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Original Contribution

Ankaflavin: a natural novel PPARγ agonist upregulates Nrf2 to attenuate methylglyoxal-induced diabetes in vivo

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ABSTRACT

Ankaflavin (AK) is an active compound having anti-inflammatory, anti-cancer, antiatherosclerotic, and hypolipidemic effects. We have previously reported that AK acts as an antioxidant and antidiabetic drug; however, the mechanism by which AK prevents diabetes remains unknown. Hyperglycemia is associated with protein glycation, which produces advanced glycation end-products (AGEs). Methylglyoxal (MG)—a metabolite of carbohydrates—is believed to cause insulin resistance by inducing inflammation and pancreas damage. In this work, diabetes was induced in Wistar rats (4 weeks of age) by treating them with MG (600 mg/kg bw) for 4 weeks. We observed that AK (10 mg/kg bw) exerted peroxisome proliferator-activated receptor-γ (PPARγ) agonist activity, thereby enhancing insulin sensitivity (as indicated by hepatic GLUT2 translocation, PTP1B suppression, and glucose uptake) by downregulating blood glucose and upregulating pancreatic and duodenal homeobox-1 and Maf-A expression and increasing insulin production in MG-induced rats. However, these effects were abolished by the administration of GW9662 (PPARγ antagonist), but the expression of hepatic heme oxygenase-1 (HO-1) and glutamate–cysteine ligase (GCL) was not suppressed in MG-induced rats. Therefore, the nuclear factor erythroid-related factor-2 (Nrf2) activation was investigated. AK did not affect hepatic Nrf2 mRNA or protein expression but significantly increased Nrf2 phosphorylation (serine 40), which was accompanied by increased transcriptional activation of hepatic HO-1 and GCL. These data indicated that AK protected rats from oxidative stress resulting from MG-induced insulin resistance. In contrast, these effects were not detected when the rats were treated with the antidiabetic drug rosiglitazone (10 mg/kg bw). Moreover, we found that AK did not inhibit the generation of AGEs in vitro; however, the glutathione (GSH) levels in liver and pancreas of MG-induced rats were elevated in rats administered AK. Therefore, we believe that GSH may lower the MG level, which attenuates the formation of AGEs in the serum, kidney, liver, and pancreas of MG-induced rats. We also found that AK treatment reduced the production of inflammatory factors, such as tumor necrosis factor-α and interleukin-1β. Taken together, the results of our mechanistic study of MG-induced rats suggest that the protective effects of AK against diabetes are mediated by the upregulation of the signaling pathway of Nrf2, which enhances antioxidant activity and serves as a PPARγ agonist to enhance insulin sensitivity.

Hyperglycemia is associated with protein glycation, and advanced glycation end-products (AGEs) are generated by nonenzymatic reactions between carbohydrates and proteins [1–3]. AGEs have a propensity to generate free radicals and undergo autoxidation to generate other reactive intermediates, thereby inducing diabetes [4]. Methylglyoxal (MG) is also known as 2-oxopropanal, pyruvaldehyde, pyruvic aldehyde, 2-ketopropionaldehyde, acetylformaldehyde, propanedione, or propionaldehyde. MG is a highly reactive dicarbonyl metabolite produced during glucose metabolism [5], and it is a major precursor of AGEs that are involved in the pathogenesis of diabetes and inflammation. Studies have suggested that AGEs and MG can generate large amounts of proinflammatory cytokines; these findings are reportedly related to the modulation of inflammatory cytokines through oxidative stress [6–10]. Oxidative stress is increased during diabetes and hyperinsulinemia; reactive oxygen species have been reported to be generated as a result of hyperglycemia, which causes many of the secondary complications of diabetes [5,8,10].

Recent studies show that acute MG administration to Sprague–Dawley rats causes glucose intolerance and reduces adipose tissue insulin-stimulated glucose uptake (MG 50 mg/kg bw; iv) and results in pancreatic dysfunction (MG 60 mg/kg bw; infusion) [11,12]. However, the in vitro studies have not established whether MG is the cause or an effect of diabetes [12], although

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MG invariably induces inflammation by the formation of AGEs and activation of the receptor for AGEs (RAGE) [8–10].

Under physiological conditions, glyoxalase converts MG to D-lactate in a reaction dependent upon reduction by glutathione. MG is also detoxified by the conversion to D-lactoylglutathione and D-lactate, which is catalyzed in the cytosol of all cells by glyoxalases I and II, suggesting that glyoxalase I completely inhibits the hyperglycemia-induced formation of AGEs; thus, the role of MG in the formation of AGEs is vital [13]. In healthy humans, plasma levels of MG are ≤ 1 μM; however, these levels are elevated two- to fourfold in patients with diabetes [14].

MG (60 mg/kg bw, infusion) efficiently induces diabetes in SD rats [12]. The administration of MG by gavage is considered suitable for inducing chronic diabetes and serves as a method to assess the anti-inflammatory activity of test drugs. However, to our knowledge, the induction of inflammation by orally administered MG has not been studied. Therefore, in this study, we attempted to induce diabetes through oral administration of MG (600 mg/kg bw). Further, the ability of various antioxidants, such as quercetin and phenolic acid, to activate nuclear factor erythroid-related factor 2 (Nrf2) and attenuate oxidative damage has been evaluated in previous studies [15,16]; the antioxidant silymarin was found to inhibit the generation of AGEs, thereby improving the symptoms of diabetes [17].

