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Kaempferol Regulates the Lipid-Profile in High-Fat-Diet Fed Rats through an Increase in Hepatic PPARα Levels

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Abstract

The aim of this study was to investigate the anti-obesity and antihyperlipidemic effects of the flavonoid kaempferol (3, 5, 7, 4'-tetrahydroxyflavone). After being fed a high-fat diet (HFD) for two weeks, rats were dosed orally with kaempferol (75, 150 or 300 mg/kg) or fenofibrate (100 mg/kg) once daily for eight weeks. Fenofibrate is an antilipemic agent that exerts its therapeutic effects through activation of peroxisome proliferator-activated receptor α (PPARα). Kaempferol (300 mg/kg/day) produced effects similar to fenofibrate in reducing body weight gain, visceral fat-pad weights, plasma lipid levels, as well as the coronary-artery-risk and atherogenic indcies of HFD-fed rats. Kaempferol also caused dose-related reductions in hepatic triglyceride and cholesterol content, and lowered hepatic lipid droplet accumulation and the size of epididymal adipocytes in HFD-fed rats. Kaempferol and fenofibrate reversed the HFD-induced down-regulation of hepatic PPARα. HFD-induced reductions in the hepatic levels of acyl-CoA oxidase (ACO) and cytochrome P450 isoform 4A1 (CYP4A1) proteins were reversed by kaempferol and fenofibrate. The elevated expression of hepatic sterol regulatory element binding proteins (SREBPs) in HFD-fed rats were lowered by kaempferol and fenofibrate. These results suggest that kaempferol reduced the accumulation of visceral fat and improved hyperlipidemia in HFD-fed obese rats by increasing lipid metabolism through the down-regulation of SREBPs and promoting the hepatic expression of ACO and CYP4A1, secondary to a direct up-regulation hepatic PPARα expression.

Keywords

kaempferol

high-fat diet

peroxisome proliferator activated receptor α

acyl-CoA oxidase

cytochrome P450 isoform 4A1

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Abbreviations

ACO: acyl-CoA oxidase

AI: atherogenic index

BW: body weight

CRI: coronary risk index

CYP4A1: cytochrome P450 isoform 4A1

FFAs: free fatty acids

H&E: hematoxylin and eosin

HDL-C: high density lipoprotein cholesterol

HFD: high-fat diet

LDL-C: low density lipoprotein cholesterol

PPAR: peroxisome proliferators activated receptor

RCD: regular rat diet

SREBP: sterol regulatory element binding protein

WAT: white adipose tissues

TC: total cholesterol

TG: triglyceride

Introduction

Obesity is a common chronic disorder of carbohydrate and fat metabolism, characterized by excessive fat deposition in adipose tissue and other internal organs, such as liver, heart, skeletal muscle, and pancreatic islets [1]. Obesity remains a major global public health issue because of its increasing prevalence, which cuts across issues of sex, age-group, ethnicity, or race [1]. Obesity alone can induce all the symptoms of metabolic syndrome, which is associated with many additional health problems, including increased risk of insulin resistance, non-alcoholic fatty liver, atherosclerosis, degenerative disorders such as dementia, some immune-mediated disorders such as asthma, and certain cancers [2].

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Pharmacological approaches to weight control have become an overriding priority [3]. Current trends for obesity management involve multiple pharmacological strategies, including blocking nutrient absorption, modulating fat metabolism, regulating adipose signals, and modulating the satiety center. However, these approaches have been associated with several serious adverse effects in the clinic, including adverse gastrointestinal effects and significant unfavorable cardiovascular effects [3]. As a result, a much safer therapeutic approach is necessary.

At present, most of the beneficial health effects of flavonoids are attributed to their ability to scavenge free radicals and chelate metals. Kaempferol (3, 5, 7, 4'-tetrahydroxyflavone), quercetin (3, 5, 7, 3', 4'-pentahydroxyflavone), and myricetin (3, 5, 7, 3', 4', 5'-hexahydroxyflavone) are structurally related members of the flavonol family, with four hydroxyl groups in kaempferol, five in quercetin, and six in myricetin. Beneficial effects of quercetin on lipid and lipoprotein profiles and the electrocardiogram have been reported in isoproterenol-treated, cardiotoxic male Wistar rats [5]. Myricetin has also been shown to normalize hypertriglyceridemia in diabetic rats [6]. Because of the structural similarity of kaempferol to quercetin and myricetin, it is of special interest to understand whether kaempferol has a positive influence on plasma lipids in a diabetic state.

Diet-induced obesity in rodents has been used as a model to investigate the interactions between the environment and genetics. Rats fed a high-fat diet (HFD) become obese and show distinctive visceral adiposity, dyslipidemia, hyperinsulinemia, and hepatic steatosis, which are typical of human obesity [7]. Therefore, this study investigated the effects of kaempferol on body fat and lipid profiles in rats with diet-induced obesity and sought possible mechanisms of action.

