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Changes in liver PPARα mRNA expression in response to two levels of high-safflower-oil diets correlate with changes in adiposity and serum leptin in rats and mice

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Abstract

The ligand-dependent transcription factor peroxisome proliferator-activated receptor α (PPARα) is known to be activated by common fatty acids and to regulate the expression of genes of various lipid oxidation pathways and transport. High-fat diets provide more fatty acids, which presumably could enhance lipid catabolism through up-regulation of PPARα signaling. However, high intake of fat could also lead to obesity. To examine PPARα signaling in high-fat feeding and obesity, this study examined the hepatic mRNA expression of PPARα and some of its target genes in Wistar rats and C57BL/6J mice fed two levels (20% or 30% wt/wt) of high-safflower-oil (SFO; oleic-acid-rich) diets until animals showed significantly higher body weight (13 weeks for rats and 22 weeks for mice) than those of control groups fed a 5% SFO diet. At the end of these respective feeding periods, only the rats fed 30% SFO and the mice fed 20% SFO among the two groups fed high-fat diets showed significantly higher body weight, white adipose tissue weight, serum leptin and mRNA expression of PPARα ($P<.05$) compared to the respective control groups. Despite elevated acyl-CoA (a PPARα target gene) protein and activity in both groups fed high-fat diets, the mRNA expression level of most PPARα target genes examined correlated mainly to PPARα mRNA levels and not to fat intake or liver lipid levels. The observation that the liver PPARα mRNA expression in groups fed high-fat diets was significantly higher only in obese animals with elevated serum leptin implied that obesity and associated hyperleptinemia might have a stronger impact than dietary SFO intake per se on PPARα-regulated mRNA expression in the liver.

Keywords: High-fat diets; PPARα; Obesity; Leptin

1. Introduction

High dietary fat intake is widely accepted to be associated with a higher risk for obesity and chronic diseases such as cardiovascular diseases and some types of cancer [1]. For example, high-fat consumption is related to the prevalence of metabolic syndrome in affluent societies [2]. It has been demonstrated in rodent models that high-fat diets could aggravate the pathological process of obesity-associated insulin resistance, atherosclerosis and some types of cancer. The metabolic changes caused by a high intake of dietary fat had not been linked to regulation at the level of gene expression until a nuclear receptor, peroxisome proliferator-activated receptors (PPARs), was discovered and shown to be activated by common fatty acids [3–7].

PPARs are ligand-activated nuclear receptors belonging to the steroid hormone receptor superfamily [8,9]. Among the three isoforms PPARα (NR1C1), PPARγ (NR1C3) and PPARδ (NR1C2), PPARα is predominantly expressed in tissues that metabolize fatty acids and regulate the expression of genes encoding for enzymes involved in peroxisome proliferation and fatty acid oxidation in peroxisomes and mitochondria and in lipid transport. Common and modified fatty acids from the diet and in the body, as well as eicosanoids, are natural ligands of PPARs [3–7]. An array of PPARα target genes (genes with PPRE in the promoter region that could be up-regulated by PPARα) identified belongs to groups of genes involved in fatty acid oxidation and transport. Regulation of gene expression through PPARα, the so-called

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PPARα signaling, is thus regarded as an adaptive metabolic system that regulates lipid homeostasis [10].

The expression of PPARα in the liver is up-regulated by glucocorticoids [11,12], $n-3$ polyunsaturated fatty acids [13] and fasting [14,15], but is down-regulated by insulin [12]. PPARα-null mice have no obvious phenotype on a normal diet, but accumulate massive amounts of lipid in their liver after fasting [14] or after eating a high-fat diet [16]. Many defects in lipid homeostasis, such as a sexually dimorphic control of circulating lipids, fat storage and obesity, were found in PPARα-null mice [17]. On the other hand, PPARα-selective agonists have been shown to be hypolipidemic, to improve insulin sensitivity and to reduce adiposity [18,19].

Based on findings from molecular studies, it is conceivable that in vivo PPAR signaling depends on ligand availability and on the expressed level of the receptor protein per se. A high-fat diet presumably provides more fatty acids as ligand for PPARs and, therefore, should be able to up-regulate those PPARα target genes and to enhance fatty acid metabolism through the PPARα signaling pathway. This, in turn, should reduce the accumulation of fat in the body. Indeed, in Wistar rats fed a high-fat diet (250 g/kg diet), there resulted increased mRNA expression of PPARα and acyl-CoA oxidase (ACO) in the liver [20]. Rats in this study [20] did not become obese since the feeding period was only 4 weeks. Feeding with a higher fat level and for a longer period is known to induce obesity in rodents [21–23]. In obese rodents, a number of studies demonstrated up-regulated PPARα mRNA [24] and protein [25] expression, as well as fatty acid oxidation enzyme protein [26] and mRNA expression [25,27]. These studies either used genetically obese mice [24,26] or did not use a well-designed diet for comparison between low-fat control and high-fat-induced obesity. The relationship between high-fat intake, PPARα signaling and obesity has not been fully addressed.

