Kaempferol ameliorates symptoms of metabolic syndrome by regulating activities of liver X receptor-β

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Abstract

Kaempferol is a dietary flavonol previously shown to regulate cellular lipid and glucose metabolism. However, its molecular mechanisms of action and target proteins have remained elusive, probably due to the involvement of multiple proteins. This study investigated the molecular targets of kaempferol. Ligand binding of kaempferol to liver X receptors (LXRs) was quantified by time-resolved fluorescence resonance energy transfer and surface plasmon resonance analyses. Kaempferol directly binds to and induces the transactivation of LXRs, with stronger specificity for the β subtype (EC50=0.33 μM). The oral administration of kaempferol in apolipoprotein-E-deficient mice (150 mg/day/kg body weight) significantly reduced plasma glucose and increased high-density lipoprotein cholesterol concentrations and insulin sensitivity compared with the vehicle-fed control. Kaempferol also reduced plasma triglyceride concentrations and did not cause liver steatosis, a common side effect of potent LXR activation. In immunoblotting analysis, kaempferol reduced the nuclear accumulation of sterol regulatory element-binding protein-1c (SREBP-1c). Our results show that the suppression of SREBP-1 activity and the selectivity for LXR-β over LXR-α by kaempferol contribute to the reductions of plasma and hepatic triglyceride concentrations in mice fed kaempferol. They also suggest that kaempferol activates LXRs and suppresses SREBP-1 to enhance symptoms in metabolic syndrome.

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1. Introduction

Metabolic syndrome is a complex disease that is characterized by insulin resistance, visceral obesity, hypertriglyceridemia, reduced high-density lipoprotein (HDL) concentrations, hypertension and hepatic steatosis; thus, patients with metabolic syndrome show markedly increased risks for cardiovascular disease and type II diabetes [1]. One potential approach to prevent and ameliorate these complex symptoms is to activate liver X receptors (LXRs). LXRs are nuclear receptors that, when activated by ligand binding, form a heterodimer with retinoid X receptor, which functions as a transcription factor, regulating the genes involved in hepatic gluconeogenesis, cholesterol transport and inflammation [2].

The activation of LXRs may lead to the induction of reverse cholesterol transport, which may protect against tissue cholesterol overload by raising HDL cholesterol concentrations and lead to the improvement of insulin sensitivity and glucose metabolism by regulating LXR target gene transcription in their corresponding biological pathways, thereby preventing and treating metabolic syndrome and atherosclerosis [3,4]. However, various synthetic LXR agonists have been reported, although not consistently, to stimulate hepatic lipogenesis, leading to hepatic steatosis and hypertriglyceridemia [5]. A previous in vivo study demonstrated that the administration of the synthetic LXR agonist T0901317 to mice significantly increased plasma triglyceride (TG) levels as a result of the activation of hepatic sterol regulatory element-binding protein-1c (SREBP-1c) transcription [6], a key transcription factor in lipogenesis that includes the genes that code for acetyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase-1 [7]. Thus, a current aim in the field is to discover moderate LXR agonists with metabolic benefits but without undesirable hepatic lipid accumulation or LXR-β-specific agonists, which may not cause fatty liver due to low expression of LXR-β in the hepatocytes.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors of which activation by ligand induces the transcription of target genes to regulate metabolisms of lipids, carbohydrates or proteins [8]. Among three PPAR isoforms, alpha (α), gamma (γ) and delta/beta (δ/β), PPAR-α is essential in regulation of genes encoding fatty acid transport, metabolism and mitochondrial and peroxisomal fatty acid oxidation (FAO) activity in the liver. Administration of PPAR-α agonists, such as fibrates widely used to treat hyperlipidemia, has
been reported ameliorates hepatic steatosis via improved mitochondrial FAO in mice [9]. On the other hand, the PPAR-α null (Ppara−/−) mice showed lower capacity of mitochondrial FAO compared to wild-type (Ppara+/+) mice [10], thereby susceptible to hepatic steatosis under high-fat diet administration [11]. Thus, activation of PPAR-α is a major strategy to ameliorate symptoms in metabolic syndrome including hypertriglyceridemia and insulin resistance.

