Available online at www.sciencedirect.com

SCIENCE DIRECT

Journal of Nutritional Biochemistry 17 (2006) 410–418

Journal of Nutritional Biochemistry

Differential action of 13-HPODE on PPARα downstream genes in rat Fao and human HepG2 hepatoma cell lines

Bettina König*, Klaus Eder

Institut für Ernährungswissenschaften, Martin-Luther-Universität Halle-Wittenberg, D-06108 Halle (Saale), Germany

Abstract

In rats, oxidized fats activate the peroxisome proliferator-activated receptor α (PPARα), leading to reduced triglyceride concentrations in liver, plasma and very low density lipoproteins. Oxidation products of linoleic acid constitute an important portion of oxidized dietary fats. This study was conducted to check whether the primary lipid peroxidation product of linoleic acid, 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), might be involved in the PPARα-activating effect of oxidized fats. Therefore, we examined the effect of 13-HPODE on the expression of PPARα target genes in the rat Fao and the human HepG2 hepatoma cell lines. In Fao cells, 13-HPODE increased the mRNA concentration of the PPARα target genes acyl-CoA oxidase (ACO), cytochrome P450 4A1 and carnitine-palmitoyltransferase 1A (CPT1A). Furthermore, the concentration of cellular and secreted triglycerides was reduced in Fao cells treated with 13-HPODE. Because PPARα mRNA was not influenced, we conclude that these effects are due to an activation of PPARα by 13-HPODE. In contrast, HepG2 cells seemed to be resistant to PPARα activation by 13-HPODE because no remarkable induction of the PPARα target genes ACO, CPT1A, mitochondrial HMG-CoA synthase and Δ9-desaturase was observed. Consequently, cellular and secreted triglyceride levels were not changed after incubation of HepG2 cells with 13-HPODE. In conclusion, this study shows that 13-HPODE activates PPARα in rat Fao but not in human HepG2 hepatoma cells.

Keywords: 13-HPODE; PPARα; Fao; HepG2

1. Introduction

Oxidized fats are generated during processing and storage of foods, and constitute an important portion of Western diets. Increased intake of oxidized fats has been linked to an enhanced incidence of coronary heart disease, endothelial dysfunction and cancer in humans [1,2]. Several studies examined the physiological effects of oxidized dietary fats in animal models [3–6]. In rats, reduced concentrations of triglycerides in liver, plasma and very low density lipoproteins (VLDL) were observed after feeding a diet rich in oxidized fats [6–9]. This is in part due to the reduced expression of lipogenic enzymes in the rat liver [6]. Furthermore, the reduced triglyceride concentrations resulted from an enhanced fatty acid oxidation in the liver. It was shown recently that dietary oxidized fats as well as cyclic fatty acid monomers, components of heated fats, lead to activation of the peroxisome proliferator-activated receptor α (PPARα) and cause an increased expression of its target genes [10–12]. This resembles the mechanism of action of fibrates, a class of hypolipidemic drugs [13].

Peroxisome proliferator-activated receptors are transcription factors belonging to the superfamily of nuclear receptors and are implicated in the regulation of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation, cancer development as well as in the control of the inflammatory response [14–16]. They can be activated by peroxisome proliferators, including fibrates, fatty acids and eicosanoids, and regulate the expression of target genes by binding to DNA sequence elements as heterodimers with the 9-*cis*-retinoic acid receptor [17]. PPARα downstream genes participate in aspects of lipid catabolism such as fatty acid uptake and binding, peroxisomal, mitochondrial and

Abbreviations: 13-HPODE, 13-hydroperoxy-9,11-octadecadienoic acid; ACO, acyl-CoA oxidase; CPT1A, carnitine-palmitoyltransferase 1A; Cyp4A1, cytochrome P450 4A1; FAS, fatty acid synthase; FCS, fetal calf serum; HMGCoAS2, mitochondrial HMG-CoA synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PPAR, peroxisome proliferator-activated receptor; PUFAs, polyunsaturated fatty acids; SREBP, sterol regulatory element-binding protein; VLDL, very low density lipoprotein.

* Corresponding author.

E-mail address: bettina.koenig@landw.uni-halle.de (B. König).

0955-2863/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2005.08.011

microsomal fatty acid oxidation and lipoprotein assembly and transport [14].

Oxidized fats include a mixture of primary and secondary lipid peroxidation products depending on their thermal treatment. Heating fats at relatively low temperatures over a long period results in high concentrations of primary lipid peroxidation products. These fats have a more pronounced effect on the lipid metabolism of rats than fats heated at a high temperature for a shorter period [6]. Linoleic acid is the most predominant fatty acid among dietary polyunsaturated fatty acids (PUFAs). Its primary autooxidation product is 13-hydroperoxy-9, 11-octadecadienoic acid (13-HPODE). In plants and mammals, 13(*S*)-HPODE is the product of linoleic acid oxidation by lipoxygenases.

This study was designed to test whether 13-HPODE might be involved in the observed activation of PPARα by oxidized fats. So far, only cyclic fatty acid monomers are suggested to act as peroxisome proliferator analogs [10]. As a model system, the rat hepatoma cell line Fao was chosen, which is commonly used for examination of interactions of different substances with PPARα [18–20]. Considering the known species-specific differences of the action of fibrates and other peroxisome proliferators [21], we used the human hepatoma cell line HepG2 to evaluate the relevance of the obtained results for humans. HepG2 and Fao cells have been already used for evaluation of species-specific differences in peroxisome proliferator action [20,22,23].

Thus, this study was focused on the ability of 13-HPODE to activate PPARα in the rat and human hepatoma cell lines, Fao and HepG2, respectively. Therefore, we analyzed the mRNA concentrations of PPARα and selected PPARα downstream genes like acyl-CoA oxidase (ACO), cytochrome P450 4A1 (Cyp4A1), carnitine-palmitoyltransferase 1A (CPT1A), mitochondrial HMG-CoA synthase (HMGCoAS2) and Δ9-desaturase using a semiquantitative PCR. Considering the observed suppression of lipogenic enzymes by oxidized fats [6], we further examined the genes coding for sterol regulatory element-binding proteins (SREBPs) and fatty acid synthase (FAS). In addition, we examined the effect of 13-HPODE on the concentrations of cellular cholesterol and cellular and secreted triglycerides in Fao and HepG2 cells.

