FEBS 25863 FEBS Letters 514 (2002) 315–322

Dual action of isoprenols from herbal medicines on both PPARγ and PPARα in 3T3-L1 adipocytes and HepG2 hepatocytes

Nobuyuki Takahashi[a,b], Teruo Kawada[a,b,*], Tsuyoshi Goto[a], Takayuki Yamamoto[a], Aki Taimatsu[a], Naoko Matsui[a], Kazuhiro Kimura[b,c], Masayuki Saito[b,c], Masashi Hosokawa[b,d], Kazuo Miyashita[b,d], Tohru Fushiki[a]

[a] *Laboratory of Nutrition Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[b] *Project for Obesity and Lipid Metabolism Regulation, Bio-oriented Research Advancement Institute (BRAIN), Tokyo 105-0001, Japan*
[c] *Laboratory of Biochemistry, Department of Biomedical Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan*
[d] *Laboratory of Biofunctional Material Chemistry, Division of Marine Bioscience, Graduate School of Fisheries Science, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*

Received 21 January 2002; accepted 24 January 2002

First published online 15 February 2002

Edited by Veli-Pekka Lehto

Abstract Several herbal medicines improve hyperlipidemia, diabetes and cardiovascular diseases. However, the molecular mechanism underlying this improvement has not yet been clarified. In this study, we found that several isoprenols, common components of herbal plants, activate human peroxisome proliferator-activated receptors (PPARs) as determined using the novel GAL4 ligand-binding domain chimera assay system with coactivator coexpression. Farnesol and geranylgeraniol that are typical isoprenols in herbs and fruits activated not only PPARγ but also PPARα as determined using the chimera assay system. These compounds also activated full-length human PPARγ and PPARα in CV1 cells. Moreover, these isoprenols upregulated the expression of some lipid metabolic target genes of PPARγ and PPARα in 3T3-L1 adipocytes and HepG2 hepatocytes, respectively. These results suggest that herbal medicines containing isoprenols with dual action on both PPARγ and PPARα can be of interest for the amelioration of lipid metabolic disorders associated with diabetes.

Key words: Lipid metabolism; Insulin resistance; Obesity; Isoprenoid; Thiazolidinedione; Fibrate

1. Introduction

It is known that several herbal medicines improve diabetes mellitus, hyperlipidemia, and cardiovascular diseases associated with an abnormality in lipid metabolism [1,2]. Such plants contain many bioactive phytochemicals. In particular, terpenoids, which include carotenoids, isoprenoids and their alcohols (isoprenols), are contained in many plants not only for herbal use but also for dietary use [3]. It is known that these dietary terpenoids have multifunctions, such as the suppression of tumor proliferation [3–5], induction of apoptosis [6] and regulation of cation channels [7]. Some terpenoids of intermediates of cholesterol synthesis regulate 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 [8,9]. Such functions of dietary terpenoids are significant for the therapy of diseases, such as cancers or cardiovascular diseases, using food factors.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of a nuclear receptor superfamily [10]. PPARs have three subtypes of α, δ and γ. PPARδ is ubiquitously expressed [11] and is thought to be involved in cell proliferation or carcinogenesis [12,13]. PPARγ is an essential transcription factor in adipogenesis [14] and the overexpression of PPARγ enhances adipogenesis in vitro [15]. On the other hand, hetero-PPARγ knockout results in the inhibition of adipogenesis in vivo, although null-PPARγ deficient mice are embryo lethal [16–18]. Moreover, PPARγ is a regulator of the insulin resistance in adipose tissues. Thiazolidinediones, which are anti-diabetic drugs, improve insulin resistance and this effect depends on the promotion of adipocyte differentiation via PPARγ activation in adipocytes. It appears that the improvement of insulin resistance by PPARγ activation is due to the increase in the number of differentiating adipocytes with a high ability to uptake and to utilize glucose [19,20]. PPARα is highly expressed in the liver, skeletal muscle and kidney [11]. PPARα regulates the expression of target genes involved in lipid catabolism [21] and plays a role in the clearance of circulating and cellular lipids as the target of fibrates, which are anti-hyperlipidemic drugs [22]. The reduction in the level of circulating or cellular lipids by PPARα activation is attributed to the stimulation of fat degradation in peripheral tissues expressing PPARα [23]. Recently, it is thought that a dual or cross activation of both PPARα in the liver and PPARγ in adipocytes is important for the improvement of hyperlipidemia and diabetes associated with obesity.

Here, we report a novel function of terpenoids, particularly

*Corresponding author. Fax: (81)-75-753-6264.
E-mail address: fat@kais.kyoto-u.ac.jp (T. Kawada).

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-response element; CBP, CREB-binding protein; CREB, cAMP-response-element-binding protein; aP2, adipocyte fatty acid-binding protein; LPL, lipoprotein lipase; CPT1A, carnitine palmitoyltransferase 1 liver; ACS, acyl-CoA synthetase; ECH, enoyl-CoA hydratase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

PII: S0014-5793(02)02390-6

isoprenols used in herbal medicines. First, we developed a highly sensitive screening system for PPAR ligands with coexpression of coactivator cAMP-response-element-binding protein (CREB)-binding proteins (CBP). Next, we elucidated that isoprenols (also called terpenols or polyprenyl alcohols), such as farnesol, geranylgeraniol and geraniol activated both PPARγ and PPARα as determined using our highly sensitive screening system. The isoprenol is a type of terpenoids, which are contained in herbal medicines and dietary food. These alcohols regulated the transcription of PPAR target genes in 3T3-L1 adipocytes and HepG2 hepatocytes. These findings show the molecular mechanism of the effects of herbal plants and the possibility to control the clearance of circulating and cellular lipids using food factors.

2. Materials and methods

2.1. Chemical reagents

All carotenoids, terpenoids and fenofibrate were from Nakalai Tesque (Kyoto, Japan) or Sigma (MO, USA). Thiazolidinedione T174, a specific ligand for PPARγ [24], was kindly provided by Tanabe Seiyaku (Osaka, Japan). These compounds were diluted with dimethyl sulfoxide (DMSO) to prepare the stock solutions (10–100 mM). All other chemicals were of guaranteed reagent grade or tissue culture grade.

