyltransferase 1 (CPT1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), and long-chain acyl-CoA dehydrogenase (LCAD) was completed using gene-specific primers. Primer sequences are available upon request. Results are expressed as mean ± S.D. after normalizing to expression of β-actin gene using the $\Delta\Delta Ct$ method.

Statistical analysis. Numerical data are reported as means ± S.D. Differences present in groups were analyzed by Student's *t*-test procedure with p values of $p < 0.05$ being considered as statistically significant.

RESULTS

Effect of melanin complex on glucose tolerance and weight gain in diet-induced obese mice

To test whether water-soluble melanin complex administration changes body weight and/or glucose tolerance, male C57BL/6J mice were placed on a chow or HF diet at 10 weeks of age. On a HF diet for 10 weeks, melanin complex-treated mice had reduced body weight although it was not a significant difference, whereas on chow diet, there were no differences in body weight between control and melanin complex-treated mice (Fig. 1A). However, fat pad weights, including epididymal and mesenteric, were reduced significantly in melanin complex-treated mice as compared to controls in an HF-fed condition (Table 1). Liver weight did not change by melanin complex treatment.

Additionally, melanin complex-treated mice on HF diet showed significant enhancement of glucose tolerance compared to the control group at 10 weeks of treatment (Fig. 1B). On chow diet, no significant difference was observed between melanin complex-treated and control groups.

Effect of melanin complex on insulin stimulated glucose uptake via PI 3-K pathway in 3T3-L1 adipocytes

To elucidate the mechanism of enhanced glucose tolerance in melanin complex-treated mice on HF-fed condition, we examined the effect *in vitro* using cultured adipocytes. To determine the effect of melanin complex on glucose uptake, a focal indicator of insulin sensitivity, differentiated 3T3-L1 adipocytes were treated with increasing concentrations of melanin complex (0.1, 0.2, 0.5, 1.0, 2.0 mg/mL). Two groups of 3T3-L1 cells were used with one being treated with acute insulin of 100 nM for 10 min to induce glucose uptake (insulin-stimulated glucose uptake) and the latter with KRP buffer only (basal glucose uptake). As shown in Fig. 2, glucose uptake in response to acute insulin was augmented significantly following incubation of 3T3-L1 adipocytes with melanin complex for 24 h. Melanin treatment stimulated both insulin-stimulated and basal glucose uptake in a dose-dependent manner with peak enhancement shown at 1 mg/mL. In an effort to ascertain whether or not the melanin complex effect on insulin-stimulated glucose uptake was attributed to activated PI 3-K pathway, 3T3-L1 cells were incubated with or without 1 mg/mL melanin complex, insulin, and acute wortmannin, a common PI 3-K inhibitor, and analyzed by glucose uptake assay. Fig. 3 shows an identical increase in glucose uptake following activation by acute insulin together with the previously observed enhancement of glucose uptake upon treatment with melanin complex without wortmannin. However, following wortmannin treatment, the augmented glucose uptake was completely abolished, indicating that the enhancing

