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X-3, a mangiferin derivative, stimulates AMP-activated protein kinase and reduces hyperglycemia and obesity in db/db mice

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ABSTRACT

Diabetes mellitus is a major health concern, affecting nearly 10% of the population. Here we describe a potential novel therapeutic agent for this disease, X-3, a derivative of mangiferin. Therapeutic administration of X-3 significantly and dose-dependently reduced plasma glucose and triglycerides in db/db mice following 8 week-treatments. Treatment with X-3 dose-dependently increased the number of insulin-positive β-cell mass. Importantly, X-3 did not cause any death or signs of toxicity in acute toxicity studies. Study of mechanism of action revealed that X-3 increased glucose uptake in parallel with increased phosphorylation of AMP-activated protein kinase (AMPK) in 3T3-L1 cells. It activates AMPK in both LKB1-dependent and -independent manner. Furthermore, administration of X-3 resulted in activation of AMPK and its downstream target, acetyl-CoA carboxylase (ACC) in the hypothalamus, liver, muscle and adipose tissues of C57BL/6 mice. An 80 mg/kg X-3 was more potent than metformin at 500 mg/kg in the hypothalamus, and interscapular fat tissues, potent than MF at the same dose in the liver. Thus, we conclude that X-3 is a promising new class of AMPK activating drug, and can potentially be used in the treatment of type 2 diabetes.

1. Introduction

Mangiferin (MF) is a xanthonoid found in mangoes and *Anemarrhena asphodeloides* rhizomes (Miura et al., 2001). Mangiferin has been used in India for the treatments of arteriosclerosis, coronary heart disease and diabetes. It has been shown that MF exhibits antidiabetic (Ichiki et al., 1998; Muruganandan et al., 2005; Yoshikawa et al., 2001), hypolipidemic and antiatherogenic properties (Guo et al., 2011; Muruganandan et al., 2002, 2005) by reducing plasma total cholesterol, triglycerides, low density lipoprotein-cholesterol (LDL-C) and increasing high density lipoprotein (HDL-C) (Muruganandan et al., 2005). Niu et al. has recently reported that MF decreased plasma FFA in hyperlipidemic rats and activated AMPK in liver, whereas there is no sufficient evidence to show that MF could alone activate AMPK at the cellular level (Niu et al., 2012). Moreover the major shortcomings of MF are its poor solubility and oral bioavailability (Cai et al., 2010).

AMPK is a major cellular energy sensor and a master regulator of metabolic homeostasis (Viollet et al., 2009; Zhang et al., 2009). AMPK is a heterotrimeric protein kinase consisting of a catalytic (α) and two regulatory subunits (β and γ) (Hardie et al., 2006; Viollet et al., 2006). AMPK are activated by two distinct signals: a Ca^{2+}-dependent pathway mediated by calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) and an AMP-dependent pathway mediated by LKB1 (Sanders et al., 2007). Under conditions of energy depletion, AMPK inhibits ATP-consuming pathways (e.g., fatty acid synthesis, cholesterol synthesis, protein synthesis and gluconeogenesis) and stimulates ATP-generating processes (e.g., fatty acid oxidation and glycolysis), thus restoring overall cellular energy homeostasis (Carling, 2004; Zhang et al., 2009). In addition, AMPK activation acutely increases glucose uptake (via Glut1 and Glut4) and glycolysis (Hue et al., 2002; Jing and Ismail-Beigi, 2006; Jones and Dohm, 1997). In lipid metabolism, AMPK activation results in the phosphorylation and inactivation of ACC (Carling et al., 1987), a direct AMPK substrate, leading to decreased conversion of acetyl-CoA to malonyl CoA. Malonyl CoA allosterically inhibits carnitine palmitoyl-CoA transferase (CPT1), the rate-limiting step in transport of long chain acyl-CoAs into mitochondria for oxidation (Lochhead et al., 2000; McGarry and Brown, 1997). Therefore, a reduction in malonyl CoA levels increases fatty acid oxidation.

AMPK, independently of insulin, is able to phosphorylate Akt substrate AS160, while inhibition of AS160 is believed to allow increased GLUT4 membrane localization and glucose uptake (Sano et al., 2007).

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In addition, AMPK inhibits the mTOR/p70S6K pathway and reduces IRS-1 phosphorylation on serine, resulting in the overphosphorylation of Akt and AS160, and then, increasing insulin sensitivity (Lansey et al., 2012; Sano et al., 2007; Wang et al., 2007).

Given the aforementioned critical actions of AMPK on glucose and lipid metabolism, targeting AMPK activation appears to be an attractive therapeutic strategy for the treatment of *type 2 diabetes mellitus (*T2DM) and related metabolic disorders. Two classes of commonly used insulin-sensitizing drugs, thiazolidinediones and biguanides, exert their therapeutic effects, at least in part, by activating AMPK (Nawrocki et al., 2010; Zhou et al., 2001). A number of natural products, including alkaloids, bitter melon extracts, berberine, and resveratrol, have been also found to activate AMPK (Hay and Sonenberg, 2004). Resveratrol has been shown to lower the blood sugar levels in both Phase Ib and Phase IIa clinical trials (Sirtris Pharmaceuticals, 2008, 2009).

