Molecular Pharmacology Fast Forward. Published on April 11, 2007 as doi:10.1124/mol.107.034447

MOL #34447

Anti-diabetes and anti-obesity effect of cryptotanshinone via activation of AMP-activated protein kinase.

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Molecular Pharmacology Fast Forward. Published on April 11, 2007 as DOI: 10.1124/mol.107.034447
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Running Title: Cryptotanshinone activates AMPK.

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Text pages : 32
Table : 1
Figures : 8
References : 40
Abstract : 206 words
Introduction : 416 words
Discussion : 1041 words

Abbreviations: AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; Cry, cryptotanshinone; ACC, acetyl-CoA carboxylase

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Abstract

Metabolic disorders, including type 2 diabetes and obesity, represent major health risks in industrialized countries. Recently, 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, as 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 which is used extensively in Oriental medicine, and which is known to exert beneficial effects on the circulatory system. For the first time, in the present study, we have described the potent anti-diabetic and anti-obesity 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.

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Introduction

AMP-activated protein kinase (AMPK) is a well-conserved heterotrimeric enzyme complex, which is comprised of an α-catalytic subunit and regulatory β and γ subunits. AMPK has been tentatively implicated as a cellular energy sensor, as it is known to be exquisitely sensitive to intracellular energy status (Carling, 2004; Kemp et al., 2003). 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 (Hardie, 2006; Kahn et al., 2005; Viollet et al., 2006). Indeed, AMPK cascades are also known to mediate, in part, the beneficial effects of anti-diabetic and anti-obese 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.

In order to identify a novel compound that harbors anti-diabetes and anti-obesity potential, we have 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 anti-diabetic and anti-obese effects, both in vitro and in vivo. Cryptotanshinone was originally isolated from the dried roots of Salvia militorrhiza Bunge, an herb which is extensively employed in Oriental medicine (Ji et al., 2000; Zhou et al., 2005). The extracts of this herb have been utilized in the treatment of several pathologies, including cardiovascular diseases, hematological

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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, anti-mutagenic, anti-tumor, anti-inflammatory, and anti-platelet aggregation effects (Wang et al., 2007; Zhou et al., 2005). 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.

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Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen. [γ-^{32}P]ATP (6000 Ci/mmol) and 2-deoxy-D-[^{3}H] glucose (6.0 Ci/mmol) were purchased from Amersham Biosciences. The phosphospecific-AMPK Thr^{172}, total AMPK, phosphospecific-PKB/Akt, total PKB/Akt, phosphospecific-ACC, phosphor-mTOR, and phosphospecific-p70 S6K antibodies used in this study were obtained from Cell Signaling Technology. Antibodies for Glut4 and IGF-IRβ were purchased from Santa Cruz Biotechnology.

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, 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 hours. Cells were used in experiments at 4-5 days after differentiation.

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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 minutes. The AMPK was immunoprecipitated with AMPK pan-α antibody, and its activity was assessed in kinase assay buffer containing 200 μM AMP, an ATP mixture (100 μM ATP, and 1.5 μCi of [γ-^{32}P] ATP), with or without 250 μM SAMS peptide (HMRSAMSGLHLVKRR) at 30 °C for 10 minutes, as previously described (Lee et al., 2003). In order to characterize the direct *in vitro* effects of cryptotanshinone, partially purified rat liver AMPK was purchased from Upstate. 20 mili-units of AMPK were incubated with cryptotanshinone in an identical reaction buffer for 10 minutes, without 200 μM AMP. PI 3-kinase activity was measured via immunoprecipitation with anti-phosphotyrosine antibody, as previously described (Kim et al., 2002).

ATP analysis

After treatment, cells were washed with ice-cold PBS, and 100mM of 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 (Molecular Probes). The assay buffer (100 μL), which contained 0.5 mM luciferin, 1.25 μg/mL of luciferase, 25 mM Tris, pH 7.8, 5 mM $MgSO_4$, 100 μM EDTA, and 1 mM DTT, was mixed with the cell lysates (5 μL). Luminescence was analyzed by VICTOR$^3{}_{TM}$ luminometer (Perkin Elmer) and normalized using the cellular proteins.

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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_2PO_4, 1.3 mM $MgSO_4$, 5 mM $NaHCO_3$, 0.07 % bovine serum albumin, and 5.5 mM glucose) and incubated for 20 minutes in KRB buffer. The cells were then incubated for 10 minutes in KRB containing 0.5 μCi 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: UCP2, sense 5`-AACAGTTCTACACCAAGGGC-3`, and antisense 5`-AGCATGGTAAGGGCACAGTG-3`; ACC1, sense 5`-GTCAGCGGATGGGCGGAATG-3`, and antisense 5`-CGCCGGATGCCATGCTCAAC-3`; ACC2, sense 5`-GCTGCGGTCAAGTGTATGCG-3`, and antisense 5`-CACTGATGCATTTGCCCTGG-3`; CPT1, sense 5`-GCTCTCGAGGCTCACTGATT-3`, and antisense 5`-CAGTCAGAGCAGCTAGGTGT-3`; PGC-1α, sense 5`-ACGAGGCCAGTCCTTCCTCC -3`, and antisense 5`-AGCTCTGAGCAGGGACGTCT-3`; Glut1, sense 5`-CGGGCCAAGAGTGTGCTAAA -3`, and antisense 5`-TGACGATACCGGAGCCAATG-3`. GAPDH: sense 5'-TGCTGAGTATGTCGTGGAGTCTA-3', anti-sense 5'-AGTGGGAGTTGCTGTTGAAGTCG-3';

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Animal Experiments

db/db and ob/ob mice on a C57BL/6 background (male, 9-11 weeks old, 30-40 g) were purchased from Jackson Lab (Bar Harbor, ME). ZDF (Zucker Diabetic Fatty male, 8 weeks, 200-300 g) type 2 diabetic rat models were purchased from Charles River Lab. 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 fed on a standard diet. Cryptotanshinone in 0.1 % solution of sodium lauryl sulfate or vehicle was administered p.o. in a volume of 10 mL/kg body weight/day in the morning (9.00~10.00 h). To measure plasma glucose level, the animals were deprived of food for 11-14 hours, 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 Boehringer Mannheim. 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 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 p.o. in a volume of 10 mL/kg body

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weight/day in the morning (9.00~10.00 h) for 28 days. Third group was pair-fed to the previous day's level of intake of the cryptotanshinone-treated animals. The body weight was measured everyday.

Statistical Analysis

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

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Results

Cryptotanshinone activates AMPK in C2C12 myotubes.

