MOL PHARM

# Antidiabetes and Antiobesity Effect of Cryptotanshinone via Activation of AMP-Activated Protein Kinase

MOLECULAR PHARMACOLOGY

Eun Ju Kim, Seung-Nam Jung, Kun Ho Son, Sung Ran Kim, Tae Youl Ha, Myoung Gyu Park, In Gun Jo, Jong Guk Park, Wonchae Choe, Sung-Soo Kim, and Joohun Ha

Department of Biochemistry and Molecular Biology, Medical Research Center for Bioreaction to Reactive Oxygen Species, Kyung Hee University School of Medicine, Seoul, Korea (E.J.K., S.-N.J., W.C., S.-S.K., J.H.); Department of Food Science and Nutrition, Andong National University, Andong, Korea (K.H.S.); Food Function Research Division, Korea Food Research Institute, Sungnam, Korea (S.R.K., T.Y.H.); and New Drug Research Division, MD Bioalpha Co., Ltd., Sungnam, Korea (M.G.P., I.G.J., J.G.P.)

## ABSTRACT

Metabolic disorders, including type 2 diabetes and obesity, represent major health risks in industrialized countries. AMP-activated protein kinase (AMPK) has become the focus of a great deal of attention as a novel therapeutic target for the treatment of metabolic syndromes, because AMPK has been demonstrated to mediate, at least in part, the effects of a number of physiological and pharmacological factors that exert beneficial effects on these disorders. Thus, the identification of a compound that activates the AMPK pathway would contribute significantly to the treatment and management of such syndromes. In service of this goal, we have screened a variety of naturally occurring compounds and have identified one compound, cryptotanshinone, as a novel AMPK pathway activator. Cryptotanshinone was originally isolated from the dried roots of *Salvia militorrhiza*, an herb that is used extensively in Asian medicine and that is known to exert beneficial effects on the circulatory system. For the first time, in the present study, we have described the potent antidiabetic and antiobesity effects of cryptotanshinone, both in vitro and in vivo. Our findings suggest that the activation of the AMPK pathway might contribute to the development of novel therapeutic approaches for the treatment of metabolic disorders such as type 2 diabetes and obesity.

AMP-activated protein kinase (AMPK) is a well-conserved heterotrimeric enzyme complex that is composed of an $\alpha$-catalytic subunit and regulatory $\beta$ and $\gamma$ subunits. AMPK has been implicated tentatively as a cellular energy sensor, because it is known to be exquisitely sensitive to intracellular energy status (Kemp et al., 2003; Carling, 2004). Recent studies have shown that the AMPK pathway performs a central function in the regulation of glucose and lipid homeostasis, body weight, food intake, insulin signaling, and mitochondria biogenesis, thereby making its cascade a promising pharmacological target for the treatment of metabolic disorders, including obesity and type 2 diabetes (Kahn et al., 2005; Hardie, 2006; Viollet et al., 2006). Indeed, AMPK cascades are also known to mediate, in part, the beneficial effects of antidiabetic and antiobese factors, including leptin, adiponectin, exercise, metformin, and rosiglitazone (Kahn et al., 2005). Therefore, the identification of a compound that activates the AMPK pathway would significantly contribute to our ability to treat obesity and type 2 diabetes.

To identify a novel compound that harbors antidiabetes and antiobesity potential, we evaluated the pharmacological effects of a number of natural single compounds. In this study, we report that cryptotanshinone is a novel activator of the AMPK pathway and describe its potent antidiabetic and antiobese effects both in vitro and in vivo. Cryptotanshinone was originally isolated from the dried roots of *Salvia militorrhiza* Bunge, an herb that is extensively used in Asian med-

**ABBREVIATIONS:** AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide-1-$\beta$-D-ribofuranoside; ACC, acetyl-CoA carboxylase; DMEM, Dulbecco's modified Eagle's medium; PI-3, phosphatidylinositol-3; PBS, phosphate-buffered saline; KRB, Krebs-Ringer phosphate buffer; Glut4, glucose transporter 4; CPT-I, carnitine palmitoyl transferase I; PGC-1$\alpha$, peroxisome proliferator-activated receptor-$\gamma$ coactivator-1$\alpha$; UCP, uncoupling protein; DIO, diet-induced obese; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; IGF-IR$\beta$, insulin-like growth factor 1 receptor $\beta$.

aspet

icine (Ji et al., 2000; Zhou et al., 2005). The extracts of this herb have been used in the treatment of several pathologies, including cardiovascular diseases, hematological abnormalities, hepatitis, and hyperlipidemia (Wang et al., 2007). More than 30 diterpene compounds, including tanshinone I, IIA, IIB, and cryptotanshinone, have been separated from the plant and identified as major chemical constituents (Zhou et al., 2005). These are unique components derivate of diterpene quinone, which are found exclusively in the genus *Salvia*, exhibiting a variety of biological activities, including antibacterial, antifungal, antioxidant, antimutagenic, antitumor, anti-inflammatory, and antiplatelet aggregation effects (Zhou et al., 2005; Wang et al., 2007). However, thus far, no studies have been conducted regarding the activity of these compounds against diabetes and obesity. In the present study, we describe the pharmacological potential of cryptotanshinone against diabetes and obesity for the first time, and the results of our mechanism studies demonstrated that the activation of AMPK pathway would contribute significantly to the development of novel therapeutic approaches for the treatment of metabolic disorders, such as type 2 diabetes and obesity.

## Materials and Methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) and 2-deoxy-D-[$^{3}$H]glucose (6.0 Ci/mmol) were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). The phosphospecific-AMPK Thr$^{172}$, total AMPK, phosphospecific-PKB/Akt, total PKB/Akt, phosphospecific-acetyl-CoA carboxylase (ACC), phosphor-mTOR, and phosphospecific-p70 S6K antibodies used in this study were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for glucose transporter 4 (Glut4) and IGF-IR$\beta$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Isolation of Cryptotanshinone.** Cryptotanshinone was isolated from dried roots of *S. militorrhiza* via an identical fractionation protocol as described previously (Hur et al., 2005). Its molecular weight was determined to be 296 using NMR and mass spectrometry. It was dissolved in 0.1% solution of sodium lauryl sulfate.

**Cell Culture.** Mouse C2C12 skeletal myoblasts were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic mixture in an atmosphere of 95% air and 5% $CO_2$ at 37°C. Differentiation of C2C12 myoblasts was induced via the transference of confluent cells to DMEM supplemented with 1% fetal bovine serum and allowing for the formation of myotubes. The medium was changed every 48 h. Cells were used in experiments at 4 to 5 days after differentiation.

**AMPK and PI-3 Kinase Activity Assay.** Cells were lysed in a digitonin buffer (50 mM Tris-HCl, pH 7.3, 50 mM NaF, 30 mM glycerol phosphate, 250 mM sucrose, 1 mM sodium metavanadate, and 0.4 mg/ml digitonin) on ice for 2 min. The AMPK was immunoprecipitated with AMPK pan-$\alpha$ antibody, and its activity was assessed in kinase assay buffer containing 200 $\mu$M AMP, an ATP mixture (100 $\mu$M ATP and 1.5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP) with or without 250 $\mu$M SAMS peptide (HMR<u>SAMS</u>GLHLVKRR) at 30°C for 10 min, as described previously (Lee et al., 2003). To characterize the direct in vitro effects of cryptotanshinone, partially purified rat liver AMPK was purchased from Upstate (Charlottesville, VA). Twenty milliunits of AMPK were incubated with cryptotanshinone in an identical reaction buffer for 10 min, without 200 $\mu$M AMP. PI 3-kinase activity was measured via immunoprecipitation with antiphosphotyrosine antibody, as described previously (Kim et al., 2002).

**ATP Analysis.** After treatment, cells were washed with ice-cold PBS, and 100 mM lysis buffer (0.5% Triton X-100 and 2 mM CaCl$_2$ in PBS) was added to the cells. Intracellular ATP was measured via the luciferin/luciferase method using an ATP Determination Kit (Invitrogen). The assay buffer (100 $\mu$l), which contained 0.5 mM luciferin, 1.25 $\mu$g/ml luciferase, 25 mM Tris, pH 7.8, 5 mM MgSO$_4$, 100 $\mu$M EDTA, and 1 mM dithiothreitol, was mixed with the cell lysates (5 $\mu$l). Luminescence was analyzed by VICTOR$^3$ luminometer (PerkinElmer, Boston, MA) and normalized using the cellular proteins.

