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Antidiabetic activities of entagenic acid in type 2 diabetic db/db mice and L6 myotubes via AMPK/GLUT4 pathway

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ABSTRACT

Ethnopharmacological relevance: *Entada phaseoloides* (L.) Merr., a traditional Chinese folk medicine, has been used in treating diabetes and other inflammatory disorders. Our previous study revealed that the triterpene saponins in *E.Phaseoloides* possessed an antidiabetic effect in type 2 diabetic rats by activating AMP-activated protein kinase (AMPK). Entagenic acid, the principal aglycon, isolated from the seed kernels of *E. phaseoloides*, has been proposed to possess a significant role in the antidiabetic effect, however, its actual effect and pertinent mechanisms are still unknown.

Aim of the study: The aim of the present study was to investigate the antidiabetic effect of entagenic acid in a type 2 diabetic animal model (C57BIKsj db/db mice) and its role in the regulation of glucose uptake in L6 myotubes, and to explore the possible molecular mechanisms.

Materials and methods: In *vivo*, average weekly body weight, daily water, food intake and postprandial blood glucose levels, the intraperitoneal insulin tolerance test, glucose tolerance test, serum lipid profiles and pancreatic histopathological changes in db/db mice treated with entagenic acid orally at different doses (5, 10 and 20 mg/kg) were assessed and compared with wild-type littermates or vehicle- and metformin-treated db/db mice. In *vitro*, effects of entagenic acid on the glucose consumption and the phosphorylation of protein kinase B (AKT) and AMPK in L6 myotubes were evaluated.

Results: In *vivo*, entagenic acid significantly lowered postprandial blood glucose levels but not the body weight, normalized the serum lipid imbalance, improved the impaired glucose tolerance, insulin resistance, as well as the pathological changes in pancreatic islets. In *vitro*, entagenic acid dose-dependently promoted glucose utilization and enhanced the translocation and expression of glucose transporter 4 (GLUT4), and phosphorylation of AMPK but not AKT.

Conclusions: The present study demonstrated that entagenic acid can markedly maintain the glucose homeostasis, improve insulin resistance and ameliorate dyslipidemia. Its antihyperglycemic effect could be caused by promoting AMPK mediated cellular signaling and GLUT4 translocation in muscles.

1. Introduction

Type 2 diabetes mellitus (T2DM), with a number of severe complications in the late stages, has been considered one of the main threats to human health. As hyperglycemia is a fundamental factor contributing to T2DM and its complications, glucose-lowering strategy is of great importance in the treatment of T2DM to reduce the risk for T2DM complications (Aronoff et al., 2004). The skeletal muscles, which account for the majority (~80%) of insulin-mediated glucose uptake in the post-prandial state, play an important role in maintaining glucose homeostasis (Saltiel and Kahn, 2001). Glucose transporter 4 (GLUT4), an important sugar transporter protein, plays a predominant role in maintenance of glucose homeostasis via its translocation and expression in muscle (Morgan et al., 2011). In skeletal muscles, insulin promotes

Abbreviations: EA, entagenic acid; T2DM, Type 2 diabetes mellitus, GLUT4, glucose transporter 4; ACC, Acetyl coenzyme A carboxylase; AMPK, AMP-activated protein kinase; AKT, protein kinase B; Met, metformin; IPGTT, Intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; TC, Total cholesterol; TG, Triglycerides; IRAP, Insulin-responsive aminopeptidase

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glucose uptake by increasing translocation of GLUT4 from intracellular vesicles to the plasma membrane through activation of the phosphatidylinositol-3 kinase (PI3K) and protein kinase B (AKT) signaling pathways (Morgan et al., 2011; Taniguchi et al., 2006). Another GLUT4 translocation promoter is 5′adenosine monophosphate-activated protein kinase (AMPK). AMPK is activated by exercise, contraction, and compounds such as metformin (Kahn et al., 2005; Hawley et al., 2002). Activation of AMPK could enhance insulin sensitivity, stimulate glucose uptake in muscle and adipose tissues, and inhibit glucose production in the liver (Hardie, 2004; Thakkar et al., 2015). Thus, studies on novel compounds that activate PI3K/AKT or AMPK pathways and stimulate skeletal muscle glucose uptake would be useful for the development of remedies against insulin resistance and T2DM.

Recently, the plant derived compounds have become an important source in discovering new drugs with possibly better diversity and minimal side effects for treating metabolic disorders such as diabetes or aging-related metabolic phenotypes (Li and Vederas, 2009; Park et al., 2012). *Entada phaseoloides* (L.) Merr., belonging to the *Entada* genus, is a traditional Chinese folk medicine and has been used to treat various diseases such as stomachache, edema, diabetes and inflammatory disorders, for a long time in minority area of China (Zhao et al., 2010). Our previous study had revealed that the triterpene saponins, as the main component in *E. Phaseoloides*, possessed an antidiabetic effect in T2DM rats (Zheng et al., 2012). Entagenic acid (referred to as “EA”), a chemotaxonomic marker in the *Entada* genus, is the principal aglycon of total saponins isolated from the seed kernels of *E. phaseoloides*, however, whether EA plays any role in the antidiabetic effect is still unknown.