In order 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 hour, and AMPK was found to have been activated in a concentration-dependent manner. An approximately 1.7-fold activation was observed at 20 μM (Fig. 1A). A well-known AMPK activator, AICAR, was used as a positive control, and this 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 AMPK α, 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 P^{32} incorporation into the SAMS peptide, as is described in the Methods section. 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). In order to understand the mechanism by which cryptotanshinone activates AMPK pathway, we next examined the effect of cryptotanshinone on the

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intracellular ATP level, as AMPK is known to be exquisitely sensitive to intracellular energy status. Indeed, cryptotanshinone (20 μM) rapidly depleted ATP level in C2C12 myotubes, resulting in 75% reductions in ATP levels in 12 h (Fig. 1D). Consequently, these results suggest that cryptotanshinone indirectly activates AMPK pathways via reduction of intracellular ATP.

As 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, as it was detected 12 hours 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 minutes 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 Salvia 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 evidenced no toxicity when the C2C12 myotubes were incubated for 24 hours 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

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most effective with regard to the induction of glucose uptake, evidencing an approximately 2-fold increase (Table 1 & Fig. 2A). We further attempted to determine whether cryptotanshinone-induced glucose uptake was mediated by AMPK. Our data indicated that an AMPK inhibitor called compound C (Zhou et al., 2001) effectively blocked cryptotanshinone-induced AMPK activity, as assessed by a direct enzyme assay, as well as a Western blot analysis for AMPK phosphorylation levels (Fig. 2B). Compound C 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.

Glucose transporter 4 (Glut4), which facilitates glucose transport, is known to be recruited to the plasma membrane in response to conditions that activates AMPK (Kurth-Kraczek et al., 1999; Mu et al., 2001). In order 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 minutes 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 β expression in the plasma membrane. Moreover, cryptotanshinone also profoundly increased Glut1 mRNA expression when applied under long-term conditions (Fig. 2D). Collectively, our data indicated that cryptotanshinone enhances glucose uptake via the stimulation both of the translocation of Glut4 to the plasma membrane, as well as Glut1 mRNA expression, and AMPK also appears to play a pivotal role in these processes.

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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 appears to enhance insulin sensitivity, both in vivo and in vitro (Buhl et al., 2001; Fisher et al., 2002). Thus, we attempted to determine whether or not 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). In order 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 hour 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

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kinase, whereas the effect of cryptotanshinone appears 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). Also, 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.

Chronic AMPK activation has been shown to regulate the genes involved in glucose uptake, fatty acid synthesis, fatty acid oxidation, energy expenditure, and mitochondrial biogenesis (Suwa et al., 2003; Zong et al., 2002). 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. Acetyl-CoA carboxylase (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,

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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 (Ruderman et al., 2003; Zhou et al., 2001). Indeed, 6-12 hours of cryptotanshinone treatment in the C2C12 myotubes resulted in a profound suppression of ACC 1 and ACC2 mRNA expression, and a concomitant increase in the expression of CPT-I mRNA (Fig. 4).

Recent studies have identified Peroxisome proliferator-activated receptor-γ (PPARγ) 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. Uncoupling proteins (UCPs) are mitochondrial inner membrane proteins which have been proposed to be central to the regulation 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-1α and UCP2 mRNA (Fig. 4). Collectively, 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 anti-obesity and anti-diabetes 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, 600 mg/kg/day) was orally administered to

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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 g ± 0.6, whereas that of cryptotanshinone-treated animal (600 mg/kg/day) was 3.9 g ± 0.5. 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 2.5~3 fold higher than in the control animals, and the AMPKα 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 appears to be due to the amount of the accumulated fat within the tissues. In order 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).

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Cryptotanshinone also exerted similar anti-obesity 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). In order 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 one week of study period, but the distinct difference between these two groups had been 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 anti-obesity 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 Zucker Diabetic Fatty (ZDF) rats. These animals were orally administered for 28 days with 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 hours prior to blood glucose level measurements. Dramatic reductions in blood glucose levels were observed in the cryptotanshinone-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).

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Discussion

In this study, we determined that cryptotanshinone, a natural product isolated from dried roots of Salvia miltiorrhiza, is a novel activator of the AMPK pathway in C2C12 myotubes, further evidencing anti-obesity and anti-diabetic potential both in vitro and in vivo. In general, the pharmacological effects of cryptotanshinone chronicled herein are in a good agreement with the reports describing the physiological outcomes of AMPK activation. The primary source of cryptotanshinone is extracts of the dried roots of Salvia miltiorrhiza, which have previously evidenced beneficial effects on the circulatory system. For example, these extracts have been utilized in Oriental 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 employed in treatments for hyperlipidemia. However, the underlying mechanisms of these effects remain almost completely unknown. As 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 anti-diabetic effect in three different experimental animals (Fig. 8). Recently, it was demonstrated that the expression 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 previously shown 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 is 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

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contrary, several lines of evidence also appear 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). Obviously, further studies will be required in order to elucidate the precise signaling mechanisms that exist between these two pathways. However, the findings of this study appear 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 leptin's effects are mediated through the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) pathway, but it remains unknown as to 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 (CAMKK) (Hawley et al., 2005), have recently been identified as novel upstream AMPK kinases, although their physiological role in the AMPK pathway will require further investigation. In order to better understand the pharmacological mechanisms underlying the activities of cryptotanshinone, we are currently assessing the effects of cryptotanshinone on Jak-STAT, LKB1, CAMKK, as well as the possibility of cross-talk between these factors and the intracellular energy status.

Interestingly, 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). Similarly, 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.

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Footnotes

This work was supported by a grant No. R13-2002-020-01004-0 from the Korea Science & Engineering Foundation and by a grant from Seoul R&BD program.

Molecular Pharmacology Fast Forward. Published on April 11, 2007 as DOI: 10.1124/mol.107.034447
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Figure Legends

Fig. 1. Cryptotanshinone activates AMPK in C2C12 myotubes.

A. C2C12 myotubes were incubated for 1 hour with the indicated concentrations of cryptotanshinone. AMPK was then immunoprecipitated with AMPK pan-α antibody, and an in vitro activity assay was conducted, using SAMS peptide as a substrate. The results are expressed as the means ± S.E. for two independent assays conducted in duplicate. AICAR (500 μM , 1h) was employed as a positive control. B. Total cell extracts were prepared under identical conditions and subjected to Western blot assays using anti-phosphospecific AMPKα Thr^{172} (P-AMPKα), anti-AMPK pan-α (AMPKα), 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 μM of AMP. D. C2C12 myotubes were treated with cryptotanshinone (20 μM) for 12 h, and then intracellular ATP levels were measured. The results are expressed as the means ± S.E. for three independent assays conducted in duplicate. E. C2C12 myotubes were treated with either cryptotanshinone (20 μM) or metformin (2 mM) for the indicated times. Western blot analysis was conducted using AMPKα Thr^{172} (P-AMPKα), anti-AMPK pan-α (AMPKα), anti-phosphospecific ACC Ser^{79} (P-ACC), and anti-ACC (ACC) antibodies. Cry, cryptotanshinone

Fig. 2. Cryptotanshinone stimulates glucose uptake via AMPK activation.