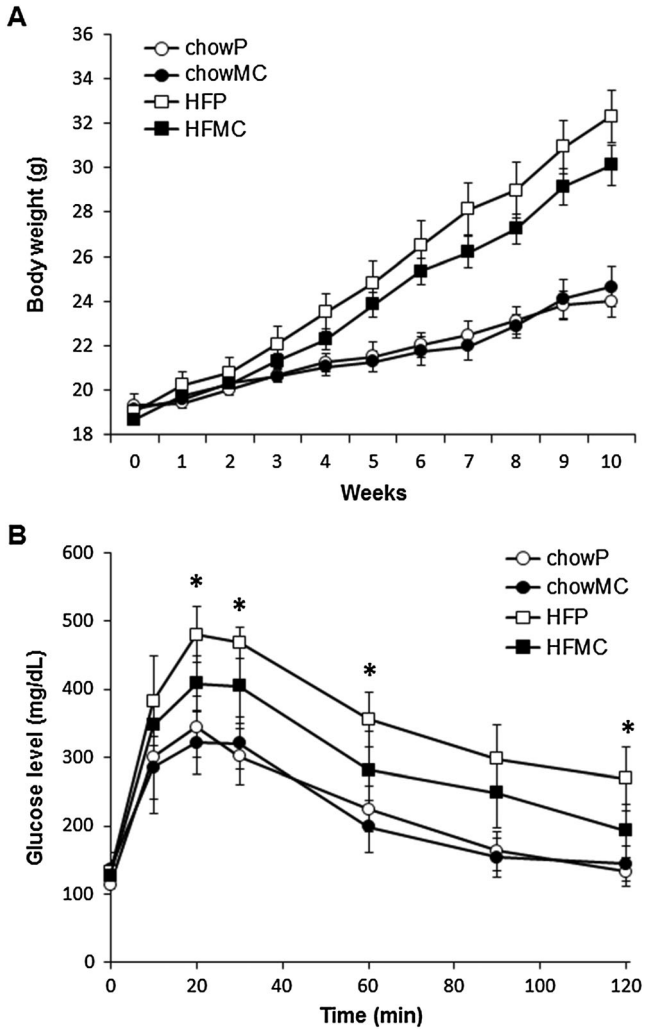

Figure 1. Effect of water-soluble melanin complex on weight gain and glucose tolerance in HF-fed mice. C57BL/6J mice were fed a normal or high-fat diet with treatment of PBS (control) or melanin complex for 10 weeks. (A) Changes of body weight after treatment of PBS or melanin complex. (B) Changes of glucose level after intraperitoneal injection of glucose (2 g/kg in DDW) in glucose tolerance tests at 10 weeks of treatment. $*p < 0.05$ compared to melanin complex-treated HF-fed (HFMC) mice. Data represent means ± S.D. for six or seven mice per group. chowP: chow diet-fed PBS-treated mice, chowMC: chow diet-fed melanin complex-treated mice, HFP: high-fat diet-fed PBS-treated mice, HFMC: high fat-diet-fed melanin complex-treated mice.

effect of melanin complex on glucose uptake occurs via a mechanism involved in the activation of PI 3-K. This analysis is further supported by Western blot analysis of Akt, a downstream protein of PI 3-K which showed a marked dose-dependent increase in phosphorylation in response to melanin complex treatment (Fig. 4). Furthermore, subcellular fractionation analysis showed a dose-dependent increase in the plasma membrane

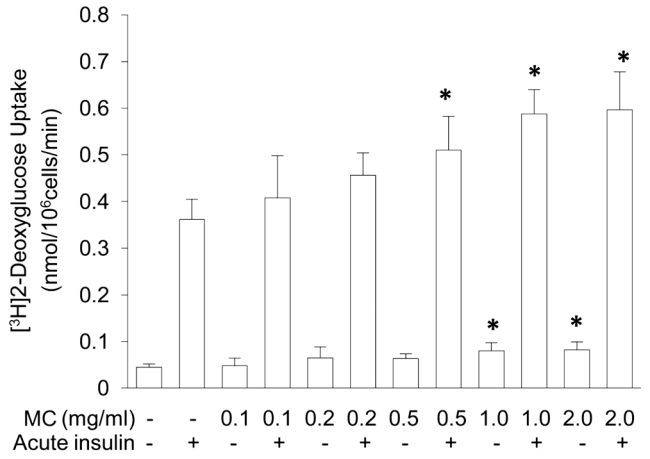

Figure 2. Effect of melanin complex on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Cells were exposed to increasing concentrations of melanin complex (0.1, 0.2, 0.5, 1.0, 2.0 mg/mL) for 24 h. Cells were then rinsed with KRP and insulin (100 nM), or vehicle was added for 10 min. [^{3}H]2-Deoxyglucose (0.2 mM, 0.2 μCi) was added for an additional 10 min. Transport activity was terminated with three washes of ice-cold PBS. Cells were lysed in 0.1% SDS and counted for radioactivity. Data represent means ± S.D. of three independent experiments of cytochalasin B-inhibitable uptake. $*p < 0.05$ compared to insulin-stimulated glucose uptake value of normal control cells. MC; melanin complex.