2. Materials and methods

2.1. Materials

Linoleic acid, soybean lipoxygenase (type V) and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Munich, Germany). F-12 Nutrient Mixture (Ham), RPMI 1640, trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA), gentamycin and fetal calf serum (FCS) were obtained from Invitrogen (Karlsruhe, Germany). Rat hepatoma Fao cell line was obtained from ECACC (Salisbury, UK) and human HepG2 hepatoma cell line was from DSMZ (Braunschweig, Germany).

2.2. Cell culture

HepG2 human hepatoma cells were grown in RPMI 1640 medium supplemented with 10% FCS and 0.05 mg ml^{-1} gentamycin. Fao rat hepatoma cells were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg ml^{-1} gentamycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, cells were seeded in 24-well cell culture plates at a density of 2.1×10^5 (Fao) and 1.7×10^5 (HepG2) cells per well and used prior reaching confluence (usually 3 days after seeding). The cells were preincubated with low-serum medium (0.5% FCS) for 16 h and then stimulated for the times indicated. Fatty acids were added to the low-serum medium from a stock solution in ethanol. Cells treated with the appropriate vehicle concentration were used as a control.

Cell viability after treatment with fatty acids was assessed by the MTT assay [24].

2.3. Preparation of 13-HPODE

13-HPODE was prepared by oxidation of linoleic acid with soybean lipoxygenase according to Funk et al. [25]. Stock solution of linoleic acid was prepared in absolute ethanol and then diluted in PBS. The linoleic acid was incubated with soybean lipoxygenase (100 U/100 nmol, 2 h at 37°C). The formation of 13-HPODE was monitored spectrophotometrically between 200 and 300 nm, using PBS as reference. The conversion of linoleic acid into 13-HPODE is observed as an increase in absorbance at 234 nm. Subsequently, the 13-HPODE was extracted twice with *n*-hexane. The *n*-hexane was evaporated and 13-HPODE was dissolved in ethanol. The concentration of the stock solution was calculated using the extinction coefficient of 23 mM^{-1} cm^{-1} for conjugated diens.

2.4. RT-PCR analysis

Measurement of mRNA concentration was done as a semiquantitative analysis using β-actin for normalization. Total RNA was isolated from cells by Trizol reagent (Life Technologies, Karlsruhe, Germany) according to the manufacturer's protocol. Total RNA (1.2 μg) was used for cDNA synthesis with 60 U Revert Aid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) and 0.5 μg oligo dT18 primer (Qiagen, Hilden, Germany) for 1 h at 42°C followed by an inactivation step at 70°C for 10 min. The PCR reaction was carried out in 20 μl PCR buffer containing 2 μl RT reaction, 1 U BioTherm DNA Polymerase (Genecraft, Lüdinghausen, Germany), 200 μM dNTPs and 20 pmol of the specific primers. Sequences and characteristics of the specific primers used for cDNA amplification are listed in Table 1. After initial denaturation at 95°C for 3 min, cycling was performed with

Table 1
Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward primer	Reverse primer	bp	Annealing temperature (°C)	No. of cycles	Genbank accession no.
ACO, rat	5′ CTTTCTTGCTTGCCTTCCTTCTCC 3′	5′ GCCGTTTCACCGCCTCGTA 3′	415	60	27	NM017340
Cyp4A1, rat	5′ CAGAATGGAGAATGGGGACAGC 3′	5′ TGAGAAGGGCAGGAATGAGTGG 3′	460	65	29	NM175837
CPT1A, rat	5′ GGAGACAGACACCATCCAACATA 3′	5′ AGGTGATGGACTTGTCAAACC 3′	416	60	28	NM031559
PPARα, rat	5′ CCCTCTCTCCAGCTTCCAGCCC 3′	5′ CCACAAGCGTCTTCTCAGCCATG 3′	555	65	29	M88592
SREBP-1c, rat	5′ GGAGCCATGGATTGCACATT 3′	5′ AGGAAGGCTTCCAGAGAGGA 3′	191	60	33	XM213329
SREBP-2, rat	5′ CCGGTAATGATGGGCCAAGAGAAAG 3′	5′ AGGCCGGGGGAGACATCAGAAG 3′	400	60	29	XM216989
FAS, rat	5′ CCTCCCCTGGTGGCTGCTACAA 3′	5′ CCTGGGGTGGGCGGTCTTT 3′	224	60	27	X62888
β-Actin, rat	5′ ATCGTGCGTGACATTAAAGAGAAG 3′	5′ GGACAGTGAGGCCAGGATAGAG 3′	429	60	22	NM031144
ACO, human	5′ GTGGGCGCATACATGAAGGAGACC 3′	5′ GTGGCTGGATGCGCTGACTGG 3′	367	65	26	X71440
CPT1A, human	5′ AATCATCAAGAAATGTCGCACGA 3′	5′ AGGCAGAAGAGGTGACGATCG 3′	309	65	32	NM001876
HMGCoAS2, human	5′ AACGCCTAGCCTCCCGAAAGTG 3′	5′ CCATAAGCCCAGGACAGTGATTGC 3′	401	60	36	NM005518
Δ9-Desaturase, human	5′ TTCCTGGCTCTACCCTGTCTGTCC 3′	5′ GGGCACCCTCACCAAGTAAGC 3′	480	60	27	NM005063
FAS, human	5′ CATCGGCGACGTGGGCATTTTG 3′	5′ CCGGGTTCACCAGCAGGGAGCG 3′	494	65	26	BC007909
PPARα, human	5′ TGTGGCTGCTATCATTTGCTGTGG 3′	5′ CTCCCCCGTCTCCTTTGTAGTGC 3′	344	60	31	L02932
SREBP-1, human	5′ GTGGCGGCTGCATTGAGAGTGAAG 3′	5′ AGGTACCCGAGGGCATCCGAGAAT 3′	362	60	30	U00968
SREBP-2, human	5′ CGCCACCTGCCCCTCTCCTTCC 3′	5′ TGCCCTGCCACCTATCCTCTCACG 3′	390	65	28	NM004599
β-Actin, human	5′ GAGCGGGAAATCGTGCGTGAC 3′	5′ GCCTAGAAGCATTTGCGGTGGAC 3′	518	60	19	NM001101

denaturation for 30 s at 95°C, annealing for 30 s at a primer-specific temperature (Table 1) and elongation for 1 min at 72°C, followed by a final extension step for 7 min at 72°C. Initially, the linearity of PCR for each specific primer pair was tested to ensure that amplification of target cDNA remained in the exponential range. The final PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. The relative intensities of the bands were quantified by densitometric analysis and corrected for the corresponding β-actin band densities. Data are expressed relatively to mRNA levels of control cells.