This study, therefore, examined the mRNA expression of PPARα and some of its target genes in the liver of C57BL/6J mice and Wistar rats fed two levels (20% and 30%, wt/wt) of high-safflower-oil (SFO) diet. Animals were fed until one of the high-SFO diet groups showed significantly higher body weight than the control group (fed a 5% SFO diet). Significantly up-regulated liver PPARα mRNA was observed in rats fed a 30% SFO diet and in mice fed a 20% SFO diet, in which adipose tissue mass and serum leptin were elevated, while the expression was not changed significantly in the nonobese animals fed high-fat diets (rats fed a 20% SFO diet and mice fed a 30% SFO diet).

2. Materials and methods

2.1. Animals and diets

Six-week-old male Wistar rats ($n=29$) and C57BL/6J mice ($n=33$) were purchased from the laboratory animal center of the National Taiwan University College of Medicine (Taipei, Taiwan). Animals were housed individually in stainless steel wire cages in a room maintained at 25±2°C, with a controlled 12-h light/dark cycle (light period: 0600–1800 h). They were acclimated for 2 weeks on a nonpurified diet (Laboratory Rodent Chow; Ralston Purina, St. Louis, MO, USA) and then randomly assigned to three diet groups. Modified from the AIN-76 formulation, the three test diets contained 5 g/100 g, 20 g/100 g or 30 g/100 g SFO (wt/wt). The amounts of casein, vitamin and mineral mixture in the high-fat diets were adjusted to ensure that the nutrient/energy ratios were equivalent among the three diets. The percentages of fat energy/total energy in the three test diets were 11.65%, 39.64% and 53.68%, respectively. The composition of the three test diets is shown in Table 1. Casein (ICN, Aurora, OH, USA), cornstarch (Samyang, Seoul, South Korea), cellulose (J. Rettenmaier and Söhne, Holzmühle, Germany), AIN-76 mineral mixture (ICN) and AIN-76 vitamin mixture (ICN) were purchased from commercial sources. SFO and sucrose were from Taiwan Sugar Co. (Tainan, Taiwan). Methionine and choline chloride were from Sigma Chemical (St. Louis, MO, USA). Animals were allowed free access to food and tap water. Body weight and food intake were recorded weekly. Animal care and handling conformed to accepted guidelines [28].

2.2. Tissue sampling and preparation

After one of the groups of animals fed a high-SFO diet showed significantly higher body weight than the group fed 5% SFO (i.e., 13 weeks for rats and 22 weeks for mice), animals were killed by carbon dioxide asphyxiation after overnight fasting. Blood was collected and placed on ice. Serum samples were obtained by blood centrifugation and stored at −20°C for lipid and leptin analyses. Liver, epididymal fat and retroperitoneal fat were excised and weighed. Aliquots of liver were quick-frozen in liquid N_2 and stored at −80°C for total RNA extraction. A portion of the fresh liver was homogenized (10% wt/vol) in 0.3 mol/L

Table 1
Composition of the 5%, 20% and 30% (wt/wt) SFO test diets

Test diets	5% SFO (g/kg diet)	20% SFO (g/kg diet)	30% SFO (g/kg diet)
Ingredients			
Casein	200	235	260
DL-methionine	3	3	3
Cornstarch	325	224	160
Sucrose	325	224	160
Cellulose	50	59	61
SFO[a]	50	200	300
Vitamin mixture	10	12	12
Mineral mixture	35	41	42
Choline	2	2	2
Energy (kJ/g)	16.31	18.98	21.03
Protein (% energy)	21.03	20.96	20.91
Fat (% energy)	11.65	39.64	53.68

[a] Fatty acid composition of SFO (C16:0, 7.0%; C16:1, 1.1%; C18:0, 2.5%; C18:1, 65.3%; C18:2, 24.1%) as analyzed by gas chromatography.

mannitol, 10 mmol/L HEPES and 1 mmol/L EGTA buffer (pH 7.2), and centrifuged at 1000×*g* (4°C) for 10 min. The resulting postnuclear supernatant (PNS) was analyzed for activity and protein expression of ACO. Another portion of the fresh liver was homogenized in 10 mmol/L phosphate buffer (pH 7.4) to prepare microsomal pellets, as described [29]. Microsomal pellets were analyzed for CYP4A (cytochrome *P*450 4A) protein expression by Western blot analysis.