Kaempferol [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one] is a flavonoid present in various plant-based foods. Several reports have proposed descriptions of the antiatherogenic mechanisms of kaempferol, including that its antioxidant activity prevents oxidation of low-density lipoprotein (LDL) [12,13]. Kaempferol has also been reported to exert cardiovascular and antidiabetic effects in animal [14–16] and human studies [17,18]. It has further been suggested that kaempferol induces ATP-binding cassette transporters A1 (ABCA1) expression and HDL formation, mediating the first step of the reverse cholesterol transport pathway in macrophages and plasma TG reductions [19]. Still, the molecular mechanisms behind the cardiovascular effects of kaempferol remain elusive. In the initial luciferase-based LXR and PPAR activity screening with approximately 900 natural compounds, it was found that kaempferol had a potent LXR agonistic activity as well as with a moderate PPAR activation (data not shown).

Natural compounds and some drug molecules may interact with multiple target proteins as shown by aspirin, which interacts directly with cyclooxygenase, protein kinase Cδ kinase isoforms and AMP-activated protein kinase, and natural compounds often regulate upstream kinase activity to result in several biological outcomes. Since kaempferol has revealed multiple biological activities without inducing hepatic steatosis, we investigated whether kaempferol is an LXR and a PPAR modulator and then also examined a potential mechanism of kaempferol on hepatic lipogenesis.

2. Methods and materials

2.1. Time-resolved fluorescence resonance energy transfer (TR-FRET) assay of LXR and PPAR ligand-binding activity

The potential LXR- and PPAR-activating capacities of kaempferol were investigated via Lanthascreen TR-FRET LXR-α and LXR-β and PPAR-α, PPAR-β/δ and PPAR-γ coactivator assays (Biotrue Inc., Carlsbad, CA, USA), according to previously described protocols [20]. The synthetic LXR agonist T0901317 was used as a positive control. Fenofibric acid, GW0741 and troglitazone were used as positive controls for PPAR-α, PPAR-β/δ and PPAR-γ, respectively. The ligand-mediated conformational change in a glutatione S-transferase-tagged LXR or PPAR ligand-binding domain (LBD) recruits the fluorescein-labeled coactivator peptide (Trp220/Dip2-2 peptide and Dip2 peptide for LXR-α and LXR-β/γ assay; PGC1-α, C-33 and Trp220/Dip2-2 peptides for PPAR-α, PPAR-β/δ and PPAR-γ assay, respectively). The terbium moiety on the glutathione S-transferase antibody is excited at 340 nm, and then, this energy is transferred to the fluorescein label on the coactivator peptide and detected at an emission wavelength of 520 nm rather than direct emission from terbium at 495 nm. The FRET signal ratio was measured using a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 340 nm, emission wavelengths of 520 and 490 nm, a delay time of 100 μs and an integration time of 400 μs. The emission ratio was calculated as the emission value at 520 nm divided by the emission value at 490 nm. The ratio of the emission signals at 520 nm to that at 495 nm was plotted against the log of the ligand concentration to generate a binding curve. To determine the concentration required to produce a 50% effect (EC50), the data were fitted with a curve (varying slope) using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

2.2. Surface plasmon resonance (SPR) analysis of LXR and PPAR ligand-binding activity

The binding of immobilized His-tagged LXR or PPAR LBD to kaempferol or the positive control compounds was analyzed using a Biacore 2000 instrument (HP/GE Healthcare, Piscataway, NJ, USA). Cloning, expression and purification of LXRα [20] and PPARα [21] were performed as described. The LBD protein was immobilized in the hydrophilic carboxyethylmethylated dextran matrix of the CMS sensor chip (Biacore) via the standard primary amine coupling reaction. The matrix containing the covalent protein was diluted in 10 mM sodium acetate (pH 4.0) to a final concentration of 45 mg/ml. After baseline equilibrium was achieved via a continuous flow of running buffer (10 mM Hepes [pH 7.4], 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20) through the chip for 1–2 h, the ligand solution was automatically injected into the flow cell. All experiments were performed at 25°C at a constant flow rate of 25 μl/min. The data were collected using the Biacore 2000 analysis program (HP/GE Healthcare).