Materials and Methods

Animal models and treatment protocols

Male Wistar rats, 8 weeks of age, were obtained from the National Laboratory Animal Center (Taipei, Taiwan). They were maintained in a temperature-controlled room ($25 \pm 1°C$) on a 12h:12h light-dark cycle (lights on at 06:00 h) in the animal center (Tajen University, Ping Tung Shien, Taiwan). Food and

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water were available *ad libitum*. Regular rat chow diet (RCD, #D12450B, Research Diets, New Brunswick, NJ) with 20 kcal% protein, 70 kcal% carbohydrate, and 10 kcal% fat from lard was used as the maintenance and control diet. A purified ingredient HFD with 20 kcal% protein, 35 kcal% carbohydrate, and 45 kcal% fat primarily from lard (#D12451, Research Diets) was used to induce a rapid increase in body weight (BW) and obesity [8]. The caloric density of the control diet was 3.85 kcal/g; that of the HFD was 4.73 kcal/g. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. These studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Tajen University (approval number: IACUC 99-16; approval date: September 9, 2010).

After being fed a HFD for two weeks, rats were dosed by oral gavage once per day for eight weeks with kaempferol (purity ≥ 97.0%, Sigma-Aldrich, St. Louis, MO) doses of 75, 150, and 300 mg/kg in a volume of 1.5 mL/kg distilled water. Another group of HFD-fed rats was treated orally for eight weeks with 100 mg/kg/day fenofibrate (purity ≥ 99.0%; Sigma-Aldrich). The dose of fenofibrate was based on studies with long term fenofibrate treatment in rats [9]. A vehicle-control group of HFD-fed and RCD-fed rats was treated with 1.5 mL/kg distilled water only over the same treatment period. Eight weeks after treatment with kaempferol or fenofibrate (total diet-fed period was 10 weeks), HFD-fed rats were weighed and anesthetized with sodium pentobarbital (30 mg/kg) administered intraperitoneally (i.p.), and blood samples were collected from the lateral tail vein. Feed and water were supplied *ad libutum* throughout the 10-week experimental period. Samples were centrifuged at $2,000 \times g$ for 10 minutes at 4°C. The plasma was then removed and placed into aliquots for the respective analytical determinations. After blood was collected, the liver and visceral and subcutaneous white adipose tissues (WAT) were removed, rinsed with physiological saline, weighed, and immediately stored at -70°C.

Biochemical parameter analysis

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Diagnostic kits for the measurement of plasma glucose (Cat. No. 10009582), total cholesterol (TC; Cat. No. 10007640), and triglycerides (TG; Cat. No. 10010303) were purchased from Cayman Chemical Company (Michigan, USA). The diagnostic kit to determine plasma and hepatic levels of high density lipoprotein cholesterol (HDL-C) was purchased from Bio-Quant Diagnostics (CA, USA; Cat. No. BQ 019CR), and low density lipoprotein cholesterol (LDL-C) levels were calculated using Friedewald's equation [10]. Plasma free fatty acid (FFA) levels were determined using an FFA-quantification kit obtained from Abcam plc (MA, USA; Cat. No. ab65341). All samples were analyzed in triplicate.

Determination of AI and CRI

Atherogenic index (AI) and coronary risk index (CRI) were calculated as LDL-C/HDL-C and TC/HDL-C, respectively [11, 12].

Extraction of hepatic lipids

A section of each liver was collected for lipid content analysis. The liver (1.25 g) was homogenized with chloroform/methanol (1:2, 3.75 mL), and then chloroform (1.25 mL) and distilled water (1.25 mL) were added to the homogenate and mixed well. After centrifugation (1,500 × g for 10 min), the lower clear organic phase solution was transferred into a new glass tube and then lyophilized. The lyophilized powders were dissolved in chloroform/methanol (1:2) as the hepatic lipid extracts and stored at -20°C for fewer than three days [13].

Hepatic pathological evaluation

Small pieces of hepatic tissues taken from experimental animals were fixed in 10% neutral formalin, dehydrated with alcohol, embedded in paraffin, and sectioned to a mean thickness of 4 μm. Hematoxylin and eosin- (H&E) stained tissues were examined histologically to evaluate the index of diabetic-induced necrosis. Liver biopsy was scored according to the following criteria: grade 0, no steatosis, normal liver; grade 1, < 25% of hepatocytes affected; grade 2, 26–50% of hepatocytes

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affected; grade 3, 51–75% of hepatocytes affected; and grade 4, >76% of hepatocytes affected [14].

Adipocyte pathological evaluation

Histological photomicrographs of adipose tissue were analyzed with a light microscope using the paraffin method. Fresh tissues were fixed immediately in Bouin's solution for 6 to 12 hours, and then fixed tissues were washed under running water. After being dehydrated through different grades of alcohol, the tissues were embedded in paraffin block at 60°C. Sections (8 μm) were cut and mounted on glass slides coated with an egg albumin, and the paraffin was removed using xylene and alcohol. The tissues were stained with H&E. After being dehydrated and cleared of alcohol and xylene, the glass slides were mounted in Canada Balsam. Photomicrographs were taken with a Zeiss Axiolab light microscope equipped with a Nikon Microflex HFX microscope camera. The sizes of epdidymal adipocytes were calculated using Image-Pro Plus 7.0 (Media Cybernetics, Maryland, USA).