A. C2C12 myotubes were incubated for 1 hour with cryptotanshinone (20 μM) in the absence or presence of AMPK inhibitor compound C (20 μM). Then, 2-[^{3}H] deoxy-D-glucose was added to

Molecular Pharmacology Fast Forward. Published on April 11, 2007 as DOI: 10.1124/mol.107.034447
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culture media for 10 minutes at the end of the exposure period, and glucose uptake was measured as described in the "Research Design" section. 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 Thr^{172} (P-AMPKα) and anti-AMPK pan-α (AMPKα) antibodies or AMPK in vitro kinase assays. C. After 1 hour 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 glucose transporter 4 (Glut4) or IGF-I receptor β (IGF-IR β) antibodies. D. Myotubes were incubated with cryptotanshinone (20 μM) for 12 hour, and total RNA was extracted for the indicated time periods and subjected to RT-PCR, using specific primers for Glut1 and GAPDH genes. Cry, cryptotanshinone; C.C, compound C

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 hour 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 μM) or insulin (100 nM) for 1 h, and then PI-3 kinase activity assays were conducted as described in the "Research Design" section. C. Myotubes were exposed to cryptotanshinone (20 μM) for the indicated time period, and phosphorylation levels of Akt (P-Akt) and total Akt (Akt) were determined via Western blot analysis. D-E. Myotubes were exposed to cryptotanshinone (20 μM), insulin (100 nM), or both in the presence or absence of compound C (20 μM) for 1 h, after which Western blot analysis was conducted to measure the phosphorylation levels of AMPKα (P-AMPKα), Akt (P-Akt),

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mTOR (P-mTOR), and p70S6K (P-p70S6K). Total amounts of AMPKα (AMPKα) and Akt (Akt) were also compared. Cry, cryptotanshinone; C.C, compound C

Fig. 4. The effects of cryptotanshinone on the mRNA levels of ACC, CPT-I, PGC-1α, and UCP in C2C12 myotubes.

C2C12 myotubes were exposed to cryptotanshinone (20 μM) for 3, 6, and 12 h. Total RNA was extracted and subjected to RT-PCR using specific primers for ACC1, ACC2, CPT-I, PGC-1α, UCP2, and GAPDH genes.

Fig. 5. Body weight, food intake, adipose tissues, serum lipids, and AMPK of control and cryptotanshinone-administered ob/ob mouse.

Cryptotanshinone (0, 200, 400, 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 were 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 (ERK) 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)

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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 (upper panels) or with hematoxylin and eosin (H&E, lower panel). These were compared with the liver sections obtained from the normal mice.

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 Zucker Diabetic Fatty (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.

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TABLE 1

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

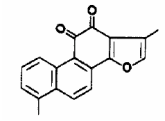
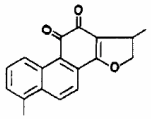
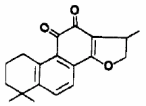
	Control	 Tanshinone-I	 Dihydrotanshinone	 Cryptotanshinone
AMPK activity (fold induction)	**1 ± 0.1**	**1 ± 0.1**	**1.3 ± 0.1**	**1.7 ± 0.1**
^{3}H-2-deoxy-glucose uptake (% of control)	**100 ± 3.1**	**97.8 ± 1.0**	**77.5 ± 1.8**	**197.2 ± 3.8**
Cell viability (% of control)	**100 ± 4.8**	**91 ± 11.3**	**80 ± 3.7**	**94 ± 3.1**

