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RESEARCH ARTICLE

Ethylacetate Extracts of the Muscles of *Anguilla japonica* Suppress Glucose Levels in *db/db* Mice via Activation of AMP-activated Protein Kinase

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Abstract Hypoglycemic effects of ethylacetate extracts of *Anguilla japonica* (EMA) muscles in *db/db* mice were investigated. To understand the mechanism responsible for the hypoglycemic effects of EMA, the effects of EMA on AMP-activated protein kinase (AMPK) activation in L6 myotubes and *in vivo* using type II diabetic *db/db* mice were analyzed. In L6 myotubes, the phosphorylation degrees of AMPK and acetyl-CoA carboxylase (ACC) were markedly increased and glucose uptake was significantly ($p<0.001$) increased by EMA, compared with untretaed L6 myotubes. However, in L6 myotubes, these effects were abolished by compound C, an AMPK inhibitor. Moreover, EMA significantly reduced non-fasting blood glucose and serum insulin levels, and strongly induced AMPK phosphorylation in skeletal muscle tissues of *db/db* mice. EMA regulates glucose levels in L6 myotubes and in diabetic mice by activation of AMPK. Beneficial effects for diabetes treatment are indicated.

Keywords: *Anguilla japonica,* AMP-activated protein kinase, *db/db* mouse, glucose uptake, L6 myotube

Introduction

Diabetes mellitus is classified as insulin-dependent diabetes mellitus (IDDM, type 1 diabetes) or non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes). Approximately 90% of patients have NIDDM and insulin resistance plays a key role in development of the disease (1). Hyperinsulinemia and insulin resistance (reduced insulin potency) are characteristics of NIDDM and usually precede the onset of hyperglycemia. Skeletal muscle is a major peripheral tissue that plays an important role in energy balance, and is the primary tissue of insulin-stimulated glucose uptake, disposal, and storage (2). Glucose transport, which is the rate-limiting step in glucose metabolism, can be activated in peripheral tissues by 2 distinct pathways, one stimulated by insulin through IRS-1/PI 3-kinase, and the other stimulated by muscle contraction/exercise through activation of AMP-activated kinase (AMPK) (3). AMPK is a phylogenetically conserved intracellular energy sensor that plays central regulatory roles in the metabolism of glucose and lipids (4). Furthermore, activation of AMPK leads to phosphorylation and regulation of a number of downstream targets involved in different pathways in numerous body tissues, such as, adipose, liver, and muscle tissues, and the hypothalamus (5). Therefore, AMPK provides an attractive target for therapeutic intervention in metabolic disorders, such as, obesity and Type 2 diabetes.

Eel is a common traditional Korean and Japanese food that contains considerable quantities of EPA, DHA, vitamin A, and essential fatty acids. Hexane extracts of eel bones

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and internal organs exhibit anti-inflammatory activity *in vitro* and *in vivo* (6). However, the regulatory effects of eel extracts on intracellular glucose levels and *in vivo* in an animal model have not been previously studied. During evaluation of the antidiabetic activities of different eel extracts, it was found that the ethylacetate extract of the muscle tissues of *Anguilla japonica*e (EMA) exhibited antidiabetic activity both *in vitro* and *in vivo*.

Materials and Methods

Preparation of extracts Muscles of *Anguilla japonica* (6.41 kg, wet weight) were extracted using ethanol (EtOH) once at room temperature for 1 week. The solution obtained was then filtered and concentrated to yield the EtOH extract (100.97 g), which was suspended in H_2O and successively partitioned using *n*-hexane, ethylacetate (EtOAc) and *n*-butanol (*n*-BuOH). After reduction and drying, the *n*-hexane-soluble fraction (4.12 g), the EtOAc-soluble fraction (2.98 g), the *n*-BuOH-soluble fraction (16.21 g), and a water-soluble residue (92.91 g) were obtained. Dried EtOAc extracts of muscle (EMA) were dissolved in dimethyl sulfoxide (DMSO) then diluted using DMEM (Invitrogen, Carlsbad, CA, USA). Final concentrations of DMSO were adjusted to 0.1% (v/v) in the culture medium. Control experiments showed that DMSO at this concentration had no effect on glucose uptake and AMPK expression in L6 myotubes (American Type Culture Collection, Manassas, VA, USA). DMSO and chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell culture L6 myotubes were maintained in an αMEM culture medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a 5% CO_2 humidified atmosphere at 37°C. After reaching confluence, cells were cultured in αMEM containing 2% FBS, and maintained for 5-7 days with a change in the medium every 48 h prior to use in experiments. αMEM, FBS, penicillin, and streptomycin were obtained from Invitrogen.