Preparation of hepatic fractions

Hepatic fractions were prepared as described previously [15]. To prepare nuclear fractions, hepatic tissue was homogenized with ice-cold lysis buffer containing 5 mmol/l Tris-HCl (pH 7.5), 2 mmol/L $MgCl_2$, 15 mmol/L $CaCl_2$, and 1.5 mol/L sucrose, 0.1 mol/L dithiothreitol (DTT), and protease inhibitor cocktail. After centrifugation (10,500 × g for 20 minutes at 4°C), the pellet was suspended in extraction buffer containing 20 mmol/L 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (pH 7.9), 1.5 mmol/L $MgCl_2$, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, and 25% (v/v) glycerol, 0.1 mol/L DTT, and protease inhibitor cocktail. The mixture was placed on ice for 30 minutes. The nuclear fraction was prepared by centrifugation at 20,500 × g for 5 minutes at 4°C. The post-nuclear fraction was extracted from the liver of each rat as described below. In brief, hepatic tissue was homogenized with ice-cold lysis buffer (pH 7.4) containing 137 mmol/L NaCl, 20 mmol/L Tris-HCl, 1% Tween 20, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail solution in DMSO. The homogenate was then centrifuged at 2,000 × g for 10 minutes at 4°C. The

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protein concentration of each fraction was determined using a commercial kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Western Blot analyses

For the determination of peroxisome proliferators activated receptor (PPAR), sterol regulatory element binding protein (SREBP)-1, and SREBP-2, 30 mg protein of each nuclear fraction was resolved using 8% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred electrophoretically to a nitrocellulose membrane, blocked with 5% (w/v) skim milk solution for 1 hour, and then incubated with primary antibodies to PPARα (Santa Cruz Biotechnology, Inc., CA, USA; Cat. No. sc-1985), SREBP-1 (Santa Cruz Biotechnology, Inc.; Cat. No. sc-367), SREBP-2 (Santa Cruz Biotechnology, Inc.; Cat. No. sc-5603), or β-actin (Santa Cruz Biotechnology, Inc.; Cat. No. sc-130656) overnight at 4°C. After the blots were washed, they were incubated with goat anti-rabbit and/or goat anti-mouse IgG HRP-conjugated secondary antibody for 1.5 hours at room temperature. The blots were stripped with Restore Western Blot Stripping Buffer (CANDOR Bioscience GmbH, Wangen, Germany) for 15 minutes and incubated with the antibodies. In addition, 30 mg protein from each post-nuclear fraction for acyl-CoA oxidase (ACO; Santa Cruz Biotechnology, Inc.; Cat. No. sc-98499) and cytochrome P450 isoform 4A1 (CYP4A1; Santa Cruz Biotechnology, Inc.; Cat. No. sc-53248) was subjected to 10% SDS-PAGE. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (Fujifilm, Tokyo, Japan). Band densities were determined using ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to β-actin. The mean value for samples from the vehicle-treated RCD-fed group on each immunoblot, expressed in densitometry units, was adjusted to a value of 1.0. All experimental sample values were then expressed relative to this adjusted mean value.

Statistical analysis

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Data are expressed as the mean ± standard deviation (S.D.) for each group of animals at the number indicated in tables. Statistical analysis was performed with one-way analysis of variance (ANOVA). The Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. A P value < 0.05 was considered statistically significant.

Results

The BWs of HFD-fed rats in the drug-treated and vehicle-treated groups were monitored over the eight week treatment period (**Table 1**). At the end of treatment, the BW of kaempferol-treated rats was significantly lower than that of rats in the vehicle-treated group. Kaempferol significantly suppressed BW gain at both the moderate (150 mg/kg/day) and high doses (300 mg/kg/day). Similar results were seen in rats treated with fenofibrate (100 mg/kg/day; **Table 1**). No significant differences in daily food intake were observed among the groups over the experimental period, despite the slightly higher water intake observed in the vehicle-treated HFD-fed group as compared to the others (**Table 1**).

The weight of fat pads from HFD-fed rats in the drug-treated and vehicle-treated groups were assessed after the treatment period (**Table 1**). Epididymal WAT, perirenal WAT, mesenteric WAT, and inguinal WAT weights were lower in kaempferol-treated rats than in their vehicle-treated counterparts. A significant reduction in fat-pad weight was seen with both the moderate (150 mg/kg/day) and high doses (300 mg/kg/day) of kaempferol (P < 0.05). Similarly, after treatment with fenofibrate, epididymal, perirenal mesenteric, and inguinal fat pads were 27%, 32%, 31%, and 33% lower, respectively, than in their vehicle-treated counterparts.

The HFD caused elevated concentrations of plasma TC, TG, and LDL-C. The moderate (150 mg/kg/day) and high doses (300 mg/kg/day) of kaempferol significantly reduced plasma total TC levels (23.8% and 31.6% reduction, respectively) compared with vehicle-treated, HFD-fed rats (**Table 2**). All doses of kaempferol decreased plasma TG levels in HFD-fed rats (**Table 2**). The low (75 mg/kg per day), moderate, and high doses of kaempferol significantly reduced plasma LDL-C levels (17.6%, 35.8%, and 51.9% reductions, respectively) (**Table 2**). Plasma TC, TG, and LDL-C concentrations

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were reduced significantly by 38.2%, 49.7%, and 60.1%, respectively, in fenofibrate-treated, HFD-fed rats compared with vehicle-treated, HFD-fed rats (**Table 2**).