Figure 1

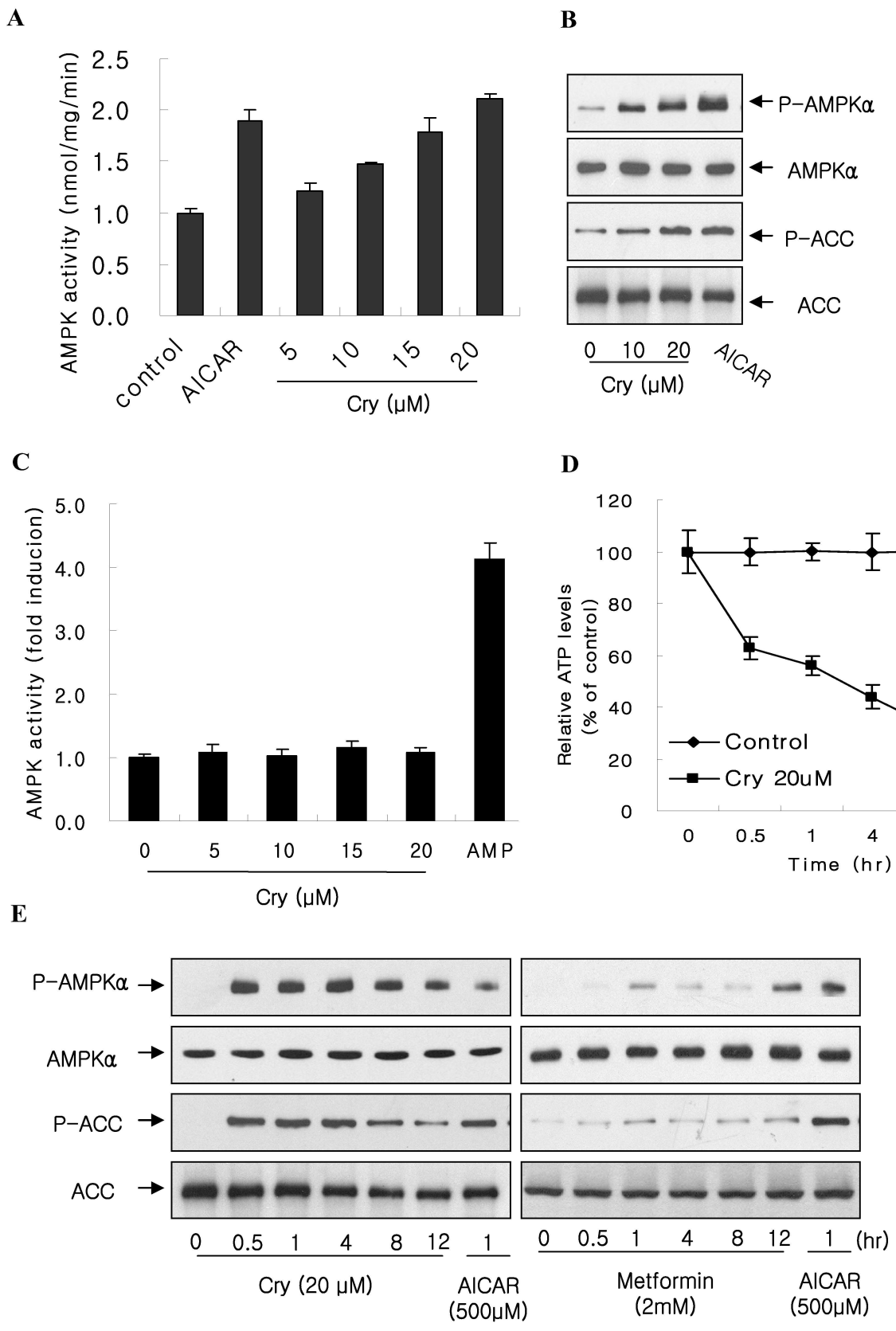

A
AMPK activity (nmol/mg/min)
2.5
2.0
1.5
1.0
0.5
0.0
control
AICAR
5
10
15
20
Cry (μM)
B
P-AMPKα
AMPKα
P-ACC
ACC
0
10
20
AICAR
Cry (μM)
C
AMPK activity (fold inducion)
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
AMP
Cry (μM)
D
Relative ATP levels (% of control)
120
100
80
60
40
20
0
Control
Cry 20uM
0
0.5
1
4
8
12
Time (hr)
E
P-AMPKα
AMPKα
P-ACC
ACC
0
0.5
1
4
8
12
1
(hr)
Cry (20 μM)
AICAR (500μM)
Metformin (2mM)
AICAR (500μM)

Figure 2

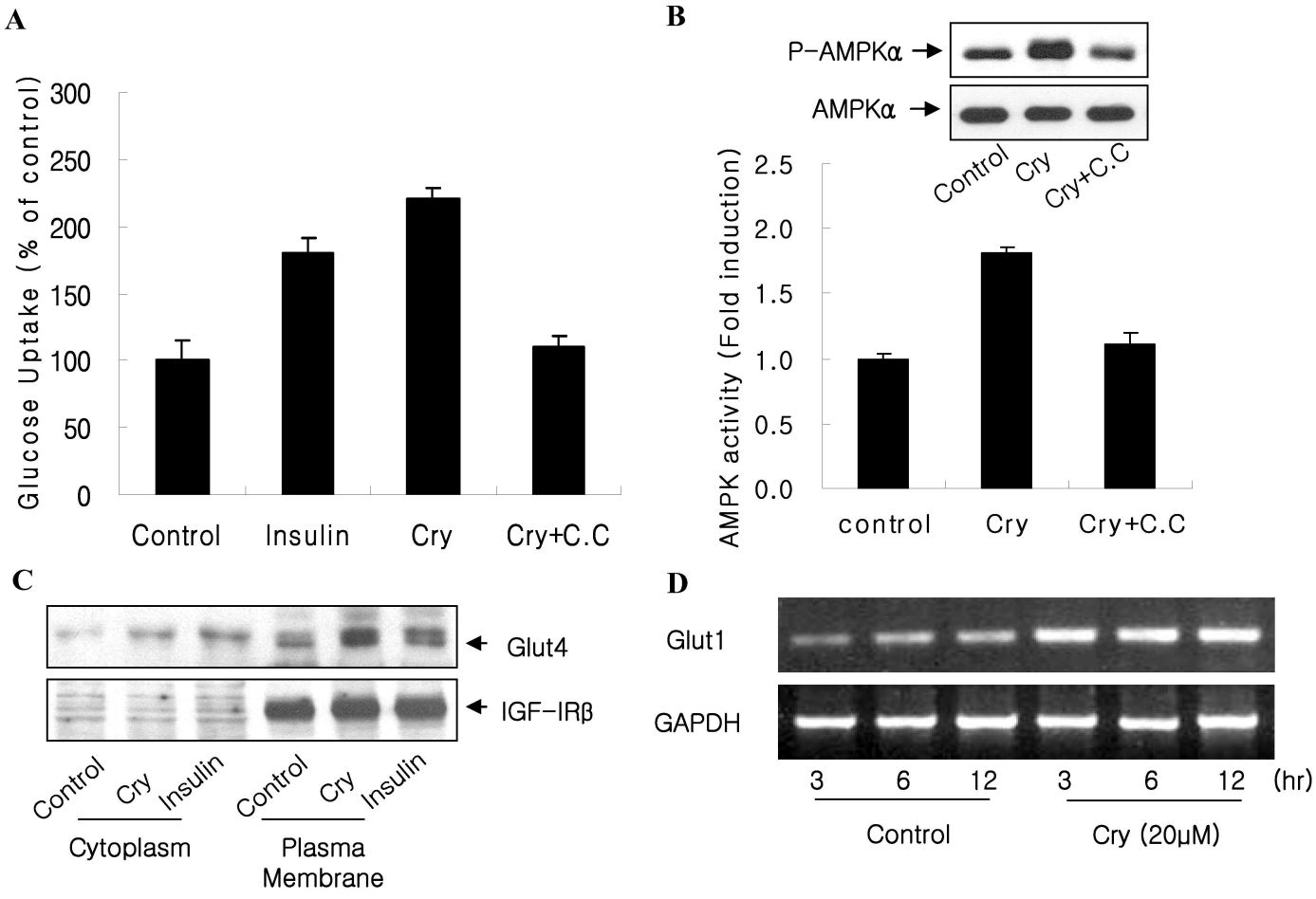

A
Glucose Uptake (% of control)
300
250
200
150
100
50
0
Control
Insulin
Cry
Cry+C.C
B
P-AMPKα
AMPKα
Control
Cry
Cry+C.C
AMPK activity (Fold induction)
2.5
2.0
1.5
1.0
0.5
0.0
control
Cry
Cry+C.C
C
Glut4
IGF-IRβ
Control
Cry
Insulin
Control
Cry
Insulin
Cytoplasm
Plasma Membrane
D
Glut1
GAPDH
3
6
12
3
6
12
(hr)
Control
Cry (20μM)

