Farnesol, an isoprenoid, improves metabolic abnormalities in mice via both

PPARα-dependent and -independent pathways

Tsuyoshi Goto¹,², Young-Il Kim¹, Kozue Funakoshi¹, Aki Teraminami¹, Taku Uemura¹, Shizuka Hirai¹, Joo-Young Lee¹, Makoto Makishima³, Rieko Nakata⁴, Hiroyasu Inoue⁴, Hiroyuki Senju⁵, Masayoshi Matsunaga⁵, Fumihiko Horio⁶, Nobuyuki Takahashi¹,², and Teruo Kawada¹,²,§

¹ Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji 611-0011, Japan
² Research Unit for Physiological Chemistry, the Center for the Promotion of Interdisciplinary Education and Research, Kyoto University, Japan
³ Department of Biochemistry, Nihon University School of Medicine, Itabashi-ku, Tokyo 173-8610, Japan
⁴ Department of Food Science and Nutrition, Nara Women's University, Nara 630-8506, Japan
⁵ Hakutsuru Sake Brewing Co., Kobe 658-0041 Japan
⁶ Department of Applied Molecular Bioscience, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

§ Corresponding Author: Teruo Kawada, Ph.D.
Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University
Uji 611-0011, Japan
TEL: +81-774-38-3751
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Abbreviations: PPAR, peroxisome proliferator-activated receptor; HFD, high-fat diet; TG, triglyceride; FXR, farnesoid X receptor; TZD, thiazolidinedione; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; SREBP-1c, sterol regulatory element binding protein-1c; FAS, fatty acid synthase; FFA, free fatty acid; OCT, optimal cutting temperature; WAT, white adipose tissue; HDL, high-density lipoprotein; aP2, adipocyte fatty-acid-binding protein; LPL, lipoprotein lipase; CPT1, carnitine palmitoyl transferase 1; ACO, acyl-CoA oxidase; UCP2, uncoupling protein 2; FAT, fatty acid translocase; ACS, acyl-CoA synthase; SHP, small heterodimer partner; LXR, liver X receptor; GTT, glucose tolerance test; ITT, insulin tolerance test; AUC, area under the curve
Peroxisome proliferator-activated receptors (PPARs) control energy homeostasis. In this study, we showed that farnesol, a naturally occurring ligand of PPARs, could ameliorate metabolic diseases. Obese KK-Ay mice fed a high-fat diet (HFD) containing 0.5% farnesol showed significantly decreased serum glucose level, glucosuria incidence, and hepatic triglyceride contents. Farnesol-containing HFD up-regulated the mRNA expressions of PPARα target genes involved in fatty acid oxidation in the liver. On the other hand, farnesol was not effective in up-regulating the mRNA expressions of PPARγ target genes in white adipose tissues. Experiments using PPARα-deficient [(-/-)] mice revealed that the up-regulation of fatty acid oxidation-related genes required PPARα function, but the suppression of hepatic triglyceride accumulation was partially PPARα-dependent. In hepatocytes isolated from the wild-type and PPARα (-/-) mice, farnesol suppressed triglyceride synthesis. In luciferase assay, farnesol activated both PPARα and the farnesoid X receptor (FXR) at similar concentrations. Moreover, farnesol increased the mRNA expression level of a small heterodimer partner known as one of the FXR target genes and decreased those of sterol regulatory element-binding protein-1c and fatty acid synthase in both the wild-type and PPARα (-/-) hepatocytes. These findings suggest that farnesol could improve metabolic abnormalities in mice via both the PPARα-dependent and -independent pathways and that the activation of FXR by farnesol might contribute partially to the PPARα-independent hepatic triglyceride content-lowering effect. To our knowledge, this is the first study on the effect of the dual activators of PPARα and FXR on obesity-induced metabolic disorders.

**Key words:** FXR, fatty acid oxidation, triglyceride biosynthesis, metabolic syndrome
INTRODUCTION

Obesity is one of the most prevalent and serious chronic disorders in industrialized societies. Frequently, it clusters with metabolic abnormalities including type-2 diabetes, hypertension, and hyperlipidemia (2, 6, 55). Obesity causes excess fat accumulation in various tissues; most notoriously in adipose tissues, but also in other insulin-responsive organs such as the liver and skeletal muscle, predisposing one to the development of insulin resistance (47, 48). Yet, the molecular mechanisms underlying insulin resistance and obesity have not been fully clarified, and effective therapeutic approaches are currently of general interest.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that serve as ligand-inducible transcription factors (4, 9, 13). PPARs have three isoforms (PPARα, PPARγ and PPARδ) with different ligand specificities and tissue distributions (1, 4, 9, 13, 19, 35). PPARα is highly expressed in the liver and regulates the mRNA expressions of target genes involved in lipid catabolism (19). Activators of PPARα such as fibrates lower circulating lipid levels and are commonly used to treat hyperlipidemia and other dyslipidemic states (19). PPARγ is abundant in adipose tissues functioning as the key transcription factor for adipogenesis (35). Synthetic full agonists for PPARγ, such as thiazolidinediones (TZDs), are clinically used to treat type-2 diabetes (35). In spite of their proven efficacy and widespread use, TZDs cause numerous deleterious side effects, including weight gain and peripheral edema (60). Recently, several PPARγ partial agonists with anti-diabetic efficacy and lower side effects have been described (60). Moreover, recent studies suggest that dual agonists or a combination of ligands to activate PPARα and PPARγ can effectively improve insulin sensitivity and correct diabetic dyslipidemia in obese diabetic animals (34, 46). PPARδ is expressed in many tissues including the skeletal muscle and
brown adipose tissue (1). Recently, it has been suggested that PPARδ activation attenuates obesity and type-2 diabetes (43, 51).