2.3. In silico molecular modeling of kaempferol and LXR LBDs

To gain insight into the mode of binding, kaempferol was docked into the LXR LBD. A total of 10 binding sites were predicted assuming the protein structure to be rigid. Coordinates for kaempferol were prepared by GRAM eye server (Global Phasing Ltd.) with Mogul-assisted modeling. In the panel, kaempferol in stick model was shown in the putative binding site as predicted by AutoDock 4. The side chains of residues lining kaempferol were also depicted in stick model.

2.3.1. Animals, diets, histological analysis and glucose tolerance

Apoe-deficient male mice on a C57BL/6J background (8 weeks old) were purchased from Central Lab, Animal Inc. (Seoul, Korea). All mice were maintained at controlled temperatures of 21–25°C and 50–60% humidity under a 12-h light/dark cycle and were fed a diet of commercial diet for a week. Thereafter, the mice were fed a high-fat diet (D12451, Supplemental Table 1; Research Diets Inc., New Brunswick, NJ, USA) for 8 weeks. After verifying the induction of dyslipidemia, then mice were treated with T0901317 (10 mg/kg/day), kaempferol (150 mg/kg/day) or vehicle/control (PEG400:Tween 80, 4:1) by oral administration for another 10 weeks. There were eight mice in each group. At the end of the feeding period, the mice were killed, and tissue samples were collected. Liver tissues were fixed in 4% formaldehyde and stained with hematoxylin and eosin (H&E). The sizes of the stained aortic area and fat pads were determined using an upright microscope and the related software (Axio Imager M1; Carl-Zeiss, Oberkochen, Germany) at the Histopathology Department of Korea University Anam Hospital (Seoul, Korea). All animal experiments were performed according to a protocol approved by the Animal Experimentation Committee of Korea University (Protocol No. KUIACUC-20090420-4). Glucose tolerance test was performed after 4 days before the end of 8 weeks of feeding periods. The mice were fasted overnight and fed glucose (1.5 g/kg body weight in phosphate-buffered saline) by oral administration. Blood glucose concentrations were measured using a portable glucose meter (Accu-Check Go, Roche) at 0, 15, 30, 60, 90 and 120 min after the glucose feeding.

2.3.2. Lipid, hormone analysis and glucose tolerance test

An automated clinical chemistry analyzer (Cobas111, Roche, Basel, Switzerland) determined the total, HDL, LDL cholesterol and TG levels in the plasma and the amount of hepatic TG and cholesterol. Lipids were extracted from livers at room temperature with 2 ml of a 2:1 (v/v) mixture of hexane and isopropanol. The organic solvent was removed by vacuum centrifugation and the lipids were resuspended in 200 μl 95% ethanol. TG and total cholesterol concentrations were enzymatically determined using the Cobas C111 analyzer and the concentrations were normalized with total protein concentrations [22].

All of the reagents were purchased from Roche, and the measurements were performed according to the manufacturer’s instructions. Plasma insulin (Alpco, Salem, NH, USA), leptin (Millipore, Bedford, MA, USA), resistin (Millipore, Bedford, MA, USA) and adiponectin (Innitrogen) concentrations were measured by enzyme-linked immunosorbent assay. Fecal cholesterol and bile acids were quantified as described [20]. The total bile acid concentration was analyzed using total bile acid assay kit (Diazyme, Poway, CA, USA). All assays were performed at least in triplicate.

2.3.3. Quantitative polymerase chain reaction (qPCR)

The total RNA from the livers was extracted, and the cDNA was synthesized as described previously [21]. The livers were homogenized in RNAiso Plus reagent (Takara Bio Korea, Seoul, Korea) and the total RNA samples were extracted according to the manufacturer’s instructions. The synthesis of cDNA was performed by mixing 2 μl of total RNA with the M-MV reverse transcriptase, oligo-dT and dNTPs (Mhibeotech, Korea). The real-time qPCR was performed with the Bio-Rad iQ5 System according to the manufacturer’s instructions using glyceraldehyde 3-phosphate dehydrogenase as a reference for normalization. Primers were designed using online OligoPerfect designer (Invitrogen) showed in Supplemental Table 2.