Leptin receptor-mutant (db/db) mice have been used widely in research as a T2DM animal model, which presented a number of complications including hyperglycemia, obesity and hyperlipidemia, perfectly resembling those syndromes in human DM diseases (Wang B et al., 2014). Also, the rat skeletal muscle (L6) cell line would present myogenic differentiation and GLUT4 expression in a culture medium, and has been widely used for novel antidiabetic compound screening. Therefore, both db/db mice and L6 myotubes were applied in this study, to investigate the antidiabetic effect of EA and explore its potential mechanisms.

2. Materials and methods

2.1. Plant material

The seed kernels of *E. phaseoloides* were collected from Xishuangbanna, Yunnan Province, China and identified by associate professor Xinqiao Liu, School of Pharmaceutical Sciences, South-Central University for Nationalities. A voucher specimen (No. EP-20131115) was deposited in the herbarium of the Institute for traditional Chinese folk medicine, South-Central University for Nationalities.

2.2. Materials

Rat L6 myoblasts were obtained from the lab of Professor Tao Xu, Chinese Academy of Sciences. FBS, α-MEM, trypsin, penicillin-streptomycin antibiotic solution were purchased from Hyclone, USA. Glucose assay kit (GLU-OX) was purchased from InTec PRODUCTS, Xiamen, China. Antibodies for phospho-AKT Ser-473(#4051), AKT (#4685), GLUT4 (#2213), phospho-AMPKα Thr-172(#2535), and phospho-ACC Ser-79 (#3661), ACC (#3676) were purchased from Cell Signaling Technology, USA. Antibody for AMPKα (A1229) and GAPDH (60004-1-lg) were purchased from ABclonal Technology, China and proteintech, USA, respectively. The goat anti-mouse (#072-01-18-06-1) and goat anti-rabbit (#14-13-06-1) secondary antibodies were purchased from Kirkegard & Perry Laboratories, USA. Biochemical assay kits for the measurement of total triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were purchased from Jiancheng Bioengineering Institute, Nanjing, China. Mercodia Mouse Insulin ELISA kits were purchased from Mercodia AB, Sylveniusgatan 8 A, Sweden.

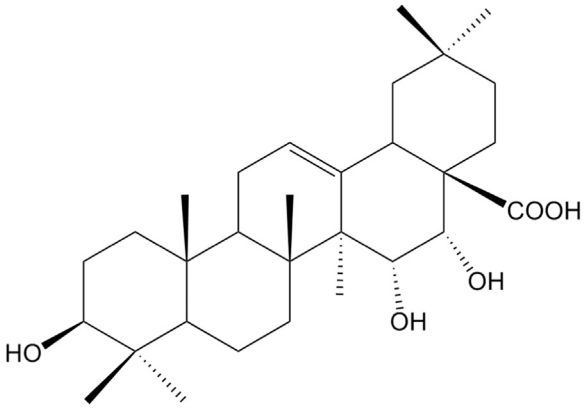

Fig. 1. Chemical structure of entagenic acid (EA).

2.3. Extraction and isolation of EA

The dried and powdered seed kernels (5.3 kg) of *E.Phaseoloides* were extracted with 70% EtOH at room temperature. The filtrate obtained was evaporated under reduced pressure to give ethanol extract (1288 g). Then the ethanol extract was submitted to a column of Diaion HP-20 eluting with different H_2O-EtOH gradients to afford a crude saponin mixture (collecting 50% H_2O-EtOH fractions).

The total saponin (100 g) was hydrolyzed in 2 N aqueous HCl (1000 ml) for 3 h at 90 °C. The resulting mixture was adjusted to pH 6.0 with a dilute NaOH solution and then extracted with EtOAc. The EtOAc extracts were further purified by MCI column with H_2O-EtOH gradients to give entagenic acid (6 g, collecting 7:3/H_2O-EtOH fractions, Fig. 1). The purity of EA was confirmed to be 98.0% by HPLC with isocratic elution of acetonitrile-water (60:40).

2.4. Animals and treatments

The 8-week-old male C57BLKS/$Lepr^{db}$ (hereinafter referred to as “db/db”) mice, and their littermate male C57BLKS (hereinafter referred to as “WT”) mice, were purchased from the National Resource Center for Mutant Mice (NRCMM) (Nanjing, China). All mice were maintained under specific-pathogen-free (SPF) conditions, and housed at 22 ± 2 °C, 45–75% relative humidity, and 12 h light/dark cycle, and kept for 1 week to acclimate before the experiment. The mice care and experimental procedures were approved by the Laboratory Animal Ethics Committee of the School of Life Sciences, South-Central University for Nationalities.

Mice were orally treated with vehicle (0.9% saline), metformin (Shanghai, China) (referred to as “Met”, 150 mg/kg) and EA (5, 10, 20 mg/kg) for 6 weeks (n = 8) and housed as stated above.

2.5. Glucose, body weight (BW) and water intake measurements

Postprandial blood glucose levels of mice were monitored weekly after removal of food for 2 h, and performed on blood drawn from the tail vein using glucose monitors (LifeScan, Inc., Milpitas, CA) (Zheng et al., 2015). To get precise data, the measurement was always conducted at the same time period. Both water and food intake of the mice were measured semiweekly (Zheng et al., 2015).