**Glucose Uptake.** The cells were cultured on 12-well cluster dishes, washed in Krebs-Ringer phosphate buffer (KRB) (25 mM HEPES, pH 7.4, 118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.3 mM MgSO$_4$, 5 mM NaHCO$_3$, 0.07% bovine serum albumin, and 5.5 mM glucose), and incubated for 20 min in KRB buffer. The cells were then incubated for 10 min in KRB containing 0.5 $\mu$Ci of 2-deoxy-D-[$^{3}$H]glucose. After three washings in PBS, the cells were dissolved in 0.5% Triton X-100. Tracer activities were assessed using a liquid scintillation counter.

**Primers Used for PCR.** cDNA fragment was PCR-amplified using the following specific primers: uncoupled protein 2 (UCP2), sense 5′-AACAGTTCTACACCAAGGGC-3′, and antisense 5′-AGCATGGTAAGGGCACAGTG-3′; ACC1, sense 5′-GTCAGCGGATGGGC-GGAATG-3′, and antisense 5′- CGCCGGATGCCATGCTCAAC-3′; ACC2, sense 5′-GCTGCGGTCAAGTGTATGCG-3′, and antisense 5′-CACTGATGCATTTGCCCTGG-3′; CPT1, sense 5′-GCTCTCGAGG-CTCACTGATT-3′, and antisense 5′-CAGTCAGAGCAGCTAGGTG-T-3′; PGC-1$\alpha$, sense 5′-ACGAGGCCAGTCCTTCCTCC -3′, and antisense 5′-AGCTCTGAGCAGGGACGTCT-3′; Glut1, sense 5′-CGGG-CCAAGAGTGTGCTAAA -3′, and antisense 5′-TGACGATACCGGA-GCCAATG-3′; and GAPDH: sense 5′-TGCTGAGTATGTCGTGGAG-TCTA-3′, and antisense 5′-AGTGGGAGTTGCTGTTGAAGTCG-3′;

**Animal Experiments.** db/db and ob/ob mice on a C57BL/6 background (male, 9–11 weeks old, 30–40 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Zucker Diabetic Fatty (ZDF) (male, 8 weeks old, 200–300 g) type 2 diabetic rat models were purchased from Charles River Breeding Laboratories (Portage, MI). All animal experiments were approved by the Ethics Committee for Animal Experimentation of Korea Food Research Institute. The animals were given free access to water and were fed on a standard diet. Cryptotanshinone in 0.1% solution of sodium lauryl sulfate or vehicle was administered orally in a volume of 10 ml/kg body weight/day in the morning (9:00 to 10:00 AM). To measure plasma glucose level, the animals were deprived of food for 11 to 14 h, and then blood samples were collected from the tail veins of mice under ether anesthesia. Glucose levels were determined using a standard glucose oxidase assay kit from Sigma. Triglyceride levels were determined using a kit manufactured by Roche Diagnostics. Cholesterol levels were determined using an assay kit produced by Roche Diagnostics (Indianapolis, IN). Food intake and body weight were monitored on a daily basis.

**Diet-Induced Obesity Mice.** Male C57BL/6J (4 weeks old) mice were obtained from The Jackson Laboratory. All animals were individually housed and maintained at 25°C with a 12-h light/dark cycle. A group of C57BL/6J mice was maintained on a high-fat diet (44.9% fat; D12451; Research Diets, New Brunswick, NJ) for 10 weeks (Jiang et al., 2005), and these animals were divided into three groups ($n = 9$): cryptotanshinone in 0.1% solution of sodium lauryl sulfate or vehicle was administered orally in a volume of 10 ml/kg body weight/day in the morning (9:00 to 10:00 AM) for 28 days. The third group was pair-fed to the previous day's level of intake of the cryptotanshinone-treated animals. The body weight was measured every day.

**Statistical Analysis.** Results are expressed as the means ± S.E. We used Student's $t$ tests for unpaired data. Differences were considered significant at a $P$ value of <0.05.

## Results

**Cryptotanshinone Activates AMPK in C2C12 Myotubes.** To identify a novel activator of the AMPK pathway,

we have applied a number of natural products to C2C12 myotubes, after which the endogenous AMPK activity was measured. During such a screening course, we determined that cryptotanshinone was a potent AMPK activator in the C2C12 myotubes (Fig. 1). The C2C12 myotubes were exposed

to the indicated concentrations of cryptotanshinone for 1 h, and AMPK was found to have been activated in a concentration-dependent manner. An approximately 1.7-fold activation was observed at 20 $\mu$M (Fig. 1A). A well-known AMPK activator, AICAR, was used as a positive control, and this
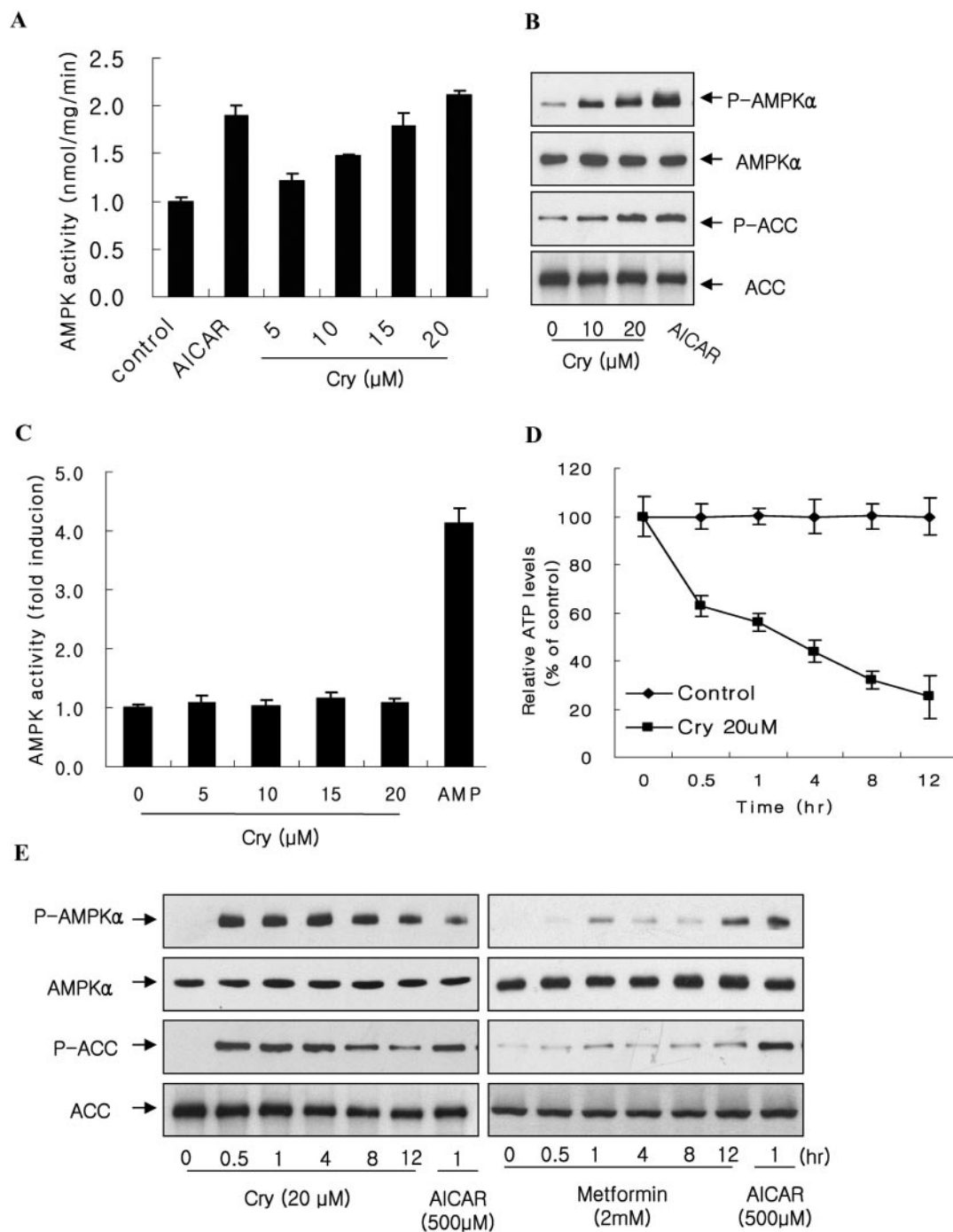
**Fig. 1.** Cryptotanshinone activates AMPK in C2C12 myotubes. A, C2C12 myotubes were incubated for 1 h with the indicated concentrations of cryptotanshinone. AMPK was then immunoprecipitated with AMPK pan-$\alpha$ antibody, and an in vitro activity assay was conducted using SAMS peptide as a substrate. The results are expressed as the means $\pm$ S.E. for two independent assays conducted in duplicate. AICAR (500 $\mu$M, 1 h) was used as a positive control. B, total cell extracts were prepared under identical conditions and subjected to Western blot assays using antiphosphospecific AMPK$\alpha$ Thr[172] (P-AMPK$\alpha$), anti-AMPK pan-$\alpha$ (AMPK$\alpha$), anti-phosphospecific ACC Ser[79] (P-ACC), and anti-ACC (ACC) antibodies. C, the purified rat liver AMPK was incubated with the indicated concentrations of cryptotanshinone in the absence of AMP, and the in vitro activity assays were conducted using SAMS peptide as a substrate. As a control, kinase assays were conducted in the presence of 200 $\mu$M AMP. D, C2C12 myotubes were treated with cryptotanshinone (20 $\mu$M) for 12 h, and then intracellular ATP levels were measured. The results are expressed as the means $\pm$ S.E. for three independent assays conducted in duplicate. E, C2C12 myotubes were treated with either cryptotanshinone (20 $\mu$M) or metformin (2 mM) for the indicated times. Western blot analysis was conducted using AMPK$\alpha$ Thr[172] (P-AMPK$\alpha$), anti-AMPK pan-$\alpha$ (AMPK$\alpha$), anti-phosphospecific ACC Ser[79] (P-ACC), and anti-ACC (ACC) antibodies. Cry, cryptotanshinone.