Attention has recently been focused on the potential uses of constituents in plants and other foods for the treatment of diabetic symptoms. In order to find novel natural ligands for PPARs, we have evaluated PPAR ligand activity for various terpenoids containing carotenoids, isoprenoids, and their alcohols (isoprenols), which are widely distributed in many plants (14, 22, 26, 33, 39-41), and we have reported that several isoprenols activate PPARα and PPARγ in hepatocytes and adipocytes, respectively (41). In our previous study, farnesol, one of the isoprenols, showed the most potent PPAR activity in cultured cells. Farnesol is contained not only in herbal plants but also in dietary fruits and berries, such as apricots, peaches, plums, blueberries, cranberries, raspberries, and strawberries and vegetables such as tomatoes (17, 44). It is known that farnesol affects lipid metabolism in various cells. Farnesol regulates the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol synthesis, by controlling the degradation of the enzyme (3, 7, 28). Farnesol has also been shown to inhibit phosphatidylcholine synthesis (49) and de novo synthesis of triglyceride (TG) (18). Moreover, it has been reported that farnesol lowers serum cholesterol and TG levels (8, 31). However, the effects of farnesol on obesity-associated metabolic disorders, such as hyperglycemia, hyperlipidemia, and hepatic steatosis, are largely unknown.

In the present study, we investigated whether farnesol functions as a dietary dual agonist to activate PPARα and PPARγ in vivo. In obese diabetic KK-Ay mice, the farnesol-containing high-fat diet (HFD) significantly decreased serum glucose and hepatic TG levels. Farnesol up-regulated the mRNA expressions of PPARα target genes in the liver. Moreover, using PPARα (-/-) mice, we demonstrated that the suppression of hepatic TG accumulation by farnesol was partially dependent on PPARα. Finally, we showed that
farnesol reduced the mRNA expression levels of sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase (FAS) and inhibited hepatic TG synthesis in primary-cultured hepatocytes in a PPARα-independent manner. In conclusion, farnesol ameliorates obesity-induced hyperglycemia and hepatic steatosis via both the PPARα-dependent and -independent pathways, suggesting that farnesol might be one of the food components that are effective for ameliorating abnormalities of lipid metabolism.

MATERIALS AND METHODS

Materials and plasmids.

Unless otherwise indicated, all chemicals including \textit{trans,trans}-farnesol were purchased from Sigma (MO, USA) or Nacalai Tesque (Kyoto, Japan).

\textit{p4xUASg-tk-Luc} (a reporter plasmid), \textit{pM-hPPARα} (an expression plasmid for chimera protein for GAL4 DNA-binding domain and human PPARα ligand-binding domain), and \textit{pRL-CMV} (an internal control for normalizing transfection efficacies) were previously described (41). \textit{p3xIR1-tk-Luc} (a reporter plasmid) and \textit{pCMX-hFXR} (an expression plasmid for human FXR) were also previously described (27).

Animals.

All mice were maintained in a temperature-controlled (23°C) facility with a constant 12 hour light/dark cycle and were given free access to water. Five-week-old male KK-Ay (CLEA Japan, Tokyo, Japan) mice were maintained for 5 weeks either on a 60% HFD (D12492 research diet, MO USA) or on a diet containing 0.25%, 0.5% farnesol, or 0.2% bezafibrate. The energy intake of all mice was adjusted by pair-feeding. For pair-feeding, we measured food intake of all mice daily. Generally, once a day, all mice were provided with
food equal to the least amount consumed by the mice on the previous day. Before fasting, all
mice were given free access to food, and food intake of each mouse was measured. Every 7
days after the feeding initiation, blood samples were collected from the tail vein to measure
serum glucose level, and glucosuria incidence was estimated using Uropiece S (Fujisawa
Pharmaceutical, Osaka). Three to four weeks after feeding, an oral glucose tolerance test was
carried out by administering D-glucose (2 g/kg body weight) through a gastric feeding tube
after fasting overnight. For the insulin tolerance test, human insulin (Eli Lilly Japan, Kobe,
Japan) was injected intraperitoneally (0.6 units/kg body weight) in non-fasted animals. Blood
samples were collected from the tail vein before and 30, 60, 90 and 120 min after injection.
Area under the curve (AUC) was calculated using the trapezoidal rule. Five weeks after
feeding, mice were fasted 1 hour before harvesting blood for metabolic characteristic analysis,
and tissues for RNA isolation, lipid measurements, and histochemistry. Serum glucose, TG,
free fatty acid (FFA), total cholesterol, and HDL levels were measured enzymatically using
glucose CII test Wako, triglyceride G test Wako, NEFA C test Wako, free cholesterol C test
Wako, and HDL cholesterol test Wako (Wako Pure Chemicals, Osaka, Japan) kits. Serum
insulin level was measured using an ELISA kit (Morinaga Institute of Biological Science,
Yokohama, Japan). Male wild-type and PPARα (-/-) mice with a Sv/129 genetic background
were used to study the contribution of PPARα to serum glucose and hepatic TG
level-lowering effects of farnesol. Eight-week-old wild-type and PPARα (-/-) mice were fed
HFD or a diet containing 0.5% farnesol or 0.2% bezafibrate for 6 weeks. The same amount of
food was given to all the mice. After fasting for 8 hours, blood samples and tissues were
harvested for each analysis. The animal care procedures and methods were approved by the
Animal Care Committee of Kyoto University.
**Hepatic lipid analysis and liver histochemistry.**

For the measurement of hepatic TG content, the liver was homogenized in chloroform/methanol (2:1 v/v) using a Polytron tissue grinder (Kinematica AG, Luzern, Switzerland). Lipid extracts were prepared by the conventional Folch method. Extracts were dried under N₂ flow and resuspended in 2-propanol. The amounts of hepatic TGs were determined enzymatically using the Wako triglyceride G-test.