Immunoblotting L6 myotubes and tissue samples were isolated and processed as described previously (7). Polyvinylidene difluoride membranes (Calbiochem; Merk, Darmstadt, Germany) were probed using AMPK, pAMPK, ACC (acetyl-CoA carboxylase), and pACC (all from Cell Signaling Technology, Beverly, MA, USA), then developed using an enhanced chemiluminescence Western blot detection kit (Amersham Bioscience, Piscataway, NJ, USA).

Glucose uptake assay Uptake of radiolabeled 2-deoxyglucose was measured for 10 min in a transport buffer containing 20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 10 μM unlabeled 2-deoxyglucose (Calbiochem), and 10 μM 2-deoxy-[^{3}H]D-glucose (1 μCi/mL; Perkin-Elmer Life Sciences, Boston, MA, USA). The reaction was stopped by washing 3 times in ice-cold 0.9% NaCl (w/v). Nonspecific uptake was determined in the presence of 10 μM cytochalasin B (Sigma). Compound C (Sigma) was used as AMPK inhibitor. Cells were collected in 0.05 N NaOH and cell-associated radioactivity was determined using liquid scintillation counters (Tri-Carb 2910TR; Perkin Elmer). Results were expressed as pmol of 2-deoxyglucose transported per min/mg of protein. All other reagents were of the highest analytical grade commercially available.

Animal care and experimental procedures Male C57BL/KsJ-$Lepr^{db}/Lepr^{db}$ mice and lean C57BL/6J*ms* mice, purchased from The Jackson Laboratories (Bar Harbor, ME, USA), were housed in a temperature-controlled room (22±2°C), with a light/dark cycle of 12 h. Water and a normal standard pellet diet (Orient Bio, Seoul, Korea) were available *ad libitum* throughout the experimental period. From 7 weeks of age, EMA dosages of 100 and 400 mg/kg per day were orally administered once a day for 4 weeks to *db/db* mice, with a saline vehicle control administered to C57BL/6J*ms* normal glycemic (lean) mice at a volume of 10 mL/kg per day. After administration of EMA, changes in body weight (BW), food intake, blood glucose, serum insulin, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, and total cholesterol levels were determined. The effect of EMA was compared to the effect of rosiglitazone (Sigma), which improved insulin resistance when administered orally at 5 mg/kg per day in *db/db* mice (The Jackson Laboratories).

For immunoblot analysis, muscle tissue was expeditiously isolated following animal sacrificed and homogenized in ice-cold buffer (50 nmol/L of HEPES, pH 7.4, 150 mmol/L of NaCl, 10 mmol/L of NaF, 1 mmol/L of sodium pyrophosphate, 0.5 mmol/L of EDTA, 250 mmol/L of sucrose, 1 mmol/L of dithiothreitol, 1% TritonX-100, 1 mmol/L of Na_3VO_4, and one Roche protease inhibitor tablet per 50 mL of buffer) using an electrical homogenizer (IKA T10; Hamburg, Germany). Lysates were prepared as previously described (8,9) and stored at −80°C until analysis. The protein content in lysates was measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). All animal experiments were approved by the Institutional Animal Care and Use Committees of Yeungnam University. All other reagents were of the highest analytical grade commercially available.

Biochemical analysis Serum triglyceride, total cholesterol, and HDL cholesterol levels were determined using

commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). Serum insulin levels were measured using an insulin-enzyme immunosorbent assay test kit (Mercodia, Uppsala, Sweden).