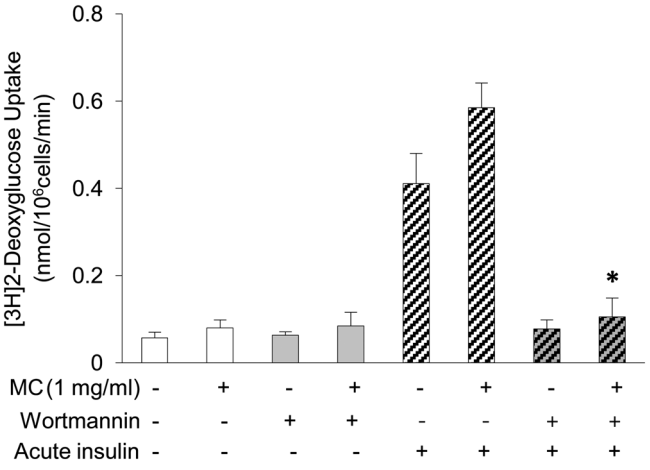

Figure 3. Effect of wortmannin on melanin complex-augmented insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Cells were exposed to 1 mg/mL melanin complex for 24 h, then rinsed in KRP and incubated with or without 100-nM wortmannin for 10 min. Insulin (100 nM) or vehicle was added for an additional 10 min before deoxyglucose uptake was assessed. Data represent means ± S.D. of three independent experiments. $*p < 0.05$ compared to insulin-stimulated glucose uptake value of melanin complex-treated cells. MC; melanin complex.

Table 1. Body and tissue weights after 10-week treatment of melanin complex. $*p < 0.05$ compared to HFP mice. Values represent means ± S.D. for six or seven mice per group

Group	Body weight (g)	Liver (g)	Epididymal fat (g)	Mesenteric fat (g)
chowP	24.01 ± 0.73	0.82 ± 0.06	0.39 ± 0.09	0.25 ± 0.09
chowMC	24.59 ± 0.90	0.84 ± 0.10	0.37 ± 0.13	0.27 ± 0.08
HFP	32.32 ± 1.17	1.63 ± 0.19	2.41 ± 0.28	1.28 ± 0.16
HFMC	30.10 ± 0.90	1.38 ± 0.16	2.04 ± 0.31*	0.76 ± 0.13*

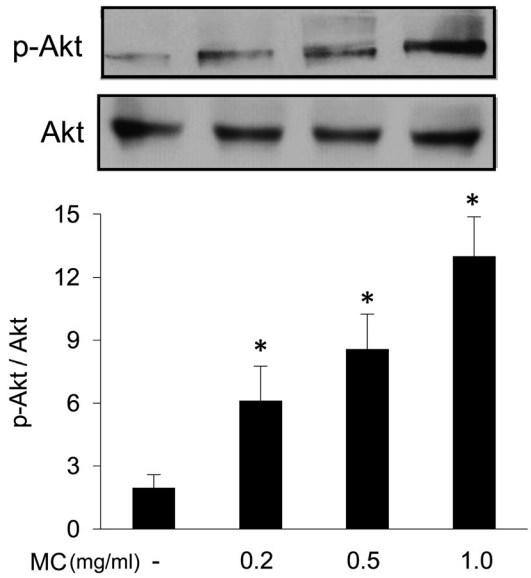

Figure 4. Effect of melanin complex on insulin-dependent Akt phosphorylation in 3T3-L1 adipocytes. Cells were treated with 1 mg/mL melanin complex for 24 h. Cells were washed in cold PBS 3 times and extracted for Akt. Equal protein was analyzed by SDS/PAGE-Western blot to measure total Akt, p-Akt (Ser473). Immunoblots are representative of three independent experiments. Data represent means ± S.D. of three independent experiments. $*p < 0.05$ compared to relative intensity values of p-Akt (ser473) in control. MC; melanin complex.