The liver was removed from each animal, and then fixed in 10% formaldehyde/PBS. The fixed samples were embedded in the optimal cutting temperature (OCT) compound in isopentane cooled with liquid nitrogen. They were cut into 8 µm sections using a cryostat at -30°C and placed on microscope slides (Matsunami glass, Osaka, Japan). Liver cryosections were fixed in 50% ethanol for a few minutes and stained with 2% Sudan III in 70% ethanol for 1 hour and subsequently washed with 50% ethanol and water. Sections were counterstained with Mayer’s hematoxylin.

**RNA preparation and real-time fluorescence monitoring RT-PCR.**

Total RNA was prepared from livers and mesenteric or inguinal white adipose tissues (WATs) or primary cultured hepatocytes using Qiazol lysis reagent (QIAGEN, CA, USA) or Sepasol(R)-RNA I Super (Nacalai Tesque) in accordance with the manufacturer’s protocol. Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, WI, USA) in accordance with the manufacturer’s instructions using a thermal cycler (Takara PCR Thermal Cycler SP: Takara, Shiga, Japan). To quantify mRNA expression, real-time RT-PCR was performed with a LightCycler system (Roche Diagnostics, Mannheim, Germany) using SYBR Green fluorescence signals, as described previously (41, 42). The oligonucleotide primers of mouse 36B4, PPARα target genes, and FXR-SHP-SREBP-1c pathway genes were
designed using a PCR primer selection program in the website of the Virtual Genomic Center
from the GenBank database. All oligonucleotide primer sets used to measure mRNA
expression levels in WATs were previously described (26, 42). The primers used for
measurements of hepatic mRNA expression levels for upstream and downstream were
respectively 5'-GCACCATTGCCATTCGATACA-3' and
5'-CCACTGCTGTGAGAATAGCCGT-3' for mouse ACO mRNA,
5'-CTGTAGGCTCAACACCGGAAC-3' and 5'-CTGTCATGGCCTTAGATGCAT-3' for
mouse CPT1, 5'-CAGTTTCACTCCGGATACC-3' and
5'-GATCCCTTCCTCTCGACAAT-3' for mouse UCP2,
5'-CAAGGGGTTGCTGAAATCATGT-3' and 5'-CTGTCATGGCCTTAGATGCAT-3' for
mouse FABP1, 5'-GATGTGGAAGGCTAGAATGGATTG-3' and
5'-GGTCCTAGCTTATCGCCACTG-3' for mouse FAT/CD36,
5'-AGTAGGGAAGTCTCCTGTCGAT-3' for mouse ACS,
5'-AGTAGGGAAGTCTCCTGTCGAT-3' for mouse SHP,
5'-GGAGCCATGGATTGCACATT-3' and 5'-GCCAGAGGCAAGAGAAGAG-3' for
mouse SREBP-1c, and 5'-TGGGTTAGGCTCCAGGAG-3' and
5'-ACCACCAGAGAGGGTATG-3' for mouse FAS. To compare mRNA expression level
among the samples, copy numbers of all transcripts were divided by that of mouse 36B4
showing a constant mRNA expression level in adipocytes and hepatocytes. All mRNA
expression levels were represented as the ratio relative to that of a control in each experiment.

Preparation of mouse primary hepatocytes.
Primary hepatocytes were prepared from 10-week-old male mice as previously described with slight modifications (52). In the light phase, animals were anesthetized with pentobarbital and livers were perfused with 40 ml of liver perfusion medium (Invitrogen, CA, USA) followed by 30 ml of liver digestion medium (Invitrogen), both at a flow rate of 5 ml/min at 37 °C. Liver cells were dispersed in Williams’ E medium (Sigma) by dissection and gentle shaking. After filtration through a 100 μm nylon mesh filter, hepatocytes were isolated by repeated centrifugation (4 times) at 50g for 2 min. Isolated hepatocytes were suspended in Williams’ E medium supplemented with 10% FBS, 0.1 μM insulin, 1 μM dexamethasone, 100 U/ml penicillin, and 100 μg/ml streptomycin, and seeded at a density of 2.5 x 10^5 cells/well in Type I collagen-coated 12-well plates (Iwaki, Tokyo, Japan). After a 2-hour incubation at 37 °C in an atmosphere of 5% CO₂, nonattached cells were removed by washing with culture medium. Farnesol was added immediately after washing, and cells were further incubated for 24 hours.

Measurement of TG biosynthesis activity of mouse hepatocytes.

TG biosynthesis activity was determined by measuring the conversion of [1-\(^{14}\)C] acetic acid (GE Healthcare, Little Chalfont, UK) into cellular TGs as previously described with slight modifications (18). Primary cultured hepatocytes were incubated with or without compounds for 22 hours. [1-\(^{14}\)C] acetic acid was added to the cells at a concentration of 370 kBq/ml, and they were incubated for an additional 2 or 24 hours. The cells were washed with Williams’ E medium twice and cellular lipids were extracted by incubating cells with 1 ml of hexane-isopropyl alcohol (3:2, v/v) for 30 min at room temperature. Aliquots were transferred and evaporated under a reduced pressure. Samples were resuspended in 30 μl of chloroform, applied onto a silica gel TLC plate (Merck, Darmstadt, Germany) and separated. Petroleum
ether/diethyl ether/acetic acid (80:30:1) was used as a developing solvent. Spots were visualized in iodine vapor atmosphere. TG spots were scraped and vortexed with 3 ml of Aquasol-2 (PerkinElmer, MA, USA). Their radioactivities were measured using a liquid scintillation counter as TG synthetic activity.

Luciferase reporter assay.