Statistical analysis Data were expressed as mean± standard error of the mean (SEM). Analysis of variance (ANOVA) and/or Student's *t* tests were used to determine the significance of differences at a *p*-value of<0.05.

Results and Discussion

EMA increased AMPK activity in, and glucose uptake by, L6 myotubes EMA significantly increased the degree of phosphorylation of AMPK and ACC in a dose-dependent manner, compared with non-EMA treated mice and a maximum activity was obtained when cells were treated with 120 mg/mL of EMA for 2 h (Fig. 1A). Metformin, a well known AMPK agonist, also elicited increases in phosphorylations of AMPK and ACC (10,11). The effect of EMA on glucose uptake in L6 myotubes was also examined. As shown in 1B, EMA also increased glucose uptake in a dose dependent manner and maximum increasing activity was occurred at 120 mg/mL of EMA (increased approximately 1.8 fold compared to non-treated group). AMPK is a highly conserved sensor of the cellular energy status and is activated by a variety of cellular stresses that deplete ATP (12) such as, exercise, muscle contraction, reduced fuel availability, reduced levels of adipocyte-derived hormones (adiponectin and leptin), and by hypoxia, oxidative stress, and osmotic shock (13). Recent studies have suggested that activators of AMPK are good candidate targets for treatment of type 2 diabetes and the insulin resistance syndrome (14). Metformin (an anti-diabetic) was recently reported to activate AMPK and to decrease glucose production and increase fatty acid oxidation in the liver (15). Accumulating evidence also suggests that AMPK mediates insulin-independent glucose uptake signaling (16). Activation of AMPK in response to muscle contraction, hypoxia, and hyperosmolarity is closely correlated with increased muscle glucose uptake (17,18).

To examine the specific effect of EMA on AMPK signaling, L6 cells were pretreated with compound C, an inhibitor of AMPK, prior to EMA treatment, and changes in the degree of phosphorylation of AMPK and ACC were