The plasma concentration of HDL-C in HFD-fed rats was reduced to 59.2% of the level in the RCD-fed group (**Table 2**). After 8 weeks of treatment with kaempferol (300 mg/kg/day) or fenofibrate, the plasma HDL-C concentration in HFD-fed rats was elevated to nearly that of the RCD-fed rats.

Plasma FFAs were significantly higher in vehicle-treated, HFD-fed rats compared to RCD-fed rats (**Table 2**). The plasma FFA level was reduced by 36.2% in HFD-fed rats treated with 300 mg/kg per day kaempferol, compared with their vehicle-treated counterparts (**Table 2**). Fenofibrate treatment reduced FFA concentrations in HFD-fed rats by 46.3% relative to the level in vehicle-treated, HFD-fed rats ($P < 0.05$) (**Table 2**).

Fenofibrate treatment arrested the elevation of AI and CRI LPL in HFD-fed rats (**Table 2**). Kaempferol treatment also caused a significant ($P < 0.05$) and dose-related reduction in the atherogenic and coronary artery risk indices in the HFD-fed rats when compared to the values recorded for their vehicle-treated counterparts (**Table 2**).

The hepatic TC level was significantly higher in HFD-fed rats than in rats from the RCD-fed group. TC levels were reduced by 25.6% in HFD-fed rats treated with 300 mg/kg per day kaempferol (**Table 2**). Similarly, kaempferol treatment (300 mg/kg per day) also produced a significant reduction in hepatic TG concentration, to 64.7% of that in vehicle-treated, HFD-fed rats (**Table 2**). Hepatic TC and TG levels were significantly reduced (by 38.1% and 47.4%, respectively) in fenofibrate-treated rats compared with vehicle-treated, HFD-fed rats (**Table 2**).

HFD-fed rats showed significantly greater hepatic lipid accumulation than RCD-fed animals (**Fig. 1A** and **1B**). The extent of hepatic lipid accumulation after 8 weeks in fenofibrate-treated, HFD-fed rats was similar to that in RCD-fed rats (**Fig. 1A** and **1C**). HFD-fed rats treated with 300 mg/kg/day kaempferol showed considerably lower hepatic lipid accumulation than their vehicle-treated counterparts (**Fig. 1B** and **1D**). The pathological grading of hepatic steatosis in each group is indicated in **Table 3**.

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The histological appearance of epdidymal adipocyte was irregular in HFD-fed rats compared to RCD-fed group; this morphological change did not appear in HFD-fed groups after 8-week of fenofibrate (100 mg/kg/day) treatment (**Fig. 2A, 2B and 2C**). The histological appearance of epdidymal adipocyte was more regular in HFD-fed rats treated with kaempferol (300 mg/kg/day) for 8 weeks (**Fig. 2D**). In addition, the sizes of epdidymal adipocytes were significantly bigger in HFD-fed group compared to RCD-fed group (**Fig. 2A and 2B**). After 8 weeks of treatment, the average size of epididymal adipocytes was approximately lower by 26.5% and 37.3%, respectively in kaempferol (300 mg/kg/day) or fenofibrate (100 mg/kg/day)-treated HFD-fed rats as compared with the vehicle-treated counterparts (**Fig. 2C and 2D; Table 3**).

The histological appearance of epdidymal adipocytes was irregular in HFD-fed rats compared with animals in the RCD-fed group. This morphological change was not evident in HFD-fed rats after fenofibrate treatment (**Fig. 2A, 2B, and 2C**). The histological appearance of epdidymal adipocytes in HFD-fed rats treated with 300 mg/kg/day kaempferol was not as normal as in fenofibrate-treated rats, but was better than in vehicle-treated controls (**Fig. 2D**). Epdidymal adipocytes were also significantly larger in HFD-fed compared to RCD-fed rats (**Fig. 2A and 2B**). The average size of epididymal adipocytes was reduced by approximately 26.5% and 37.3%, respectively, in HFD-fed rats treated with kaempferol (300 mg/kg/day) or fenofibrate compared with their vehicle-treated counterparts (**Fig. 2C and 2D; Table 3**).

Hepatic PPARα protein expression in HFD-fed rats was lower than that in RCD-fed animals, but was elevated significantly by treatment with kaempferol (300 mg/kg/day) (**Fig. 3**). In addition, the hepatic expression levels of ACO and CYP4A proteins in HFD-fed rats were markedly lower than in RCD-fed rats, but were significantly elevated in kaempferol-treated (300 mg/kg/day) HFD-fed rats (**Fig. 3**). Similar results were seen in rats treated with fenofibrate (**Table 3**).

The expression levels of hepatic SREBP-1 and SREBP-2 proteins in HFD-fed rats were significantly higher in RCD-fed rats. The levels were decreased by 53.7% and 49.3% in HFD-fed rats treated with kaempferol (300 mg/kg/day), relative to those in vehicle-treated, HFD-fed rats (**Fig. 3**). Hepatic

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SREBP-1 and SREBP-2 protein expression levels in HFD-fed rats treated with fenofibrate were 50.2% and 42.2%, respectively, lower than those in their vehicle-treated counterparts (**Fig. 3**).