CV-1 monkey kidney cells were from the American Type Culture Collection (ATCC). CV-1 cells were grown in DMEM supplemented with 10% FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO₂. Luciferase ligand assay was performed using the dual luciferase system (Promega), as previously described (41). For PPARα activity assay, we transfected p4xUASg-tk-Luc, pM-hPPARα, and pRL-CMV into CV-1 cells. For FXR activity assay, p3xIR1-tk-Luc, pCMX-hFXR, and pRL-CMV were transfected into CV-1 cells. Briefly, transfections into CV-1 cells cultured on 100 mm dishes were performed using LipofectAMINE (Invitrogen) in accordance with the manufacturer’s protocol. Five hours after transfection, the transfected cells were seeded into 96-well plates in the medium containing farnesol. After 24-hour incubation, luciferase activity was measured.

Statistical analyse.

The data, presented as means ± SEM, were statistically analyzed using ANOVA followed by Bonferroni’s test and Student’s t-test. Differences were considered significant at $P < 0.05$.

RESULTS

Farnesol-containing diet prevented development of diabetic conditions in KK-Ay mice.
We first determined whether farnesol, one of the dietary PPARα and PPARγ dual agonists in vitro, ameliorates metabolic disorders in the obese model, KK-Ay mice. Since strong PPARα agonists have been shown to reduce food intake in rodents (11), all mice were housed in pair-fed conditions to test for this. However, we could not observe any difference in food intake between the acclimation period (ad lib feeding) and the experimental period. Thus, neither farnesol (0.25% and 0.5%) nor bezafibrate (0.2%) treatment seemed to cause anorexia under our experimental conditions. Throughout the experimental period (35 days), there was no significant difference in serum glucose level between the control HFD-fed and 0.25% farnesol-containing HFD-fed groups. However, the treatment with 0.5% farnesol for 21 days significantly decreased serum glucose level, and this anti-hyperglycemic effect was also observed in the group fed HFD supplemented with 0.2% bezafibrate (Fig. 1A). This effect of 0.5% farnesol HFD appeared to continue for another 10 days (22 and 25% reduction on days 21 and 30, respectively). On day 28, the glucosuria incidence in the control HFD-fed mice increased to 67%, whereas no 0.5% farnesol HFD-fed mice showed glucosuria (Fig. 1B). To understand better the effect of farnesol on the glucose metabolism in the obesity model mice, we performed an oral glucose tolerance test (GTT) and an insulin tolerance test (ITT) using mice fed with a 0.5% farnesol diet for 3-4 weeks. Plasma glucose levels in both tests were significantly lower in the farnesol-treated mice than in the pair-fed control mice (Fig. 1C and E). The AUC for the glucose responses of the 0.5% farnesol diet group were significantly lower than that of the control diet group in both tests (19% and 26% decrease in GTT and ITT, respectively). Throughout the experimental period, we could not observe any differences in body weight between the control HFD-fed group and the farnesol- or bezafibrate-fed group (Supplemental Table 1). After 35 days of feeding, there were no significant changes in the weights of WATs, liver, or pancreas with by the farnesol treatment, whereas treatment with
bezafibrate, a synthetic PPAR\(\alpha\) agonist, decreased WAT weight and increased liver weight, as shown in Supplemental Table 1. In the farnesol-treated mice, serum TG levels tended to be lower than those in the control mice, but this difference was not significant as shown in Supplemental table 2. No significant changes following farnesol treatment were observed in several other metabolic characteristics such as serum levels of insulin, FFA, total cholesterol, and high-density lipoprotein (HDL) (Supplemental Table 2). These findings demonstrate that farnesol treatment decreases the level of serum glucose and improves obesity-induced glucose intolerance and insulin resistance.

**Farnesol treatment prevented development of hepatic triglyceride accumulation in KK-Ay mice.**

Next, we examined the effects of farnesol on hepatic TG accumulation. Although hepatic TG levels in the 0.25% farnesol-treated mice were equivalent to those in the control HFD-fed mice, hepatic TG contents in the 0.5% farnesol-treated mice were significantly lower than those in the control HFD-fed mice (Fig. 2A). A similar TG content lowering effect was observed in the 0.2% bezafibrate-treated mice. This effect of farnesol was confirmed by histological analysis. As in the case of the bezafibrate-treated mice, visible lipid droplets stained with Sudan III orange dye markedly decreased in the liver of the 0.5% farnesol-treated mice (Fig. 2B). This finding indicates that farnesol treatment inhibits the HFD-induced TG accumulation in the liver.

**Farnesol treatment induced expression of PPAR\(\alpha\) target genes in the liver but did not affect that of PPAR\(\gamma\) target genes in white adipose tissues.**

To determine whether farnesol acts *in vivo* as a dual agonist of PPAR\(\alpha\) and PPAR\(\gamma\),
we measured the mRNA expression levels of PPARα and PPARγ target genes in the liver and WATs, respectively. Unexpectedly, farnesol treatment did not change the mRNA expression levels of PPARγ target genes such as those encoding adipocyte fatty-acid-binding protein (aP2), lipoprotein lipase (LPL), and adiponectin in both mesenteric (Fig. 3A-C) and inguinal (data not shown) WATs. On the other hand, the mRNA expression levels of PPARα target genes such as carnitine palmitoyl transferase 1 (CPT1), acyl-CoA oxidase (ACO), and uncoupling protein 2 (UCP2) significantly increased in the liver of the farnesol-treated mice in a dose-dependent manner (Fig. 3D-F). These findings suggest that farnesol serves as a PPARα activator but not as a PPARγ activator in vivo.

Hepatic TG content lowering effect of farnesol was not entirely dependent on PPARα activation.