activator induced a 2-fold increase in AMPK activity. The cryptotanshinone-induced activation of AMPK was also associated with the phosphorylation level of Thr[172] of AMPKα, which is essential for AMPK activity (Fig. 1B). The phosphorylation level of Ser[79] of ACC, an intracellular substrate of AMPK, was also increased as the result of cryptotanshinone treatment (Fig. 1B), which indicated that cryptotanshinone does indeed activate AMPK in C2C12 myotubes. We then attempted to determine whether cryptotanshinone could directly activate AMPK in a test tube. After purified AMPK was incubated in the presence of the indicated concentration of cryptotanshinone, we assessed the AMPK activity via the evaluation of $^{32}P$ incorporation into the SAMS peptide, as is described under *Materials and Methods*. Our results indicated that cryptotanshinone does not directly activate AMPK in a test tube, whereas its activity was increased 4-fold in the presence of AMP (Fig. 1C). To understand the mechanism by which cryptotanshinone activates the AMPK pathway, we next examined the effect of cryptotanshinone on the intracellular ATP level, because AMPK is known to be exquisitely sensitive to intracellular energy status. Indeed, cryptotanshinone (20 μM) rapidly depleted ATP levels in C2C12 myotubes, resulting in 75% reductions in ATP levels in 12 h (Fig. 1D). As a consequence, these results suggest that cryptotanshinone indirectly activates AMPK pathways via reduction of intracellular ATP.

Because the beneficial effects of metformin on type 2 diabetes are known to be mediated, in part, by AMPK (Zhou et

al., 2001), we then conducted a comparison of the effects of cryptotanshinone on AMPK activity with those of metformin. Metformin-induced AMPK activation was found to be relatively slow, because it was detected 12 h after treatment when 2 mM was applied to the experimental C2C12 cells (Fig. 1E). By way of contrast, clear AMPK activation was observed within 30 min of cryptotanshinone treatment (20 μM), thereby indicating that cryptotanshinone is a more rapid and potent AMPK activator than metformin in C2C12 cells (Fig. 1E).

In addition to cryptotanshinone, other forms of tanshinone, including tanshinone-I and dihydrotanshinone, were also isolated from the dried roots of *S. miltiorrhiza*. We then compared the effects of these tanshinones on AMPK activity (Table 1). Among the tanshinones tested herein, cryptotanshinone was the most effective with regard to the induction of AMPK activity in the C2C12 myotubes. Moreover, cryptotanshinone showed no toxicity when the C2C12 myotubes were incubated for 24 h at a concentration of 20 μM.

**Cryptotanshinone Stimulates Glucose Uptake via AMPK Activation.** We next evaluated the effects of cryptotanshinone on glucose uptake in the C2C12 myotubes. Among the tested tanshinones, cryptotanshinone was found to be most effective with regard to the induction of glucose uptake, evidencing an approximately 2-fold increase (Table 1 and Fig. 2A). We further attempted to determine whether cryptotanshinone-induced glucose uptake was mediated by AMPK. Our data indicated that an AMPK inhibitor called

TABLE 1
The chemical structures of tanshinone-I, dihydrotanshinone, and cryptotanshinone and their effects on AMPK activity, glucose uptake, and cell viability of C2C12 myotubes

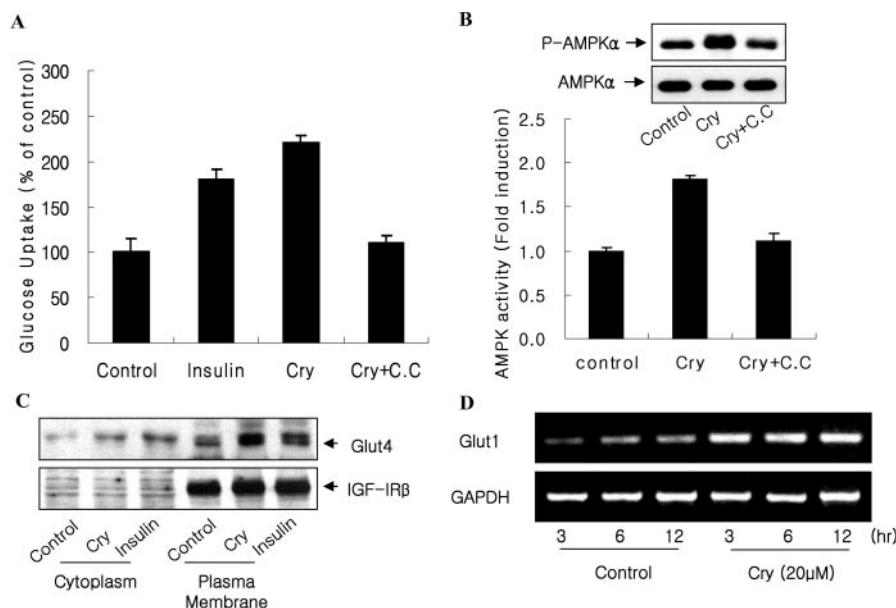| | Control | Tanshinone-I | Dihydrotanshinone | Cryptotanshinone |
|---|---|---|---|---|
| AMPK activity, -fold induction | 1 ± 0.1 | 1 ± 0.1 | 1.3 ± 0.1 | 1.7 ± 0.1 |
| [³H]2-Deoxy-glucose uptake, % of control | 100 ± 3.1 | 97.8 ± 1.0 | 77.5 ± 1.8 | 197.2 ± 3.8 |
| Cell viability, % of control | 100 ± 4.8 | 91 ± 11.3 | 80 ± 3.7 | 94 ± 3.1 |