2.2. Reporter plasmids and luciferase assays

To generate a fusion protein expression vector (pM-PPARγ and pM-PPARα) containing residues 1–147 of the GAL4 DNA-binding domain (DBD) and 204–505 of the human PPARγ ligand-binding domain (LBD) or 167–467 of the human PPARα LBD, the LBDs of hPPARγ and hPPARα from human adipose tissue or liver cDNA libraries (Clontech, CA, USA) were amplified by polymerase chain reaction (PCR) and subcloned into the pM mammalian expression plasmid (Clontech). The reporter plasmid (p4xUASg-tk-luc) contains four copies of a 17-mer upstream activating sequence (UAS) for GAL4 DBD and a thymidine kinase gene promoter (tk-promoter) in front of luciferase cDNA. p4xPPRE-tk-luc contains four copies of the PPAR-response element followed by a tk-promoter and a luciferase reporter gene. pRL-CMV (Promega, WI, USA) was used as an internal control for normalizing transfection efficiency. pCMX-CBP (a CBP expression vector) and pSK-CBP (a control plasmid not expressing CBP proteins) were kindly provided by Dr. Goodman.

CV1 monkey kidney cells were from the American Type Culture Collection (ATCC). CV1 cells were grown in maintenance medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and 10 mg/ml penicillin and streptomycin) at 37°C in 5% CO_2. In the case of the GAL4 chimera system, pM-hPPARγ or pM-PPARα (0.2 μg/well), p4xUASg-tk-luc (0.2 μg/well), pRL-CMV (0.2 ng/well) and pCMX-CBP or pSK-CBP (0.2 μg/well) were transfected into CV1 cells cultured on 24-well tissue culture plates. In the case of a full-length PPAR system, p4xPPRE-tk-luc (0.2 μg/well) and pDEST-hPPARγ or pDEST-hPPARα (full-length PPAR expression vectors) (0.2 μg/well) were also transfected into CV1 cells cultured on 24-well tissue culture plates. In both cases, transfection was performed with a LipofectAMINE system (Invitrogen, CA, USA). After transfection, the cells were cultured for 24 h, and compounds for ligand assay were added into the medium at appropriate concentrations. After an additional 24-h incubation, the cells were lysed for luciferase assay performed using a Dual-Luciferase Reporter Gene Assay system (Promega) according to the manufacturer's protocol.

2.3. Adipocyte and hepatocyte cultures

Differentiation assays on adipocytes were performed using materials and reagents as previously described [25]. In brief, 3T3-L1 murine preadipocytes (from ATCC) were maintained in the maintenance medium with 200 μM ascorbic at 37°C in 5% CO_2. After 4 days when confluence was reached, the cells were incubated in the differentiation medium (DM) containing 0.25 μM dexamethazone, 10 μg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine as in the maintenance medium. After 40 h, the cell culture medium was changed to post-DM containing 5 μg/ml insulin as in the maintenance medium, and fresh post-DM was refed every 2 days. 7 days after differentiation induction, the cells were washed with phosphate-buffered saline and total RNA was prepared using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

HepG2, a human hepatocarcinoma cell line (from ATCC), was maintained on six-well tissue culture plates containing the maintenance medium at 37°C in 5% CO_2. At 80% confluent, 1 μg/well of a human PPARα expression vector, pDEST-hPPARα, or control vector was transfected into cells using a LipofectAMINE Plus system (Invitrogen) and the cells were then cultured in the maintenance medium for 24 h. The transfected cells were incubated in an assay medium containing isoprenols as samples or fenofibrate as a positive control for PPARα activation. After 48 h, total RNA was isolated as described above.

2.4. RNA preparations and real-time fluorescence monitoring reverse transcription (RT)-PCR

Using M-MLV Reverse Transcriptase (Invitrogen), the total RNA was reverse-transcribed according to the manufacturer's instructions with a thermal cycler (Takara PCR Thermal Cycler SP: Takara Shuzo Co., Shiga, Japan). To quantify mRNA expressions, PCR was performed using a fluorescence temperature cycler (LightCycler System: Roche Diagnostics, Mannheim, Germany). The oligonucleotide primers of mouse or human PPAR target genes were designed using a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database as follows: mouse lipoprotein lipase (*LPL*), adipocyte fatty acid-binding protein (*aP2*), human liver carnitine palmitoyltransferase 1 (*CPT1A*), acyl-CoA synthetase (*ACS*), enoyl-CoA hydratase (*ECH*), and mouse/human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Table 1). Amplification was performed according to a published protocol [26]. Briefly, the reaction solution (10 μl final volume) contained 3 μM $MgCl_2$, 2.0 μl of LightCycler DNA Master SYBR Green I dye, and 5 μM of each primer. The standard amplification program included 30 cycles of three steps each, which involved heating the product to 95°C at 20°C/s with a 30 s hold, annealing to 55°C at 20°C/s with a 5 s

Table 1
Sequences of PCR primers and GenBank accession numbers

Strain	Gene	Forward (F), Reverse (R)	GenBank accession number
Mouse	*GAPDH*	F: GAAGGTCGGTGTGAACGGATT	M32599
		R: GAAGACACCAGTAGACTCCACGACATA	
	aP2	F: AAAGACAGCTCCTCCTCGAAGGTT	K02109
		R: TGACCAAATCCCCATTTACGC	
	LPL	F: ATCCATGGATGGACGGTAACG	J03302
		R: CTGGATCCCAATACTTCGACCA	
Human	*GAPDH*	F: TCATCCATGACAACTTTGGTATCG	M33197
		R: GAGCTTGACAAAGTGGTCGTTGA	
	CPT1A	F: AATCATCAAGAAATGTCGCACGA	NM001876
		R: AGGCAGAAGAGGTGACGATCG	
	ACS	F: AGCAGAGCTTCGCAGCGGC	L09229
		R: CTGCTGTTTTCGCTGGGTCC	
	ECH	F: TCTGCAGGTGCTGATATTCGTG	NM001966
		R: AATTGGCTTGTTGCAGAGTCTACG	

hold, and extension to 72°C at 20°C/s with a 10 s hold. The fluorescence at 530 nm was recorded on line at the end of the extension phase. Standards of PCR products amplified from the mRNA of adipocytes or hepatocytes were prepared by the same method as described above using the LightCycler. Each PCR product was subcloned into T-easy vectors (Promega) and used as standard plasmids for standard templates. The copy number of each standard plasmid was calculated from the absorbance at 260 nm and the molecular weight of each plasmid. The copy numbers of standards and samples were amplified simultaneously in the LightCycler. The first cycle number indicated specific fluorescence against noise, and the logarithm of the concentration of the PCR product standard, the external standard curve, was calculated with the LightCycler software version 3. To confirm the amplification of specific transcripts, melting curve profiles were produced at the end of each run.