To verify whether the effects of farnesol, namely, the reduction of serum glucose level and hepatic TG accumulation, are mediated by PPARα activation, we treated HFD-fed wild-type and PPARα-deficient (PPARα (-/-)) mice with farnesol or bezafibrate for 6 weeks and measured fasting serum glucose level and hepatic TG contents. As previously reported [19], hypoglycemia was observed in the PPARα (-/-) mice after fasting (Fig. 4A). The treatment with 0.5% farnesol or 0.2% bezafibrate significantly decreased serum glucose level in the wild-type mice (Fig. 4A). Importantly, this decrease was completely abolished in the PPARα (-/-) mice. The treatments with both farnesol and bezafibrate dramatically decreased hepatic TG contents in the wild-type mice (36% reduction in 0.5% farnesol-treated group). In contrast to serum glucose level, hepatic TG contents were reduced by approximately 17% in the farnesol-treated PPARα (-/-) mice (Fig. 4B), whereas bezafibrate treatment did not affect the hepatic TG contents in the PPARα (-/-) mice. These findings suggest that PPARα
activation is only partially involved in the hepatic TG lowering effect of farnesol.

To determine whether the effects of farnesol on the PPARα (-/-) mice are accompanied by similar changes at the molecular level, we measured the hepatic mRNA expression levels of PPARα target genes. The mRNA expression levels of ACO, fatty acid translocase (FAT)/CD36, acyl-CoA synthase (ACS), and UCP2 significantly increased in the farnesol-treated wild-type mice (Fig. 5A-E). The mRNA expression levels of ACO, FAT, ACS, and UCP2 increased approximately 1.9-, 1.9-, 1.8-, and 1.6-fold, respectively. However, such increases were not detected in the farnesol-treated PPARα (-/-) mice (Fig. 5A-E). These data indicate that PPARα plays an essential role in the induction of hepatic PPAR target genes involved in fatty acid catabolism in the farnesol-treated mice.

Farnesol suppressed hepatic TG synthesis through a PPARα-independent pathway.

To determine whether PPARα is involved in the effect of farnesol to suppress hepatic TG synthesis, we performed experiments to examine TG synthesis using primary hepatocytes isolated from the wild-type and PPARα (-/-) mice. Farnesol treatment inhibited the incorporation of [1-14C] acetic acid into the TG fraction by 31% in comparison with vehicle treatment of the wild-type hepatocytes (Fig. 6A). Moreover, farnesol treatment significantly induced the mRNA expressions of PPARα target genes such as CPT1, ACO, and UCP2 in a dose-dependent manner (Fig. 6B-D). In PPARα (-/-) hepatocytes, farnesol also inhibited the incorporation of [1-14C] acetic acid by 48% (Fig. 6A), whereas no up-regulation of PPARα target genes was observed in the PPARα (-/-) hepatocytes (Fig. 6B-D). These findings suggest that farnesol suppresses hepatic TG synthesis via a PPARα-independent pathway.

Farnesol activated FXR pathway in vivo and in vitro independently of PPARα activation.
It has been reported that farnesol is a weak ligand of the farnesoid X receptor (FXR) (10), whose activation inhibits hepatic TG synthesis via the regulation of mRNA expressions of molecules involved in lipid metabolism, such as small heterodimer partner (SHP) (induced by FXR activation), SREBP-1c (suppressed by SHP activation), and fatty acid synthase (FAS; induced by SREBP-1c activation) (52). In our luciferase reporter assay, farnesol activated both PPARα and FXR at similar concentrations in CV-1 cells (Fig. 7A and B). In the assay, the addition of farnesol at 30 μM induced 5.0- and 3.6-fold increases in PPARα and FXR activities, respectively. These findings indicate that farnesol serves as an activator for both PPARα and FXR.

According to the findings, we studied whether farnesol activates the FXR-SHP-SREBP-1c pathway. In the liver of the wild-type mice, 0.5% farnesol treatment significantly increased hepatic SHP mRNA expression level by approximately 1.9-fold and decreased the mRNA expression levels of SREBP-1c and FAS (30% and 40%, respectively) (Fig. 7C). In the PPARα (-/-) mice, farnesol treatment also increased the hepatic SHP mRNA expression level and tended to decrease the mRNA expression levels of SREBP-1c and FAS (Fig. 7C). In the primary-cultured wild-type hepatocytes, farnesol treatment significantly increased the mRNA expression level of SHP (1.4-fold) and decreased those of SREBP-1c and FAS (63 and 13%, respectively) at a concentration of 100 μM farnesol (Fig. 7D). In the PPARα (-/-) hepatocytes, similar changes were observed (1.7-fold increase in SHP mRNA expression level and 36% and 33% decreases in SREBP-1c and FAS mRNA expression levels, respectively) (Fig. 7D). Furthermore, in the presence of the FXR antagonist, guggulsterone, the extent to which farnesol induced a decrease in TG synthesis was attenuated (Fig. 7E). These findings indicate that PPARα is not necessary for farnesol to exert its effect of activating the FXR-SHP-SREBP-1c pathway, suggesting that the farnesol-dependent
activation of this pathway might be, at least partly, involved in the inhibitory effect of farnesol on hepatic TG synthesis.