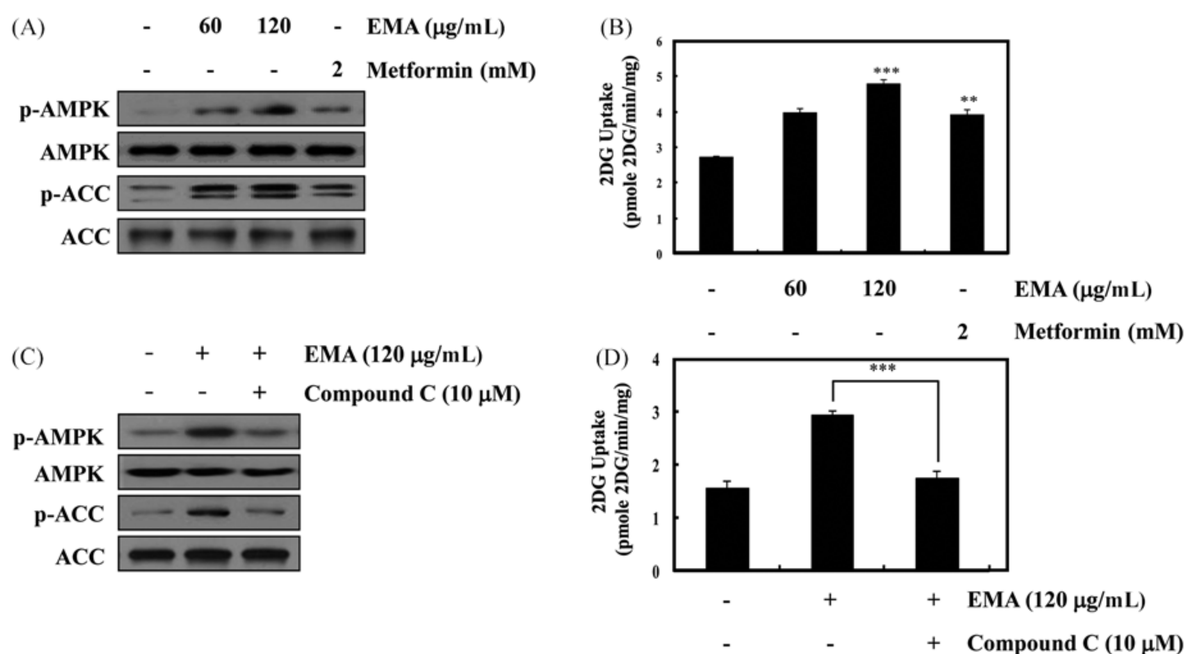

Fig. 1. Effect of EMA on the phosphorylations of AMPK and ACC and on glucose uptake in L6 myotubes. (A) L6 myotubes were exposed to different concentrations of EMA or metformin (2 mM) for 2 h, and the phosphorylation of AMPK and ACC were then analyzed using Western blotting. EMA increased the phosphorylation of AMPK and ACC dose-dependently. Metformin was used as a positive control for AMPK phosphorylation. (B) Cells were serum-deprived and incubated with 60 or 120 μg/mL of EMA for 2 h, glucose uptake was then measured. As a positive control, L6 myotubes were treated with 2 mM metformin for 2 h. The glucose uptake shown are representative of five independent experiments. Results are expressed as means±SEM (*n*=4). ***p*<0.1 and ****p*<0.01 versus untreated controls. (C) Increased phosphorylation degrees of AMPK and ACC in L6 myotubes treated with 120 μg/mL of EMA for 2 h were prevented by compound C pretreatment (10 μM for 20 min). Phosphorylated AMPK and ACC were immunoblotted with specific antibodies as described in Materials and Methods. (D) Increased glucose uptake after treatment with 120 μg/mL of EMA for 2 h was significantly inhibited by pretreating with compound C (10 μM for 20 min). Results are expressed as means±SEM (*n*=4). ****p*<0.01 versus EMA-treated cells (Student's *t* test or ANOVA)