**Fig. 2.** Cryptotanshinone stimulates glucose uptake via AMPK activation. A, C2C12 myotubes were incubated for 1 h with cryptotanshinone (20 μM) in the absence or presence of AMPK inhibitor compound C (20 μM). Then, 2-[³H]deoxy-D-glucose was added to culture media for 10 min at the end of the exposure period, and glucose uptake was measured as described under Research Design. The results are expressed as the means ± S.E. of at least six determinations. As a control, the myotubes were exposed to insulin (100 nM) for 1 h. B, under identical conditions, total cell extracts were subjected to Western blot analyses using AMPKα Thr¹⁷² (P-AMPKα) and anti-AMPK pan-α (AMPKα) antibodies or AMPK in vitro kinase assays. C, after 1 h of exposure to cryptotanshinone (20 μM) or insulin (100 nM), the cytoplasmic and plasma membrane fractions were collected and subjected to Western blot analysis using Glut4 or IGF-IRβ antibodies. D, myotubes were incubated with cryptotanshinone (20 μM) for 12 h, and total RNA was extracted for the indicated time periods and subjected to reverse transcription-polymerase chain reaction using specific primers for Glut1 and GAPDH genes. Cry, cryptotanshinone; C.C, compound C.
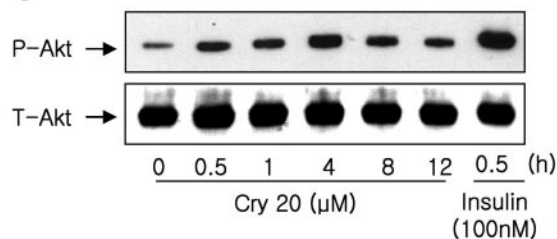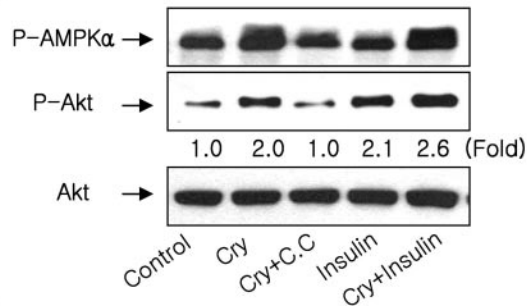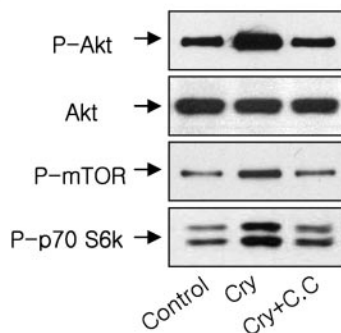
**A**
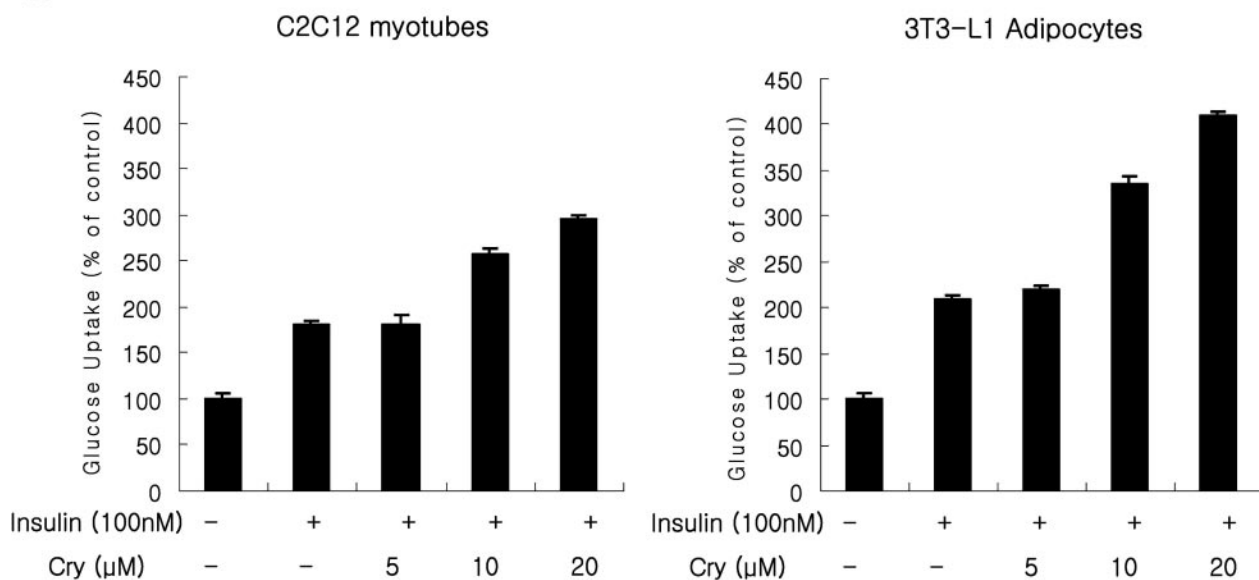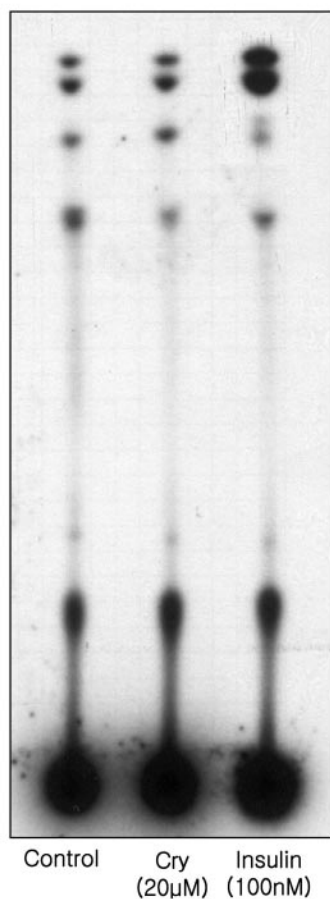
**Fig. 3.** Cryptotanshinone enhances insulin-derived glucose uptake in C2C12 myotubes and 3T3-L1 adipocytes via AMPK and Akt activation. A, C2C12 myotubes or 3T3-L1 adipocytes were incubated for 1 h with insulin in the presence of the indicated concentrations of cryptotanshinone, and then the glucose uptake was measured. Results are expressed as the means ± S.E of at least six determinations. B, C2C12 myotubes were exposed to cryptotanshinone (20 $\mu$M) or insulin (100 nM) for 1 h, and then PI-3 kinase activity assays were conducted as described under Research Design. C, myotubes were exposed to cryptotanshinone (20 $\mu$M) for the indicated time period, and phosphorylation levels of Akt (P-Akt) and total Akt (Akt) were determined via Western blot analysis. D and E, myotubes were exposed to cryptotanshinone (20 $\mu$M), insulin (100 nM), or both in the presence or absence of compound C (20 $\mu$M) for 1 h, after which Western blot analysis was conducted to measure the phosphorylation levels of AMPK$\alpha$ (P-AMPK$\alpha$), Akt (P-Akt), mTOR (P-mTOR), and p70S6K (P-p70S6K). Total amounts of AMPK$\alpha$ (AMPK$\alpha$) and Akt (Akt) were also compared. Cry, cryptotanshinone; C.C, compound C.

compound C (Zhou et al., 2001) effectively blocked cryptotanshinone-induced AMPK activity, as assessed by a direct enzyme assay and a Western blot analysis for AMPK phosphorylation levels (Fig. 2B). Compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl-4-yl-pyrrazolo[1,5-$a$]-pyrimidine) significantly blocked cryptotanshinone-induced glucose uptake, thereby indicating the critical role of AMPK in the process (Fig. 2A). Moreover, cryptotanshinone enhanced glucose uptake as effectively as insulin under our experimental conditions.

Glut4, which facilitates glucose transport, is known to be recruited to the plasma membrane in response to conditions that activate AMPK (Kurth-Kraczek et al., 1999; Mu et al., 2001). To determine the cellular mechanisms by which cryptotanshinone stimulates the glucose transport rate, we assessed the changes in the levels of Glut4 in the plasma membrane upon the activation of AMPK by cryptotanshinone. When the C2C12 myotubes were treated for 30 min with either cryptotanshinone or insulin, both reagents significantly enhanced the Glut4 contents within the plasma membrane (Fig. 2C). The purity of the membrane fractions was verified via determinations of the levels of IGF-IR$\beta$ expression in the plasma membrane. Moreover, cryptotanshinone also profoundly increased Glut1 mRNA expression when applied under long-term conditions (Fig. 2D). Together, our data indicate that cryptotanshinone enhances glucose uptake via the stimulation both of the translocation of Glut4 to the plasma membrane and Glut1 mRNA expression, and AMPK also seems to play a pivotal role in these processes.