2.5. Lipid extraction and concentration determination

Total cellular lipids were extracted with a mixture of *n*-hexane and isopropanol (3:2). Lipid extracts were dried and dissolved in a small volume of Triton X-100 [26]. For determination of triglyceride concentration in VLDL, cell supernatants were collected after 24 h incubation and centrifuged for 5 min at 350×*g* to remove residual cells. Concentrations of cholesterol and triglycerides were determined using an enzymatic reagent kit (Ecoline S+, Merck, Darmstadt, Germany).

2.6. Statistical analysis

Effects of various concentrations of 13-HPODE or linoleic acid were analyzed using one-way ANOVA using the Minitab Statistical Software (Minitab, State College, PA). For significant *F* values, means were compared by Fisher's multiple range test. Furthermore, the direct comparison of the effects of 13-HPODE and linoleic acid at equal concentration was done by pairwise Student's *t* test. Differences with $P<.05$ were considered to be significant.

3. Results

3.1. Effect of 13-HPODE and linoleic acid on mRNA concentrations of selected genes involved in lipid metabolism of Fao cells

Cell viability of Fao cells was not reduced by 24 h incubation with 13-HPODE up to a concentration of 50 μM as demonstrated by the MTT assay. Incubation of Fao cells with increasing amounts of 13-HPODE led to a significant and concentration-dependent increase of mRNA levels of the PPARα downstream genes ACO, Cyp4A1 and CPT1A (Fig. 1). At the highest 13-HPODE concentration used, mRNA levels of these genes were about 50–70% higher than those of the control cells. mRNA concentration of PPARα itself did not change. At 50 μM 13-HPODE, a slight but significant increase of mRNA concentration was further found for SREBP-1c and SREBP-2, whereas FAS mRNA was not influenced at all (Fig. 1). For comparison, Fao cells were stimulated with linoleic acid under identical conditions. After 6 h incubation, a significant increase of 10–27% of ACO and Cyp4A1 mRNA levels was observed. Pairwise comparison of the relative mRNA levels of cells treated with equal concentrations of 13-HPODE and linoleic acid, respectively, revealed that the effects of 13-HPODE are significantly stronger than those of linoleic acid. mRNA concentrations of all other genes tested were not affected by incubation with linoleic acid (Fig. 1). After 12 h incubation of Fao cells with 13-HPODE, Cyp4A1 and CPT1A mRNA levels remained elevated over control, but to a lesser extent, compared to 6 h incubation, whereas mRNA concentrations of ACO, PPARα, SREBP-1c, SREBP-2 and FAS remained constant (data not shown). No significant changes in mRNA levels of all examined