2.3. Biochemical analyses

Liver lipids were extracted by a mixture of chloroform/methanol (2:1, vol/vol). Serum and liver lipids were analyzed for triglyceride (TG), nonesterified fatty acid (NEFA) and cholesterol by enzymatic methods using commercial kits (Randox Laboratory, Crumlin, Northland, UK). Serum leptin was measured by enzyme-linked immunosorbent assay using a goat antimouse leptin antibody (R&D Systems, Minneapolis, MN, USA) with high affinity for rat and mouse. The peroxisomal ACO activity of liver PNS samples was assayed according to the method of Small et al. [30].

2.4. Western blot analysis

Liver PNS containing 5 μg of proteins was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride transfer membranes (NEN Life Science, Boston, MA, USA). The protein level of ACO was detected with antirat ACO antiserum (polyclonal antibody generated by a synthetic peptide: NPDLRKERASATF) and biotinylated goat antirabbit IgG (Amersham Bioscience, Buckinghamshire, UK). This, in turn, was detected by streptavidin horseradish peroxidase (HRP; 1:5000) and ECL-Plus detection reagents (Amersham Bioscience). A liver microsomal suspension containing 2.5 μg of proteins was subjected to 12.5% SDS-PAGE and transferred. The blot was detected using goat antirat CYP4A (Daiichi Pure Chemicals, Tokyo, Japan) as the primary antibody and antigoat IgG-conjugated HRP (Calbiochem, USA) as the secondary antibody. To avoid nonspecific binding, transferred membranes were preincubated overnight in a 5% skim milk phosphate-buffered saline (PBS) solution at 4°C before reacting with the primary antibody. All antibodies were dissolved in PBS containing 0.5% nonfat dry milk. Emitting light was captured on film (BioMax Light film; Kodak, Rochester, NY, USA). The film was then subjected to a microcomputer imaging device (MCID; Fuji, Tokyo, Japan) analysis system. Protein was quantified by Bio-Rad protein assay dye (Bio-Rad, Hercules, CA, USA). The protein sequences of ACO and CYP4A (from the National Center for Biotechnology Information protein database) are highly identical (~92%) between rats and mice. The reactivity of the primary antibodies used across rat and mouse species was validated by successful detection of CYP4A and ACO in clofibrate-treated rat and mouse livers.

2.5. Preparation of cDNA probes of some target genes of PPARα

The cDNA of CYP4A and ACO used were as described [31]. The cDNA of fatty acid binding protein (FABP), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), carnitine palmitoyl-transference 1A (CPT1A) and 18*S* cDNA were synthesized by reverse transcriptase–polymerase chain reaction (RT-PCR). Rat liver cDNA was used as template, and the sequence of primers used was as follows: 5-TGCCACCATGAACTTCTCC-3 (forward) and 5-TCTCTTGTAGACGATGTCACC-3 (reverse) for FABP (accession no. M35991; bases 33–405); 5-GCTGATGGAACGCACAAA-3 (forward) and 5-ACACTAGACACCAGCTTCTC-3 (reverse) for HMGS (accession no. M33648; bases 385–1374); 5-TGGTGGTGGGTGTGATTTC-3 (forward) and 5-ATACAGCAGTATGGCGTGG-3 (reverse) for CPT1A (accession no. NM_031559; bases 194–846); 5-TACCTGGTTGATCCTGCCAG-3 (forward) and 5-TGTCAATCCTGTCCGTGTCC-3 (reverse) for 18*S* (accession no. M11188; bases 1–1297). The PCR products were ligated into pGEM-T easy vector (Promega, Madison, WI, USA) or yT&A vector (Yeastern Biotech, Taipei, Taiwan) and then transformed to *Escherichia coli* (JM109). After blue/white screening, cDNA targets were cut from the plasmid by appropriate restriction enzymes. All of the cDNA targets were confirmed by sequence analysis. For the preparation of probes, the cDNA was labeled by random primer method using a cDNA labeling kit (Amersham Bioscience, Piscataway, NJ, USA) and α-^{32}P-dCTP. Unincorporated nucleotides were removed by MicroSpin G-50 Microcolumn (Amersham Bioscience).