Discussion

In the this study, BW loss in HFD-fed rats was accompanied by a depletion of body fat stores, since treatment with kaempferol also significantly reduced the weight of the visceral and subcutaneous WAT compared with that of vehicle-treated HFD-fed rats. Excessive growth of adipose tissue results in obesity, which involves two growth mechanisms: hyperplasia (cell number increase) and hypertrophy (cell size increase) [19]. The histological appearance of white adipocytes in HFD-fed animals supplemented with fenofibrate or kaempferol was more regular, and adipocytes were similar in size to those in RCD-fed rats. This suggests that kaempferol suppresses the HFD-induced increase in adipose tissue mass and BW gain and that it may inhibit lipid accumulation in adipose tissue in particular.

Obesity, especially abdominal obesity, is associated with dyslipidemia, characterized by elevated TG and reduced HDL-C concentrations [20]. TGs are involved in the ectopic accumulation of lipid stores in the liver and is associated with a number of diseases such as metabolic syndrome and type 2 diabetes [21]. High TC levels increase the risk of developing coronary heart disease, and high levels of LDL-C are a risk factor for coronary heart disease, while high HDL-C is helpful in transporting excess cholesterol to the liver for excretion in the bile [22]. As a result, HDL-C levels are inversely related to coronary heart disease risk [23]. Similar to fenofibrate treatment, the oral administration of kaempferol significantly lowered plasma TC, TG, and LDL-C levels in rats with HFD-induced obesity. Thus, kaempferol may be of benefit to patients with hypercholesterolemia and hypertriglyceridemia.

The effect of kaempferol on the atherogenic and coronary artery risk indices is also notable. The ratio of total cholesterol to HDL-C (i.e., the atherogenic index) and the ratio of LDL-C to HDL-C (i.e., the coronary artery index) are strong and reliable indicators of whether or not cholesterol is deposited into tissues or metabolized and excreted [24]. The results of this study show that treatment with kaempferol

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or fenofibrate causes profound reductions in the atherogenic and coronary indices in experimental hyperlipidemic rats. This strongly suggests that kaempferol has therapeutic potential for the management of obesity and hyperlipidemia, and for the prevention of atherogenic cardiovascular diseases.

It has been reported that kaempferol is a weak partial agonist of PPARγ with increasing insulin sensitivity [25]. Due to the ability of kaempferol to reduce serum levels of TGs and total cholesterol, as well as adipose tissue mass and BW gain-functions similar to those of PPARα activation-we hypothesized that the actions of kaempferol are related to the regulation of hepatic expression of PPARα-target genes involved in lipid metabolism. In hepatocytes and other tissues (e.g., heart), ligand- (natural long chain fatty acids) activated PPARα binds to peroxisome proliferator response elements of DNA and increases the transcription of genes encoding enzymes involved in fatty acid oxidation and lipoprotein metabolism [26, 27]. The outcome is an increase in hepatic fatty acid oxidation and ketogenesis, decreased lipid levels in tissues, and protection against lipotoxicity. We found that kaempferol-treated HFD-fed rats had significantly higher hepatic PPARα protein, and that the effect of kaempferol was equivalent to that of fenofibrate. It appears that kaempferol regulates the lipid-profile in HFD-fed rats through an increase in hepatic PPARα levels. The discovery of kaempferol as a PPARα activator may offer the promise of a novel class of anti-diabetic drug. A metabolism coefficient of 6.25 was employed to convert the effective daily oral dosage of kaempferol for rats (300 mg/kg) into people, assuming that average body weight of an adult is 60 kg [28], the recommended daily oral dosage of kaempferol applied in humans is nearly 2.8 g.

Although reductions in lipogenic activity and in dietary lipid absorption have been suggested as causes of the reduced liver lipid content, an increased capacity of peroxisomal β-oxidation (ACO) and microsomal ω-oxidation (CYP4A) of fatty acids could also be contributing factors [29]. Elevated hepatic levels of ACO and CYP4A proteins imply an enhanced oxidation of fatty acids in the peroxisomes and microsomes of kaempferol-treated, HFD-fed rats. These results support the contention that kaempferol, by directly or indirectly activating PPARα, can up-regulate the expression

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of genes downstream of PPARα, which may lead to enhanced hepatic fatty acid oxidation and reduced TG content.

The sterol regulatory element binding proteins (SREBPs) are a family of three basic helix-loop-helix leucine zipper transcription factors (SREBP-1a, -1c, and -2) that have been identified as transacting factors involved in the maintenance of intracellular cholesterol homeostasis, the control of fatty acid metabolism, and the differentiation of adipocytes [30]. The SREBP-2 isoform activates genes of the cholesterogenic pathway, whereas the SREBP-1 isoforms are more active in regulating the synthesis of fatty acids [31]. It has been documented that SREBPs were the up-regulate genes related to fatty acid synthase and cholesterol levels [32]. In the current study, the elevated expressions of SREBP-1 and SREBP-2 in HFD-fed rats were significantly decreased by the kaempferol (300 mg/kg per day) treatment. These results suggest that kaempferol has an ameliorating effect on dyslipidemia through the impaired hepatic SREBPs as well.