2.5. Statistical analysis

The data are presented as means ± S.E.M. and were statistically analyzed using the unpaired *t*-test or the Welch *t*-test when variances were heterogeneous. Differences were considered significant when P was < 0.05.

3. Results

3.1. Cotransfection of coactivator CBP improves the sensitivity of PPARγ ligand assay

To study the effects of a coactivator in luciferase assays, we performed PPARγ ligand assays using the GAL4 chimera system with or without the coexpression of CBP, a major coactivator of nuclear receptors including PPARs. Cotransfection of 0.4 μg of pCMX-CBP, a CBP expression vector, induced a higher level of PPARγ/GAL4 transactivation than that of pSK-CBP, a CBP cloning vector (does not express the CBP protein) as shown in Fig. 1A. CBP coexpression induced about 1.8-fold activation at 10 μM T174 thiazolidinedione. Under the same conditions, luciferase activity was stimulated by adding oleic acid. Oleic acid is a ligand of PPARγ as well as polyunsaturated fatty acids [27] and a major component of dietary fatty acids. The concentration of total fatty acids in human serum is 200–500 μM [28]. Thus, oleic acid is a significant food-derived ligand of PPARγ at a physiologically enough concentration to bind to PPARγ. In our luciferase assays, oleic acid activated PPARγ in a dose-dependent manner (Fig. 1B). The activation of PPARγ by addition of oleic acid was enhanced by CBP coexpression as well as that of TZD (1.6-fold at 100 μM). This result suggests the possibility that CBP coexpression can improve the sensitivity of PPARγ/GAL4 luciferase assays.

3.2. Several isoprenols stimulate the activity of PPARγ

We carried out the screening for novel PPAR ligands in natural compounds present in herbal plants using novel luciferase assays with CBP coexpression. As shown in Fig. 2A, we used several terpenoids or carotenoids for this screening, because these compounds are present in many herbal plants [3]. Some terpenoids called isoprenols, such as geraniol, farnesol and geranylgeraniol (their chemical structures are shown in Fig. 2B), activated PPARγ/GAL4 chimera transactivations at 100 μM. At this concentration, the activities induced by geraniol, farnesol and geranylgeraniol were about 2.2-, 4.1- and 3.7-fold as compared to vehicle control, respectively. The isoprenols exerted their effects in a dose-dependent manner (Fig. 3A). The ED_{50}s, the concentration showing a half activity of the maximum in ligand-dependent activation, for PPARγ activated by geraniol, farnesol and geranylgeraniol

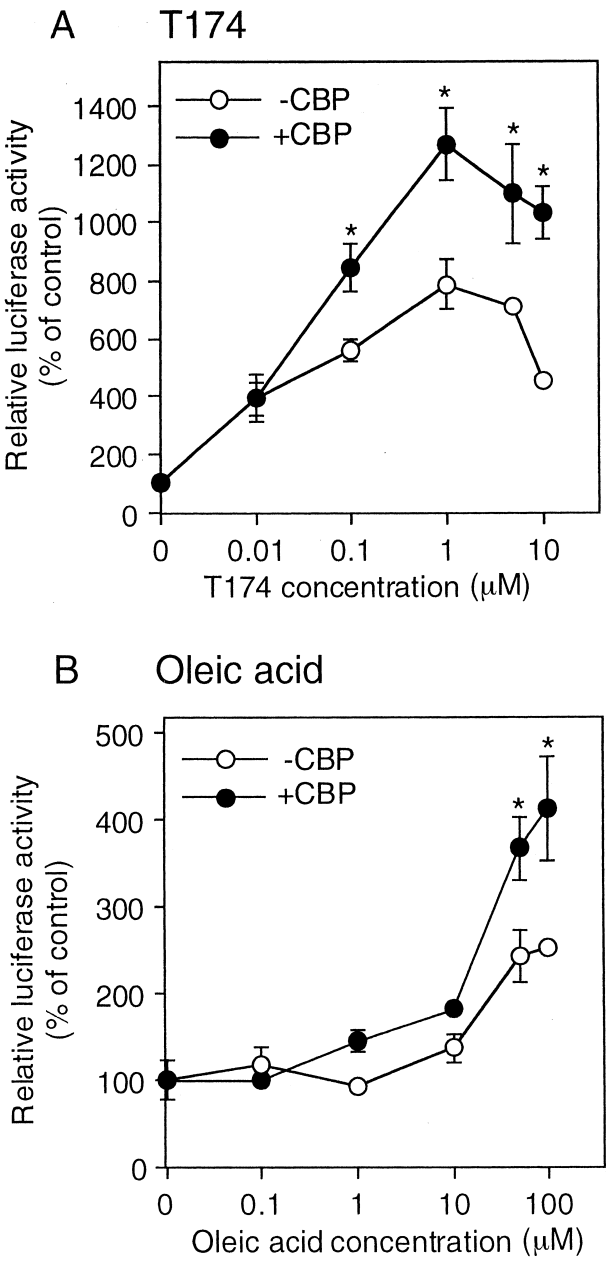

Fig. 1. Effects of coactivator CBP coexpression determined by luciferase ligand assays. CV1 cells were cotransfected with p4xUASg-tk-luc (a reporter plasmid regulated by GAL4/PPARγ chimera protein), pM-hPPARγ (a expression plasmid for GAL4/PPARγ chimera protein) and pCMX-CBP expression vectors (a expression plasmid for CBP, closed circles) or pSK-CBP control plasmids (a control plasmid containing *CBP* cDNA, open circles) and pRL-CMV (an internal control not regulated by compound additions). The activity of a vehicle control was set at 100% and the relative luciferase activities were presented as fold induction to that of the vehicle control. All values are represented as means ± S.E.M. of 3–4 tests. $*P < 0.05$ versus controls without CBP coexpression at the same concentrations of each compound. A: Transfected CV1 cells were treated with T174 thiazolidinedione at various concentrations for 24 h, and cell extracts were subsequently assayed for luciferase activities as described in Section 2. B: Transfected CV1 cells were treated with oleic acid. Closed circles indicate CV1 cells transfected with pCMX-CBP; open circles, CV1 cells transfected with pSK-CBP, a control plasmid to make the amount of DNA transfected constant.

