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# Compound K protects pancreatic islet cells against apoptosis through inhibition of the AMPK/JNK pathway in type 2 diabetic mice and in MIN6 β-cells

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ABSTRACT

*Aims:* Compound K (CK) is known to possess anti-diabetic activities but the mechanism for this action is unknown. The present study observed the protective effect of CK on islet cell apoptosis through the AMP-activated protein kinase (AMPK) mediated C-Jun N-terminal kinase (JNK) pathway.
*Main methods:* Treatment effect of CK on type 2 diabetic (T2D) mice and palmitate-induced MIN6 β-cells injury was observed. Fasting plasma glucose, triacylglycerol, total cholesterol, insulin levels and glucose tolerance test were evaluated. The expression of AMPK and JNK was detected in islet and MIN6 cells.
*Key findings:* CK treatment (30 mg/kg) decreased fasting plasma glucose, triacylglycerol, total cholesterol, elevated plasma insulin levels and improved glucose tolerance in T2D mice. CK treatment attenuated islet cell apoptosis and caspase-3 activity accompanied by a decrease in AMPK and JNK activation. Meanwhile, CK treatment attenuated the palmitate-induced reduction in MIN6 β-cell viability, apoptosis and caspase-3 activity and activation of AMPK and JNK. The AMPK activator AICAR attenuated the CK-mediated inhibition of palmitate-induced apoptosis.
*Significance:* These data suggest that CK treatment provides a beneficial anti-diabetic effect in mice with T2D and this protective effect may be mediated through prevention of β-cell apoptosis via inhibition of the AMPK-JNK pathway.



## Introduction

Type 2 diabetes (T2D) is the most common endocrine disease. T2D patients develop insulin resistance and β-cell failure leading to inadequate insulin secretion, finally presenting as elevated blood glucose level. Experimental evidence indicates that a decrease in pancreatic β-cell mass and function is a major contributing factor to the reduction in circulating insulin (Chang-Chen et al., 2008). Destruction of pancreatic β-cells is caused by several metabolic stressors including endoplasmic reticulum stress (Fonseca et al., 2011) and elevated reactive oxygen species (Giacca et al., 2011) which occur in hyperlipidemic conditions. Hence, novel strategies to enhance β-cell survival would be of clear therapeutic benefit for patients with T2D.

AMP-activated protein kinase (AMPK) is a regulator of cellular and systemic energy homeostasis in mammalian cells, activated by metabolic stressors including hypoxia, low glucose and nutrient deprivation (Ryu et al., 2009). Activation of AMPK in skeletal muscle, liver, and adipose tissue was shown to enhance metabolism and improve insulin sensitivity, and may be favorable for the treatment of diabetes (Dziewulska et al., 2010). However, the beneficial effect of AMPK activation in vivo in diabetes is complicated. For example, AMPK activation in β-cells reduced glucose-stimulated insulin secretion (Okazaki et al., 2010) and increased β-cell death through apoptosis (Fu et al., 2009; Kefas et al., 2004; Riboulet-Chavey et al., 2008; Santos et al., 2011). In addition, AMPK activation induced apoptosis of insulin-producing MIN6 cells through stimulation of c-Jun-N-terminal kinase (JNK) (Kefas et al., 2003). Thus AMPK might serve as an important pharmacological target for enhancement of β-cell function.

Although several drugs are available for the treatment of diabetes there are many side effects which limit the effectiveness of existing drugs. Recent research has examined the role of natural products or dietary interventions in the prevention and treatment of diabetes.

*Abbreviations:* T2D, type 2 diabetes; AMPK, AMP-activated protein kinase; JUN, C-Jun N-terminal kinase; CK, compound K; HFD, high fat diet; FBG, fasting blood glucose; ISI, insulin-sensitivity index; TG, triglyceride; TC, total cholesterol; FINS, fasting blood insulin level.

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Compound K (CK) (also known as M1, IH901) is a terminal metabolite of protopanaxadiol ginsenosides. CK exhibits various biological activities as an anti-cancer (Kim do et al., 2009a; Lee et al., 2010) and anti-inflammation (Joh et al., 2011) and it has hepatoprotective effects (Lee et al., 2005). In *db*/*db* mice CK increased insulin secretion in β-cells through its action on an ATP-sensitive $K^+$ channel and improved insulin sensitivity and enhanced plasma adiponectin levels (Han et al., 2007). In addition, CK attenuated hepatic lipid accumulation via AMPK activation in HepG2 (Kim do et al., 2009b). Previously we demonstrated that CK down-regulates the key gluconenogenesis enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. Reduction in the activities of these enzymes may serve as one of the hypoglycemic mechanisms mediated by CK treatment (Li et al., 2012). However, the effect of CK on AMPK activity in β-cell function and survival remains unclear. In the present study, we examined the protective effects of CK on pancreatic β-cell function in T2D mice and in MIN6 β-cells following palmitic acid induced cell injury and examined whether modulation of AMPK activation via CK was involved in this protective effect.