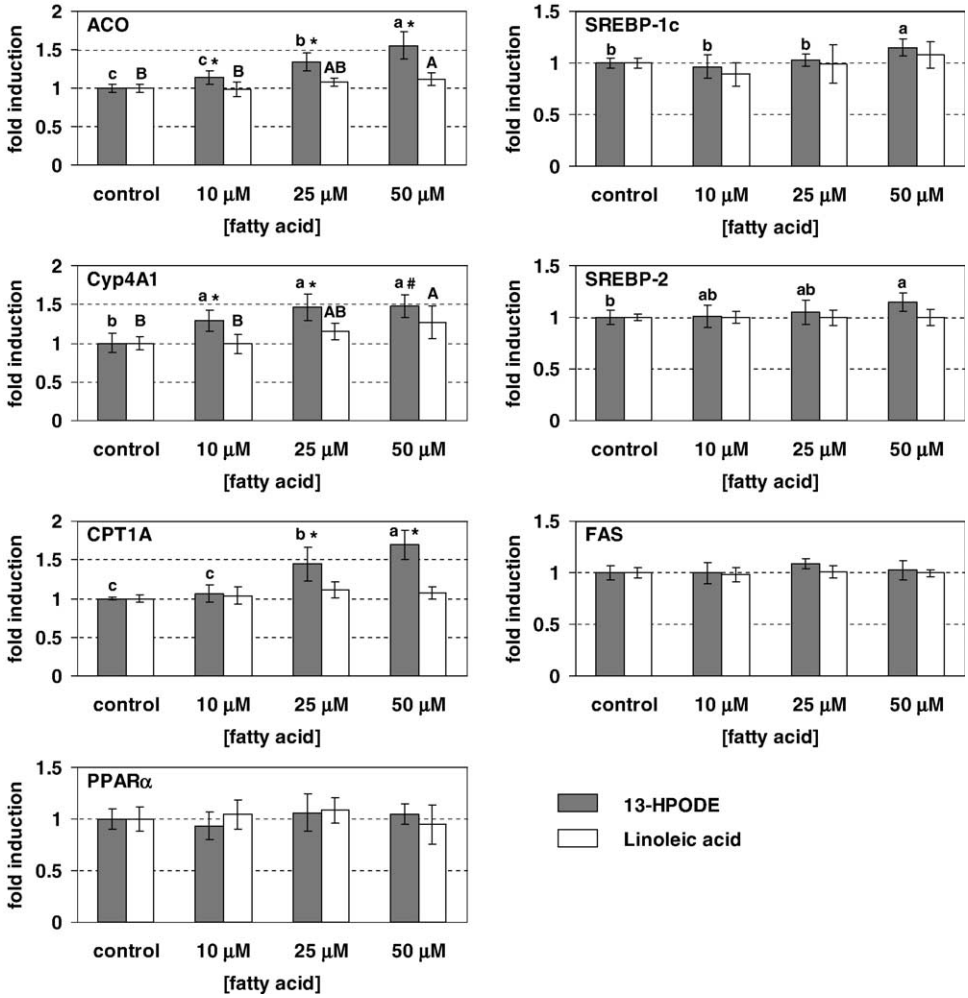

Fig. 1. Effect of 13-HPODE and linoleic acid on mRNA concentrations of selected genes involved in lipid metabolism of Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% FCS). After 16 h preincubation in this medium, 13-HPODE or linoleic acid were added from ethanolic stock solution to give final concentrations of 10, 25 and 50 μM, respectively, and cells were incubated for 6 h. Control cells were incubated with low-serum medium containing vehicle alone. Acyl-CoA oxidase, Cyp4A1, CPT1A, PPARα, SREBP-1c, SREBP-2 and FAS mRNA levels were determined by semiquantitative RT-PCR analysis using β-actin mRNA concentration for normalization as described in the experimental procedures section. Values are means±S.D. ($n=6$). Data are expressed relative to mRNA levels of control cells. Small letters (a, b, c) denote differences in 13-HPODE; capital letters (A, B) denote differences in linoleic acid ($P<.05$). The symbols * and # indicate significant differences at $P<.05$ and $P<.1$, respectively, from linoleic acid at equal concentration.

genes were observed after 24 h incubation. Stimulation of Fao cells with linoleic acid for 12 and 24 h did not influence mRNA concentrations of ACO, Cyp4A1, CPT1A, PPARα, SREBP-1c, SREBP-2 and FAS (data not shown). β-Actin mRNA that was used as a control for normalization was not influenced at all by treatment of the cells with 13-HPODE and linoleic acid, respectively.

3.2. Effect of 13-HPODE on mRNA concentrations of selected genes involved in lipid metabolism of HepG2 cells

Incubation of HepG2 cells with 13-HPODE concentrations above 25 μM strongly reduced cell viability as tested by the MTT assay (data not shown). Consequently, HepG2 cells were incubated with 13-HPODE concentrations up to 25 μM for mRNA analysis. As with Fao cells, the PPARα downstream genes ACO and CPT1A were analyzed. Cyp4A11, the gene corresponding to rat Cyp4A1, was not examined because no Cyp4A11 mRNA was found in HepG2 cells [27,28]. Furthermore, the PPARα target genes Δ9-desaturase and HMGCoAS2 were included. The mRNA concentrations of ACO, CPT1A and Δ9-desaturase were not affected after stimulation of HepG2 cells with 13-HPODE for 6 h. For HMGCoAS2, a slight but significant concentration-dependent induction of mRNA level after 6 h incubation was observed (Fig. 2). No changes in mRNA levels of all examined PPARα downstream genes could be seen after 24 h incubation of HepG2 cells with 13-HPODE (Fig. 2). Peroxisome proliferator-activated receptor α mRNA was readily detectable by RT-PCR in HepG2 cells used in this study, indicating that the absence of effects on PPARα downstream genes was not due to lacking expression of PPARα.

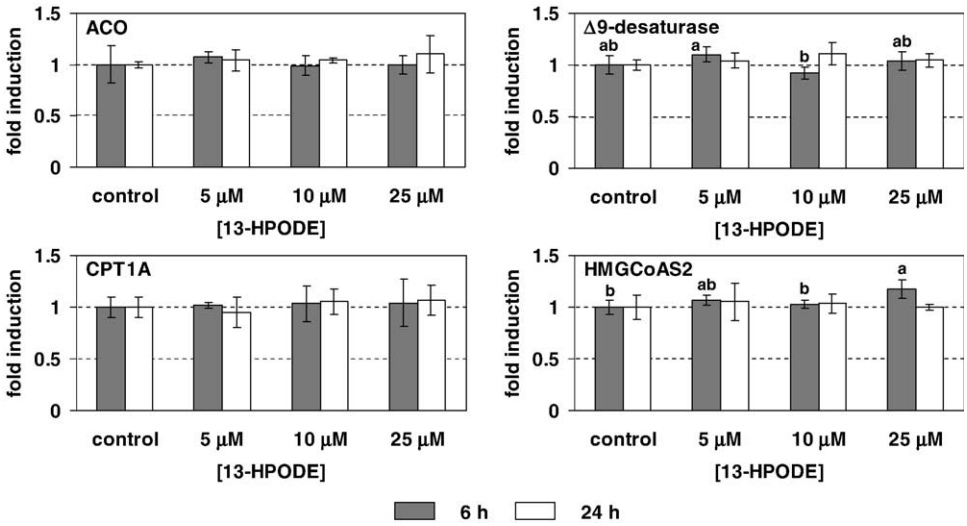

Fig. 2. Effect of 13-HPODE on mRNA concentrations of selected genes involved in lipid metabolism of HepG2 cells. HepG2 cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% FCS). After 16 h preincubation in this medium, 13-HPODE was added from ethanolic stock solution to give final concentrations of 5, 10 and 25 μM, respectively, and cells were incubated for 6 and 24 h. Control cells were incubated with low-serum medium containing vehicle alone. Acyl-CoA oxidase, CPT1A, Δ9-desaturase and mitochondrial hydroxymethylglutaryl-CoA synthase (HMGCoAS2) mRNA levels were determined by semiquantitative RT-PCR analysis using β-actin mRNA concentration for normalization as described in the Materials and Methods. Values are means±S.D. ($n=4$). Data are expressed relative to mRNA levels of control cells. Means with unlike letters differ, $P<.05$.

Furthermore, no alterations of SREBP-1, SREBP-2 and FAS mRNA levels occurred after stimulation of HepG2 cells with 13-HPODE for 6 and 24 h (data not shown). Finally, no effect on mRNA concentrations of PPARα downstream genes was observed after incubation of HepG2 cells with linoleic acid at concentrations up to 25 μM (data not shown).