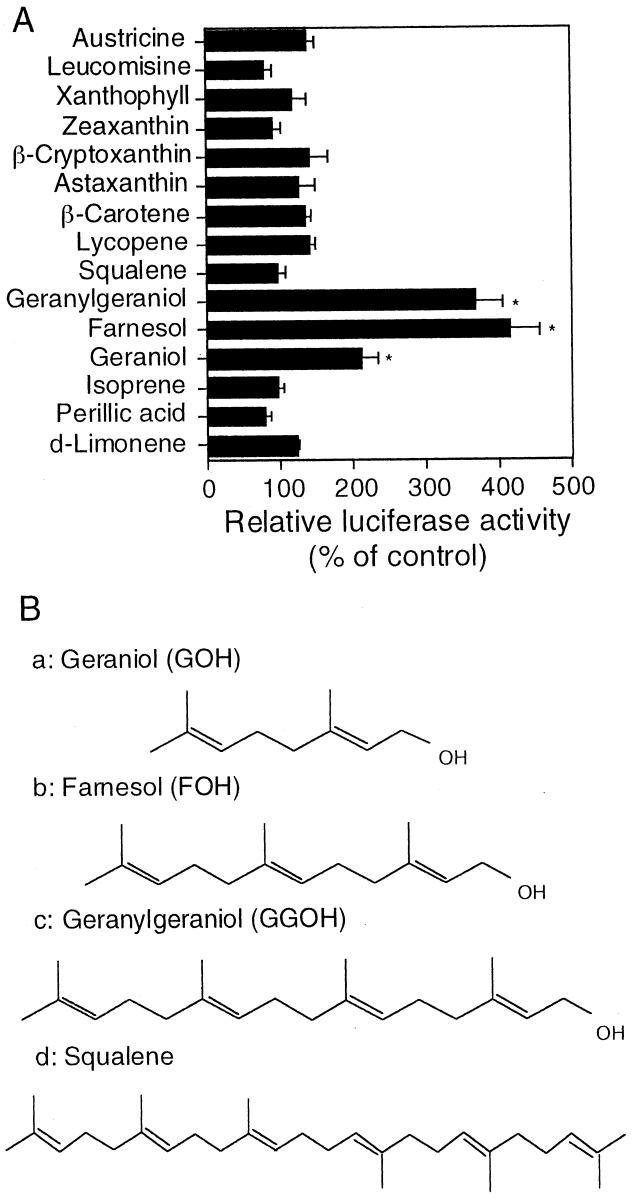

Fig. 2. Effects of various carotenoids and terpenoids on the activation of the PPARγ ligand and the chemical structures of isoprenols. A: pM-hPPARγ, p4xUASg-tk-luc and pRL-CMV were transfected into CV1 cells together with pCMX-CBP and, 24 h after the transfection, the cells were treated with various terpenoids at 100 μM for 24 h. These compounds are classified into several groups: non-acylic isoprenoids (austricine and leucomisine); carotenoids (xanthophylls, zeaxanthin, β-cryptoxanthin, astaxanthin and β-carotene); long-chain isoprenoids (lycopene and squalene); isoprenols (geranylgeraniol, farnesol and geraniol); short-chain isoprenoids (isoprene, perillic acid and *d*-limonene). The activity of a vehicle control was set at 100% and the relative luciferase activities were presented as fold induction to that of the vehicle control. The values are means ± S.E.M. of 3–4 tests. $*P < 0.05$ compared with vehicle controls (DMSO only). B: The chemical structures of isoprenols showing PPAR activation; a, geraniol (GOH); b, farnesol (FOH); c, geranylgeraniol (GGOH); d, squalene. Isoprenols have several isoprenoid units in their structures and one hydroxyl group.

were about 135, 28 and 60 μM, respectively. On the other hand, squalene had no effect on PPARγ transactivation (Fig. 3A). These results indicate that some isoprenols induced the transactivation of PPARγ.

Next, we tested whether isoprenols can induce PPARα stimulation in our luciferase assay system. These PPARα ligand assays were performed with CBP coexpression because this coexpression was found to improve the sensitivity of PPARα ligand assays as well as PPARγ ligand assays (data not shown). Although geraniol had no effect, farnesol and geranylgeraniol dose-dependently activated PPARα/GAL4 chimera transactivation (at 100 μM, 10- and 8.6-fold