## Materials and methods

### *Materials*

Streptozotocin, AMPK activator AICAR and inhibitor Compound C were purchased from Sigma; insulin was purchased from Eli Lilly, Changchun, China; glucose, total cholesterol (TC) and triglyceride (TG) test kits were obtained from BHKT Clinical Reagent Co., Ltd, Beijing, China; Iodine [$^{125}$I] Insulin Radioimmunoassay Kit was purchased from Tianjin Nine Tripods Medical & Bioengineering Co., Ltd, Tianjin, China; and other reagents were purchased from Beijing Chemical Factory, Beijing, China. Antibodies (AMPK, p-AMPK, JNK, p-JNK, BAX, BCL-2, cytochrome-c, caspase-3, GAPDH) were purchased from Santa Cruz, CA, USA. CK used in this study was isolated and purified from *Panax ginseng* roots by a series of chromatography procedures in our laboratory, and their structures were elucidated by comparison of spectral data. Its purity was determined to be more than 98.5% by HPLC–UV analysis. The solution of palmitate was prepared by mixing and heating to 90 °C with equal molar amounts of NaOH and palmitic acid to a concentration of 400 mM. It was further diluted with distilled water and 5% BSA (fatty acid free) to 50 mM, sterilized and stored at 4 °C. A suitable amount of palmitate was slowly added to 37 °C culture medium before use.

### *Animal models*

Male ICR mice (18-22 g) were purchased from the experimental animal center of Jilin University (Jilin, China). After one week of regular feeding, the animals were randomly divided into 2 groups: a control group (CON, $N = 12$) and a high-fat diet fed group (HFD, $N = 50$). After 4 weeks of high-fat diet feeding, 100 mg/kg streptozotocin dissolved in citrate buffer was injected (i.p.) into HFD animals. The control animals were injected with citrate buffer alone. 4 weeks after injection, fasting blood glucose (FBG) was measured. HFD fed mice with FBG above 7.8 mmol/L were considered T2D and were then randomly divided into 2 groups: an untreated group (DM) ($n = 12$) and a CK-treated group ($n = 12$). Other mice with FBG less than 7.8 mmol/L were sacrificed. CK (30 mg/kg) dissolved in saline was given by gavage. CON and DM were gavaged with saline alone. Mice were treated daily for 4 weeks. All the mice were housed in standard polypropylene cages (6 mice/cage) and maintained under controlled room temperature and humidity with 12 h light and dark cycles. Food intake was recorded daily and body weight was recorded once per week. At the end of the study, animals were fasted overnight and blood samples obtained from the tails were collected into EDTA containing tubes and placed on ice. After centrifugation at 3500 ×*g* for 10 min, plasma was collected and stored at −80 °C. The pancreases were immediately separated, collected and stored in liquid nitrogen until further analysis.

### *Measurement of fasting blood glucose, fasting insulin, triglyceride, total cholesterol, insulin sensitivity index (ISI) and oral glucose tolerance test (OGTT)*

FBG, TC, and TG in the plasma collected above were measured according to the instructions of the corresponding commercial kits. Fasting insulin was assayed by RIA according to the instructions provided by the manufacturer. The ISI was calculated by the formula [$ISI = Ln(FBG \times FINS)^{-1}$] according to the fasting insulin and glucose concentration of each mouse. An OGTT was conducted after an overnight fast (12 h). Mice were gavaged with glucose (2 g/kg BW). Blood samples were subsequently obtained at 2 h after gavage and the blood glucose was measured according to the instructions of the corresponding commercial kit.

### *Cell experiments*

MIN6 cell line was purchased from XiangYa School of Medicine, Central South University. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM 25 mmol/L glucose) equilibrated with 5% $CO_2$ and 95% air at 37 °C. The medium was supplemented with 10% fetal calf serum, 100 U/ml penicillin sulfate and 50 μg/ml gentamycin. All experiments were performed when cells reached 80% confluence. Cell viability was assessed by the MTT assay. Briefly, MIN6 cells were seeded in 96 well plates at $1 \times 10^4$ cells/well. Cells were incubated with 0–1.0 mM palmitate for 24 h. Cells were then washed twice with cold PBS and then MTT solution was added to the cells at a final concentration of 0.5 mg/ml. After incubating for 4 h at 37 °C, with 5% $CO_2$, the solution was removed, and 150 μl dimethylsulfoxide was added. The precipitate in each well was dissolved for 10 min and the optical density (OD) was determined at 570 nm using a microplate reader. The cell viability of CK-treated cells was performed as above except cells were incubated with 2–32 μM CK. The optimum concentration of palmitate and CK used for subsequent experiments was determined after cell viability assays. CK and/or AICAR (1 mM) was added simultaneously with palmitate for 24 h. Cells were pre-incubated with 10 μM Compound C for 1 h prior to treatment for 24 h with palmitate. Total protein was extracted and probed with antibodies specific for AMPK, pThr172-AMPK, JNK and p-JNK.

### *Determination of caspase-3 activity*

Cell apoptosis was assessed by caspase-3 activity and annexin V/PI staining. Caspase-3 activity was measured using a Caspase-3 Colorimetric Assay kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. Briefly, cells were washed with cold PBS, resuspended in lysis buffer and left on ice for 15 min. The lysate was centrifuged at 16,000 ×*g* at 4 °C for 15 min. The production of p-nitroaniline was measured at 405 nm using a microplate reader. Caspase-3 activity was normalized to the total extracted protein concentration.

### *Apoptosis determination*

Quantitative evaluation of apoptosis was performed by flow cytometry after double staining with the Annexin V-FITC apoptosis detection kit (Tianjin Sungene Biotech Co., Ltd. China). Intact (normal) cells (FITC−/PI−), early-stage apoptotic cells (FITC+/PI−), late-stage apoptotic cells (FITC+/PI+) and necrotic cells (FITC−/PI+) were quantified by flow cytometry. In brief, following treatment of MIN6 cells under various conditions, cells were harvested and pelleted by centrifuging at 1000 rpm for 5 min at room temperature. Cells were