3.3. Influence of 13-HPODE on the concentrations of cellular and secreted lipids of Fao and HepG2 cells

To test whether the observed induction of PPARα downstream genes resulted in alterations of cellular and secreted lipids, we examined the concentrations of cellular cholesterol and cellular and secreted triglycerides after

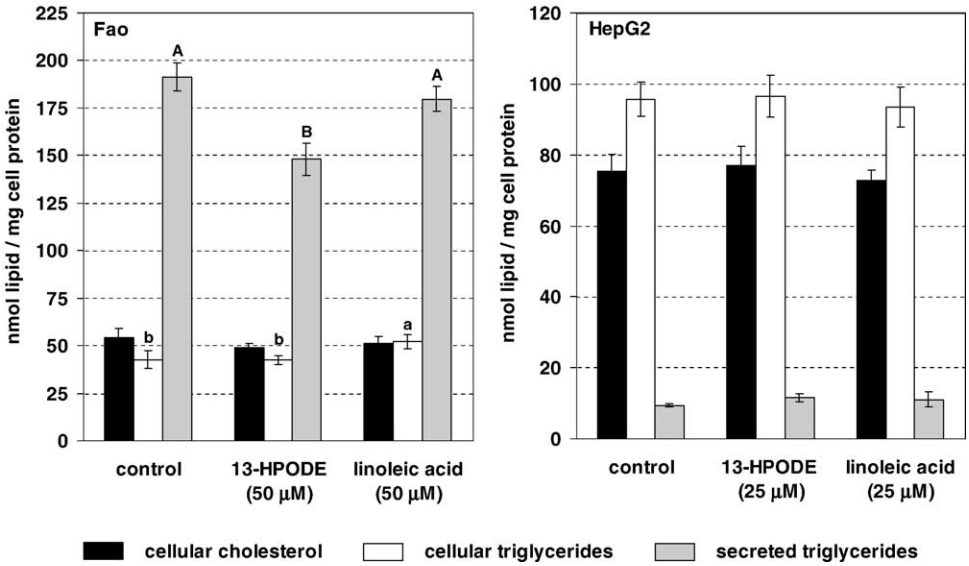

Fig. 3. Influence of 13-HPODE and linoleic acid on concentrations of cellular cholesterol and cellular and secreted triglycerides in Fao and HepG2 cells. Cells were incubated with 13-HPODE and linoleic acid, respectively, for 24 h in low-serum medium. Final fatty acid concentrations were 50 μM (Fao) and 25 μM (HepG2). Control cells were treated with low-serum medium containing vehicle alone. Cell supernatant after incubation was used for determination of triglyceride concentration in VLDLs. Total cellular lipids were extracted with a mixture of *n*-hexane and isopropanol (3:2), extracts were dried and lipids were dissolved in a small volume of Triton X-100. Cholesterol and triglyceride concentrations were determined using an enzymatic reagent kit. Values are means±S.D. ($n=4$). Small letters (a, b) denote differences in cellular triglycerides, capital letters (A, B) denote differences in secreted triglycerides ($P<.05$).

stimulating the cells with 13-HPODE. For comparison, cells were incubated with linoleic acid at equal concentration. Control cells were treated with vehicle alone. Final concentrations of fatty acids during incubation were 50 μM for Fao and 25 μM for HepG2 cells, respectively.

Cholesterol concentration in Fao cells was not influenced by incubation with 13-HPODE and linoleic acid, respectively (Fig. 3). The cellular triglyceride concentration was about 20% higher in cells treated with linoleic acid compared to 13-HPODE-treated and control cells. 13-HPODE-treated cells secreted about 20% less triglycerides compared to cells incubated with linoleic acid and control cells (Fig. 3).

Remarkably, the ratio of cellular to secreted triglyceride concentration was much higher in HepG2 compared to Fao cells (Fig. 3). We could not find any difference in cellular cholesterol and cellular and secreted triglyceride concentrations of HepG2 cells treated with 13-HPODE and linoleic acid, respectively, for 24 h.

4. Discussion

Lipid hydroperoxides are the fundamental primary products of autooxidation of PUFA. They can further react with oxygen and decompose to form a great variety of secondary oxidation products. Linoleic acid oxidation products represent an important portion of oxidized dietary fats. The aim of this study was to evaluate a possible involvement of the primary lipid peroxidation product of linoleic acid, 13-HPODE, in the observed activation of PPARα by oxidized fats in rat liver. Our data demonstrate that incubation of rat hepatoma Fao cells with 13-HPODE for 6 h led to a significant and concentration-dependent increase of mRNA concentrations of the PPARα downstream genes ACO, Cyp4A1 and CPT1A. Linoleic acid, the precursor of 13-HPODE, did also slightly enhance ACO and Cyp4A1 mRNA levels, but clearly less effective than its oxidized form. Acyl-CoA oxidase catalyses the rate-limiting step of peroxisomal β-oxidation and represents a direct target of PPARα. Induction of ACO is generally considered as marker of peroxisome proliferation [29]. Cyp4A1 catalyses the microsomal ω-hydroxylation of fatty acids [30]. CPT1A is involved in the first limiting step of mitochondrial β-oxidation, the entry flux of fatty acids into the mitochondria by catalyzing the formation of fatty acyl carnitine for translocation across the inner mitochondrial membrane. Both Cyp4A1 and CPT1A are regulated by PPARα and strongly induced by fibrates and other peroxisome proliferators [30,31]. Because PPARα mRNA was not affected by 13-HPODE and linoleic acid, respectively, the observed increased expression of PPARα downstream genes was not due to a higher PPARα expression. Hence, the up-regulation of ACO, Cyp4A1 and CPT1A genes should be the result of an activation of PPARα by 13-HPODE and linoleic acid, respectively. The observed changes in mRNA levels of PPARα downstream genes are very small. This is consistent with the described PPAR-mediated effects of other fatty acids [32,33]. Compared to lipid- and glucose-lowering drugs, PPAR binding and activation by fatty acids are weaker, which is also in accordance with the high plasma concentrations of fatty acids [34]. The finding that linoleic acid, which was used as a control, had a negligible effect on expression of PPARα downstream genes further supports that the observed effect was specific for 13-HPODE.