DISCUSSION

A pharmacological treatment of metabolic syndrome aims to reduce insulin resistance and other risk factors by the modulation of PPARs. Fibrates (agonists for PPARα) and TZDs (agonists for PPARγ) are often used to treat hyperlipidemia and hyperglycemia, respectively (19, 35). A combination of these medications is an attractive option for the treatment of obesity accompanied by type-2 diabetes (46). Compounds with dual agonistic activity on both of these receptors have been shown to improve insulin resistance and dyslipidemia in obese diabetic animals (30, 34). In this context, farnesol is considered to be valuable for the treatment of metabolic abnormalities, because this compound has been reported as a naturally occurring PPARα and PPARγ dual agonist in cultured cells (41). Indeed, farnesol treatment caused improvement of hyperglycemia and hepatic steatosis under the HFD-feeding conditions. On the basis of the dual agonistic effect of farnesol in cultured cells, it was hypothesized that the serum glucose level and hepatic TG content-lowering effects of farnesol were due to the activation of PPARγ and PPARα, respectively. However, in WATs of the farnesol-treated mice, there was no change in the mRNA expression levels of several well-known PPARγ target genes, whereas the mRNA expression levels of PPARα target genes increased in the liver of farnesol-treated mice. These findings were similar to those after treatment with isocohumulone, which activates both PPARα and PPARγ in vitro as efficiently as farnesol. Although no induction of PPARγ target gene expression is observed in WATs of the isocohumulone-treated mice, obesity-induced hyperglycemia is improved (56). In our luciferase reporter assay, the addition of farnesol activated PPARα and PPARγ with ED50
values of 5.5 and 28 μM, respectively (41). This indicates that farnesol activates PPARα a little more potently than PPARγ, suggesting that this slight divergence might be reflected in vivo. Moreover, the in vivo distribution of farnesol administered orally might influence its effect on PPARγ activity in WAT. Several drugs, including fibrates, administered orally distribute more abundantly in the liver than in WAT (50, 53). Farnesol is oxidized to farnesolic acid in the liver via ω- and β-oxidation, which is converted into several dicarboxylic acids, which are then excreted in urine (21). Thus, hepatic first pass effect might be important. These reports raise the possibility that dietary farnesol exists more abundantly in the liver than in WAT. In this study, the effective dose of 0.5% of farnesol translates into about 15-20 g intake/adult human/day. While this amount seems high, the pharmacokinetic properties of farnesol, such as its gastrointestinal absorption rate and bioavailability in humans, should also be taken into consideration. Because information on pharmacokinetic properties of farnesol is very limited, it is necessary to perform pharmacokinetic studies of farnesol to clarify these issues. Furthermore, pharmacokinetic studies would reveal the concentration of farnesol in serum and tissues. Thus, they might also show why 0.25% farnesol treatment has no effect on hyperglycemia.

In addition to the suppression of hepatic TG accumulation, farnesol treatment significantly increased the mRNA expression levels of hepatic PPARα target genes in both the KK-Ay and wild-type mice but not in the PPARα (-/-) mice. These findings indicate that farnesol activates PPARα, suggesting that the treatment with farnesol subsequently enhances fatty acid oxidation in the liver. This probably causes the suppression of hepatic TG accumulation in the farnesol-treated mice. However, farnesol treatment could not significantly decrease serum TG levels in the KK-Ay mice, although the agonists for PPARα usually improve hyperlipidemia. In contrast to our findings, another recent report has shown that the
administration of farnesol for 7 days significantly decreases serum TG levels in nonobese Sprague-Dawley rats (8). Therefore, if several conditions of farnesol treatment such as applied dose and injection period are optimized, farnesol might be effective for obesity-induced hyperlipidemia.

While bezafibrate treatment resulted in hepatomegaly, farnesol did not cause hepatomegaly, despite the apparent responses mediated by PPARα. Most pure PPARα agonists, including bezafibrate, cause hepatomegaly in rodents in a species-specific manner due to peroxisome proliferation (20). However, there are studies showing that coactivation of PPARα/δ (29) or PPARα/γ (58) does not induce hepatomegaly. Thus, it might be possible that activation of FXR interacts to prevent PPARα-induced hepatomegaly. Alternatively, the PPARα stimulation by farnesol was not strong enough to produce hepatomegaly. In our previous study, the KK-Ay mice fed with HFD containing 0.2% bezafibrate, using the same protocol as given in Fig. 3, demonstrated increases in the hepatic mRNA expression levels with 6.8-, 1.4-, and 8.4-fold increase in ACO, CPT1, and UCP2, respectively (data not shown) in comparison to those for the control HFD-fed mice (with 2.4-, 4.3-, 1.6-fold increases in ACO, CPT1, and UCP2, respectively, in mice fed a 0.5% FOH diet; Fig. 3).

In contrast to the differences in PPARγ target gene expression and serum TG levels, the treatment with farnesol decreased serum glucose level as well as that with bezafibrate. It has been shown that PPARα plays an important role in carbohydrate metabolism in addition to lipid metabolism. Treatment of obese mice with a PPARα agonist also improved insulin resistance, thus decreasing fasting blood glucose level (46). This contradiction seems to be due to the multiple metabolic actions of PPARα, suggesting that PPARα is a key player in whole-body metabolic regulation. Severe hypoglycemia is observed in the PPARα (-/-) mice under fasting conditions (23). Several mechanisms may account for fasting hypoglycemia in
PPARα (-/-) mice, including defective hepatic glucose production (54). Moreover, PPARα directly up-regulates TRB3 (25), which is an inhibitor of Akt, a positive regulator of the cellular response to insulin. On the other hand, although the molecular mechanism underlying the improvement in carbohydrate metabolism induced by a PPARα agonist is not fully clarified, it is at least partly due to an enhanced fatty acid clearance from insulin-sensitive organs, such as the liver and skeletal muscles (5, 15, 24, 38, 57). Recently, Orellana-Gavaldà et al. reported that a long-term increase hepatic CPT1 expression (with a 1.6-fold increase in CPT1 mRNA level) enhanced hepatic fatty acid oxidation and improved obesity-induced carbohydrate dysfunctions, such as hyperglycemia (32). Regarding TG levels in PPARα (-/-) mouse livers, we found that hepatic TG levels in PPARα (-/-) significantly decreased (17% decrease) compared to those in WT, indicating that the farnesol action on the reduction of hepatic TG could be independent on PPARα. Using primary hepatocytes from PPARα (-/-), we further examined whether the ligand action of farnesol is abolished by PPARα (-/-) hepatocytes, and found that farnesol inhibited the incorporation of [1-14C] acetic acid by 48%, and did not alter PPARα target genes in the PPARα (-/-) hepatocytes. These findings suggest that farnesol suppresses hepatic TG synthesis via a PPARα-independent pathway, and this may be attributed to the reduction of hepatic TG by farnesol treatment in PPARα (-/-) mice. Further study may be necessary to clarify the detail mechanisms.

