



Kaempferol alleviates insulin resistance via hepatic IKK/NF- κ B signal in type 2 diabetic rats

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ABSTRACT

Recent studies show that inflammation underlies the metabolic disorders of insulin resistance and type 2 diabetes mellitus. Since kaempferol, a naturally occurring flavonoid, has been described to have potent anti-inflammatory properties, we investigated whether kaempferol could ameliorate insulin resistance through inhibiting inflammatory responses. The model of diabetic rat was induced by 6-week high-fat diet plus streptozotocin. Animals were orally treated with kaempferol (50 or 150 mg/kg) and aspirin (100 mg/kg) for 10 weeks. The results showed that kaempferol ameliorated blood lipids and insulin in an dose-dependent manner. Kaempferol effectively restored insulin resistance induced alteration of glucose disposal by using an insulin tolerance test and the euglycemic–hyperinsulinemic clamp method. Western blotting results showed that KPF inhibited the phosphorylation of insulin receptor substrate-1 (IRS-1), I κ B kinase α (IKK α) and I κ B kinase β (IKK β). These effects were accompanied with reduction in nucleic and cytosol levels of nuclear factor kappa- β (NF- κ B), and further tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) levels. Aspirin had similar effects. These results provide in vivo evidence that kaempferol-mediated down-regulation of IKK and subsequent inhibition of NF- κ B pathway activation may be associated with the reduction of hepatic inflammatory lesions, which is contributing to the improvement of insulin signaling defect in diabetes.

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1. Introduction

Inflammation is independently and collectively correlated with the development of insulin resistance, type 2 diabetes mellitus (T2DM) and metabolic syndrome, and subacute hepatic inflammation through NF- κ B activation causes insulin resistance both locally in liver and systemically [1]. Accumulating evidences reveal that I κ B kinase β (IKK β) plays a crucial role in the pathogenesis of insulin resistance in obesity and type II diabetes mellitus [2,3]. The anti-diabetic effects of salicylate have been known for years which refer to the IKK- β /nuclear factor kappa- β (NF- κ B) pathway [3]. The I κ B kinase (IKK) complexes, including IKK α and IKK β , are essential to control the stimulated activation of NF- κ B, and the activation of IKK much depends on phosphorylation of its IKK β subunit [4]. NF- κ B is a nuclear transcriptional activator that can induce a large range of transcription genes of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-1 α (IL-1 α), and interleukin-1 β (IL-1 β), which can induce the development of insulin resistance [5].

Kaempferol (KPF, Fig. 1) is a typical dietary flavonoid which was found to have anti-diabetic properties of anti-oxidative [6], anti-inflammatory [7] and promoting functions of pancreatic β -cell [8]. In addition, some reports have demonstrated that KPF could work as a

regulator of lipid metabolism [9]. Several studies demonstrated that KPF could stimulate insulin-dependent glucose uptake [10,11] and other flavonoids including quercetin [12] and rutin [13] were reported to ameliorate insulin resistance through inhibiting inflammatory responses. It is significant that previous studies raised the possibility that KPF suppressed the inflammatory IKK and NF- κ B, thereby significantly decreasing the proinflammatory cytokines [7,14,15]. So we hypothesize that KPF may possess therapeutic properties for insulin resistance by inhibiting IKK/NF- κ B signal. Researches about the anti-inflammatory effect of KPF on insulin resistance in T2DM and its mechanisms have not been reported previously. Thus, the present study was aimed to investigate the protective effect and mechanisms of KPF on the pathological process of insulin resistance in diabetic rats.

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley (SD) rats (140–160 g) were obtained from the Laboratory Animal Center, ChongQing Medical University (Chongqing, China). Rats were used after at least one-week quarantine and acclimation period. They were kept in the departmental animal house under controlled conditions of temperature of 23 °C $\pm$ 2 °C, relative humidity of 60% $\pm$ 5%, a light:dark cycle of 12 h each, and given free access to normal pellet diet and water. The guidelines of the committee for the

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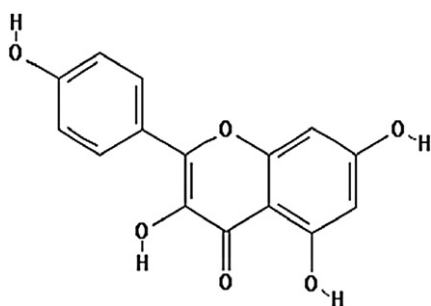


Fig. 1. The molecular structure of kaempferol.

purpose of control and supervision of experiments on animals (CPCSEA), Govt. of China were followed for conducting the study.

2.2. Drugs and reagents

Kaempferol (KPF, purity 98%) was purchased from ZeLang Medicine Science and Technology Co., Ltd. (Nanjing, China). Streptozotocin (STZ) was purchased from Sigma Chemical (St Louis, Mo, USA). Normal pellet diet (NPD) and high-fat diet (HFD) were obtained from the Laboratory Animal Center of ChongQing Medical University (Chongqing, China). Aspirin was purchased from Bayer (Leverkusen, Germany). Anti-IRS1 antibody (bs-0172R), anti-phospho-IRS-1 antibody (Ser307) (bs-2736R), anti-IKK- α antibody (bs-2907R), anti-IKK- β antibody (bs-4880R), anti-phospho-IKK- α (Ser180) + IKK- β (Ser181) antibody (bs-3236R), and anti-NF- κ B p65 antibody (bs-0465R) were all purchased from Bioss (Beijing, China). Anti-IL-6 antibody (sc-1265) and anti-TNF- α antibody (sc-1350) were purchased from ZSGB-BIO (Beijing, China).