GLUT4, the primary glucose transporter protein responsible for facilitating insulin-mediated glucose uptake, indicating an increase in GLUT4 translocation to the plasma membrane from cytoplasmic vesicles (Fig. 5).

Effect of melanin complex on adiponectin production in adipose tissue

To further analyze the metabolic effect of melanin complex on energy metabolism, real-time RT PCR analysis was performed to determine the gene expression of adiponectin, a key hormone involved in activation of AMPK in liver and skeletal muscle. On a chow diet, there was no significant difference in adiponectin mRNA expression in epididymal adipose tissue between melanin complex-treated and control groups. However, melanin complex-treated mice on HF diet showed a significant increase of adiponectin expression compared to the control group (Fig. 6A). In addition, gene expression of adiponectin in 3T3-L1 adipocytes in response to 24-h incubation with melanin complex was increased dose dependently with a near two-fold increase at 1 mg/mL melanin complex concentration (Fig. 6B). We next examined the correlation of the increase of adiponectin production in adipocytes with the associated responses of metabolically relevant cells such as hepatocytes and skeletal muscle cells. HepG2 hepatoma cells and C2C12 myotubes were incubated with the conditioned media from 3T3-L1 adipocyte cultures. 3T3-L1 cells were incubated in increasing concentrations of melanin complex for 24 h; afterwards the conditioned media were collected and treated on HepG2 and C2C12 cells for 24 h. The conditioned media incubation caused a marked increase in AMPK activation in both HepG2 and C2C12 cells (Fig. 7A and 7B, respectively), which is indicative of positive adipokine-mediated AMPK activation. These results showed a metabolic correlation between 3T3-L1 and peripheral tissue cells in response to melanin complex treatment.

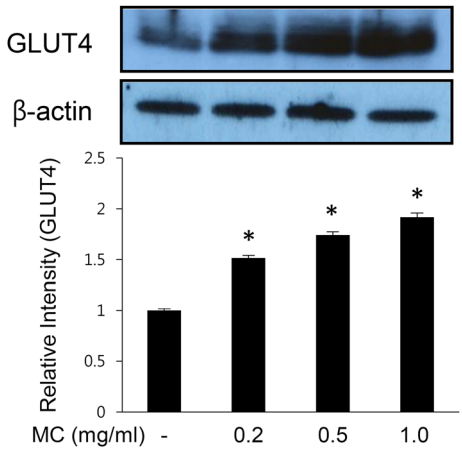

Figure 5. Effect of melanin complex on insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. Cells were treated with increasing concentration of melanin complex for 24 h. Cells were washed in cold PBS 3 times. Subcellular fractions were prepared, and GLUT4 proteins were resolved by SDS-PAGE and analyzed by Western blotting. MC; melanin complex. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Effect of melanin complex on expression of lipogenic and fatty acid oxidative genes

To determine the effect of melanin complex on lipid metabolism, real-time RT PCR analysis of lipogenic and fatty acid oxidative gene expression was performed on melanin complex-treated 3T3-L1 cells. As shown in Fig. 8A, mRNA expression of lipogenic genes ACC, FAS, PPAR-γ and SREBP1-c in response to incremental dose of melanin complex showed an increase in FAS expression but showed insignificant alteration of the remaining lipogenic genes, indicating that melanin complex does not have a significant effect on lipogenesis. In contrast, expression of fatty acid oxidation genes including CPT-1, AOX, and LCAD was significantly increased in response to melanin complex (Fig. 8B). Similar to 3T3-L1 cells, the epididymal adipose tissue of melanin complex-treated HF-fed mice showed higher expressions of fatty acid oxidative genes (CPT-1, AOX, and PGC1α) relative to their controls, but lipogenic gene expressions were not significantly different between the two groups (data not shown). This suggests a mechanism of oxidative depletion of fatty acid in adipose tissue in response to water-soluble melanin complex.