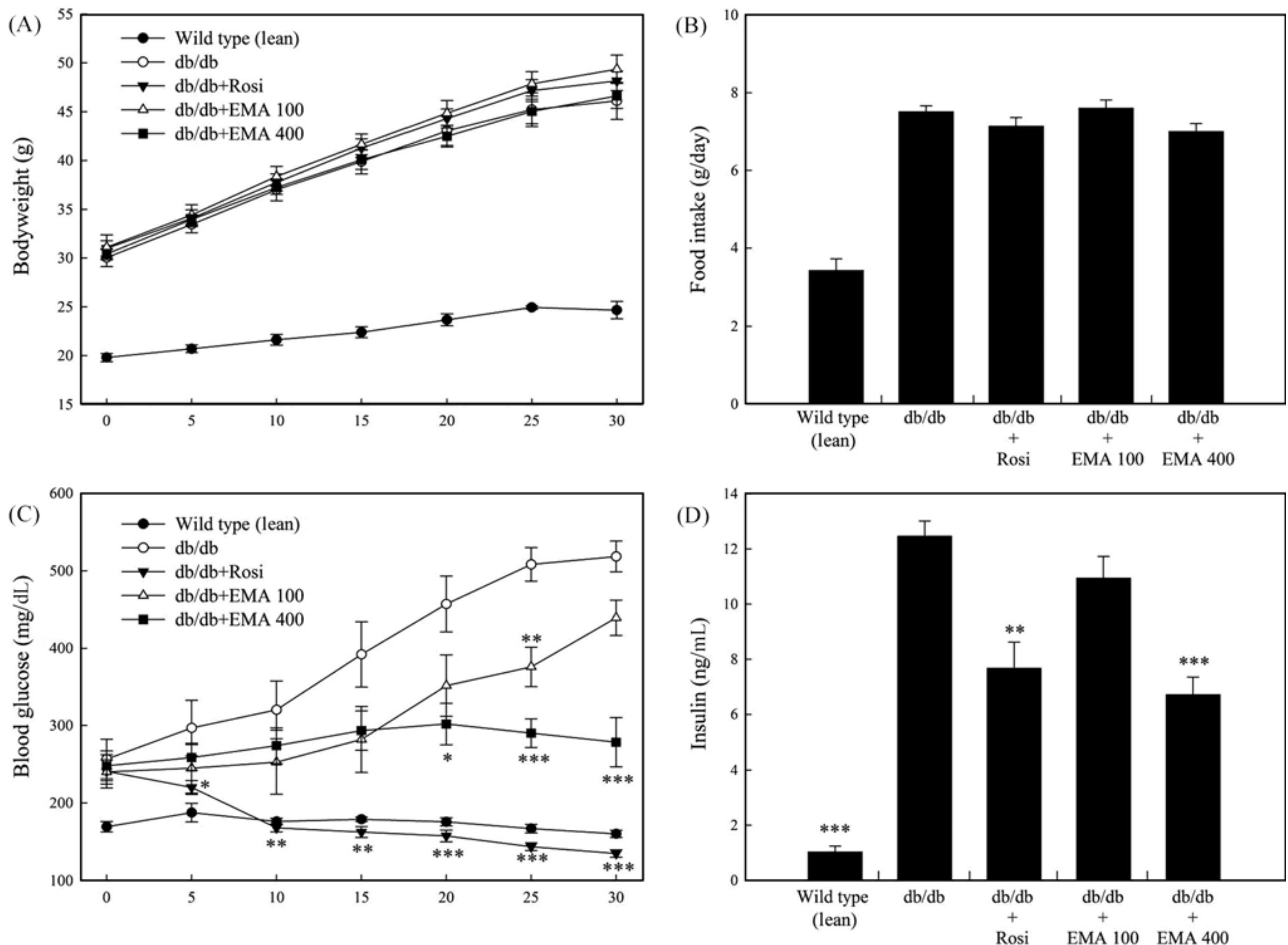

Fig. 2. Effects of EMA in *db/db* mice. (A) Effect of EMA on body weight. ●, wild type (*Lean*); ○, *db/db*; ▼, *db/db*+Rosi (rosiglitazone 5 mg/kg of BW); △, *db/db*+EMA 100 (EMA 100 mg/kg of BW); ■, *db/db*+EMA 400 (EMA 400 mg/kg of BW). (B) Effect of EMA on food intake. Wild type (*Lean*), *db/db*, *db/db*+Rosi (rosiglitazone 5 mg/kg of BW), *db/db*+EMA 100 (EMA 100 mg/kg of BW), *db/db*+EMA 400 (EMA 400 mg/kg of BW). (C) Effect of EMA on non-fasting blood glucose levels. ●,Wild type (*Lean*); ○, *db/db*; ▼, *db/db*+Rosi (rosiglitazone 5 mg/kg of BW); △, *db/db*+EMA 100 (EMA 100 mg/kg of BW); ■, *db/db*+EMA 400 (EMA 400 mg/kg of BW). (D) Effect of EMA on insulin levels in the serum. Wild type (*Lean*), *db/db*, *db/db*+Rosi (rosiglitazone 5 mg/kg of BW), *db/db*+EMA 100 (EMA 100 mg/kg of BW), *db/db*+EMA 400 (EMA 400 mg/kg of BW). Results are expressed as means±SEM (n=6). *p<0.05, **p<0.01, and ***p<0.01 *versus db/db* control groups

examined. Pretreatment with compound C at 10 mM almost completely abrogated the phosphorylation of AMPK and ACC that was due to EMA (Fig. 1C), and inhibited EMA-induced glucose uptake (Fig. 1D). These beneficial effects of EMA might have been due to activation of AMPK signaling, because compound C pretreatment abrogated EMA-mediated increases in the phosphorylation of AMPK and ACC and glucose uptake in L6 myotubes. The upstream kinase required for EMA-mediated activation of AMPK signaling has yet to be identified, although it has been reported that LKB1 and calcium/calmodulin-dependent protein kinase kinase (CaMKK) are upstream kinases of AMPK (19).