**Cryptotanshinone Sensitizes C2C12 Myotubes to Insulin-Induced Glucose Uptake via the Activation of Akt and AMPK.** The results of recent studies suggest that AMPK also seems to enhance insulin sensitivity, both in vivo and in vitro (Buhl et al., 2001; Fisher et al., 2002). Thus, we attempted to determine whether cryptotanshinone enhances insulin sensitivity. Insulin effected an approximately 2-fold increase in glucose uptake in C2C12 myotubes and 3T3-L1 adipocytes, and insulin-induced glucose uptake was further enhanced by cryptotanshinone in a dose-dependent manner (Fig. 3A). To characterize the underlying mechanisms, we then attempted to ascertain whether cryptotanshinone would affect the PI-3 kinase/Akt pathway in C2C12 myotubes. As had been expected, insulin treatment of C2C12 myotubes for 1 h resulted in a marked increase in PI-3 kinase activity, whereas cryptotanshinone had no effect (Fig. 3B). However, under identical conditions, the phosphorylation level of Akt-Ser[473], which is downstream of PI-3 kinase, was rapidly increased by cryptotanshinone in a time-dependent manner (Fig. 3C), thereby suggesting that cryptotanshinone activates insulin signaling downstream in a PI-3 kinase-independent manner. Cryptotanshinone-induced Akt activation was attenuated significantly in the presence of AMPK inhibitor (Fig. 3D, third lane), thereby suggesting that AMPK functions as an upstream signaling component under cryptotanshinone-treatment conditions. Moreover, cryptotanshinone effected a further increase in insulin-induced Akt activation (Fig. 3D, fifth lane). Insulin failed to activate AMPK under our experimental conditions (Fig. 3D, fourth lane). These results suggest that Akt can be activated via at least two different upstream factors, namely PI-3 kinase and AMPK. Insulin signals are transmitted to Akt via PI-3 kinase, whereas the effect of cryptotanshinone seems to be

transmitted to Akt via AMPK, and a cross-link between AMPK and the Akt signaling pathway is probably responsible for the functions of cryptotanshinone as an insulin-sensitizer (Fig. 3A).

Akt has also been reported to transmit its signal to mTOR, which results in several physiological events, including the regulation of protein translation and cell size (Hay and Sonenberg, 2004). Indeed, cryptotanshinone effected an increase in the phosphorylation level of mTOR-Ser[2448], which is known to be phosphorylated by Akt (Nave et al., 1999) (Fig. 3E). In addition, the phosphorylation of Thr[389]of p70[S6K], one of the downstream targets of mTOR kinase (Burnett et al., 1998), was also increased as the result of cryptotanshinone treatment. Therefore, it is possible that, in addition to its effects on glucose uptake, cryptotanshinone may regulate both muscle cell growth and protein translation.

**The Effects of Cryptotanshinone on the Expressions of Genes Involved in Fatty Acid Metabolism and Mitochondrial Biogenesis.** Long-term AMPK activation has been shown to regulate the genes involved in glucose uptake, fatty acid synthesis, fatty acid oxidation, energy expenditure, and mitochondrial biogenesis (Zong et al., 2002; Suwa et al., 2003). We then attempted to determine the long-term effects of cryptotanshinone by assessing the mRNA expression levels of genes involved in fatty acid metabolism and mitochondrial biogenesis. ACC catalyzes the biosynthesis of malonyl-CoA from acetyl-CoA, and malonyl-CoA functions as an initial substrate for de novo fatty acid biosynthesis (Kim, 1997). In addition, malonyl-CoA allosterically inhibits carnitine palmitoyl transferase I (CPT-I), which is a rate-limiting step in fatty acid oxidation. Thus, decreased malonyl-CoA concentrations, resulting from the suppression of ACC 1 and ACC 2 gene expression, might induce a reduction in lipid synthesis and an increased rate in fatty acid oxidation, respectively (Zhou et al., 2001; Ruderman et al., 2003). Indeed, 6 to 12 h of cryptotanshinone treatment in the C2C12 myotubes resulted in a profound suppression of ACC 1 and ACC 2 mRNA expression and a concomitant increase in the expression of CPT-I mRNA (Fig. 4).

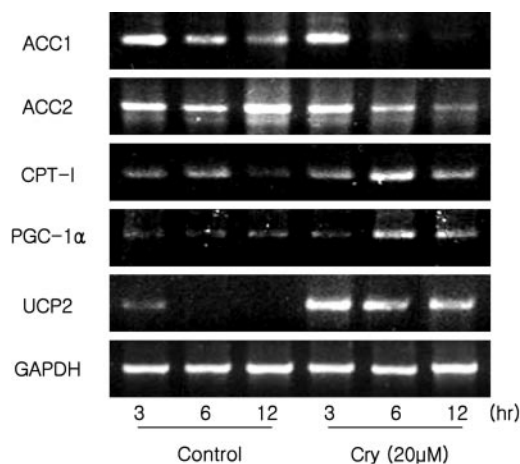Recent studies have identified peroxisome proliferator-ac-



**Fig. 4.** The effects of cryptotanshinone on the mRNA levels of ACC, CPT-I, PGC-1$\alpha$, and UCP in C2C12 myotubes. C2C12 myotubes were exposed to cryptotanshinone (20 $\mu$M) for 3, 6, and 12 h. Total RNA was extracted and subjected to reverse transcription-polymerase chain reaction using specific primers for ACC1, ACC2, CPT-I, PGC-1$\alpha$, UCP2, and GAPDH genes.

tivated receptor-γ coactivator-1α (PGC-1α) expression as one of the mechanisms involved in the control of glucose uptake and insulin sensitivity in muscle cells (Michael et al., 2001).

Moreover, PGC-1α performs a critical function in mitochondrial biogenesis. UCPs are mitochondrial inner membrane proteins that have been proposed to be central to the regu-
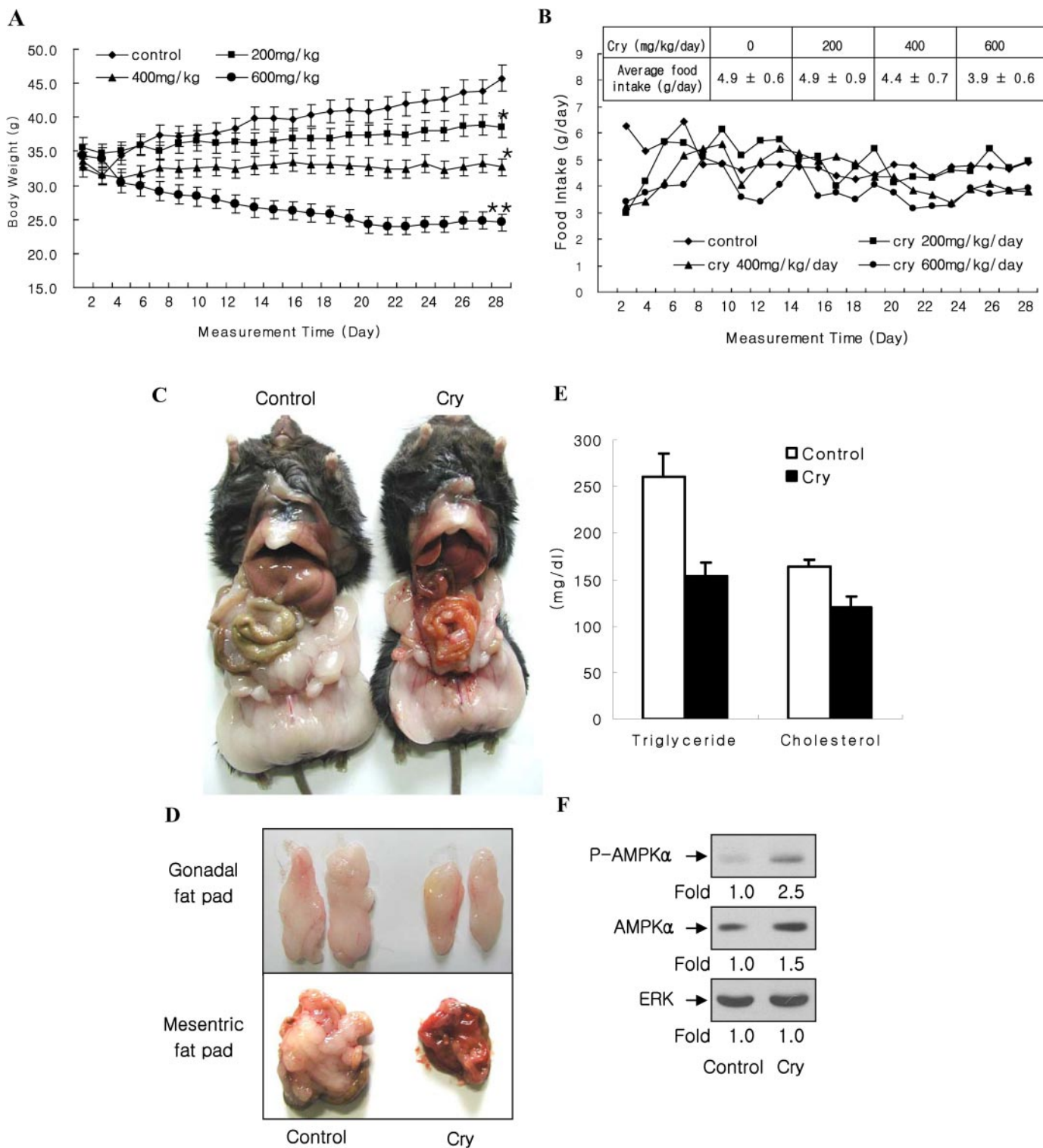
**Fig. 5.** Body weight, food intake, adipose tissues, serum lipids, and AMPK of control and cryptotanshinone-administered ob/ob mouse. Cryptotanshinone (0, 200, 400, and 600 mg/kg/day) was orally administered to ob/ob mice (C57BL/6J-Lep[ob]) for a month, and the body weight (A) and food intake (B) were monitored on a daily basis. C, control and cryptotanshinone (600 mg/kg/day)-administered ob/ob mice were sacrificed on day 28, and abdominal view of the fat pad under the skin was presented. D, gonadal and mesenteric fat pads were isolated from control and cryptotanshinone-fed mice. E, the amount of triglyceride and cholesterol present in sera was measured and compared. F, protein extracts were isolated from the hind limbs of each animal, and phosphorylation levels (P-AMPKα) and total amounts of AMPKα (AMPKα) were determined via Western blot analysis. Extracellular signal-regulated protein kinase was used as a loading control. Each experimental group consisted of 10 animals, and the data are expressed as the means ± S.D. (∗, $P < 0.05$; ∗∗, $P < 0.01$; compared with control).