DISCUSSION

Recent discovery of severely debilitating side effects of the most common treatments for diabetes such as further weight gain and increased risk of cardiovascular disorder has encouraged the pursuit of alternative sources of metabolically beneficial remedies. Previously reported data suggested there to be a correlation of antihyperglycemia to extracts of *I. obliquus* (Mizuno *et al.*, 1999), a traditional remedy which has garnered acceptance as having antidiabetic properties despite the

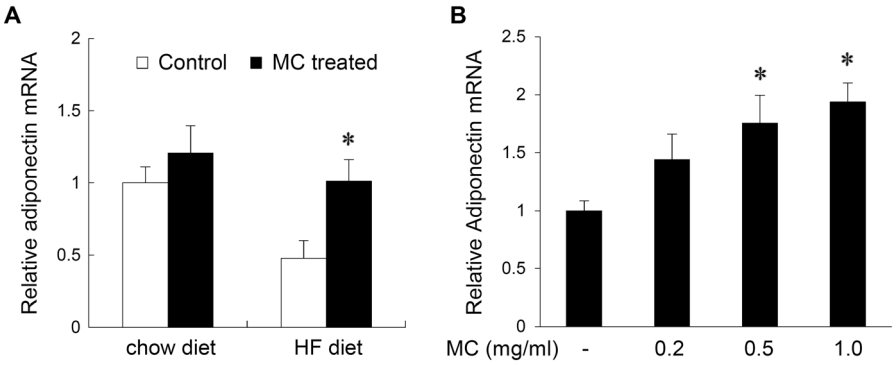

Figure 6. Effect of melanin complex on adiponectin gene expression in (A) epididymal adipose tissue and (B) 3T3-L1 adipocytes. Mice were fed a chow or high-fat (HF) diet with treatment of PBS (control) or melanin complex for 10 weeks. 3T3-L1 adipocytes were treated with melanin complex in serum-free DMEM with incremental increase in concentration for 24 h. Total RNAs were extracted from epididymal adipose tissue and 3T3-L1 cells with TRI reagents for reverse transcription and real-time PCR analysis. Data represent the means ± S.D. of three independent experiments. $*p < 0.05$ compared to control. MC; melanin complex.