Rosiglitazone (Rosi)—a peroxisome proliferator-activated receptor-γ (PPARγ) ligand—belongs to the thiazolidinedione (TZD) class of antidiabetic drugs. The activation of PPARγ is known to affect pancreatic β-cell function and insulin production [18]. However, TZD use is limited by side effects that are mediated through ectopic activation of PPARγ, including weight gain, edema, and increased risk of fractures. These side effects of TZDs have limited their potential application for a variety of inflammatory and obesity-related metabolic diseases. The future widespread use of TZDs as insulin sensitizers and for treatment of other metabolic and inflammatory diseases clearly requires a breakthrough that would allow better therapeutic profiles [19].

Monascus species have been used as traditional food fungi in eastern Asia for several centuries. *Monascus*-fermented rice was gradually developed as a popular functional food for hypolipidemia. Ankaflavin (AK) is a water-insoluble polyketide metabolite isolated and identified from *Monascus.* This yellow pigment has an azaphilonoid structure. Recently, it was proven to be the functional ingredient for anti-inflammatory actions [20]. AK has been reported to downregulate hyperlipidemia and lipid peroxidation; and no toxicity or side effects were found in our previous study [21]. However, the interactions of AK with other compounds are as yet unknown. In this study, we found that AK acts as a novel natural PPARγ agonist. However, the influence of AK on PPARγ activity involved in the prevention of MG-induced diabetes by PPARγ agonists in vivo remained unclear.

Experimental procedures

Chemicals

Glucose, MG, insulin, rosiglitazone, and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) was from Invitrogen (Carlsbad, CA, USA). PPARγ antagonist (GW9662) and glutathione (GSH) ELISA kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-glucose transporter 2 (GLUT2) antibody, anti-protein-tyrosine phosphatase 1B (PTP1B) antibody, anti-pancreatic and duodenal homeobox-1 (PDX-1) antibody, anti-Maf-A antibody, and anti-CCAAT/enhancer binding protein β (C/EBPβ) antibody for rat were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-p-Nrf2, anti-Nrf2, and anti-heme oxygenase-1 (HO-1) antibodies for rat were purchased from Bioss (Woburn, MA, USA). Anti-glutamate–cysteine ligase (GCL) antibody for rat was purchased from Epitomics (Burlingame, CA, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). RPMI 1640 medium, L-glutamine, sodium pyruvate, and antibiotics (penicillin/streptomycin) were purchased from Gibco (Grand Island, NY, USA). Preparation of AK ($>95\%$ purity) was identified by nuclear magnetic resonance (Varian Gemini, 200 MHz, FT-NMR; Varian, Palo Alto, CA, USA) and electrospray ionization–mass spectrometry (Thermo Electron, Waltham, MA, USA) analysis.

Agonist activity of AK for PPARγ

The PPARγ agonist activity of AK was confirmed by LanthaScreen TR-FRET PPARγ coactivator assay kit (Invitrogen). The assay was carried out following the normative manual.

Animals and diabetes induction

Male Wistar rats (4 weeks of age) were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Animals were acclimatized for 1 week before use; they were divided at random into six treatment groups (six rats per group) and provided with food and water ad libitum. Animals were subjected to a 12-h light/dark cycle with a maintained relative humidity of 60% and a temperature at 25 °C (protocol complied with guidelines described in the *Animal Protection Law,* amended on 17 January 2001, Hua-Zong-(1)-Yi-Tzi-9000007530, Council of Agriculture, Executive Yuan, Taiwan, ROC). Rats were divided into the following treatment groups: (1) control (saline administration), (2) MG (600 mg/kg bw), (3) MG + rosiglitazone (a synthetic PPARγ agonist; 10 mg/kg bw), (4) MG + rosiglitazone + GW9662 (a synthetic PPARγ antagonist; 10 mg/kg bw), (5) MG + ankaflavin (a novel natural PPARγ agonist; 10 mg/kg bw), and (6) MG+ankaflavin+GW9662. MG, Rosi, and AK were administered to Wistar rats by oral administration for 28 days, and GW9662 was administered by intraperitoneal (ip) injection for 28 days.

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

OGTT and ITT were performed at day 24 and day 26. After overnight fasting, an OGTT was performed. Briefly, rats were anesthetized, a basal blood sample was collected, and an oral glucose load (2 g/kg bw) was given by oral administration. Subsequently, blood was collected (0–120 min), and the blood glucose levels were determined using a glucose assay kit (BioAssay Systems, Hayward, CA, USA) and insulin levels were measured with a rat insulin ELISA kit (Mercodia, Winston Salem, NC, USA). For ITT, rats were given an ip injection of insulin solution (0.5 U/kg bw) after 4 h of fasting. Blood glucose level was determined.

Glucose uptake of hepatic cells

Hepatic cell glucose uptake was performed according a previous study [22]. The 2-NBDG was chosen as a glucose uptake indicator.

Isolation of hepatic plasma membrane for GLUT2

Plasma membrane extracts of liver were isolated using an isolation kit (BioVision, Mountain View, CA, USA).