2.3.4. Immunoblotting analysis

Tissues were lysed in an ice-cold lysis buffer containing 10 μM Tris–HCl (pH 7.4), 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100 and a protease inhibitor cocktail (Roche, Mannheim, Germany). The lysate was clarified by centrifugation at 14,000 rpm for 10 min at 4°C. To quantify SREBP-1, the proteins were isolated from the nuclear and membrane fractions using a kit (Cayman Chemical, Ann Arbor, MI, USA). Protein concentrations were determined using a Bio-Rad Protein Assay kit with bovine serum albumin (Sigma) as the standard. SDS-PAGE and immunoblotting were performed, as described previously [21]. SREBP-1 antibody was purchased from Santa Cruz Biotechnology (#sc-8984, Santa Cruz, CA, USA) and diluted to 1:500 with TBS and 0.1% Tween. The secondary antibodies (antimouse and antirabbit immunoglobulin G) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted to 1:8000 prior to incubation.
2.3.5. Statistical analysis

All data are expressed as the means±standard error. Multiple group comparisons were performed using one-way analysis of variance followed by Tukey tests and the unpaired Student t test. Differences were considered to be statistically significant at P<0.05.

3. Results

3.1. Kaempferol interacts directly with LXRs with a higher affinity with β-subtype

Results from the TR-FRET assay revealed that kaempferol induced the recruitment of the Trap220/Drip-2 coactivator peptide to LXR-α-LBD and the D22 coactivator peptide to LXR-β-LBD in a dose-dependent manner, with the EC50 of 116 μM and 0.33 μM for LXR-α and LXR-β, respectively (Fig. 1A). Next, the results from the SPR analysis confirmed the direct binding of kaempferol to both LXR subtypes; the Kd values, which reflect the concentration at which a compound dissociates from the immobilized protein after the association phase, were 0.24 μM and 0.16 μM for LXR-α and LXR-β, respectively (Fig. 1B).

In silico virtual modeling analysis showed that kaempferol forms hydrogen bonds with LXR-α at Met298, Thr302 and Glu267 while with LXR-β at Met312, Thr316, Ser242 and Glu267; this finding indicates that the two LXRs are similarly interacting with kaempferol (Fig. 1C). However, Glu267 (α1) forms a hydrogen bond with kaempferol at 2.6 Å in LXR-β, whereas Glu267 (α2) bonds at 3.0 Å; this relative distance from kaempferol in LXR-α compared with LXR-β may explain the higher binding affinity and transactivation of LXR-β over LXR-α with kaempferol. These data collectively demonstrate that kaempferol binds directly to the LBD of LXRs with a higher affinity to β-subtype.

3.2. Kaempferol ameliorates dyslipidemia and improves insulin sensitivity in ApoE-deficient mice fed high-fat diet

Next, animal feeding experiments were performed. Food intake was unaltered with kaempferol feeding (Supplemental Table 3). Kaempferol administration (150 mg/kg/day) increased HDL cholesterol concentrations but reduced total, LDL and hepatic cholesterol concentrations compared to controls (Fig. 2A and B). T0901317, a synthetic agonist for LXRs, did not affect total and LDL cholesterol levels. Both T0901317 and kaempferol significantly increased the fecal output of cholesterol and bile acid compared with the control group (Fig. 2B). The fasting glucose concentrations and glucose tolerance in the kaempferol group were significantly improved in kaempferol-fed mice compared with the controls; this result represents a greater degree of improvement than was achieved with T0901317 (Fig. 2C). Plasma insulin and leptin concentrations were significantly reduced and the insulin sensitivity index was significantly increased while the homeostasis model assessment of insulin resistance (HOMA-IR) index was reduced in the kaempferol groups over the values in the controls (Fig. 2D). These effects were not found in T0901317 group. Collectively, these results demonstrated that kaempferol feeding
ameliorated dyslipidemia and improved insulin sensitivity in mice, which are known metabolic effects of LXR agonism.