In conclusion, the results of this study show that kaempferol suppresses BW gain and body fat accumulation by increasing fatty acid oxidation, an effect which is likely mediated via up-regulation of PPARα and down-regulation of SREBP expression in the liver of HFD-fed rats. We suggest that supplemental treatment with kaempferol may prevent or improve obesity by modulating lipid metabolism and preventing metabolic syndrome, a representative, lifestyle-related cluster of diseases caused by an excessively HFD.

Conflicts of interests

No competing financial interests exist.

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Legends for Figures

Fig 1. Histological photographs of livers from rats treated with kaempferol, fenofibrate, or vehicle for 8 weeks. Vehicle-treated rats were given the same volume of vehicle (distilled water) used to dissolve the test medications. Photomicrographs are of tissues isolated from vehicle-treated RCD-fed rats (A), vehicle-treated HFD-fed rats (B), kaempferol (300 mg/kg/day)-treated HFD-fed rats (C) or fenofibrate (100 mg/kg/day)-treated HFD-fed rats (D). Photomicrographs were taken at a magnification of ×400.

Fig 2. Histological photographs of epididymal fat pads from rats treated with kaempferol, fenofibrate, or vehicle for 8 weeks. Vehicle-treated rats were given the same volume of vehicle (distilled water) used to dissolve the test medications. Photomicrographs are of tissues isolated from vehicle-treated RCD-fed rats (A), vehicle-treated HFD-fed rats (B), kaempferol (300 mg/kg/day)-treated HFD-fed rats (C) or fenofibrate (100 mg/kg/day)-treated HFD-fed rats (D). Photomicrographs were taken at a magnification of ×400.

Fig 3. Representative immunoblots of protein expression of PPARα, ACO, CYP4A, and SREBPs in livers of HFD-fed rats receiving 8-week treatment with kaempferol, fenofibrate, or vehicle. Vehicle-treated rats were given the same volume of vehicle (distilled water) used to dissolve the test medications. Similar results were obtained with an additional 4 replications. Quantification of the data is shown in Table 4.

Table 1. Changes on body weight, food intake and fat-pad weight in HFD-fed rats receiving 8-weeks treatment with kaempferol or fenofibrate.

Variable (s)	RCD-fed Vehicle	HFD-fed				
		Vehicle	Kaempferol (mg/kg/day)			Fenofibrate
			75	150	300	(100 mg/kg/day)
Initial BW (g/rat)	181.6 ± 8.1	182.5 ± 7.3	180.4 ± 7.5	179.3 ± 8.2	182.7 ± 6.8	181.4 ± 7.2
BW gain (g/rat)	18.6 ± 6.5^d	53.4 ± 7.4^b	41.4 ± 7.1^b	$32.3 \pm 7.3^{a,c}$	24.9 ± 5.6^d	20.4 ± 5.8^d
Food intake (g/rat/day)	20.3 ± 6.3	21.4 ± 7.1	20.9 ± 6.8	21.8 ± 6.9	21.4 ± 8.2	20.9 ± 7.5
Water intake (ml/rat day)	70.5 ± 8.5^c	92.9 ± 7.8^a	86.8 ± 9.7	85.1 ± 10.2	82.3 ± 8.3	80.7 ± 9.5
Epididymal WAT (mg/100 g BW)	303.6 ± 15.8^d	422.1 ± 21.3^b	391.5 ± 17.9^a	$360.2 \pm 25.4^{a,c}$	$328.1 \pm 21.3^{a,c}$	312.7 ± 16.2^d
Perirenal WAT (mg/100 g BW)	172.9 ± 12.7^d	263.8 ± 14.2^b	243.2 ± 15.0^a	$211.6 \pm 15.4^{a,c}$	197.2 ± 13.4^c	181.1 ± 13.1^d
Mesenteric WAT (mg/100 g BW)	129.8 ± 9.6^c	191.7 ± 13.6^a	182.4 ± 14.5^a	166.3 ± 11.5^a	145.7 ± 9.8^c	133.6 ± 10.2^c
Inguinal WAT (mg/100 g BW)	146.9 ± 11.2^c	223.6 ± 14.2^a	206.7 ± 13.1^a	$181.5 \pm 12.3^{a,c}$	160.7 ± 10.2^c	151.3 ± 11.7^c

Kaempferol or fenofibrate was dissolved in distilled water for oral administration at the desired doses in a volume of 10 ml/kg once a day into HFD-fed rats. The vehicle (distilled water) used to dissolve the tested medications was given at the same volume. Values (mean ± SD) were obtained from each group of 7 animals in each group after 8 weeks of the experimental period.

[a]P< 0.05 and [b]P < 0.01 compared to the values of vehicle-treated RCD-fed rats in each group, respectively.

[c]P < 0.05 and [d]P < 0.01 compared to the values of vehicle-treated HFD-fed rats in each group, respectively.