Some bile acids activate the transcription of FXR target genes as endogenous ligands (27, 16). FXR activation regulates the mRNA expressions of various transport proteins and synthetic enzymes crucial to the homeostasis of cholesterol and bile acid (27, 16, 36). In addition, the activation of FXR lowers both hepatic TG contents (52) and plasma glucose level (61). Farnesol treatment induces the mRNA expression of SHP (FXR target gene) irrespective of PPARα function. In the present study, farnesol decreased SREBP-1c and FAS
mRNA expression levels, which was accompanied by the suppression of TG synthesis in hepatocytes. Although it has been reported that PPARα suppresses SREBP-1c expression through the inhibition of the liver X receptor (LXR) (59), farnesol suppressed SREBP-1c mRNA expression independently of PPARα in this study. These findings indicate that FXR activation by farnesol is involved in the down-regulation of SREBP-1c and the suppression of hepatic TG synthesis. Recently, Duncan et al. have described an SHP- and SREBP-1c-independent mechanism of suppression of FAS mRNA expression by farnesol (8). They have shown that farnesol does not affect SHP and SREBP-1c mRNA expressions but significantly suppresses FAS mRNA expression. The reason for these differences in findings is unclear, but it is possible that the differences may be due to the use of different cell types. Duncan et al. used Clone-9 rat hepatocytes, whereas we used primary mouse hepatocytes (8). In addition, they have reported that farnesol does not alter SREBP-1c expression at the concentration of 30 μM (8). In the present study, we were not able to detect the decrease in SREBP-1c mRNA expression level at 30 μM, but were able to do so at 100 μM. Following the treatment with farnesol, SREBP-1c mRNA expression level significantly decreased in the wild-type mice and tended to be lower in the PPARα (-/-) mice. Thus, we speculate that the alteration of SREBP-1c mRNA expression is one of the causes of the suppression of FAS mRNA expression and hepatic TG accumulation in vivo, although several other mechanisms cannot be excluded.

Farnesol activated both PPARα and FXR at almost similar concentrations in luciferase reporter assay, and induced mRNA expressions of their target genes in vitro and in vivo. This is a very unique feature of farnesol. Farnesol has been shown to elicit a single ligand activity for PPARα or FXR, respectively (16, 19, 46, 61). However, its dual ligand action for both PPARα and FXR, especially in whole animal system, has never been studied. To our
knowledge, this is the first study showing that farnesol acts as a dual activator for PPARα and FXR in vitro and in vivo. The dual action, which is a very unique feature of farnesol, may contribute to its protective effect against obesity-induced metabolic disorders. Because the activation of either PPARα or FXR is useful for the improvement in metabolic abnormalities, the dual activation of PPARα and FXR by farnesol seems to be more suitable. In humans, the activation of FXR induces the expression of PPARα via direct binding to the farnesoid-X-response element (FXRE) in the PPARα promoter (45). Although FXRE is absent from the mouse promoter (45), PPARα expression is induced in mice by FXR activation (37). This crosstalk between PPARα and FXR might synergistically enhance the effect of farnesol.

In summary, we reported here the effects of dietary farnesol on carbohydrate and lipid metabolisms. Dietary farnesol prevented HFD-induced hyperglycemia and hepatic steatosis. The dual activation of PPARα and FXR by farnesol seems to be involved in these effects. Dietary patterns rich in vegetables and fruit are associated with a lower prevalence of metabolic syndrome (12). Because farnesol is contained in vegetables and fruit, the present study suggests that farnesol may contribute to decreasing the risk of metabolic syndrome.
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FIGURE LEGENDS

Figure 1. Effects of farnesol treatment on carbohydrate metabolism in KK-Ay mice fed HFD. Obese diabetic KK-Ay mice were fed the control HFD with or without 0.25% or 0.5% farnesol (FOH) or 0.2% bezafibrate (Beza) for 35 days under pair-fed conditions. A: Nonfasting blood samples were drawn from tail veins once a week. Serum glucose level was measured by an enzymatic colorimetric assay. B: Glucosuria incidence (over than 250 mg/dl glucose) in mice fed each experimental diet for 28 days. Glucosuria was not detected (ND) in either 0.5% farnesol- or bezafibrate-treated mice. C-F: Serum glucose levels during oral glucose tolerance test (GTT) (C) and insulin tolerance test (ITT) (E). The area under the curve (AUC) for glucose response in each group (C and E) is shown (D and F, respectively). Both GTT and ITT were performed on KK-Ay mice that were fed HFD or HFD containing 0.2% bezafibrate (Beza) for 3-4 weeks under pair-fed conditions. Serum glucose levels were measured using the enzymatic colorimetric assay. Data are represented as mean ± SEM of 5-7 animals per group. * P < 0.05, ** P < 0.01, as compared with control diet group.