Antidiabetic effects of EMA in type II diabetes To test the antidiabetic effect of EMA in an animal model, EMA was administered to type 2 diabetic *db/db* mice. Different doses EMA (100, 400 mg/kg of body weight/day) were orally administered to *db/db* mice for 4 weeks and blood glucose levels were analyzed every 5 days. The antidiabetic effects of EMA were compared to the effects of the AMPK activator rosiglitazone at 5 mg/kg of body weight. Rosiglitazone was used as a positive control. Mice treated with EMA or vehicle had similar body weights (Fig. 2A) and food intakes (Fig. 2B) during the experimental period. Treatment of *db/db* mice with EMA (100 and 400 mg/kg) for 30 days decreased non-fasting blood glucose levels by 25.9 % (100 mg/kg) and 46.3% (400 mg/kg) (p<0.001), and also decreased insulin levels by 46.1% (p<0.001), compared to untreated *db/db* mice (Fig. 2C, 2D). In rosiglitazone treated mice, non-fasting blood glucose and insulin levels were significantly lower than in untreated *db/db* mice (Fig. 2C,

Table 1. Effects of EMA on lipid profiles in *db/db* mice

Group	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL cholesterol (mg/dL)	LDL cholesterol (mg/dL) [1)]
Wild type (lean)	110.8±3.6***[2)]	90.0±3.0*	88.1±7.0	4.7±6.0***
db/db	217.6±8.1	190.0±41.2	83.1±11.5	96.5±14.7
db/db+Rosi (5 mg/kg)	250.7±12.6	63.4±9.2*	93.0±4.4	145.0±9.9*
db/db+EMA (100 mg/kg)	208.5±8.6	127.7±19.4	105.5±6.2	77.5±8.0
db/db+EMA (400 mg/kg)	274.2±9.0**	110.6±15.0	138.7±6.2**	113.4±5.7

[1)]Total cholesterol-HDL cholesterol-triglyceride/5
[2)]Each value is a mean±SEM for 6 mice. *p<0.05, **p<0.01, and ***p<0.001 compared to *db/db* control.