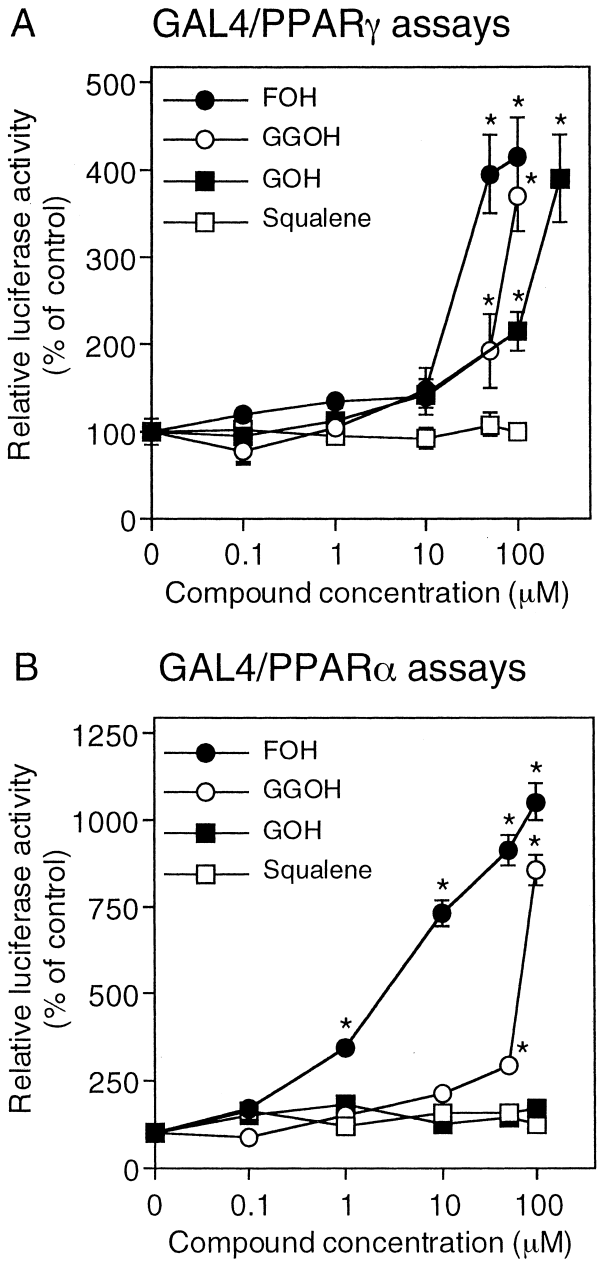

Fig. 3. Isoprenols activated PPARs as determined by luciferase ligand assays using the PPAR chimera system. p4xUASg-tk-luc, pRL-CMV and pCMX-CBP were transfected into CV1 cells together with pM-hPPARγ (A) or pM-hPPARα (B) and then, 24 h after the transfection, the cells were treated with farnesol (FOH; closed circles), geranylgeraniol (GGOH; open circles), geraniol (GOH; closed squares), and squalene (open squares) at various concentrations for 24 h. The activity of a vehicle control was set at 100% and the relative luciferase activities were presented as fold induction to that of the vehicle control. The values are means ± S.E.M. of 3–4 tests. $*P < 0.05$ compared with vehicle controls.

increases compared with that of the vehicle control, respectively) (Fig. 3B). These activities were almost the same as that of 10 μM fenofibrate, one of the fibrates (anti-hyperlipidemia drugs) used as a positive control for PPARα (data not shown) [29]. The ED_{50}s for PPARα activation by farnesol and geranylgeraniol were about 5.5 and 62 μM, respectively. In this regard, these isoprenols have the effects of dual or cross activation of both PPARγ and PPARα.

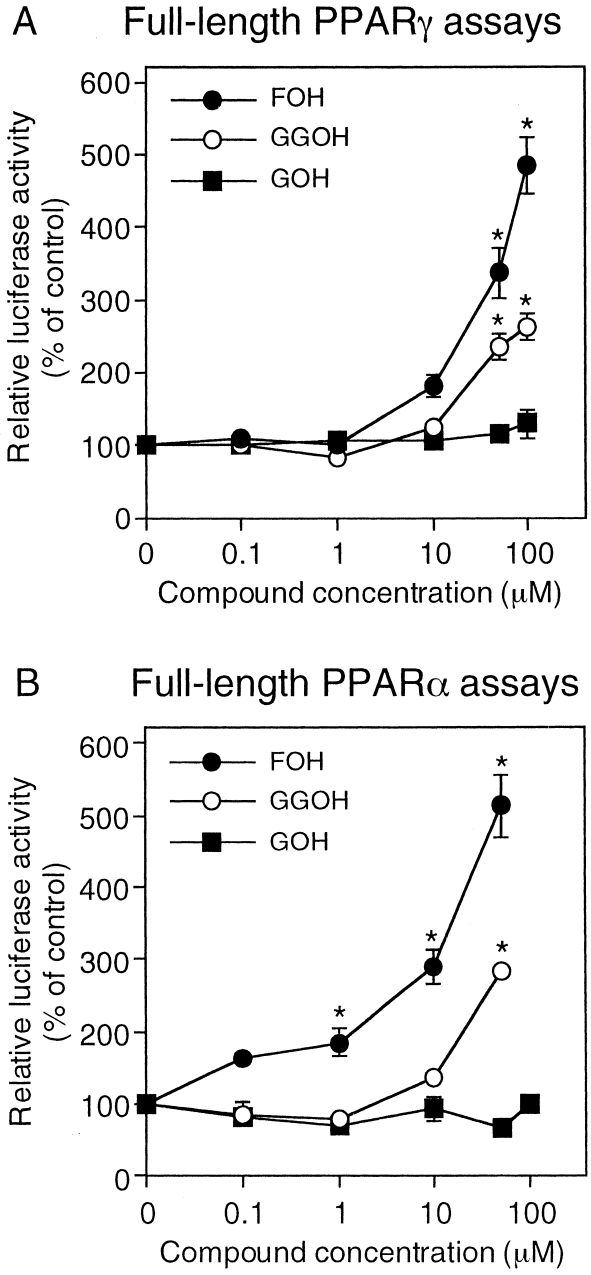

Fig. 4. Farnesol and geranylgeraniol efficiently activated full-length PPARs in cells. p4xPPRE-tk-luc was transfected into CV1 cells together with pDEST-hPPARγ (A) or pDEST-hPPARα (B). 24 h after the transfection, the cells were treated for 24 h with isoprenols at various concentrations; closed circles, farnesol (FOH); open circles, geranylgeraniol (GGOH); closed squares, geraniol (GOH); open squares, squalene. The activity of a vehicle control was set at 100% and the relative luciferase activities were presented as fold induction to that of the vehicle control. The values are means ± S.E.M. of 3–4 tests. $*P < 0.05$ compared with vehicle controls.