Table 2 Changes in the plasma lipids, hepatic lipids, atherogenic index (AI) and coronary artery index (CRI) in HFD-fed rats receiving 8-weeks treatment with kaempferol or fenofibrate

Variable (s)	RCD-fed	HFD-fed				
	Vehicle	Vehicle	Kaempferol (mg/kg/day)			Fenofibrate
			75	150	300	(100 mg/kg/day)
Plasma TC (mg/dL)	72.9 ± 5.8^d	130.2 ± 12.3^b	116.7 ± 10.3^b	$99.6 \pm 8.3^{a,c}$	$89.1 \pm 7.9^{a,c}$	80.2 ± 8.7^d
Plasma TG (mg/dL)	56.8 ± 6.6^d	124.5 ± 6.3^b	110.9 ± 6.8^b	$95.0 \pm 5.2^{b,c}$	$80.3 \pm 4.8^{b,c}$	62.5 ± 5.3^d
Plasma LDL-C (mg/dL)	31.8 ± 3.2^d	100.2 ± 4.2^b	$82.5 \pm 5.7^{b,c}$	$64.3 \pm 5.1^{b,c}$	$48.1 \pm 4.5^{a,d}$	39.2 ± 4.7^d
Plasma HDL-C (mg/dL)	46.9 ± 3.4^d	27.8 ± 3.8^b	32.1 ± 4.2^b	$39.1 \pm 2.9^{a,c}$	43.9 ± 3.2^d	45.9 ± 3.5^d
Plasma FFAs (mg/dL)	28.2 ± 2.8^d	61.9 ± 5.3^b	55.3 ± 4.6^b	$47.1 \pm 3.8^{b,c}$	$39.5 \pm 3.4^{a,d}$	33.2 ± 4.1^d
AI	0.7 ± 0.1^d	3.6 ± 0.3^b	2.6 ± 0.2^b	$1.6 \pm 0.2^{a,c}$	$1.1 \pm 0.1^{a,d}$	0.9 ± 0.2^d
CRI	1.6 ± 0.3^d	4.7 ± 0.4^b	3.6 ± 0.5^b	$2.5 \pm 0.3^{b,c}$	2.0 ± 0.2^d	1.7 ± 0.3^d
Hepatic TC (µmol/g liver)	18.3 ± 2.7^d	36.2 ± 3.1^b	33.9 ± 2.5^b	31.7 ± 2.6^a	26.9 ± 2.5^c	22.4 ± 2.4^c
Hepatic TG (µmol/g liver)	12.4 ± 2.2^d	27.2 ± 2.4^b	24.3 ± 1.9^b	20.8 ± 2.1^a	17.6 ± 1.5^c	14.3 ± 1.8^c

Kaempferol or fenofibrate was dissolved in distilled water for oral administration at the desired doses in a volume of 10 ml/kg once a day into HFD-fed rats. The vehicle (distilled water) used to dissolve the tested medications was given at the same volume. Values (mean ± SD) were obtained from each group of 8 animals in each group during after 8 weeks of the experimental period.

[a]P < 0.05 and [b]P < 0.01 compared to the values of vehicle-treated RCD-fed rats in each group, respectively.

[c]P < 0.05 and [d]P < 0.01 compared to the values of vehicle-treated HFD-fed rats in each group, respectively.

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Table 3 Results of histopathological findings in livers and epididymal adipocyte**s** of HFD-fed rats receiving 8-weeks treatment with kaempferol or fenofibrate

Findings	RCD-fed	HFD-fed		
	Vehicle	Vehicle	Kaempferol (300 mg/kg/day)	Fenofibrate (100 mg/kg/day)
Hepatic steatosis (0-4)	0^b	3.1 ± 0.4^a	$1.8 \pm 0.6^{a,b}$	$1.2 \pm 0.3^{a,b}$
Average sizes of epididymal adipocytes (μm)	30.1 ± 8.2^b	51.2 ± 7.1	$37.6 \pm 6.2^{a,b}$	$32.1 \pm 5.9^{a,b}$

Kaempferol or fenofibrate was dissolved in distilled water for oral administration at the desired doses in a volume of 10 ml/kg once a day into HFD-fed rats. The vehicle (distilled water) used to dissolve the tested medications was given at the same volume. Values (mean ± SD) were obtained from each group of 8 animals in each group during after 8 weeks of the experimental period.

[a]$P < 0.05$ compared to the values of vehicle-treated RCD-fed rats in each group, respectively.

[b]$P < 0.05$ compared to the values of vehicle-treated HFD-fed rats in each group, respectively.

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Table 4 Quantification of the protein expression of PPARα, ACO, CYP4A and SREBPs in PPARα,