Up-regulation of ACO, Cyp4A1 and CPT1A genes by PPARα activation results in an enhanced fatty acid oxidation in the cells followed by reduced triglyceride levels. This phenomenon is also part of the mechanism of hypolipidemic action of fibrates [13,35]. Reduced triglyceride levels in liver, plasma and VLDL were also observed in rats fed oxidized fats and could be attributed to activation of PPARα [6,12]. The cellular triglyceride level was elevated in Fao cells incubated 24 h with linoleic acid compared to vehicle-treated cells. This may be attributed to enhanced triglyceride synthesis by addition of a fatty acid to the cells incubated in low-serum medium. In 13-HPODE-treated cells, triglyceride level was reduced compared to cells treated with linoleic acid. We suggest that this is due to the strong activation of PPARα downstream genes by 13-HPODE leading to enhanced fatty acid oxidation. Furthermore, the level of secreted triglycerides was lower when cells were incubated with 13-HPODE instead of linoleic acid. This result corresponds also well with the stronger induction of genes implicated in β-oxidation of fatty acids by 13-HPODE compared to its nonoxidized form. These data further support our finding that 13-HPODE enhances PPARα downstream genes by activation of PPARα in Fao cells. The weak induction of PPARα downstream genes by linoleic acid was not sufficient to cause measurable changes in triglyceride concentrations.

Several endogenous oxidized lipids were recently identified as PPAR ligands. 8*S*-hydroxy-5,9,11,14-eicosatetraenoic acid [8(*S*)-HETE], an arachidonic acid metabolite from lipoxygenation, has been shown to be a strong activator of the human PPARα [36] and plays an important role in keratinocyte differentiation [37]. Lipoxygenase products of linoleic acid are constituents of oxidized LDL and have been shown to be present in atherosclerotic lesions [38]. 9*S*-hydroxy-10,12-octadecadienoic acid and 13*S*-hydroxy-9,11-octadecadienoic acid (9- and 13-HODE) are activators of PPARγ [39,40] that is involved in the differentiation of several cell types including adipocytes, monocytes and macrophages [14]. Furthermore, 9- and 13-HODE were found to activate PPARα in human primary endothelial cells in transient transfection experiments [41]. To our knowledge, 13-HPODE has not been described as a ligand of PPARs in liver cells. The implication of 13-HPODE in the pathogenesis of atherosclerosis has been reported. It increased the expression of cell adhesion molecules and inflammatory chemokines in vascular smooth muscle cells via activation of nuclear factor-kappa

B [42,43]. Furthermore, 13-HPODE activated NAD(P)H oxidases in smooth muscle cells [44] and affected NO synthase activity in endothelial cells and macrophages [45,46]. Considering these literature data on PPAR activation by structurally related compounds, we suggest that the observed induction of PPARα downstream genes in Fao cells is the result of a direct activation of PPARα by 13-HPODE. Nevertheless, we cannot exclude the possibility that PPARα was not directly activated by 13-HPODE, but by another compound that has been intermediately formed from 13-HPODE.

In our experiments, linoleic acid at the highest concentration of 50 μM also slightly enhanced ACO and Cyp4A1 mRNAs in Fao cells, but the effect of 13-HPODE at the same concentration on PPARα downstream genes was much stronger than that of its nonoxidized form (Fig. 1). Linoleic acid is known to bind all PPAR subtypes at micromolar concentrations, but with the highest affinity to PPARα [47]. Thus, we can conclude that 13-HPODE is a more potent activator of PPARα than its precursor. Analogously, 8(*S*)-HETE has a 50-fold higher affinity for *Xenopus laevis* PPARα than its precursor, arachidonic acid [34].

As mentioned above, feeding of rats with oxidized fats did also suppress the expression of lipogenic enzymes like FAS in the liver [6]. The SREBP family of transcription factors plays a key role in the regulation of lipogenic enzymes and cholesterol synthesis [48,49]. SREBP-1 has emerged as a regulator of fatty acid and triglyceride synthesis, whereas SREBP-2 regulates cholesterol synthesis [49]. At the highest 13-HPODE concentration used, we observed a small but significant increase of SREBP-1c and SREBP-2 mRNA concentration in Fao cells. Increase of SREBP-1c and SREBP-2 after fibrate treatment was observed in rats and mice and was abolished in PPARα null mice [50–52]. Possibly, the depletion of fatty acids due to stimulation of β-oxidation by fibrates causes an increase in SREBP-1c, which is required for stimulating fatty acid synthesis [52]. Nevertheless, the level of FAS mRNA, which is regulated by SREBP-1c, was not changed in our experiments.

To assess the impact of the observed effect of PPARα activation by 13-HPODE in human cells, we further examined the influence of 13-HPODE on human hepatoma HepG2 cells. In contrast to Fao cells, no induction of PPARα downstream genes was observed after stimulating HepG2 cells for 6 h with 13-HPODE. Only HMGCoAS2 mRNA was slightly enhanced after 6 h incubation at the highest 13-HPODE concentration used. HMGCoAS2 is the main enzyme involved in ketone body formation and is directly controlled by PPARα [53]. Also, longer incubation times (24 h) did not result in changes of the mRNA levels of the examined genes. Furthermore, no change of SREBP-1 and SREBP-2 mRNA levels was observed after 13-HPODE treatment in contrast to Fao cells. It is well established that species-specific differences exist in PPARα function (reviewed in Ref. [21]). Mediated by PPARα, fibrates and other peroxisome proliferators cause hypolipidemic, peroxisome proliferation and liver carcinogenic effects [13,14,35]. Rats and mice are extremely responsive to these effects, whereas humans are resistant to the proliferative and carcinogenic effects of these drugs, but the hypolipidemic effect still manifests [21,29]. There is no conclusive evidence that humans are not responsive to peroxisome proliferation. Only few studies exist demonstrating a weak induction of the peroxisome proliferation marker enzyme ACO by fibrates and other peroxisome proliferators in HepG2 cells [22,54,55]. Most studies revealed that ACO expression is not induced in HepG2 cells and human hepatocytes after treatment with these substances [20,23,27,28,56]. On the other hand, genes encoding enzymes that catalyze branch points or rate-limiting steps in the utilization of fatty acids for ketone body formation, the long chain fatty acyl-CoA synthetase, CPT1A and HMGCoAS2, are responsive to PPARα activation in HepG2 cells and human hepatocytes [23,27,28]. Controversial data exist on PPARα-mediated activation of Δ9-desaturase, a key enzyme in fatty acid biosynthesis, in HepG2 cells by fibrates [28,55]. Δ9-desaturase gene itself is regulated by PPARα in rodents [57]. Considering the much more potent action of fibrates on PPARα compared to fatty acids [34], the lack of effects of 13-HPODE on PPARα downstream genes of HepG2 cells is consistent with previous reports and reflects the known species-specific differences of peroxisome proliferators action.