3.3. Isoprenols activate full-length PPARγ and PPARα

It was investigated whether isoprenols activate full-length PPARs using the p4xPPRE-tk-luciferase reporter plasmid. T174 thiazolidinedione stimulated PPAR-response element (PPRE)-dependent luciferase activities in human PPARγ-transfected CV1 cells (about 8.3-fold at 10 μM) (data not shown). Farnesol and geranylgeraniol also upregulated luciferase activities in a dose-dependent manner (Fig. 4A). The addition of 100 μM farnesol strongly induced luciferase activity 4.8-fold, although geranylgeraniol weakly induced luciferase activity 2.6-fold. Moreover, these isoprenols activated the transcription of pPPRE luciferase in human PPARα-transfected CV1 cells (Fig. 4B). Farnesol and geranylgeraniol induced luciferase activities 5.1- and 2.8-fold, respectively, at 100 μM. On the other hand, the addition of geraniol only very weakly induced luciferase activity at 100 μM in either human PPARγ- or PPARα-transfected cells. These results were consistent with those using the GAL4/PPAR chimera system. Therefore, taken together, these results indicate that isoprenols activate both full-length PPARγ and PPARα.

3.4. Isoprenols induce the expressions of PPAR target genes in adipocytes and hepatocytes

We attempted to elucidate the effects of isoprenols on cell functions via PPAR activation. First, we used differentiation-induced 3T3-L1 cells as differentiated adipocytes. 7 days after differentiation induction, 3T3-L1 cells differentiated into adipocytes with high glycerophosphate dehydrogenase activity, a marker of adipocyte differentiation, and high-level expression of PPARγ (data not shown) [30]. At this point, PPARγ ligands can efficiently induce PPARγ target genes in the cells. T174 thiazolidinedione, farnesol, or geranylgeraniol was added into the medium of the 3T3-L1 adipocytes and the culture was incubated for 24 h. The addition of 10 μM T174 thiazolidinedione resulted in 7.7- and 2.9-fold increase in the expression levels of both *aP2* and *LPL*, respectively, under this condition. Although there was no increase in *LPL* expression level, the addition of 50 μM farnesol and geranylgeraniol resulted in 6.0- and 5.2-fold increases in the expression level of *aP2* in 3T3-L1 adipocytes compared with that of the vehicle control (Fig. 5A). These data indicate that isoprenols regulate an endogenous promoter region of *aP2* in 3T3-L1 adipocytes.

Second, we used HepG2 human hepatocarcinoma cells to investigate whether isoprenols can induce expressions of PPARα target genes in hepatocytes. We transfected pDEST-hPPARα (a human PPARα expression vector) into HepG2 cells and the transfected HepG2 cells were treated for 48 h with 20 μM fenofibrate as a positive control and with isoprenols (100 μM) after 24 h after transfection. Transfection efficiencies of pDEST-hPPARα into HepG2 cells were not different from each well in preliminary experiments (data not shown). Among the target genes that were already reported to be upregulated by PPARα in HepG2 cells [21], the expression levels of *CPT1A* and *ACS* increased 6.2- and 4.8-fold, respectively, following the addition of fenofibrate compared with that of the vehicle control. The addition of farnesol resulted in a 4.2-fold increase in the *CPT1A* expression level and a 6.0-fold increase in the *ACS* expression compared with that of the vehicle control (Fig. 5B). The addition of geranylgeraniol resulted in no difference in the *CPT1A* expression level but a 5.4-fold increase in the *ACS* expression

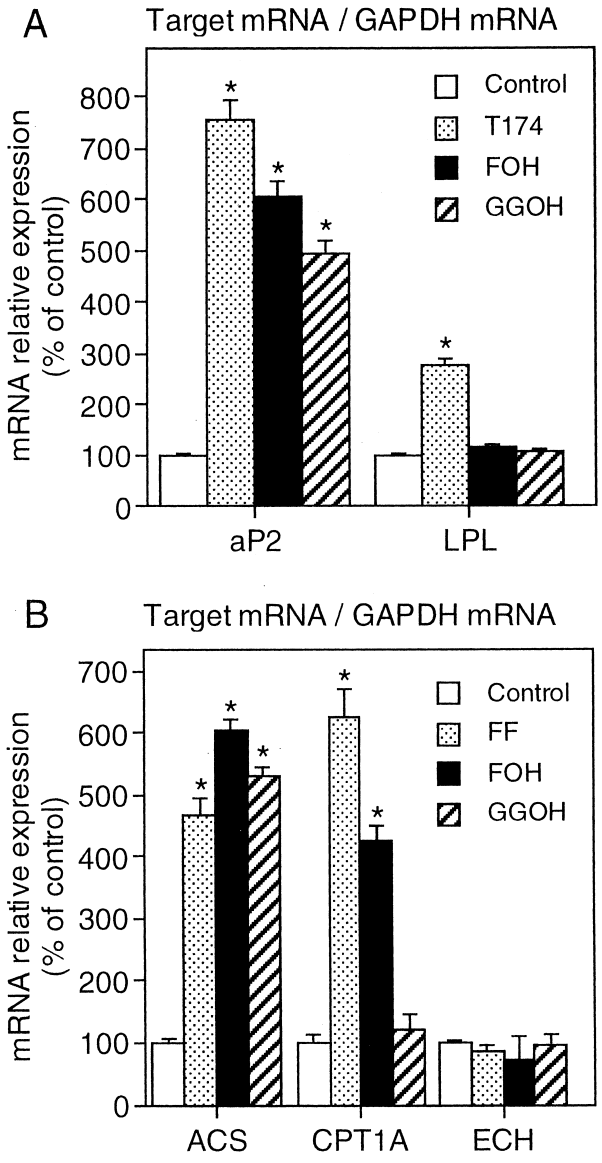

Fig. 5. Farnesol and geranylgeraniol induced PPAR target genes in 3T3-L1 and HepG2 cells. A: Effects of isoprenols on differentiated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with vehicle control (DMSO), 0.5 μM T174 (thiazolidinedione), 50 μM farnesol, and 50 μM geranylgeraniol for 24 h 7 days after the differentiation induction. Expression of mRNAs was estimated by using LightCycler, a quantitative real-time RT-PCR (see Section 2). B: Effects of isoprenols on HepG2 hepatocytes. HepG2 cells were transfected with pDEST-hPPARα and treated with vehicle control (DMSO), 20 μM fenofibrate, 100 μM farnesol, and 100 μM geranylgeraniol, respectively, 24 h after transfection. Total RNAs were prepared 48 h after the addition of compounds. Expression level of a vehicle control in each experiments was set at 100% and the relative expression levels were presented as fold induction to that of the vehicle control. All values are means ± S.E.M. of 5–6 tests. $*P < 0.05$ compared with vehicle controls.

level compared with that of the vehicle control (Fig. 5B). On the other hand, the expression level of *ECH* was not influenced by the addition of any compounds. These data show that isoprenols can regulate the expression of promoters of PPARα target genes in human PPARα-transfected HepG2 hepatocytes.