Figure 3

A

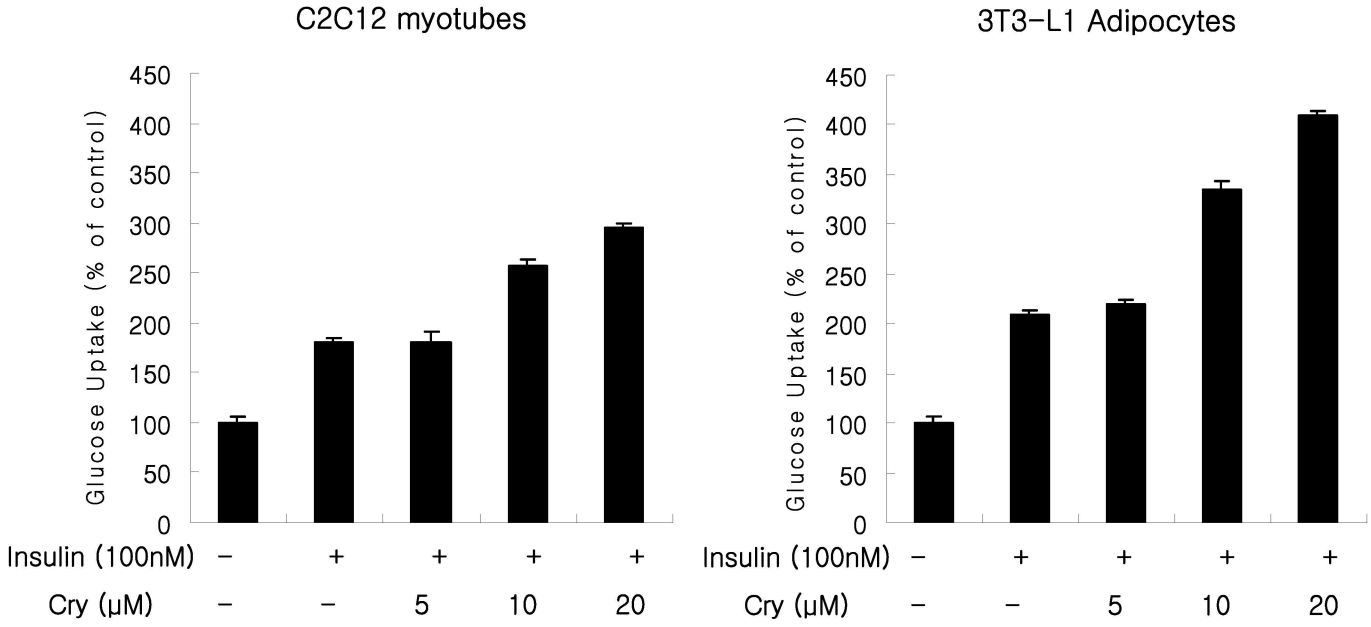

C2C12 myotubes
3T3-L1 Adipocytes
Glucose Uptake (% of control)
450
400
350
300
250
200
150
100
50
0
Insulin (100nM) – + + + +
Cry (μM) – – 5 10 20

Figure 3

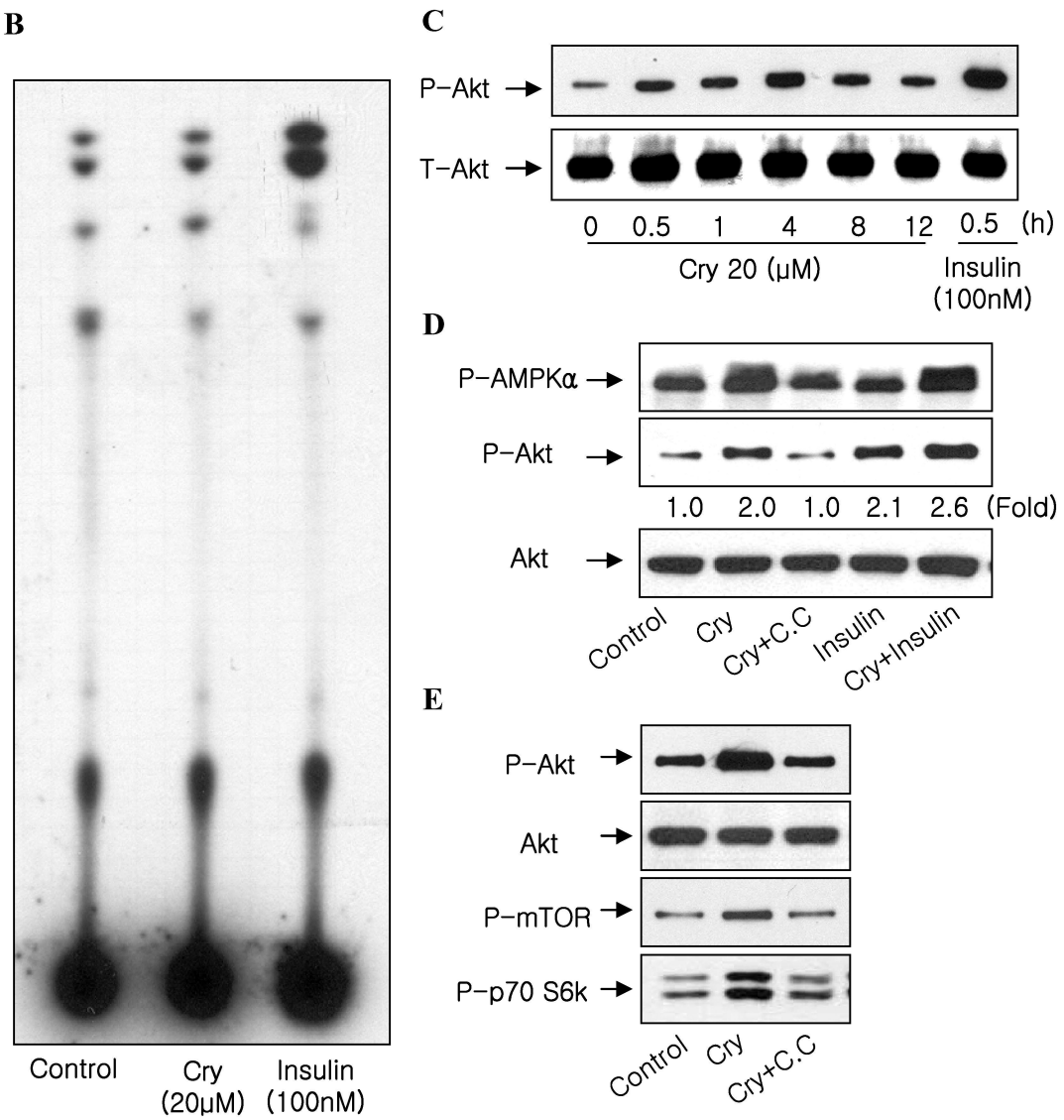

B
Control
Cry (20μM)
Insulin (100nM)
C
P-Akt
T-Akt
0 0.5 1 4 8 12 0.5 (h)
Cry 20 (μM)
Insulin (100nM)
D
P-AMPKα
P-Akt
1.0 2.0 1.0 2.1 2.6 (Fold)
Akt
Control
Cry
Cry+C.C
Insulin
Cry+Insulin
E
P-Akt
Akt
P-mTOR
P-p70 S6k
Control
Cry
Cry+C.C