In summary, we present indirect evidence that 13-HPODE, the primary lipid peroxidation product of linoleic acid, activates PPARα in rat hepatoma Fao cells. Thus, 13-HPODE might be one of the components that are responsible for the observed PPARα-activating effect of oxidized fats in rat liver. In contrast, HepG2 cells did not respond to a stimulation with 13-HPODE indicating that also the effect of oxidized fats in humans may be different from that observed in rats.

Acknowledgement

B.K. was supported by a grant from Land Sachsen-Anhalt.

References

[1] Cohn JS. Oxidized fat in the diet, postprandial lipaemia and cardiovascular disease. Curr Opin Lipidol 2002;13:19–24.
[2] Yang CM, Kendall CW, Stamp D, Medline A, Archer MC, Bruce WR. Thermally oxidized dietary fat and colon carcinogenesis in rodents. Nutr Cancer 1998;30:69–73.
[3] Corcos Benedetti P, D'Aquino M, Di Felice M, Gentili V, Tagliamonte B, Tomassi G. Effects of a fraction of thermally oxidized soy bean oil on growing rats. Nutr Rep Int 1987;36:387–401.
[4] Blanc P, Revol A, Pacheco H. Chronical ingestion of oxidized oil in the rat: effect on lipid composition and on cytidylyl transferase activity in various tissues. Nutr Res 1992;12:833–44.
[5] Hochgraf E, Mokady S, Cogan U. Dietary oxidized linoleic acid modifies lipid composition of rat liver microsomes and increases their fluidity. J Nutr 1997;127:681–6.

[6] Eder K, Suelzle A, Skufca P, Brandsch C, Hirche F. Effects of dietary thermoxidized fats on expression and activities of hepatic lipogenic enzymes in rats. Lipids 2003;38:31–8.
[7] Huang CJ, Cheung NS, Lu VR. Effects of deteriorated frying oil and dietary protein levels on liver microsomal enzymes in rats. J Am Oil Chem Soc 1988;65:1796–803.
[8] Eder K, Kirchgessner M. The effect of dietary vitamin E supply and a moderately oxidized oil on activities of hepatic lipogenic enzymes in rats. Lipids 1998;33:277–83.
[9] Eder K. The effects of a dietary oxidized oil on lipid metabolism in rats. Lipids 1999;34:717–25.
[10] Martin JC, Joffre F, Siess MH, Vernevaut MF, Collenot P, Genty M, et al. Cyclic fatty acid monomers from heated oil modify the activities of lipid synthesizing and oxidizing enzymes in rat liver. J Nutr 2000;130:1524–30.
[11] Chao PM, Chao CY, Lin FJ, Huang CJ. Oxidized frying oil up-regulates hepatic acyl-CoA oxidase and cytochrome P-450 4 A1 genes in rats and activates PPAR alpha. J Nutr 2001;131:3166–74.
[12] Sülzle A, Hirche F, Eder K. Thermally oxidized dietary fat upregulates the expression of target genes of PPAR alpha in rat liver. J Nutr 2004;134:1375–83.
[13] Staels B, Dallongeville J, Auwerx J, Schoonjans K, Leitersdorf E, Fruchart JC. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 1998;98:2088–93.
[14] Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev 1999;20:649–88.
[15] Chinetti G, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. Inflamm Res 2000;49:497–505.
[16] Duval C, Chinetti G, Trottein F, Fruchart JC, Staels B. The role of PPARs in atherosclerosis. Trends Mol Med 2002;8:422–30.
[17] Schoonjans K, Martin G, Staels B, Auwerx J. Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr Opin Lipidol 1997;8:159–66.
[18] Poirier H, Braissant O, Niot I, Wahli W, Besnard P. 9-*cis*-Retinoic acid enhances fatty acid-induced expression of the liver fatty acid-binding protein gene. FEBS Lett 1997;412:480–4.
[19] Moya-Camarena SY, Vanden Heuvel JP, Blanchard SG, Leesnitzer LA, Belury MA. Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARalpha. J Lipid Res 1999; 40:1426–33.
[20] Vanden Heuvel JP, Kreder D, Belda B, Hannon DB, Nugent CA, Burns KA, et al. Comprehensive analysis of gene expression in rat and human hepatoma cells exposed to the peroxisome proliferator WY14,643. Toxicol Appl Pharmacol 2003;188:185–98.
[21] Cattley RC, DeLuca J, Elcombe C, Fenner-Crisp P, Lake BG, Marsman DS, et al. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? Regul Toxicol Pharmacol 1998;27:47–60.
[22] Duclos S, Bride J, Ramirez LC, Bournot P. Peroxisome proliferation and beta-oxidation in Fao and MH1C1 rat hepatoma cells, HepG2 human hepatoblastoma cells and cultured human hepatocytes: effect of ciprofibrate. Eur J Cell Biol 1997;72:314–23.
[23] Ammerschlaeger M, Beigel J, Klein KU, Mueller SO. Characterization of the species-specificity of peroxisome proliferators in rat and human hepatocytes. Toxicol Sci 2004;78:229–40.
[24] Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[25] Funk MO, Isacc R, Porter NA. Preparation and purification of lipid hydroperoxides from arachidonic and gamma-linolenic acids. Lipids 1976;11:113–7.
[26] De Hoff JL, Davidson JH, Kritchevsky V. An enzymatic assay for determining free and total cholesterol in tissues. Clin Chem 1978; 24:433–5.
[27] Lawrence JW, Li Y, Chen SY, DeLuca JG, Berger JP, Umbenhauer DR, et al. Differential gene regulation in human versus rodent hepatocytes by peroxisome proliferator-activated receptor (PPAR) alpha—PPAR alpha fails to induce peroxisome proliferation-associated genes in human cells independently of the level of receptor expression. J Biol Chem 2001;276:31521–7.
[28] Hsu MH, Savas U, Griffin KJ, Johnson EF. Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor alpha in HepG2 cells. J Biol Chem 2001;276:27950–8.
[29] Bentley P, Calder I, Elcombe C, Grasso P, Stringer D, Wiegand HJ. Hepatic peroxisome proliferation in rodents and its significance for humans. Food Chem Toxicol 1993;31:857–907.
[30] Aldridge TC, Tugwood JD, Green S. Identification and characterization of DNA elements implicated in the regulation of CYP4A1 transcription. Biochem J 1995;306:473–9.
[31] Brandt JM, Djouadi F, Kelly DP. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. J Biol Chem 1998;273:23786–92.
[32] Moya-Camarena SY, Van den Heuvel JP, Belury MA. Conjugated linoleic acid activates peroxisome proliferator-activated receptor alpha and beta subtypes but does not induce hepatic peroxisome proliferation in Sprague–Dawley rats. Biochim Biophys Acta 1999;1436: 331–42.
[33] Schild RL, Schaiff WT, Carlson MG, Cronbach EJ, Nelson DM, Sadovsky Y. The activity of PPAR gamma in primary human trophoblasts is enhanced by oxidized lipids. J Clin Endocrinol Metab 2002;87:1105–10.
[34] Krey G, Braissant O, Lhorset F, Kalkhoven E, Perroud M, Parker MG, et al. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol 1997;11:779–91.
[35] Watts GF, Dimmitt SB. Fibrates, dyslipoproteinaemia and cardiovascular disease. Curr Opin Lipidol 1999;10:561–74.
[36] Yu K, Bayona W, Kallen CB, Harding HP, Ravera CP, McMahon G, et al. Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J Biol Chem 1995;270:23975–83.
[37] Muga SJ, Thuillier P, Pavone A, Rundhaug JE, Boeglin WE, Jisaka M, et al. 8*S*-Lipoxygenase products activate peroxisome proliferator-activated receptor alpha and induce differentiation in murine keratinocytes. Cell Growth Differ 2000;11:447–54.
[38] Folcik VA, Cathcart MK. Predominance of esterified hydroperoxylinoleic acid in human monocyte-oxidized LDL. J Lipid Res 1994; 35:1570–82.
[39] Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell 1998;93:229–40.
[40] Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 1998;93:241–52.
[41] Delerive P, Furman C, Teissier E, Fruchart JC, Duriez P, Staels B. Oxidized phospholipids activate PPAR alpha in a phospholipase A2-dependent manner. FEBS Lett 2000;471:34–8.
[42] Natarajan R, Reddy MA, Malik KU, Fatima S, Khan BV. Signaling mechanisms of nuclear factor-kappab-mediated activation of inflammatory genes by 13-hydroperoxyoctadecadienoic acid in cultured vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2001; 21:1408–13.
[43] Dwarakanath RS, Sahar S, Reddy MA, Castanotto D, Rossi JJ, Natarajan R. Regulation of monocyte chemoattractant protein-1 by the oxidized lipid, 13-hydroperoxyoctadecadienoic acid, in vascular smooth muscle cells via nuclear factor-kappa B (NF-kappa B). J Mol Cell Cardiol 2004;36:585–95.
[44] Li WG, Stoll LL, Rice JB, Xu SP, Miller Jr FJ, Chatterjee P, et al. Activation of NAD(P)H oxidase by lipid hydroperoxides: mechanism of oxidant-mediated smooth muscle cytotoxicity. Free Radic Biol Med 2003;34:937–46.