4. Discussion

Nuclear receptors including PPARs require regulatory proteins such as coactivators for their activation [31,32]. It is possible to consider that the amount of coactivators in cells could be a limiting factor in defining the activity of nuclear receptors. In particular, when a transient overexpression system of nuclear receptors was used, the expression level of coactivators could be one of the rate-limiting factors. Therefore, we thought that the amount of coactivators was insufficient for maximizing the ligand-dependent activity of PPARs in luciferase assays. In such a case, coexpression of coactivators could improve the sensitivity of luciferase assays. Indeed, the coexpression of CBP, one of the major coactivators, increased luciferase activity induced by T174 and oleic acid as shown in Fig. 1. The effect of coactivator coexpression depended on the amount of pCMX-CBP, a CBP expression vector (data not shown). It is consistent with reports of other groups in which the activities of several nuclear receptors or transcriptional factors depended on the amount of coactivator proteins [31–33]. The increase in what of transfected CBP expression vectors might result in the decrease in transfecting efficiency because *CBP* cDNA is very long (about 9k bp). Our condition reported here was the most effective as determined by several experiments. Generally, activation of PPARs by natural compounds is not as strong as that by synthetic compounds such as thiazolidinediones or fibrates [34,35]. Therefore, it is valuable for the screening of natural compounds that the coactivator coexpression system be used to improve the sensitivity of luciferase reporter assays. It is thought that coactivator coexpression allows easy detection and estimation of moderate effects of natural compounds on PPAR activation.

We have here shown that several isoprenols, which are components of medicinal plants used for managing hyperlipidemia or diabetes mellitus, can activate both PPARγ and PPARα. In luciferase assays using the GAL4 chimera system, farnesol and geranylgeraniol were effective activators for both PPARγ and PPARα, whereas geraniol was weaker than the two. This was confirmed by analysis using full-length PPARγ and PPARα proteins. PPARγ activation in adipose tissues improves insulin resistance [19,20,36] and PPARα activation in the liver induces a decrease in circulating lipid levels [22,23]. These effects are due to the regulation at the mRNA level of target genes of PPARs. The addition of these isoprenols upregulated several PPAR target genes in 3T3-L1 and HepG2 cells. In 3T3-L1 cells, *LPL* was not induced by isoprenols. This might be because the effect of isoprenols on gene induction was not so strong or different from that of synthetic compounds due to the difference in ligand type-specific interactions with coactivators [37]. However, isoprenols can regulate an adipocyte-specific gene, *aP2*, which is regulated by PPARγ in 3T3-L1 cells. *aP2* was found to be central to the pathway linking obesity to insulin resistance in a study using *aP2* mutant (−/−) mice [38]. Therefore, it is possible that isoprenols could regulate insulin resistance and/or circulating lipid levels. The finding of the dual or cross-effect of isoprenols in the activation of both PPARγ and PPARα is very important in terms of clarifying the mechanisms by which herbal plants exert their effects and is valuable for managing diabetes and hyperlipidemia using herbal medicine. Moreover, this induction of PPAR target genes suggests that isoprenols at appropriate concentrations could be effective even in vivo.

Indeed, farnesol and geraniol were effective in suppressing tumor growth in vitro at 50 and 150 μM IC_{50}s, respectively [39], which are similar to ED_{50}s obtained in our luciferase assays. Almost the same effects were observed in rats fed with diets containing 0.5–2.0% farnesol or geraniol [3–5,37]. Therefore, it is thought that isoprenols regulate the expression of PPAR target genes in vivo as well as in vitro.

Furthermore, it is noteworthy that pyrophosphate forms of farnesol and geranylgeraniol are intermediates in cholesterol de novo synthesis from mevalonate. Interestingly, a recent study showed that lavastatin, which is one of the inhibitors of a rate-limiting enzyme, HMG-CoA reductase, in cholesterol de novo synthesis, downregulated glucose transporter 4 (*Glut4*) expression and suppressed glucose uptake into 3T3-L1 cells to induce insulin resistance [40]. This suggests that cholesterol de novo synthesis from mevalonate, which is stimulated under the condition of cholesterol starvation, regulates *Glut4* expression in 3T3-L1 cells. Moreover, Spiegelman et al. reported that the overexpression of steroid-response element-binding proteins (SREBPs) in 3T3-L1 generated ligands that activated PPARγ [41]. This suggested that PPARγ ligands were generated in cholesterol de novo synthesis, because SREBPs were activated under the condition of cholesterol starvation and promoted cholesterol synthesis. Although further studies are needed, it is possible that pyrophosphate and/or pyrophosphate-free forms of farnesol and/or geranylgeraniol stimulate PPARγ to regulate the expression of *Glut4* at the basal level or under the condition of cholesterol starvation in 3T3-L1 cells.

Our previous study demonstrated that carotenoids and retinoids regulated adipogenesis by changing PPARγ activity [42]. In this study, we showed that isoprenols were also activators of PPARα and PPARγ, which controlled lipid metabolism. Therefore, it is thought that some phytochemicals, which include carotenoids, terpenoids and isoprenoids, are possible activators of PPARs. Our findings reported here provide not only a significant molecular basis of how herbal medicines containing phytochemicals such as isoprenols improve diabetes or hyperlipidemia, but also suggest the possibility that phytochemicals have therapeutic applications in lipid abnormalities, such as obesity, diabetes mellitus and hyperlipidemia.

Acknowledgements: This work was supported for a large part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (13460058), and PROBRAIN Project from Bio-oriented Technology Research Advancement Institution, Japan.