3.3. Quantification of LXR-responsive gene expressions in mouse livers and intestines by qPCR

In the livers of ApoE-deficient mice fed kaempferol, the Abca1, Abcg1 and Cyp7a1 mRNA expression levels were significantly up-regulated, whereas the Hmgcr mRNA expression was down-regulated (Fig. 3). In the intestine (ilium), kaempferol induced Abca1 mRNA expressions. Npc1l1 expression was reduced in the kaempferol group compared with controls. The mRNA expressions of Pepck and G6pc, two key genes in hepatic gluconeogenesis, were marginally affected in the livers of the kaempferol groups (Fig. 3). We concluded that kaempferol feeding regulated LXR-responsive genes (Abca1, Abcg1 and Cyp7a1) as well as key genes in cholesterol metabolism (Hmgcr and Npc1l1). Therefore, kaempferol showed hypolipidemic and hypoglycemic activities in mouse feeding experiments.

3.4. Kaempferol does not induce hepatic steatosis and hypertriglyceridemia

Potent LXR agonists often induce hepatic steatosis primarily by inducing SREBP-1c gene expression, which is a target gene of LXRs. However, mice of the kaempferol group showed a normal liver morphology and reduced liver weight, plasma and hepatic TG accumulation compared with high-fat-fed livers while T0901317 group increased plasma and hepatic TG levels compared with controls (Fig. 4A and B). Kaempferol significantly up-regulated hepatic FAO genes in mouse livers including carnitine palmitoyltransferase-1 (Cpt-1) and acyl-CoA oxidase (Acox) compared with controls (Fig. 4C). Meanwhile, the expressions of hepatic lipogenic genes (Srebp-1c, Fas and Scd-1) were unaffected by kaempferol, whereas the expression levels of those genes were significantly induced by T0901317 (Fig. 4C). In immunoblotting analysis, kaempferol slightly induced precursor SREBP-1 (pSREBP-1), while nuclear SREBP-1 (nSREBP-1), an active form of SREBP-1, was significantly repressed in the kaempferol group compared to controls (Fig. 4D). These findings suggest that kaempferol
inhibits the activation of SREBP-1c and, thus, may not induce hepatic lipogenic gene expressions (Ldlr and Hmgrc) and lipid accumulation.

4. Effects of kaempferol on PPAR-α

Induction of FAO genes (Cpt-1a and Acox-1; Fig. 4C) can also be regulated by PPARs, especially the α-subtype [23,24]. Therefore, we further investigated whether kaempferol activates PPARs to elucidate the role of kaempferol in reducing hepatic steatosis. Based on cell-free FRET assays, kaempferol induced the recruitment of PPAR-α and PPAR-δ/β coactivator peptides, PPAR-γ coactivator 1α and C33, respectively (EC50 for PPAR-α and PPAR-δ/β, 15.5 and 341 μM, respectively; Fig. 5A and C). In SPR analysis, kaempferol directly interacted with the PPAR-α-LBD and PPAR-δ/β-LBD (Fig. 5B). No significant binding of kaempferol to the PPAR-γ-LBD was detected. These results suggest that kaempferol interacts directly with PPAR-α; however, the EC50 for PPAR-α (15.5 μM) may be too high to achieve with dietary intake, and thus, PPAR-α may have a marginal role in the hypolipidemic effect of kaempferol.

5. Discussion

Kaempferol is a flavonoid found in various fruits, especially berries and vegetables. Epidemiologic studies and human trials have demonstrated that a diet containing kaempferol has multiple beneficial effects on metabolic syndrome symptoms, including dyslipidemia [25]. Epidemiological studies have detected a positive association between the consumption of foods containing kaempferol and a reduced risk of cardiovascular diseases and type II diabetes [26]. In addition, numerous preclinical animal studies have demonstrated that kaempferol displays a wide range of pharmacological activities, including hypolipidemic, antidiabetic and antiobesity effects; however, the direct molecular targets of kaempferol have been elusive potentially due to the involvement of several protein targets.

Here, we demonstrated that kaempferol directly interacts with both the LXR-α and LXR-β proteins, with a higher affinity to the LXR-β subtype. In obese ApoE-deficient mice fed high-fat diet, oral administration of kaempferol significantly reduced the plasma glucose TG concentrations and elevated the HDL cholesterol levels and improved insulin sensitivity and glucose tolerance. Moreover, kaempferol did not induce hepatic steatosis, a typical side effect of LXR agonism, as it inhibited posttranslational activation of pSREBP-1 to form nSREBP-1. Selective activation of LXR-β, whose expression level is low in the liver, may contribute to the avoidance of hepatic lipogenesis [27]. Collectively, we, for the first time, demonstrate that kaempferol, functioning as a direct ligand of LXR-β, ameliorates the major clinical symptoms of metabolic syndrome in mice.