lation of energy expenditure, thereby suggesting that UCP2 may prove important with regard to the determination of basal metabolic rate and may possibly also be involved in resistance to obesity (Erlanson-Albertsson, 2003). The incubation of myotubes with cryptotanshinone resulted in a pronounced increase in the expression of both PGC-1$\alpha$ and UCP2 mRNA (Fig. 4). Together, these observations suggest that in general, the gene expression patterns seen in conjunction with long-term cryptotanshinone treatment (Fig. 4) are quite consistent with the above reports describing gene expression profiles as the result of AMPK activation.

**Cryptotanshinone Exerts Antiobesity and Antidiabetes Effects in Vivo.** We next attempted to determine whether the selected effects of cryptotanshinone, as described above, would also occur in vivo using animal models. In service of this objective, cryptotanshinone (0, 200, 400, and 600 mg/kg/day) was orally administered to ob/ob mice (C57BL/6J-Lep[ob]) for a month, and we monitored the body weight on a daily basis (Fig. 5A). During the study period, the body weights in the control group increased steadily, but cryptotanshinone resulted in a dose-dependent reduction in body weight, resulting in an approximately 40% decrease of the body weight at the maximum dosage used in the study (600 mg/kg/day). Cryptotanshinone also reduced food intake (Fig. 5B). The average food intake of control animal was $4.9 \pm 0.6$ g, whereas that of cryptotanshinone-treated animal (600 mg/kg/day) was $3.9 \pm 0.5$ g. In fact, the cryptotanshinone-administered animals accumulated noticeably less fat in their adipose tissues (Fig. 5C). For example, the gonadal fat tissue (Fig. 5D) in the cryptotanshinone-administered animals weighed an average of 1.9 g, far less than what was seen in the control ob/ob mice (3.5 g). Similar effects were observed in the mesenteric fat tissues of the animals (Fig. 5D). Cryptotanshinone administration also caused significant reductions of serum triglycerides and cholesterol levels by 40 and 20%, respectively, at the end of the monitoring periods (Fig. 5E). The AMPK activity of the skeletal muscles

of the cryptotanshinone-administered mice was 2.5- to ~3-fold higher than in the control animals, and the AMPK$\alpha$ expression levels were also increased slightly (Fig. 5F).

The effects of cryptotanshinone were also apparent in the livers of the animals. The livers of the control ob/ob mice were lighter in color and more yellowish than the livers of the cryptotanshinone-fed animals (Fig. 6A), and this difference seems to be due to the amount of the accumulated fat within the tissues. To verify this, we stained liver sections with Oil Red-O to detect lipids. As expected, lipid droplets, which are primarily triglycerides, were significantly reduced as the result of cryptotanshinone administration (Fig. 6B).

Cryptotanshinone also exerted similar antiobesity effects in diet-induced obese (DIO) mice, and oral administration of cryptotanshinone (200 mg/kg/day) for 28 days resulted in an approximately 30% decrease of body weight (Fig. 7A). The average food intake was also significantly decreased by administration of cryptotanshinone (Fig. 7B). To separate the effect of cryptotanshinone on AMPK versus the effects caused by decreased food consumption, we also observed the body weight of the pair-fed animals (Fig. 7A). In fact, the pattern of body weight loss between cryptotanshinone- and the pair-fed animals was very similar during the first 1 week of study period, but the distinct difference between these two groups was observed after 1 week; the body weight of cryptotanshinone-treated group was significantly lowered than that of the pair-fed group after 1 week (Fig. 7A). These data suggest that cryptotanshinone exerts the antiobesity effects via activation of AMPK pathway.

We also assessed the effects of cryptotanshinone on the blood glucose levels of ob/ob mice (C57BL/6J-Lep[ob]), db/db mice (C57BL/KsJ-Lepr[db]), and ZDF rats. For 28 days, these animals were orally administered cryptotanshinone (600 mg/kg/day) or vehicle, after which we measured the blood glucose levels of the animals every 3 days. The animals were starved for 12 h before blood glucose level measurements. Dramatic reductions in blood glucose levels were observed in the cryp-
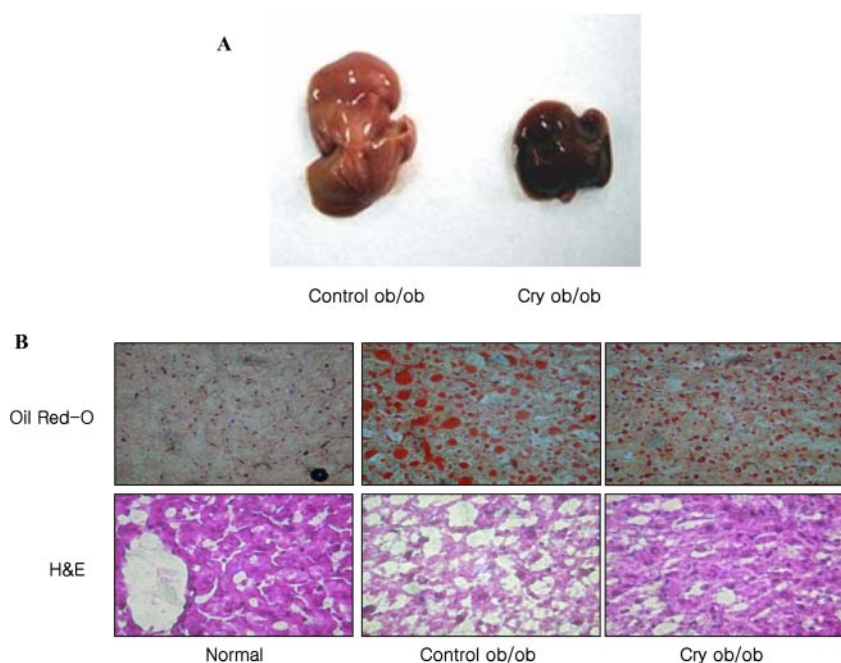


**Fig. 6.** Histological analysis of livers of control and cryptotanshinone-administered ob/ob mouse. A, livers of the control (left) and cryptotanshinone (600 mg/kg/day)-administered ob/ob mice. B, liver sections of the control and cryptotanshinone-fed ob/ob mice were stained with Oil Red-O to detect lipids (top) or with hematoxylin and eosin (H&E; bottom). These were compared with the liver sections obtained from the normal mice.

totanshinone-treated groups of three different animals after 3 days, and such effects persisted over the entirety of the monitoring period in all cases (Fig. 8).