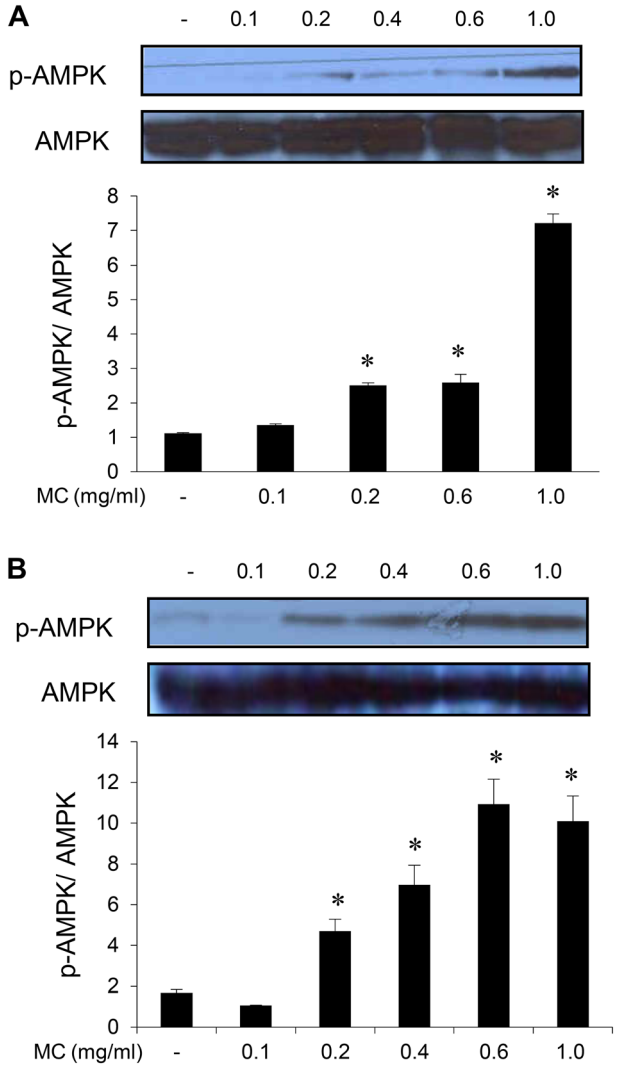

Figure 7. Effect of melanin complex on conditioned media activation of AMPK in HepG2 hepatocytes and C2C12 myotubes. 3T3-L1 adipocytes were treated with melanin complex in serum-free DMEM with incremental increase in concentration for 24 h. The conditioned media were then transferred to (A) HepG2 hepatocytes and (B) differentiated C2C12 myotubes which were then incubated for 24 h prior to protein extraction. Finally, cells were washed in cold PBS 3 times and extracted for AMPK and analyzed by SDS/PAGE-Western blot to measure total AMPK and p-AMPK. MC; melanin complex. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

fact that the exact mechanism is poorly understood. In this study, we have utilized water-soluble melanin complex extracted from *I. obliquus* and discovered that it has multiple beneficial effects on glucose and lipid metabolism and would control hyperglycemia without the adverse effect of weight gain. Consistent with the previous reports (Mizuno *et al.*, 1999; Sun *et al.*, 2008), we observed that the melanin complex extract exerted an antihyperglycemic effect in HF-fed obese mice showing an enhanced glucose tolerance. Interestingly, the adiposity estimated by measuring fat pad weight was also reduced in melanin complex-treated mice, but body weight was not reduced significantly (Table 1). Although no significance was observed, the average body weight of HFMC group (30.10 ± 0.90 g) was lower than that of the HFP group (32.32 ± 1.17 g), which was potentially caused by reduction of fat pad weights in the HFMC group. However, despite significant reductions in epididymal and mesenteric fat weights of HFMC group mice, the amount of fat reduction as compared to the HFP group (about 0.37 g epididymal fat and 0.52 g mesenteric fat) might not contribute to a significant reduction in weight gain on HF diet.

In defining the mechanism underlying the effects of melanin complex, we tested the stimulatory effect of melanin complex on insulin-stimulated glucose uptake using differentiated 3T3-L1 adipocytes. Following the treatment of wortmannin, a specific inhibitor of PI 3-K, a PI 3-K pathway-dependent mechanism was clearly shown in the total ablation of glucose uptake upon inhibition of PI 3-K. In addition to this inhibition analysis, a dose-dependent increase in Akt phosphorylation in response to melanin complex treatment confirmed a definite relation between PI 3-K pathway and melanin complex-mediated increase of glucose uptake. As PI 3-K pathway signaling of glucose uptake is carried out by translocation of GLUT4 from cytoplasmic vesicles into the plasma membrane (Taniguchi *et al.*, 2006), subcellular fractionation analysis of GLUT4 in plasma membrane was required to directly link melanin complex to insulin-mediated glucose uptake. We observed that the extent of GLUT4 translocation into the plasma membrane was increased dose dependently in melanin complex. It is therefore believed that the increase in insulin-stimulated glucose uptake is directly linked to the PI 3-K pathway which increases glucose uptake through conjunction with the translocation of GLUT4 to the plasma membrane.