References

[1] Elson, C.E., Underbakke, G.L., Hanson, P., Shrago, E., Wainberg, R.H. and Qureshi, A.A. (1989) Lipids 24, 677–679.
[2] Miller, A.L. (1998) Altern. Med. Rev. 3, 422–431.
[3] He, L., Mo, H., Hadisuslio, S., Qureshi, A.A. and Elson, C.E. (1997) J. Nutr. 127, 668–674.
[4] Burke, Y.D., Stark, M.J., Roach, S.L., Sen, S.E. and Crowell, P.L. (1997) Lipids 32, 151–156.
[5] Yu, S.G., Hildebrandt, L.A. and Elson, C.E. (1995) J. Nutr. 125, 2763–2767.
[6] Mo, H. and Elson, C.E. (1999) J. Nutr. 129, 804–813.
[7] Roullet, J.B., Luft, U.C., Xue, H., Champan, J., Bychkov, R., Roullet, C.M., Luft, F.C., Haller, H. and McCarron, D.A. (1997) J. Biol. Chem. 272, 32240–32246.
[8] Bradfute, D.L. and Simoni, R.D. (1994) J. Biol. Chem. 269, 6645–6650.
[9] Correll, C.C., Ng, L. and Edwards, P.A. (1994) J. Biol. Chem. 269, 17390–17393.
[10] Kersten, S., Desvergne, B. and Wahli, W. (2000) Nature 405, 421–424.
[11] Braissant, O. and Wahli, W. (1998) Endocrinology 139, 2748–2754.
[12] He, T.C., Chan, T.A., Vogelstein, B. and Kinzler, K.W. (1999) Cell 99, 335–345.
[13] Hansen, J.B., Zhang, H., Rasmussen, T.H., Petersen, R.K., Flindt, E.N. and Kristiansen, K. (2001) J. Biol. Chem. 276, 3175–3182.
[14] Tontonoz, P., Hu, E. and Spiegelman, B.M. (1994) Cell 79, 1147–1156.
[15] Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I. and Spiegelman, B.M. (1994) Genes Dev. 8, 1224–1234.
[16] Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., Koder, A. and Evans, R.M. (1999) Mol. Cell 4, 585–595.
[17] Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M. and Mortensen, R.M. (1999) Mol. Cell 4, 611–617.
[18] Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., Nagai, R., Tobe, K., Kimura, S. and Kadowaki, T. (1999) Mol. Cell 4, 597–609.
[19] Lehman, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M. and Kliewer, S.A. (1995) J. Biol. Chem. 270, 12953–12956.
[20] Willson, T.M., Cobb, J.E., Cowan, D.J., Wiethe, R.W., Correa, I.D., Prakash, S.R., Beck, K.D., Moore, L.B., Kliewer, S.A. and Lehnmann, J.M. (1996) J. Med. Chem. 39, 665–668.
[21] Hsu, M.H., Savas, U., Griffin, K.J. and Johnson, E.F. (2001) J. Biol. Chem. 276, 27950–27958.
[22] Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E. and Fruchart, J.C. (1998) Circulation 98, 2088–2093.
[23] Peters, J.M., Hennuyer, N., Staels, B., Fruchart, J.C., Fievet, C., Gonzalez, F.J. and Auwerx, J. (1997) J. Biol. Chem. 272, 27307–27312.
[24] Mizukami, J. and Taniguchi, T. (1997) Biochem. Biophys. Res. Commun. 240, 61–64.
[25] Kawada, T., Kamei, Y. and Sugimoto, E. (1996) Int. J. Obes. Relat. Metab. Disord. 20, S52–S57.
[26] Rasmussen, R. (2001) in: Quantification on the LightCycler (Meuer, S., Wittwer, C. and Nakagawara, K., Eds.), Rapid Cycle Real-time PCR, pp. 21–34, Springer, Berlin.
[27] Xu, H.E., Lambert, M.H., Montana, V.G., Parks, D.J., Blanchard, S.G., Brown, P.J., Sternbach, D.D., Lehmann, J.M., Wisely, G.B., Willson, T.M., Kliewer, S.A. and Milburn, M.V. (1999) Mol. Cell 3, 397–403.
[28] Simizu, S. and Yamada, H. (1985) in: Colorimetric Method (Bergmeyer, H.U., Bergmeyer, J. and Grabl, M., Eds.), Methods of Enzymatic Analysis, Vol. VIII, Metabolites 3, pp. 19–25, VCH Verlagsgesellschaft, Weinheim.
[29] Dowell, P., Ishmael, J.E., Avram, D., Peterson, V.J., Nevrivy, D.J. and Leid, M. (1997) J. Biol. Chem. 272, 33435–33443.
[30] Hida, Y., Kawada, T., Kayahashi, S., Ishihara, T. and Fushiki, T. (1998) Life Sci. 62, 205–211.
[31] Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J.C. and Auwerx, J. (1999) J. Biol. Chem. 274, 7681–7688.
[32] Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1996) Cell 85, 403–414.
[33] Takemaru, K.I. and Moon, R.T. (2000) J. Cell Biol. 149, 249–254.
[34] Forman, B.M., Chen, J. and Evans, R.M. (1997) Proc. Natl. Acad. Sci. USA 94, 4312–4317.
[35] Ferry, G., Bruneau, V., Beauverger, P., Goussard, M., Rodriguez, M., Lamamy, V., Dromaint, S., Canet, E., Galizzi, J.P. and Boutin, J.A. (2001) Eur. J. Pharmacol. 417, 77–89.

[36] Willson, T.M., Lambert, M.H. and Kliewer, S.A. (2001) Annu. Rev. Biochem. 70, 341–367.
[37] Kodera, Y., Takeyama, K., Murayama, A., Suzawa, M., Masuhiro, Y. and Kato, S. (2000) J. Biol. Chem. 275, 33201–33204.
[38] Hotamisligil, G.S., Johnson, R.S., Distel, R.J., Ellis, R., Papaioannou, V.E. and Spiegelman, B.M. (1996) Science 274, 1377–1379.
[39] Elson, C.E. (1995) J. Nutr. 125, 1666S–1672S.
[40] Chamberlain, L.H. (2001) FEBS Lett. 507, 357–361.
[41] Kim, J.B., Wright, H.M., Wright, M. and Spiegelman, B.M. (1998) Proc. Natl. Acad. Sci. USA 95, 4333–4337.
[42] Kawada, T., Kamei, Y., Fujita, A., Hida, Y., Takahashi, N., Sugimoto, E. and Fushiki, T. (2000) BioFactors 13, 103–109.