ACO, CYP4A and SREBPs in livers of HFD-fed rats receiving 8-week treatment with kaempferol or
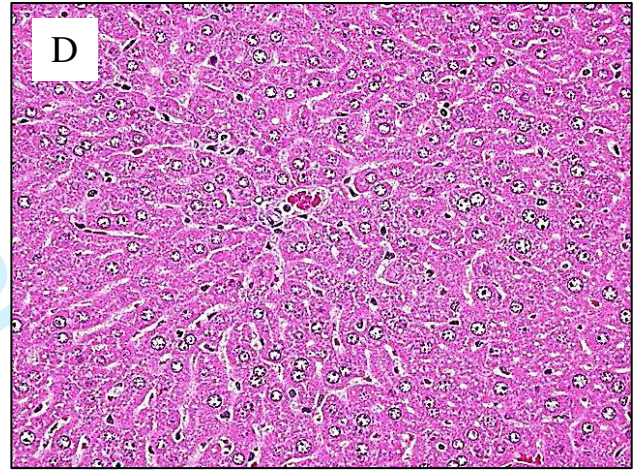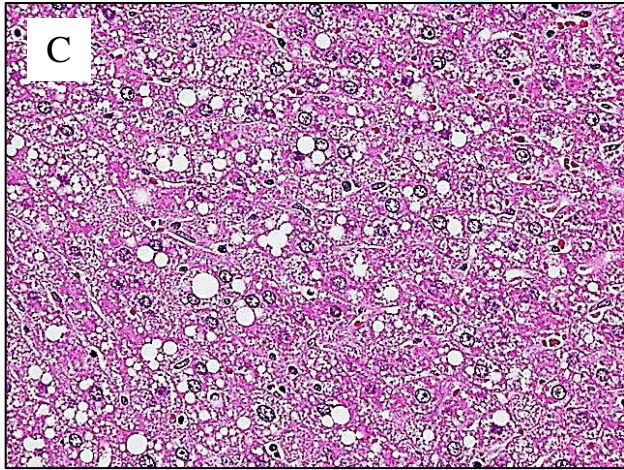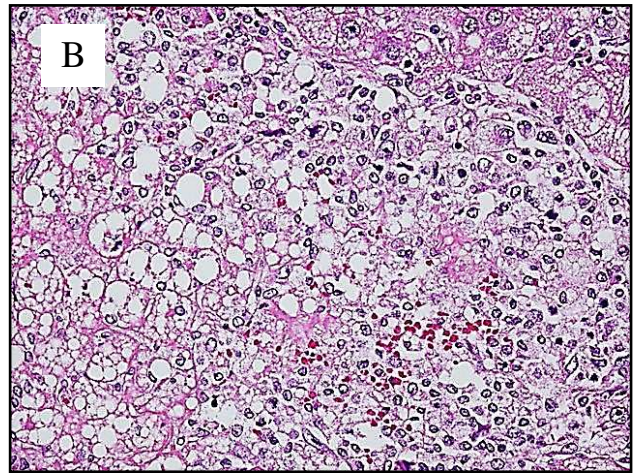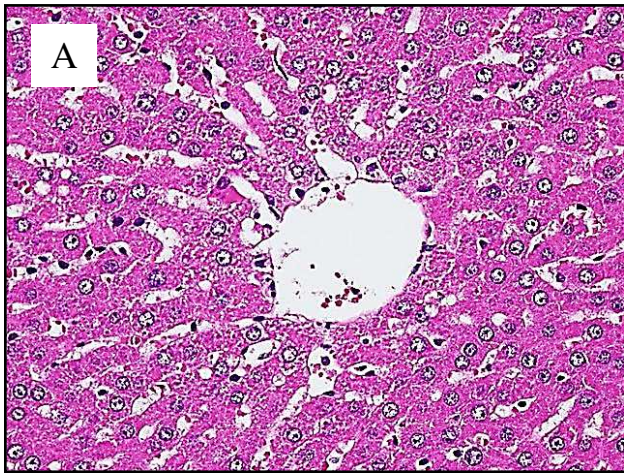
fenofibrate

Relative units	RCD-fed	HFD-fed		
	Vehicle	Vehicle	Kaempferol (300 mg/kg/day)	Fenofibrate (100 mg/kg/day)
PPARα/β-actin	1.00 ± 0.05^{b}	0.58 ± 0.08^{a}	$0.78 \pm 0.05^{a,b}$	$0.85 \pm 0.06^{a,b}$
ACO/β-actin	1.01 ± 0.04^{b}	0.45 ± 0.06^{a}	$0.76 \pm 0.06^{a,b}$	$1.13 \pm 0.04^{a,b}$
CYP4A/β-actin	1.00 ± 0.05^{b}	0.43 ± 0.07^{a}	$0.81 \pm 0.09^{a,b}$	$1.09 \pm 0.05^{a,b}$
SREBP-1/β-actin	1.02 ± 0.06^{b}	2.92 ± 0.08^{a}	$1.35 \pm 0.06^{a,b}$	$1.46 \pm 0.09^{a,b}$
SREBP-2/β-actin	1.01 ± 0.07^{b}	3.08 ± 0.09^{a}	$1.56 \pm 0.07^{a,b}$	$1.78 \pm 0.08^{a,b}$

Kaempferol or fenofibrate was dissolved in distilled water for oral administration at the desired doses

in a volume of 10 ml/kg once a day into HFD-fed rats. The vehicle (distilled water) used to dissolve

the tested medications was given at the same volume. Values (mean ± SD) were obtained from each

group of 8 animals in each group during after 8 weeks of the experimental period.

[a]P < 0.05 compared to the values of vehicle-treated RCD-fed rats in each group, respectively.

[b]P < 0.05 compared to the values of vehicle-treated HFD-fed rats in each group, respectively.

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Fig. 2

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Fig. 3

Fig. 4