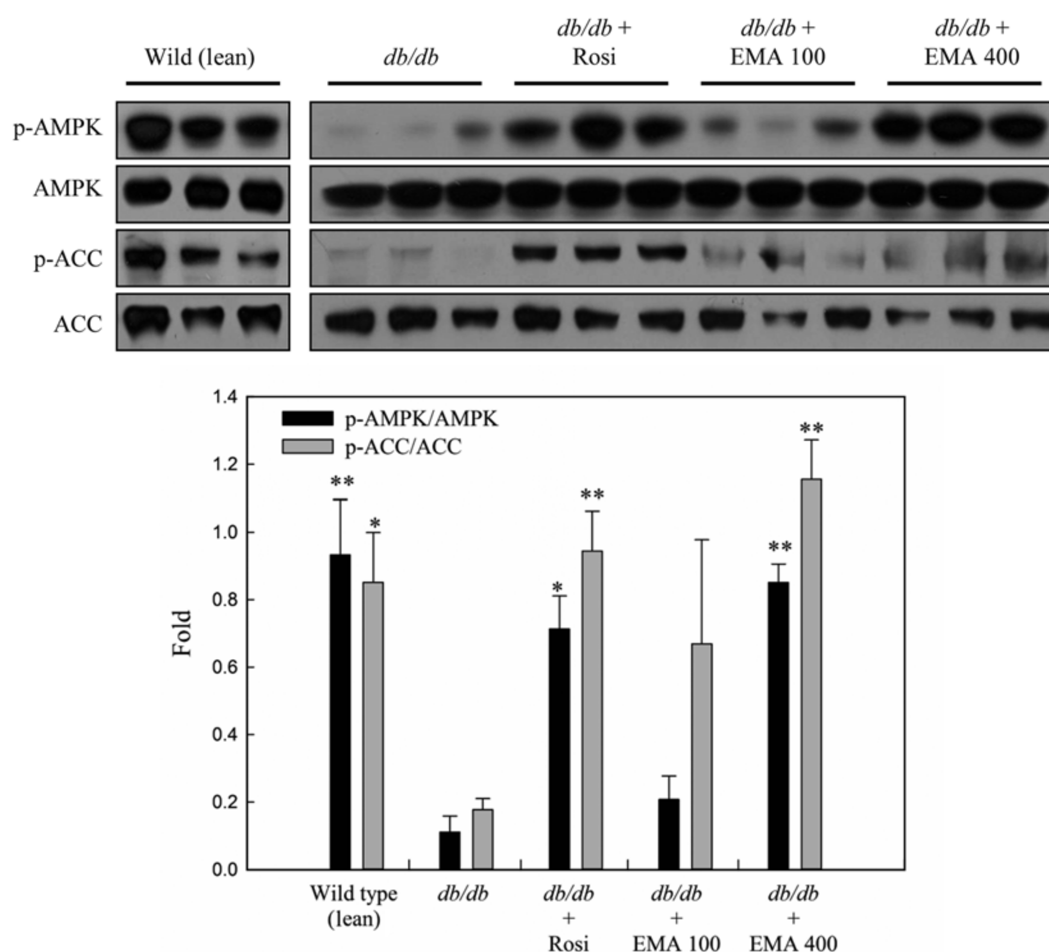

Fig. 3. Effects of EMA on AMPK activity in the soleus muscle of *db/db* mice. EMA increased the degree of phosphorylation of AMPK and ACC in the soleus muscle. Phosphorylated AMPK levels were examined using immunoblotting. Results represent the mean±SEM (n=4). *p<0.05, **p<0.01 *versus db/db* control groups; wild type (*Lean*), *db/db*, *db/db*+Rosi (rosiglitazone 5 mg/kg of BW), *db/db*+EMA 100 (EMA 100 mg/kg of BW), *db/db*+EMA 400 (EMA 400 mg/kg of BW)

2D). Next, the effects of EMA on the levels of plasma total cholesterol, triglycerides, and HDL cholesterol in *db/db* mice were investigated and summarized in Table 1. EMA (400 mg/kg) increased total plasma cholesterol by 26.0%, LDL cholesterol by 17.5, and 66.9% HDL cholesterol levels, but triglyceride levels were reduced by 41.8% compared with untreated *db/db* mice. The most critical problems associated with hyperlipidemia are increases in serum LDL, triglyceride, and total cholesterol levels and decreases in HDL levels (20). In support of the beneficial effects of EMA on blood glucose levels due to increased phosphorylation of AMPK in skeletal muscle, the muscle tissue was isolated 30 days following EMA or rosiglitazone treated with measurement by immunoblotting. As shown in Fig. 3, EMA and rosiglitazone significantly increased the degree of phosphorylation of AMPK and ACC in the

soleus muscle compared to lean control mice in a dose-dependent manner (Fig. 3). Stimulated glucose uptake in muscle cells occurred via the sequential activation of AMPK, which enhances insulin sensitivity by increasing glucose uptake and fatty acid oxidation in skeletal muscle (21). Many previous reports have shown that AMPK reduces lipogenesis by attenuating the sterol regulatory element-binding protein (SREBP)-1c and/or alleviates cholesterol synthesis by down-regulation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (22). However, levels of high-density lipoprotein cholesterol are increased in different tissues by up-regulation of AMPK (23,24). Moreover, Sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor α (PPARα) display decrease hepatic steatosis and cholesterol metabolism activities with corresponding increases in the AMPK level in different tissues (25,26). For this reason, up-regulation of SIRT1 and PPARá by EMA involvement in regulation of the lipid metabolism cannot be ruled out. Therefore, additional research is required to clarify the molecular mechanism of EMA in metabolic diseases.

The molecular signaling that is initiated by EMA improves glucose and lipid profiles in *db/db* mice and induces antidiabetic effects. These mice are hyperleptinemic and become obese, and have been used to test the efficacy of pharmaceuticals in the contexts of obesity, Type 2 diabetes, and insulin resistance (27,28). Moreover, hyperglycemia is a major feature of diabetes and hyperglycemia control is mandatory in diabetic patients (29). EMA improves glucose and lipid profiles in diabetic *db/db* mice by increasing AMPK activity in skeletal muscle. This extract should be considered for therapeutic use in the contexts of metabolic syndrome and type 2 diabetes.

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Disclosure The authors declare no conflict of interest.

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