[45] Ramasamy S, Parthasarathy S, Harrison DG. Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid. J Lipid Res 1998;39:268–76.
[46] Huang AN, Li CF, Kao RL, Stone WL. Lipid hydroperoxides inhibit nitric oxide production in RAW264.7 macrophages. Free Radic Biol Med 1999;26:526–37.
[47] Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, et al. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell 1999;3:397–403.
[48] Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89:331–40.
[49] Osborne TF. Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. J Biol Chem 2000;275:32379–82.
[50] Roglans N, Peris C, Verd JC, Alegret M, Vazquez M, Sanchez RM, et al. Increase in hepatic expression of SREBP-2 by gemfibrozil administration to rats. Biochem Pharmacol 2001;62: 803–9.
[51] Knight BL, Patel DD, Humphreys SM, Wiggins D, Gibbons GF. Inhibition of cholesterol absorption associated with a PPAR alpha-dependent increase in ABC binding cassette transporter A1 in mice. J Lipid Res 2003;44:2049–58.
[52] Le Jossic-Corcos C, Duclos S, Ramirez LC, Zaghini I, Chevillard G, Martin P, et al. Effects of peroxisome proliferator-activated receptor alpha activation on pathways contributing to cholesterol homeostasis in rat hepatocytes. Biochim Biophys Acta 2004;1683:49–58.
[53] Rodriguez JC, Gil-Gomez G, Hegardt FG, Haro D. Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids. J Biol Chem 1994;269:18767–72.
[54] Brocard C, Es-Souni M, Ramirez LC, Latruffe N, Bournot P. Stimulation of peroxisomal palmitoyl-CoA oxidase activity by ciprofibrate in hepatic cell lines: comparative studies in Fao, MH1C1 and HepG2 cells. Biol Cell 1993;77:37–41.
[55] Rodriguez C, Cabrero A, Roglans N, Adzet T, Sanchez RM, Vazquez M, et al. Differential induction of stearoyl-CoA desaturase and acyl-CoA oxidase genes by fibrates in HepG2 cells. Biochem Pharmacol 2001;61:357–64.
[56] Rodriguez C, Noe V, Cabrero A, Ciudad CJ, Laguna JC. Differences in the formation of PPAR alpha-RXR/acoPPRE complexes between responsive and nonresponsive species upon fibrate administration. Mol Pharmacol 2000;58:185–93.
[57] Miller CW, Ntambi JM. Peroxisome proliferators induce mouse liver stearoyl-CoA desaturase 1 gene expression. Proc Natl Acad Sci U S A 1996;93:9443–8.