Figure 4

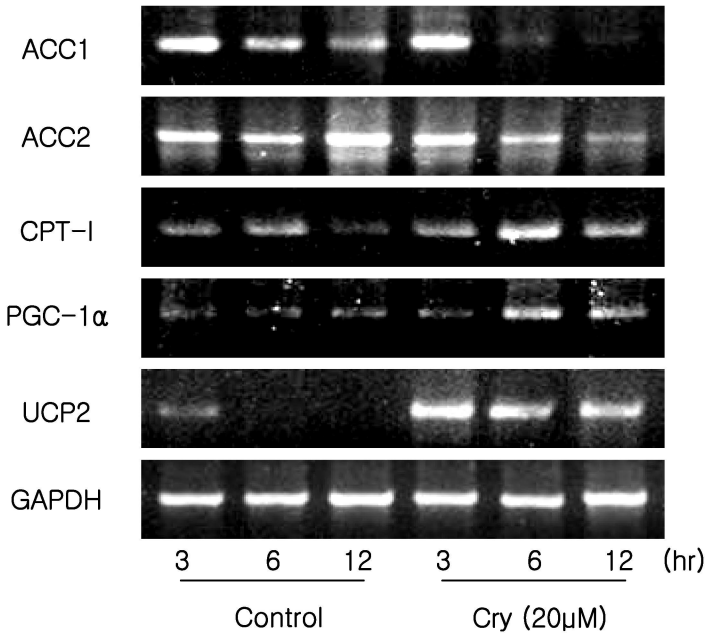
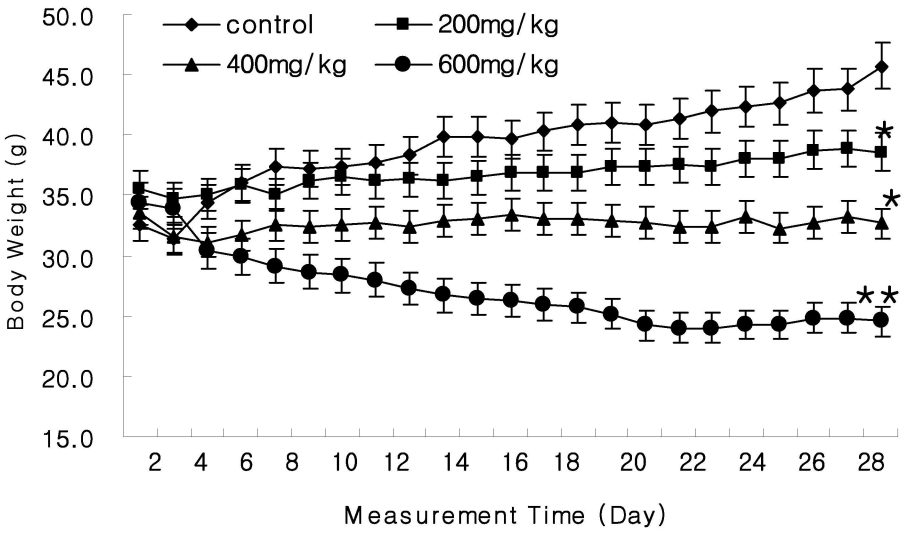

ACC1
ACC2
CPT-I
PGC-1α
UCP2
GAPDH
3
6
12
3
6
12
(hr)
Control
Cry (20μM)

Figure 5

A



B

Cry (mg/kg/day)	0	200	400	600
Average food intake (g/day)	4.9 ± 0.6	4.9 ± 0.9	4.4 ± 0.7	3.9 ± 0.6