## Discussion

In this study, we determined that cryptotanshinone, a natural product isolated from dried roots of *S. miltiorrhiza*, is a novel activator of the AMPK pathway in C2C12 myotubes, further evidencing antiobesity and antidiabetic potential both in vitro and in vivo. In general, the pharmacological effects of cryptotanshinone chronicled herein are in good agreement with the reports describing the physiological outcomes of AMPK activation. The primary source of cryptotanshinone is extracts of the dried roots of *S. miltiorrhiza*, which have evidenced previously beneficial effects on the circulatory system. For example, these extracts have been used in Asian medicine to reduce the size of myocardial infarcts, to increase coronary blood flow, and to protect the heart against ischemia-reperfusion injuries (Zhou et al., 2005). They have also been used in the treatment of hyperlipidemia. However, the underlying mechanisms of these effects remain almost completely unknown. Because cryptotanshinone is one of the primary constituents of this herb, such beneficial effects of the extracts may be mediated, in part, by the activation of AMPK. In fact, AMPK activation has been shown to protect cells from ischemia reperfusion (Dyck and Lopaschuk,

2006), and AMPK also suppresses cholesterol synthesis via the inhibition of HMG-CoA reductase. In conclusion, our findings support the notion that the activation of the AMPK pathway might result in the development of therapeutic approaches for the treatment of metabolic disorders.

Accumulating evidence suggests that AMPK is likely to mediate the effect of insulin-independent stimuli for glucose uptake. Indeed, the activation of AMPK in response to muscle contraction, hypoxia, and hyperosmolarity can be closely correlated with an increase in glucose uptake in the muscles (Hayashi et al., 2000; Mu et al., 2001). Here, we have demonstrated that cryptotanshinone induces glucose uptake in an AMPK-dependent manner (Fig. 2) in C2C12 myotubes and that it also exerted an insulin-sensitizing effect (Fig. 3). Moreover, cryptotanshinone was also found to exert a profound antidiabetic effect in three different experimental animals (Fig. 8). It has been demonstrated that the expression
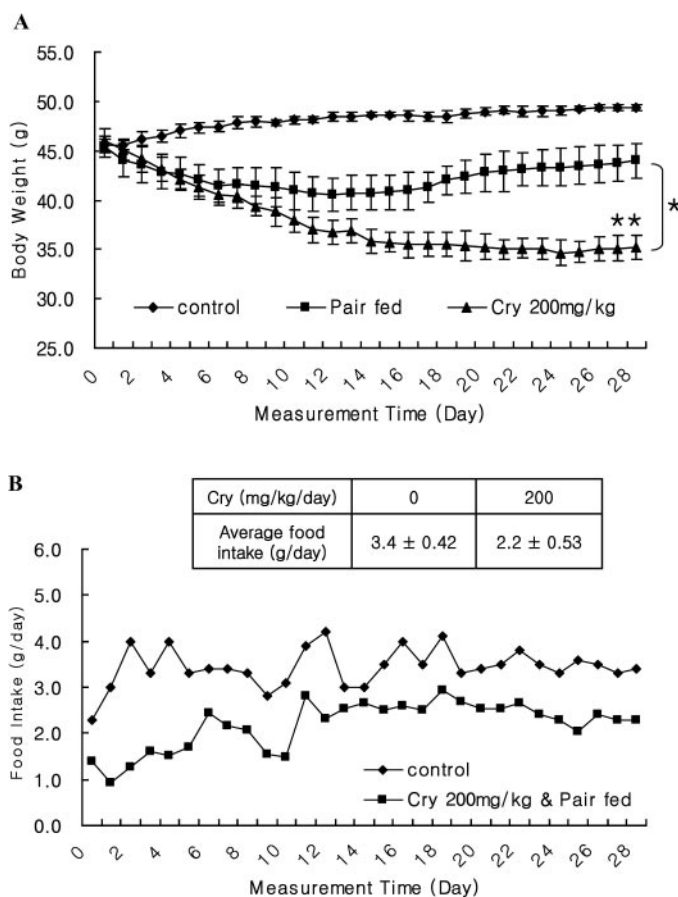


**Fig. 7.** Body weight and food intake of vehicle, cryptotanshinone, and pair-fed DIO mice. Changes in body weight (A) and daily food intake (B) of DIO mice. DIO mice were divided into three groups: vehicle, cryptotanshinone (200 mg/kg/day), and pair-fed groups ($n = 9$). (*, $P < 0.05$; **, $P < 0.01$; compared with control, or between two groups as indicated).
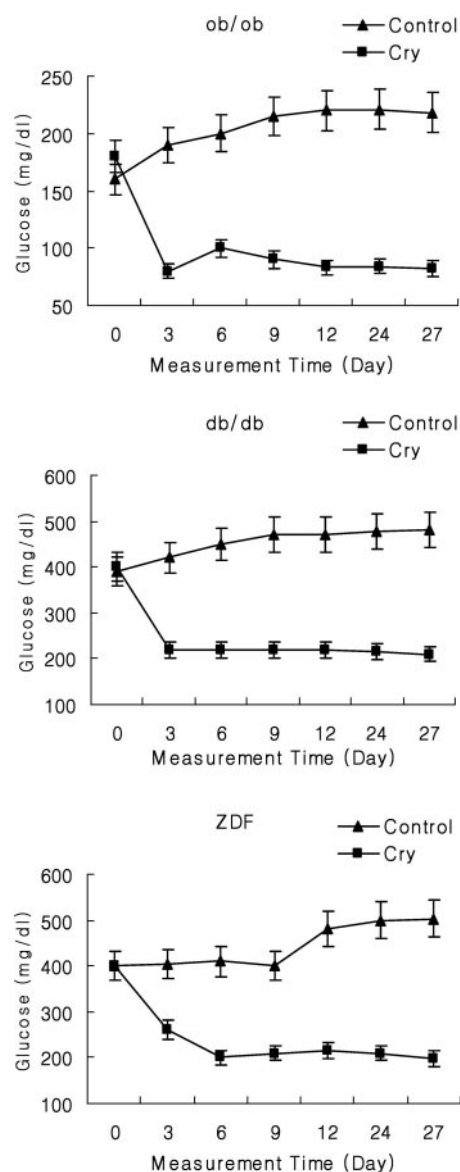


**Fig. 8.** Hypoglycemic effects of cryptotanshinone on ob/ob and db/db mice and ZDF rats. Cryptotanshinone (600 mg/kg/day) was orally administered to ob/ob and db/db mice and ZDF rats for 28 days. Every 3 days, blood glucose levels were determined ($n = 10$ per group). The data are expressed as the means ± S.D.

of a constitutively active form of AMPK-stimulated glucose uptake into the cells in association with the enhanced translocation of GLUT4 to the plasma membrane (Fryer et al., 2002). Consistent with these reports, cryptotanshinone-induced AMPK activation was associated with a rapid translocation of GLUT4 into the plasma membrane (Fig. 2C). Moreover, long-term treatment with cryptotanshinone induced mRNA expression of Glut1 (Fig. 2D).

Several studies have suggested that AMPK may also enhance insulin sensitivity, both in vitro and in vivo. The administration of AICAR to Wistar rats was shown previously to enhance insulin-stimulated GLUT4 translocation and glucose uptake in isolated rat muscles (Buhl et al., 2001). Similar results have been obtained in vitro using isolated rat muscles (Fisher et al., 2002). Indeed, our results also demonstrated that cryptotanshinone increased insulin-stimulated glucose uptake into both myotubes and adipocytes (Fig. 3A). Considering the well-established role of the PI-3 kinase pathway in the activity of insulin, there is indeed substantial interest in characterizing and elucidating the cross-talk occurring between insulin and the AMPK signaling pathways. However, recent data on this issue are highly conflicting and controversial. Initial work regarding the role of AMPK in the control of glucose uptake suggested that the activation of AMPK promotes the translocation of GLUT4 into the plasma membrane via a PI-3 kinase-independent pathway (Russell et al., 1999). On the contrary, several lines of evidence also seem to suggest that AMPK functions upstream of Akt signaling (Ouchi et al., 2004), and another report has shown that AMPK is activated in a PI-3 kinase-dependent manner upon the exposure of endothelial cells to oxidants such as ONOO$^-$ (Zou et al., 2003). It is clear that further studies are required to elucidate the precise signaling mechanisms that exist between these two pathways. However, the findings of this study seem to support the notion that the AMPK signal may be interconnected with the downstream of insulin (Fig. 3). Our data indicate that cryptotanshinone mildly activates Akt but not its upstream PI-3 kinase in an AMPK-dependent manner (Fig. 3). In fact, a recent report supports this observation, demonstrating that Akt but not PI-3 kinase is potentiated by AMPK in rat hearts in vivo (Longnus et al., 2005).