2.3. Development of T2DM and experimental design

After acclimation, the rats were fed normal pellet diet (NPD) or high-fat diet (HFD) for the initial period of 6 weeks. The compositions of HFD were as follows: powdered NPD 57.3%, sugar 20%, lard 10%, cholesterol 2.5%, egg yolk powder 10%, and sodium cholate 0.1% [16]. After 6-week dietary feeding, rats fed HFD were injected intraperitoneally with freshly prepared streptozotocin (STZ, Sigma, St. Louis, MO, USA) dissolved in 0.1 mmol/L cold citrate buffer (pH 4.2) at the dose of 30 mg/kg body weight [17,18]. Rats fed NPD were only given the citrate buffer. Blood glucose levels were monitored by glucose meter (Accu-Chek Active, Roche). After 72 h, rats with fasted blood glucose level above 200 mg/dL were categorized as diabetic model [16]. The diabetic model rats were further randomly divided into four groups: diabetic control group (DC; $n = 10$), low-dose kaempferol group (KPF + L; 50 mg/kg; $n = 10$), high-dose kaempferol group (KPF + H; 150 mg/kg; $n = 10$) and aspirin group (aspirin; 100 mg/kg; $n = 10$). The rats fed NPD were considered as the normal control group (NC; $n = 10$), which were given vehicle (CMC-Na, 1 mL/100 g) by intragastric administration. The animals were allowed to feed on their respective diets for 10 weeks.

2.4. Insulin tolerance test

An intraperitoneal insulin tolerance test was performed at the end of the 8th week. Rats fasted over night were injected intraperitoneally with recombinant human insulin injection (1 IU/kg BW; Humulin R, Eli Lilly and Company, Indianapolis, IN). Animals were not anesthetized for this procedure with removing water and food. The blood samples were taken from the tail vein at 0 (prior to insulin administration), 15, 30, 45 and 60 min after administration with insulin.

2.5. Euglycemic–hyperinsulinemic clamp

Five rats were selected randomly from each group for an euglycemic–hyperinsulinemic clamp experiment following an overnight fast. The rats were anesthetized with intraperitoneal chloral hydrate (300 mg/kg BW), and the right jugular vein and left femoral artery were catheterized for infusion and blood sampling, respectively. The glucose and insulin solutions were stored in double channel syringe pump and were joined by pipe tee to the jugular catheter. Recombinant human insulin injection (Humulin R, Eli Lilly and Company, Indianapolis, IN) was infused at a constant rate of 20 MU/kg/min. Blood samples (5 μ L) for blood glucose were taken from the femoral artery. The blood glucose concentration was clamped at the basal level by estimating blood glucose concentration at 5 min intervals in samples taken from the femoral artery and adjusting the rate of infusion of a 20% glucose solution. When blood glucose had stabilized for at least 20 min, we defined this condition as steady state. Metabolic clearance rate for glucose (MCR, mL/kg/min) was obtained from glucose infusion rate (GIR, mg/kg/min) divided by the corresponding blood glucose concentration taken as a response parameter indicating the whole body insulin sensitivity [19,20].

After the euglycemic–hyperinsulinemic clamp experiment, the remaining rats were anesthetized and sacrificed following animal ethical guidelines. The liver was removed and washed in cold saline, shock frozen in liquid nitrogen and stored at -80°C for molecular analyses.

2.6. Serum biochemical assays

Fasting blood glucose (FBG) and body weight were measured at 0th, 3rd and 9th weeks of the experiment. Blood samples collected from the tail vein without anticoagulant were centrifuged at 3000 g for 10 min at 4°C , and the serum was stored at -20°C . The serum total cholesterol (TC), total triglyceride (TG), total low density lipoprotein cholesterol (LDL-C) and nonesterified fatty acids (NEFAs) were assayed spectrophotometrically using standard kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were examined for liver function with kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Immunoreactive insulin was determined by a specific RIA kit (Beijing North Institute of Biology Technology, Beijing, China). Insulin resistance was evaluated by the homeostasis model assessment insulin resistance (HOMA-IR) index, calculated as follows: $\text{FPG (mg/dL)} \times \text{IRI (}\mu\text{U/mL)} / 405$ [20,21].

2.7. Enzyme-linked immunosorbent assay (ELISA)

Serum levels of TNF- α and IL-6 were quantified using commercially available ELISA kits (Beijing Keyingmei Biotechnology Company, Beijing, China). Assays were performed following the protocols recommended by the manufacturer.

2.8. Western blotting analysis

Total proteins were isolated from the homogenate of liver tissue using the RIPA buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% sodium deoxycholate, 0.1% SDS, 0.15 mmol/L NaCl and 1% NP-40) (BI Yuntian Co., China). For NF- κ B p65 detection, cytosolic and nuclear fractions were extracted using specific kits (Nanjing Keygen Biotech, Nanjing, China). Protein concentrations were assayed using the bicinchoninic acid (BCA) protein assay kit (BI Yuntian Co., China). Subsequently, equal amounts of protein (30 μ g) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and then, transferred onto a PVDF membrane (Millipore Co., USA). The membranes were incubated with primary antibodies: IRS-1 (137 kD), p-IRS-1 (Ser307), IKK α (85 kD), IKK β (87 kD), p-IKK α (Ser180) + IKK β (Ser181), and NF- κ B p65 (61 kD) overnight at 4°C followed by blocking with 5% skimmed milk 2 h at room temperature with gentle agitation. Then

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