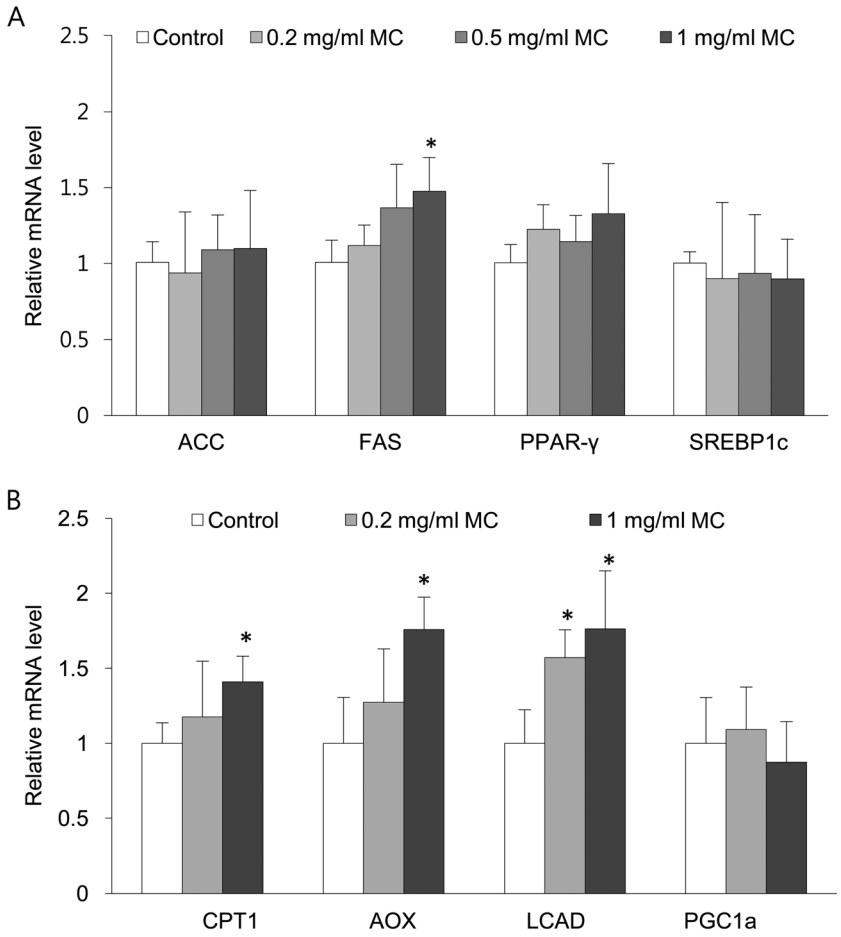

Figure 8. Effect of melanin complex on the expression of genes involved in lipogenesis (A) and fatty acid oxidation (B) in 3T3-L1 adipocytes. Cells were treated with melanin complex in serum-free DMEM with incremental increase in concentration. Total RNA were extracted from the cells with TRI reagents for reverse transcription and real-time PCR analysis. Data represent the means ± S.D. of three independent experiments. $*p < 0.05$ compared to control. MC; melanin complex.