In the present study, we demonstrated that AMPK activity was increased by cryptotanshinone in cell cultures (Fig. 1) and in the skeletal muscles of cryptotanshinone-fed animals (Fig. 5). However, we believe that it is not likely to directly activate AMPK. As was demonstrated in Fig. 1C, cryptotanshinone was not able to activate AMPK in a test tube, but it rapidly depleted the intracellular ATP level, which led to activation of AMPK (Fig. 1D). In addition to the intracellular energy change, AMPK is also known to be rapidly activated by leptin (Minokoshi et al., 2002) or adiponectin (Yamauchi et al., 2002). In this regard, cryptotanshinone may share a signaling pathway in common with leptin or adiponectin for the activation of AMPK as well. Many of the effects of leptin effects are mediated through the Janus kinase and signal transducer and activator of transcription pathway, but it remains unknown whether such pathways can be associated with the activation of AMPK. Indeed, the AMPK upstream pathways have, thus far, remained largely enigmatic. However, two different kinases, LKB1 (Hawley et al., 2003; Woods et al., 2003) and calmodulin-dependent protein kinase kinase (Hawley et al., 2005), have been identified recently as

novel upstream AMPK kinases, although their physiological role in the AMPK pathway will require further investigation. To better understand the pharmacological mechanisms underlying the activities of cryptotanshinone, we are currently assessing the effects of cryptotanshinone on Janus kinase-signal transducer and activator of transcription, LKB1, and calmodulin-dependent protein kinase kinase and the possibility of cross-talk between these factors and the intracellular energy status.

It is interesting that leptin is known to achieve anorexic action via AMPK inhibition within the hypothalamus (Minokoshi et al., 2004), whereas it activates AMPK in the skeletal muscle, resulting in enhanced fatty acid oxidation (Minokoshi et al., 2002). Likewise, α-lipoic acid treatment also reduces hypothalamic AMPK activity and food intake (Kim et al., 2004). In this study, we have determined that food intake was also significantly reduced by cryptotanshinone treatment (Fig. 5B). Thus, we are currently attempting to characterize the pharmacological effects of cryptotanshinone on AMPK in the hypothalamus.

### References

Buhl ES, Jessen N, Schmitz O, Pedersen SB, Pedersen O, Holman GD, and Lund S (2001) Chronic treatment with 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside increases insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type-specific manner. *Diabetes* **50:**12–17.

Burnett PE, Barrow RK, Cohen NA, Snyder SH, and Sabatini DM (1998) RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A* **95:**1432–1437.

Carling D (2004) The AMP-activated protein kinase cascade–a unifying system for energy control. *Trends Biochem Sci* **29:**18–24.

Dyck JR and Lopaschuk GD (2006) AMPK alterations in cardiac physiology and pathology: enemy or ally? *J Physiol* **574:**95–112.

Erlanson-Albertsson C (2003) The role of uncoupling proteins in the regulation of metabolism. *Acta Physiol Scand* **178:**405–412.

Fisher JS, Gao J, Han DH, Holloszy JO, and Nolte LA (2002) Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *Am J Physiol Endocrinol Metab* 282:E18–E23.

Fryer LG, Foufelle F, Barnes K, Baldwin SA, Woods A, and Carling D (2002) Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. *Biochem J* **363:**167–174.

Hardie DG (2006) AMP-activated protein kinase as a drug target. *Annu Rev Pharmacol Toxicol* **47:**185–210.

Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, and Hardie DG (2003) Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* **2:**28.

Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, and Hardie DG (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* **2:**9–19.

Hay N and Sonenberg N (2004) Upstream and downstream of mTOR. *Genes Dev* **18:**1926–1945.

Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, and Goodyear LJ (2000) Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* **49:**527–531.

Hur JM, Shim JS, Jung HJ, and Kwon HJ (2005) Cryptotanshinone but not tanshinone IIA inhibits angiogenesis in vitro. *Exp Mol Med* **37:**133–137.

Ji XY, Tan BK, and Zhu YZ (2000) Salvia miltiorrhiza and ischemic diseases. *Acta Pharmacol Sin* **21:**1089–1094.

Jiang T, Wang Z, Proctor G, Moskowitz S, Liebman SE, Rogers T, Lucia MS, Li J, and Levi M (2005) Diet-induced obesity in C57BL/6J mice causes increased renal lipid accumulation and glomerulosclerosis via a sterol regulatory element-binding protein-1c-dependent pathway. *J Biol Chem* **280:**32317–32325.

Kahn BB, Alquier T, Carling D, and Hardie DG (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1:**15–25.

Kemp BE, Stapleton D, Campbell DJ, Chen ZP, Murthy S, Walter M, Gupta A, Adams JJ, Katsis F, van Denderen B, et al. (2003) AMP-activated protein kinase, super metabolic regulator. *Biochem Soc Trans* **31:**162–168.

Kim KH (1997) Regulation of mammalian acetyl-coenzyme A carboxylase. *Annu Rev Nutr* **17:**77–99.

Kim MS, Lee J, Ha J, Kim SS, Kong Y, Cho YH, Baik HH, and Kang I (2002) ATP stimulates glucose transport through activation of P2 purinergic receptors in C₂C₁₂ skeletal muscle cells. *Arch Biochem Biophys* **401:**205–214.

Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS, Yun JY, Namgoong IS, Ha J, Park IS, et al. (2004) Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nat Med* **10:**727–733.

Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, and Winder WW (1999) 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* **48:**1667–1671.

Lee M, Hwang JT, Lee HJ, Jung SN, Kang I, Chi SG, Kim SS, and Ha J (2003) AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *J Biol Chem* **278:**39653–39661.

Longnus SL, Segalen C, Giudicelli J, Sajan MP, Farese RV, and Van Obberghen E (2005) Insulin signalling downstream of protein kinase B is potentiated by 5′AMP-activated protein kinase in rat hearts in vivo. *Diabetologia* **48:**2591–2601.

Michael LF, Wu Z, Cheatham RB, Puigserver P, Adelmant G, Lehman JJ, Kelly DP, and Spiegelman BM (2001) Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci U S A* **98:**3820–3825.

Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Foufelle F, Ferre P, Birnbaum MJ, et al. (2004) AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* **428:**569–574.

Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, and Kahn BB (2002) Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* **415:**339–343.

Mu J, Brozinick JT Jr, Valladares O, Bucan M, and Birnbaum MJ (2001) A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* **7:**1085–1094.

Nave BT, Ouwens M, Withers DJ, Alessi DR, and Shepherd PR (1999) Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* **344 (Pt 2):**427–431.

Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, and Walsh K (2004) Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J Biol Chem* **279:**1304–1309.

Ruderman NB, Saha AK, and Kraegen EW (2003) Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity. *Endocrinology* **144:**5166–5171.

Russell RR 3rd, Bergeron R, Shulman GI, and Young LH (1999) Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol Heart Circ Physiol* **277:**H643–H649.

Suwa M, Nakano H, and Kumagai S (2003) Effects of chronic AICAR treatment on fiber composition, enzyme activity, UCP3, and PGC-1 in rat muscles. *J Appl Physiol* **95:**960–968.

Viollet B, Foretz M, Guigas B, Horman S, Dentin R, Bertrand L, Hue L, and Andreelli F (2006) Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. *J Physiol* **574:**41–53.

Wang X, Morris-Natschke SL, and Lee KH (2007) New developments in the chemistry and biology of the bioactive constituents of tanshen. *Med Res Rev* **27:**133–148.

Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, and Carling D (2003) LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13:**2004–2008.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, et al. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8:**1288–1295.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108:**1167–1174.

Zhou L, Zuo Z, and Chow MS (2005) Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J Clin Pharmacol* **45:**1345–1359.

Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, and Shulman GI (2002) AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci U S A* **99:**15983–15987.

Zou MH, Hou XY, Shi CM, Kirkpatick S, Liu F, Goldman MH, and Cohen RA (2003) Activation of 5′-AMP-activated kinase is mediated through c-Src and phosphoinositide 3-kinase activity during hypoxia-reoxygenation of bovine aortic endothelial cells. Role of peroxynitrite. *J Biol Chem* **278:**34003–34010.

**Address correspondence to:** Dr. Joohun Ha, Department of Biochemistry and Molecular Biology, Kyung Hee University School of Medicine, Tongdaemun-gu, Hoegi-dong 1, Seoul 130-701, Korea. E-mail: hajh@khu.ac.kr