In the current study we explored whether derivatization of MF, X-3, could improve in vivo effects in the treatment of insulin-resistant diabetic mice and delineated modes of action.

2. Materials and methods

2.1. Reagents

Mangiferin, 5-aminoimidazole-4-carboxamide ribonucleoside, Compound C, STO-609, and pioglitazone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Wortmannin was from Selleck Chemicals (Houston, TX, USA). AMPK, pAMPK (Thr 172), ACC, pACC (Ser 79), AKT, pAKT (Ser 473) antibodies were from Cell Signaling Technology (Danvers, MA, USA). GLUT1, GLUT4 antibodies were from Abcam PLC (Cambridge, UK).

2.2. Animal studies

Mouse experiments were conducted in 6- to 8-week-old female db/db (C57BL/KsJ) and db/(+) (C57BL/KsJ) mice (Qingzilan Tcchnology Co. Ltd, Nanjing, China) and normal lean littermates (C57BL/6J). Experimental animals unless noted were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China). All animal procedures were performed in accordance with the guidelines of the institutional animal care and use committee of the Second Military Medical University. X-3 was administered once daily by oral gavage for 8 weeks at 40, 80, and 120 mg/kg, respectively. One percent carboxymethyl cellulose sodium in distilled water was used as the vehicle. Pioglitazone (Pio) 75 mg/kg/d was used as a positive control. Blood samples were obtained by tail snipping. Six-hour fasting plasma glucose, body weight, and food consumption were monitored weekly. Plasma insulin levels were measured by enzyme-linked immunosorbent assay using kits from Mercodia (St. Charles, MO). Serum non-esterified fatty acid (NEFA) was measured enzymatically using Wako reagents (Richmond, VA).

2.3. Intraperitoneal glucose tolerance tests

Mice were fasted for 6 h before glucose tolerance tests. Intraperitoneal glucose load was administered at 2 g/kg of body weight. Glucose levels were measured by tail bleeds.

2.4. Determination of serum and liver triglyceride content

Liver triglyceride content was assayed as described (Atkinson et al., 2003). Liver and serum triglyceride contents were quantified colorimetrically with the enzymatic assay kit L-Type Triglyceride M (Wako Pure Chemical Industries, Richmond, VA).

2.5. Determination of adipocyte size

Total adipocyte area was manually traced and analyzed with image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). White adipocyte area was measured in more than 200 cells per mouse in each group according to methods described previously (Kubota et al., 1999).

2.6. Immunohistochemistry

A commercial staining kit was utilized following the manufacturer's instructions (SuperPicture™ 3rd Gen IHC Detection Kit, Invitrogen). Primary antibodies: Guinea Pig anti-insulin (DAKO Co., Carpinteria, CA, USA) was used at 1:50 for immunohistochemistry. Morphometric evaluation of the β-cell area was performed on insulin-stained sections using Image-Pro Plus 6.0 as described (Conarello et al., 2003).

2.7. Acute Toxicity Study

Experiments were conducted in ICR mice (18–22 g, n = 20) and Wistar rats (180–220 g, n = 20), half male and half female. Mice were treated with X-3 (3.57 g/kg/d p.o.), rats were given X-3 (1.785 g/kg/d p.o.). Vehicle control animals were administered the same volume of 0.5% carboxymethyl cellulose sodium in distilled water. The treatment period is 7 days. All animals were observed daily for mortality and signs of toxicity such as changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, autonomic activity, changes in gait, posture and response to handling, as well as the bizarre behavior during the entire period of the study.

2.8. Cell lines and cell culture

Murine 3T3-L1 preadipocytes, LKB1-deficient Hela cells, and HEK293 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

2.9. The 3T3-L1 differentiation and Oil Red O staining

Culture and differentiation of 3T3-L1 cells were described previously (Choi et al., 2009). In brief, the cells were grown and maintained in high-glucose DMEM containing 10% FBS in a 5% CO2 environment. The cells were allowed to grow for 2 days postconfluency and then differentiated by the addition of IBMX (500 μM), dexamethasone (1 μM), and insulin (10 μg/ml) for 3 days. The medium was changed every 2 days (Supplementary Fig. S1).

Fat staining with Oil Red O was described previously (Choi et al., 2009). 3T3-L1 cells were fixed with 10% formaldehyde for 30 min and then stained with Oil Red O for 2 h, followed by washing with 60% methanol.

2.10. Glucose uptake assay

2-Deoxyglucose uptake was estimated in a 96-well plate by an enzymatic NADPH amplifying system assay (ab136955, Abcam, UK). Briefly, 3T3-L1 preadipocytes or adipocytes were serum starved for overnight, and then the cells were incubated with 100 μl KRPH buffer containing 2% BSA for 40 min, then treated with indicated compounds for 2 h at 37 °C. Compound C (CC, 40 μM) was added 30 min before the initiation of treatment. Wortmannin (W, 300 nM) was added 1 h before the end of the treatment. After the addition of 100 μl low-glucose DMEM (21885, Life Technologies) containing 1 mM 2-deoxyglucose for 20 min, cells were washed 3 times with PBS, and lysed to prepare an 50 μl reaction system. For 3T3-L1 preadipocytes, cell lysates were diluted 1:1, for adipocytes, they were diluted 1:10. After a series of reactions, samples were measured at