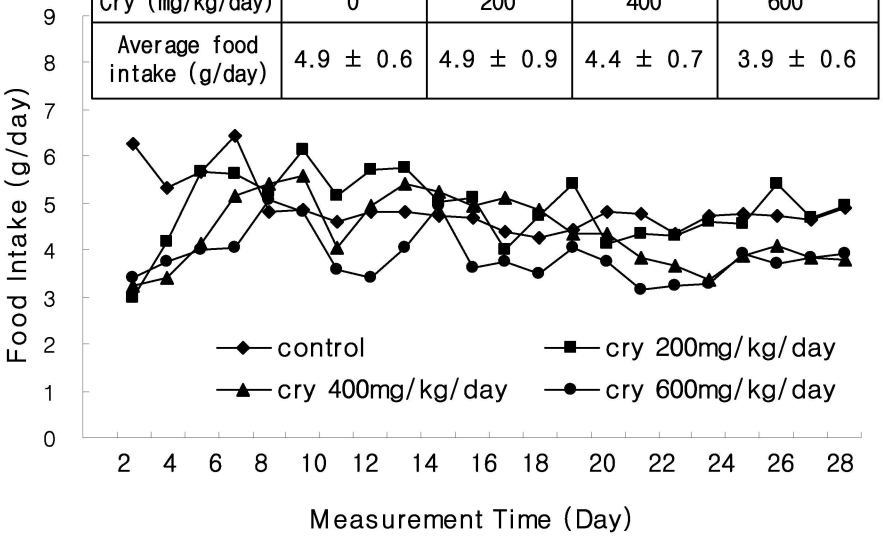


Figure 5

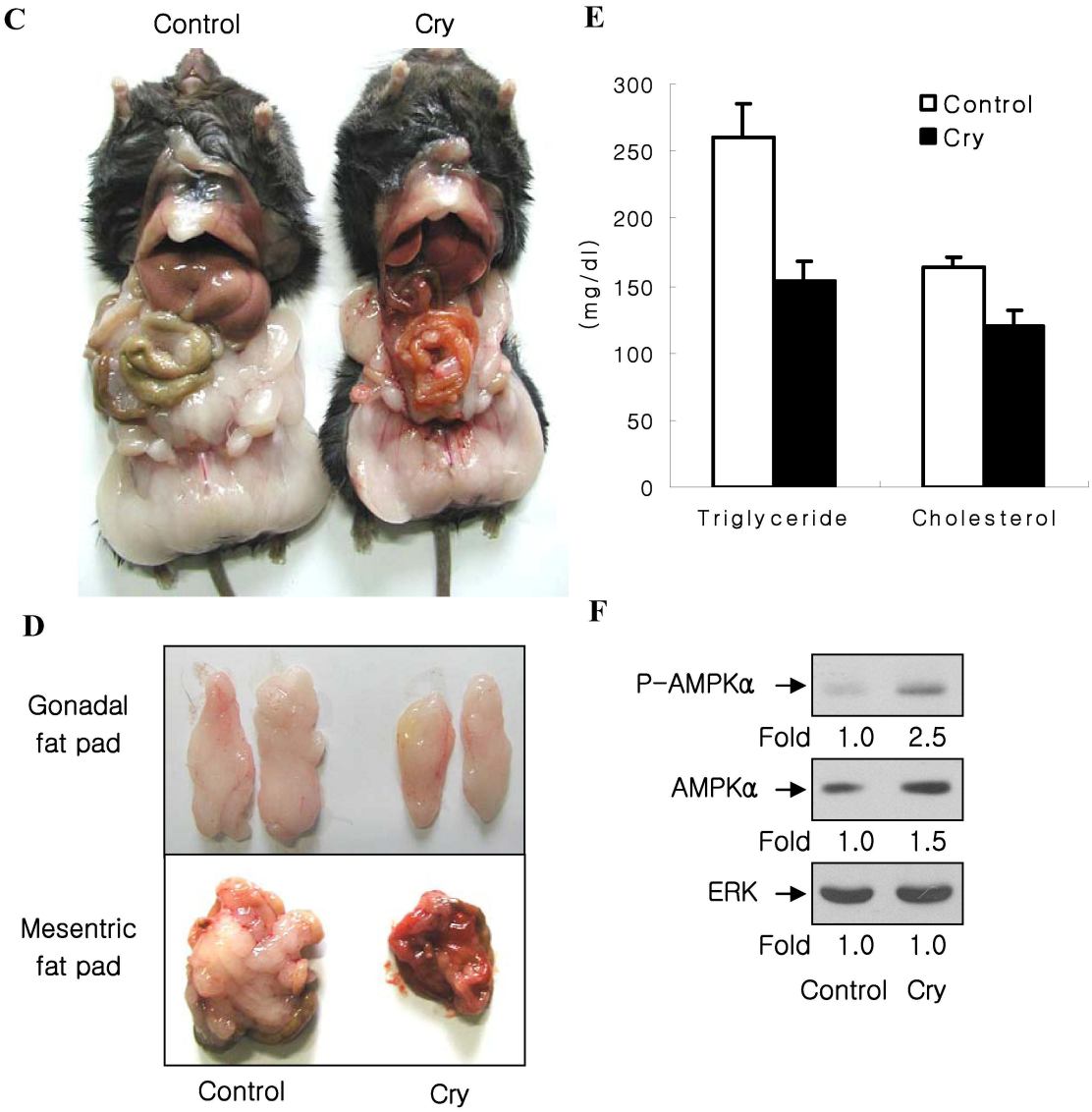

C
Control
Cry
E
300
250
200
150
100
50
0
(mg/dl)
Control
Cry
Triglyceride
Cholesterol
D
Gonadal fat pad
Mesentric fat pad
Control
Cry
F
P-AMPKα
Fold 1.0 2.5
AMPKα
Fold 1.0 1.5
ERK
Fold 1.0 1.0
Control Cry

Figure 6


A
Control ob/ob
Cry ob/ob

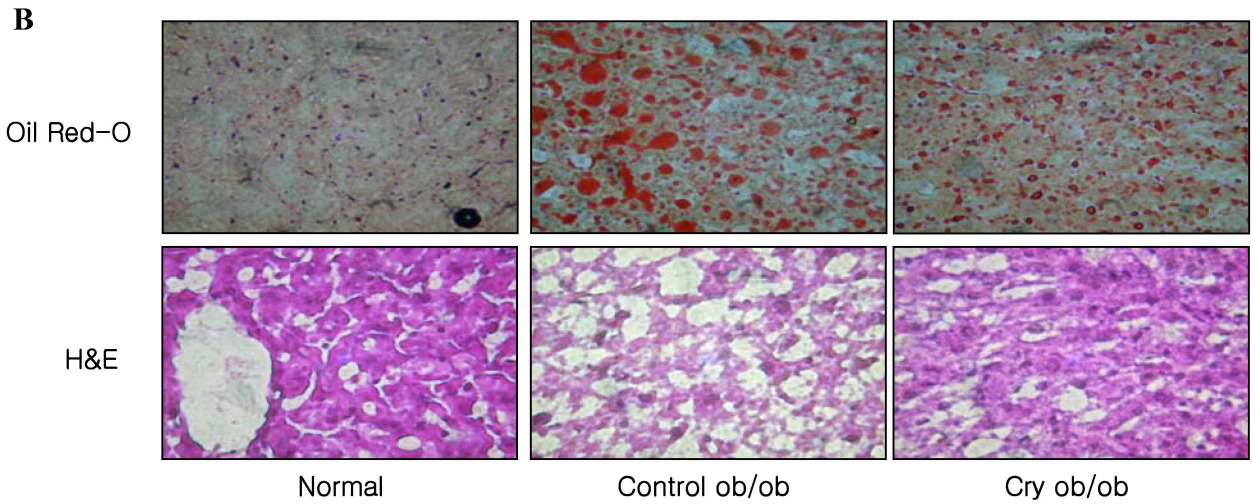
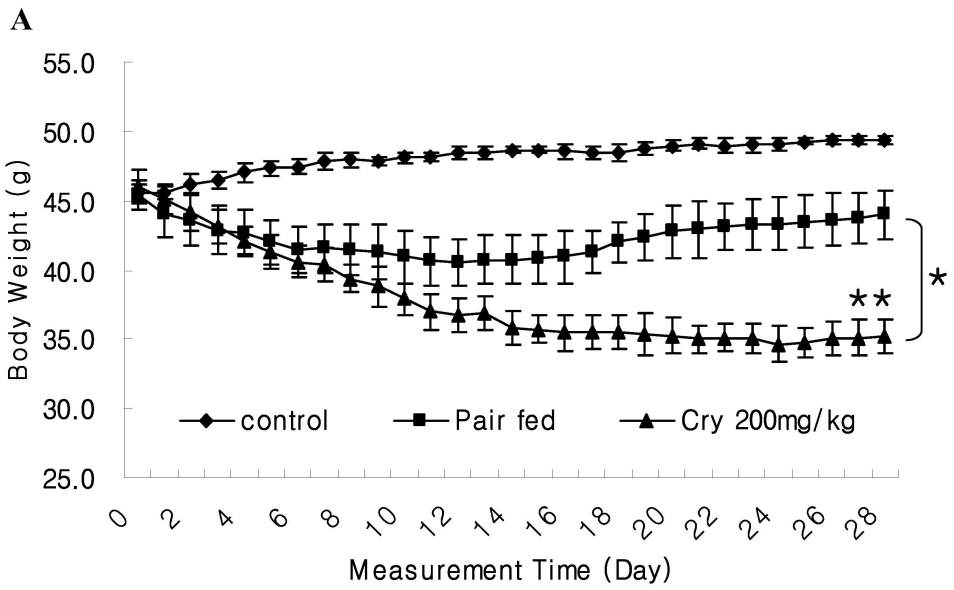