The bioavailability of kaempferol has been examined in vitro and in vivo in rats and humans [13]. In plant-based foods, kaempferol glycosides are primarily chemical forms, although aglycone kaempferol is known to be a major absorbable form from the intestine. After intestinal uptake, kaempferol can undergo further metabolism to generate its glucurono-conjugated and sulfo-conjugated forms [28,29]. Thus, the percentage of kaempferol excreted in urine has been found to be approximately 4% of the total amount of kaempferol ingested [30]. Several human studies have demonstrated that, after oral ingestion, kaempferol can be detected in plasma at concentrations in the hundred-nanomolar range [31]. Thus, kaempferol may function...
as an agonistic ligand of at least LXR-β (EC50=0.33 μM) but may not of PPAR-α (EC50=15.5 μM) under physiological conditions. Our study has several limitations, one of which is the dose of kaempferol used in the mouse experiment. Because a very high dose was used, the results cannot be applied directly to humans. A dose–response experiment is needed before clinical trials can be conducted to determine the efficacy of kaempferol.

The induction of hypertriglyceridemia by LXR agonists is controversial. Increases in the concentration of plasma TGs by LXR agonists have been reported [32]. However, other studies have reported no change [5] or only a transient increase in plasma TG concentrations [7]. The reason for this inconsistency is unclear at present. LXR agonists, such as T0901317, can also induce undesirable hepatic lipid accumulation by increasing fatty acid synthesis via the up-regulation of SREBP-1c, a key transcription factor in hepatic lipogenesis [33]. However, in the current study, the natural flavonol kaempferol did not induce the expression of genes related to hepatic lipogenesis despite activating LXRs. In addition, reduced nSREBP-1 also explains the down-regulation of Hmgcr and reduction of LDL cholesterol concentrations in mice fed kaempferol.

SREBP-1c is a major lipogenic transcription factor in endoplasmic reticulum (ER) and its posttranslational regulation has been well characterized. When intracellular sterol concentrations fall, the precursor SREBP-1 protein (pSREBP) in ER is chaperoned by the SREBP-cleavage-activating protein (SCAP) to the Golgi, where two proteases, S1P and S2P, cleave pSREBP-1 to release N-terminal SREBP-1 that is delivered to the nuclear nSREBP while activating LXRs. In addition, reduced nSREBP-1 also explains the down-regulation of Hmgcr and reduction of LDL cholesterol concentrations in mice fed kaempferol.

SREBP-1c directly phosphorylates SREBPs, which also facilitate the association of SREBP-1 with coat protein complex II vesicles to promote ER-to-Golgi transport [36]. Third, AKT phosphorylates to inhibit glycogen synthase kinase-3β activity, which mediates ubiquitin-dependent degradation of nSREBP [37]. Thus, AKT plays the role of a positive regulator for SREBP-1 nuclear translocation to induce hepatic lipogenesis. Thus, suppression of AKT activity could contribute to lower intracellular lipogenesis. Our preliminary results showed that kaempferol suppresses phosphorylation activation of AKT. This mechanism will be further investigated in the future study.

It has been demonstrated that natural compounds and some drugs can interact with multiple target proteins or regulate upstream kinases to modulate multiple biological activities. In the current study, our results indicate that kaempferol independently regulates LXR-β and PPAR-α, resulting in overall metabolic benefits. Finally, our investigation supports that the consumption of kaempferol from fruits and vegetables or as an isolated compound may be useful for the prevention and treatment of metabolic syndrome.

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References

Fig. 5. Kaempferol activates PPARs. (A) TR-FRET assay. (B) SPR analysis. (C) Binding constants of kaempferol for three PPARs. EC₅₀ and Kᵦ values are calculated from TR-FRET and SPR assay, respectively. Data represent the mean values±S.E.M.