Some other key regulators in lipid and glucose metabolism are adiponectin and AMPK. Active AMPK mediates multiple beneficial effects of modulation of insulin sensitivity and energy homeostasis (Kahn *et al*., 2005; Lim *et al*., 2010), therefore situating AMPK as a potential target for diabetes, obesity, and related metabolic diseases. The effects of AMPK activation include inhibition of de novo synthesis of fatty acids as well as mediation of fatty acid oxidation. AMPK phosphorylates and inactivates ACC1, which is a rate-determining enzyme for the synthesis of malonyl-CoA, both a critical substrate for fatty acid biosynthesis and a potent inhibitor of fatty acid oxidation, leading to inhibition of de novo fatty acid and cholesterol synthesis. Phosphorylation of ACC2 by AMPK, on the other hand, causes an increase in fatty acid oxidation (Viollet *et al*., 2009). AMPK also mediates suppression of expression of lipogenic genes such as FAS and ACC by disruption of critical transcription factors (Zhang *et al*., 2009; Foretz *et al*., 2005). Adiponectin, an insulin-sensitizing adipokine released by adipocytes, is responsible for alleviating the effects of insulin resistance with activation of AMPK, which stimulates GLUT4 translocation to the plasma membrane, decreases lipogenesis, and induces fatty acid oxidation in skeletal muscle and liver (Zhang *et al*., 2009; Viollet *et al*., 2009). In this study, it was observed that melanin complex treatment significantly enhanced adiponectin expression in white adipose tissue of HF-fed obese mice. We also tested the enhancing effect of melanin complex on adiponectin production in adipocytes using *in vitro* model systems. First, it was possible to conclude that the treatment of melanin complex on differentiated 3T3-L1 cells induced a dose-dependent increase in adiponectin. Next, 3T3-L1 cells were treated with melanin complex for 24 h to promote adiponectin secretion, and the resulting conditioned media was collected and treated on C2C12 myotubes and HepG2 hepatocytes, which are typically used myotubes and liver cells as models of skeletal muscle and liver tissue, respectively, to determine the effect on AMPK activation. Although C2C12 and HepG2 cells showed different extent of AMPK activation in response to the conditioned media, it was clear that melanin complex-mediated secretion of adiponectin in adipocytes activated AMPK in those cells. These results indicated that the treatment of melanin complex increases adiponectin expression in adipose tissue and causes a beneficial consequence, i.e. AMPK activation, in metabolically important tissues, skeletal muscle, and liver.

The expression of several genes that act as indicators of lipogenesis and fatty acid oxidation was also examined. In particular, ACC action is the first irreversible step into lipid synthesis which in conjunction with FAS is responsible for synthesis of saturated fatty acids which are essential for triglyceride production (Zhang *et al*., 2009). PPAR-γ and SREBP1-c are transcription factors which play critical roles in lipogenesis (Kodera *et al*., 2000; Roberts *et al*., 2011; Ferré and Foufelle, 2007). As

for fatty acid oxidation, CPT1 is responsible for transport of fatty acyl-CoA into the mitochondria for β-oxidation and is moderated by the presence of malonyl-CoA, a product of ACC which is prone to inhibition by AMPK activation (Zhang *et al*., 2009; Viollet *et al*., 2009). AOX and LCAD are both responsible for oxidizing acyl-CoA (Schuler and Wood, 2002). PGC1α is a transcriptional coactivator which plays a part in energy balance and is involved in mitochondria biogenesis (Puigserver and Spiegelman, 2003; Liang and Ward, 2006). In this study, real-time PCR analysis of 3T3-L1 cells with 24-h melanin complex treatment showed a lipogenic gene expression profile which had no relevance to the dose-dependent melanin complex treatment with insignificant change in ACC, PPAR-γ as well as SREBP1-c. Oil-red-O analysis (data not shown) of 3T3-L1 cells treated with dose-dependent melanin complex did not show any change in the overall lipid content of the adipose tissues, which supports the real-time PCR data showing an unchanged expression of lipogenic genes. In contrast, the expression of fatty acid oxidative genes including CPT1, AOX, and LCAD has shown a dose-dependent increase in response to melanin complex treatment. Same analysis of the epididymal adipose tissue of HF-fed mice showed similar results. While the expression of ACC, FAS, PPAR-γ, and SREBP1-c were not altered by melanin complex treatment, the expression of CPT1, AOX, and PGC1α was increased significantly in the adipose tissue of melanin complex-treated mice. The reason why PGC1α and LCAD expressions remained unchanged in 3T3-L1 cells and epididymal adipose tissue, respectively, is currently under investigation.

In conclusion, this study shows the water-soluble melanin complex of *I. obliquus* to have insulin-sensitizing activity as well as AMPK activating effect without the adverse effect of weight gain. This effect being present in tandem with increased insulin-stimulated glucose uptake with the beneficial effects on lipid metabolism in the form of increased fatty acid oxidation with little change in lipogenic gene expression presents the melanin complex of *I. obliquus* as a potential antidiabetic agent for study and further development.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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