B
Oil Red-O
H&E
Normal
Control ob/ob
Cry ob/ob

Figure 7



B

Cry (mg/kg/day)	0	200
Average food intake (g/day)	3.4 ± 0.42	2.2 ± 0.53

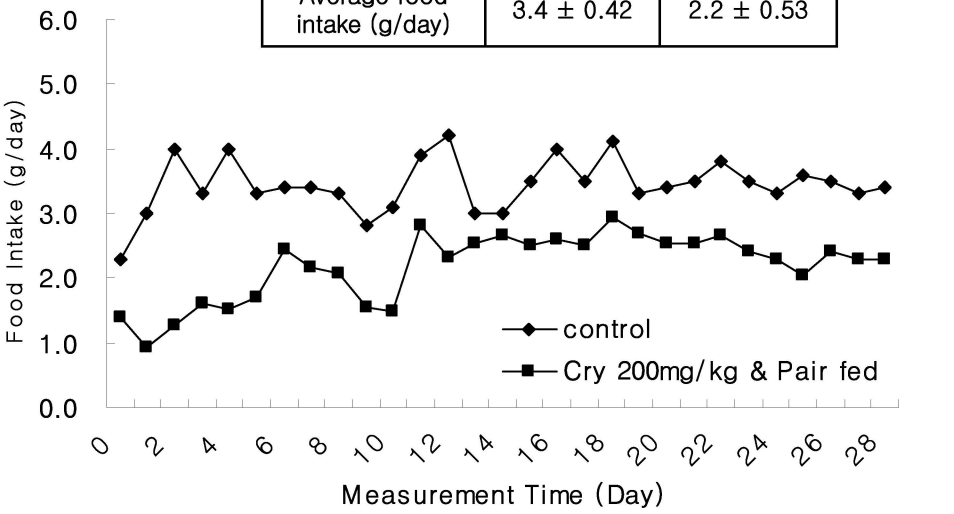
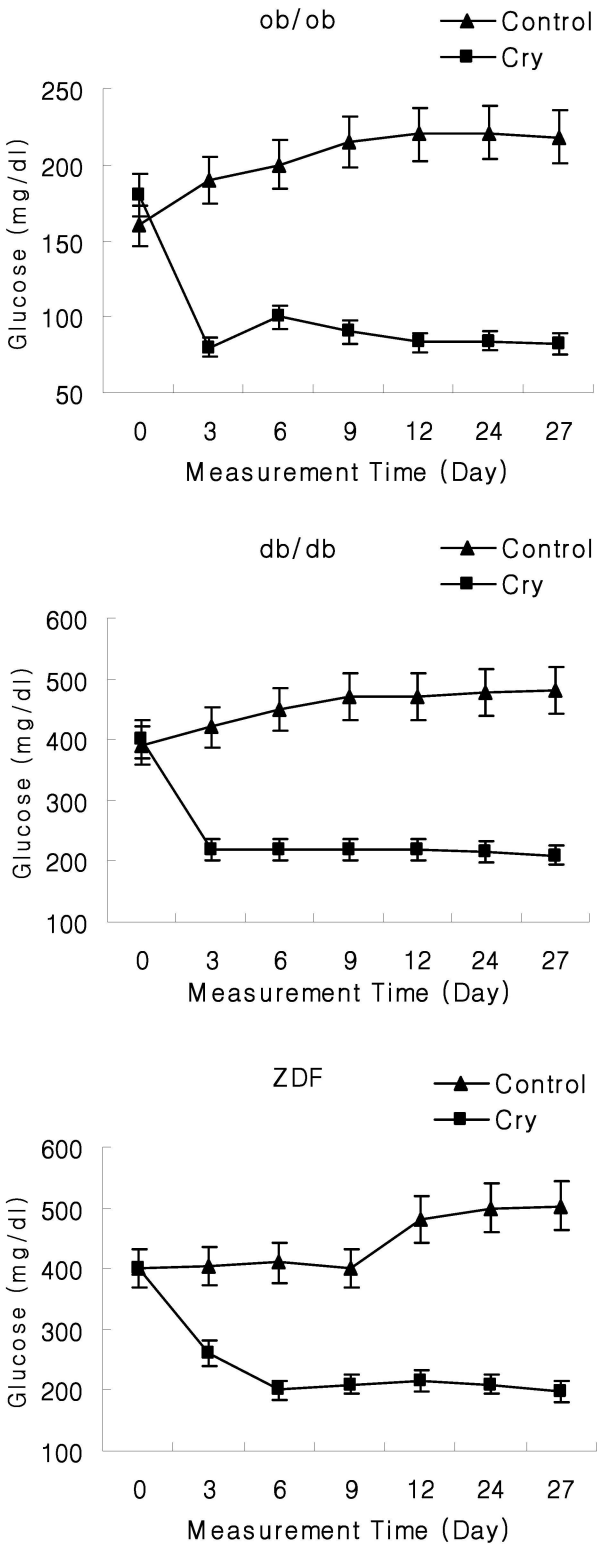


Figure 8


ob/ob
Control
Cry
Glucose (mg/dl)
250
200
150
100
50
0
3
6
9
12
24
27
Measurement Time (Day)
db/db
Control
Cry
Glucose (mg/dl)
600
500
400
300
200
100
0
3
6
9
12
24
27
Measurement Time (Day)
ZDF
Control
Cry
Glucose (mg/dl)
600
500
400
300
200
100
0
3
6
9
12
24
27
Measurement Time (Day)