Figure 2. Effect of farnesol treatment on hepatic lipid accumulation. The liver was isolated from mice fed each experimental diet as mentioned in Fig. 1. A: TG levels were measured by an enzymatic colorimetric assay. Data are represented as mean ± SEM of 5-6 animals per group. * P < 0.05, ** P < 0.01, as compared with control diet group. B: Histochemistry of liver. The isolated livers were fixed in 10% formalin for more than 24 hours, and embedded in the OCT compound in isopentane cooled with liquid nitrogen. The frozen OCT-embedded sections were cut at 8 µm thickness and placed on microscope slides. The liver sections were stained with Sudan III and hematoxylin. The original magnification is x 200.
Figure 3. Effects of farnesol treatment on mRNA expression levels of PPARγ target genes in mesenteric adipose tissue and PPARα target genes in the liver. A-F: KK-Ay mice were treated with the control HFD or 0.25% or 0.5% farnesol-containing HFD. mRNA expression levels of PPARγ target genes in mesenteric adipose tissue (A-C) and mRNA expression levels of PPARα target genes in the liver (D-F) were measured by real-time monitoring RT-PCR. The mRNA expression level of each gene was normalized to the mRNA expression level of the ribosomal 36B4 gene. The mRNA expression level in control HFD-fed mice was set at 100% and relative expression levels are presented as fold inductions over a vehicle control. A: aP2, B: LPL, and C: adiponectin mRNA expression levels in mesenteric adipose tissue. D: CPT1, E: ACO, and F: UCP2 mRNA expression levels in liver. Results are represented as mean ± SEM, normalized to the mRNA expression levels of the 36B4 gene of 4-5 animals per group. * P < 0.05, ** P < 0.01, compared with control diet group.

Figure 4. Effects of farnesol treatment on hepatic TG accumulation and serum glucose level in the wild-type and PPARα (-/-) mice. Wild-type PPARα (+/+) mice and PPARα (-/-) mice were fed the control HFD (Cont) or HFD with 0.5% farnesol (FOH)- or 0.2% bezafibrate (Beza), for 6 weeks under pair-fed conditions. A: Serum was collected from each mouse after 8 hours of fasting. Serum glucose level was measured by an enzymatic colorimetric assay. B: The levels of TG extracted from the liver in mice fed each diet were measured by an enzymatic colorimetric assay. Data are represented as mean ± SEM of 4-5 animals per group. * P < 0.05, ** P < 0.01, compared with a control diet group of the same genotype.
Figure 5. Effects of farnesol treatment on mRNA expression levels of PPARα target genes in the liver of the wild-type and PPARα (-/-) mice. A-E: The liver was isolated from mice fed each experimental diet as mentioned in Fig. 4. The mRNA expression levels of PPARα target genes involved in fatty acid metabolism (ACO, FAT, FABP1, ACS, and UCP2) in the liver of the wild-type PPARα (+/+) mice or PPARα (-/-) mice were measured as described before. The mRNA expression level in control HFD-fed PPARα (+/+) mice was set at 100%, and the relative expression levels are presented as fold inductions. mRNA expression levels are shown for ACO (A), FAT (B), FABP1 (C), ACS (D), and UCP2 (E). Results are represented as mean ± SEM, normalized to the mRNA expression levels of the 36B4 gene of 3-4 animals per group. * P < 0.05 between the farnesol-enriched diet-fed group and the control diet fed group of the same genotype. N.S., not significant.

Figure 6. Effects of farnesol treatment on TG synthesis and mRNA expression levels of PPARα target genes in primary cultured hepatocytes isolated from the wild-type and PPARα (-/-) mice. A: Primary cultured hepatocytes isolated from the wild-type PPARα (+/+) and PPARα (-/-) mice were treated with 100 μM farnesol (FOH) for 24 hours. Then, the cells were incubated in the presence of [1-14C] acetic acid (370 kBq/ml) for 2 hours. Cellular lipid was extracted and separated by thin layer chromatography, and radioactivity in cellular TGs was determined. TG synthesis level in vehicle-treated (Cont) PPARα (+/+) hepatocytes was set at 100%, and relative TG synthesis levels are presented as fold inductions. B-D: Total RNA was extracted from the hepatocytes, which were isolated from the PPARα (+/+) and PPARα (-/-) mice, treated with 30 μM or 100 μM farnesol for 24 hours. The mRNA expression levels of PPARα target genes of CPT1 (B), ACO (C), and UCP2 (D) were measured as described before. The mRNA expression level in vehicle-treated (Cont) PPARα
(+/-) hepatocytes was set at 100% and relative expression levels are presented as fold inductions. Data are represented as means ± SEM (n = 3-4). * P < 0.05, ** P < 0.01, as compared with vehicle treatment in the same genotype.

Figure 7. Effects of farnesol treatment on FXR activity. A, B: CV-1 monkey kidney cells were transfected with pCMX-hFXR, pIR1-tk-Luc, and pRL-CMV (A) or pM-hPPARα, p4xUASg-tk-Luc, and pRL-CMV (B). Four hours after the transfection, the cells were incubated with 30 μM or 100 μM farnesol (FOH) for 24 hours. The activity of a vehicle control (Cont) is set at 100% and relative luciferase activities are presented as fold inductions over the vehicle control. C-F: The liver (C) and primary cultured hepatocytes (D) were isolated from the wild-type PPARα (+/+) or the PPARα (-/-) mice as mentioned in Fig. 4 and Fig. 6, respectively. The mRNA expression levels of genes involved in the FXR-SHP-SREBP-1c pathway (SHP, SREBP-1c and FAS) were measured as described before. Data are represented as mean ± SEM (n = 3-4). * P < 0.05, ** P < 0.01, as compared with a control in the same genotype. E: TG biosynthetic activity was determined in primary cultured hepatocytes isolated from C57BL/6 male mice, treated with or without 100 μM farnesol and 60 μM guggulsterone (GS) as described in MATERIAL AND METHODS. The activity of the vehicle control is set to 100%, and the relative TG synthesis activities are presented as fold inductions over the vehicle control. Data are represented as mean ± SEM (n = 3-4). * P < 0.05. N.S., not significant.

Figure 8. Regulation of carbohydrate and lipid metabolisms by farnesol in liver. Dietary farnesol enhanced fatty acid oxidation via the activation of PPARα in the liver, whereas it suppressed hepatic TG biosynthesis through the mechanism that depends on the
activation of the FXR-SHP-SREBP-1c pathway. This dual action of farnesol in the liver might lead to the improvement in hepatic steatosis and hyperglycemia.
Figure 1.
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