2.6. Northern blot analysis

Liver total RNA was extracted with TRIzol reagent (Life Technologies, Rockville, MD, USA). Total RNA (20 μg) from each sample was separated by electrophoresis in denaturing formaldehyde 1% agarose gel, transferred to Hybond-N$^+$ passively charged nylon membrane (Amersham Bioscience) and cross-linked to the membrane by ultraviolet irradiation. The blots were prehybridized at 42°C for 3 h in the hybridization buffer with salmon sperm DNA. They were then hybridized at 42°C for 12–15 h with ^{32}P-labeled cDNA probes of CPT1A, ACO, HMGS, CYP4A or FABP, in succession. To correct for possible difference in transfer and loading, the blots were also hybridized with ^{32}P-labeled 18*S* probe as an internal control. Afterward, the blots were washed at the appropriate stringency to remove nonspecific binding and then exposed to X-OMAT AR film (Kodak) at −80°C. The amounts of each mRNA were quantitated with an image analyzer (BAS2000; Fuji).

2.7. Real-time RT-PCR

As the abundance of PPARα mRNA after 12 h of fasting was relatively low, real-time RT-PCR was employed for the detection of this mRNA species. Liver total RNA (20 ng/μl)

was reverse-transcribed by High Capacity cDNA Archive Kit (PE Applied Biosystems, USA). The reverse transcription program was: 25°C for 10 min, 37°C for 120 min and 4°C for 30 min. cDNA was stored at −20°C. The PPARα and 18*S* primers used were part of commercial kits (TaqMan Assays-on-Demand Gene Expression Products; Applied Biosystems, USA). Amplification of each target cDNA was performed with TaqMan PCR Reagents Kits (Applied Biosystems) in the ABI PRISM 7700 Sequence Detection System, according to the protocols provided by the manufacturer (PE Applied Biosystems). The PCR condition used was: 95°C for 10 min, 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. Data were calculated by relative cycle threshold (C_t) standard method and were normalized by 18*S* expression in each sample.

2.8. Statistical analyses

All experimental data were expressed as mean±S.E.M. Data from Wistar rats and C57BL/6J mice were analyzed by one-way ANOVA and Duncan's multiple range test, respectively, to justify the significance of differences among the means of the three groups. For image analysis data obtained by Northern blot analysis, the difference between any two groups was analyzed by Student's *t* test. Correlations between parameters were analyzed by Pearson correlation coefficient test. In addition, forward stepwise multiple linear regressions were used to determine which of the measured physiological/biochemical parameters (independent variables) may account for the mRNA expression and activity level of ACO (dependent variable). For all statistical analyses, data were transformed logarithmically if the variances were not homogeneous. The SAS 8.2 System (SAS Institute, Cary, NC, USA) was employed for statistical analyses, and differences were considered significant at $P<.05$.

3. Results

3.1. Food intake, body weight and adipose tissue weight

Wistar rats fed the 30% SFO diet consumed less food but gained significantly higher body weights than control rats fed the 5% SFO diet ($P<.05$; Table 2). In spite of fat intake being significantly increased with increases in dietary fat content, the total energy intakes of the three groups of rats were comparable ($P>.05$), indicating that higher body weight gains in the 30% SFO group may be related to higher fat intakes but not to higher energy intakes. On the other hand, C57BL/6J mice fed the 20% SFO diet had significantly higher body weight gains than control mice fed the 5% SFO diet ($P<.05$; Table 2). Despite the fat intake of mice also being increased with increases in dietary SFO level, energy intake decreased with increases in dietary SFO level ($P<.05$; Table 2). Noticeably, mice fed the 20% SFO diet gained higher body weights regardless of significantly lower total energy intakes compared to control mice.

The epididymal and retroperitoneal adipose tissue weights of Wistar rats fed the 30% SFO group were 147% and 152%, respectively, those of rats fed the 5% SFO group ($P<.05$; Table 2). The C57BL/6J mice fed the 20% SFO diet also had significantly higher epididymal and retroperitoneal adipose tissue weights than the remaining two groups of mice ($P<.05$; Table 2). These data indicated that rats fed the 30% SFO diet and mice fed the 20% SFO diet became obese at the end of the respective feeding periods, while rats fed the 20% SFO diet and mice fed the 30% SFO diet did not become obese at the end of the respective feeding periods.

3.2. Lipid level

The serum TG and NEFA of Wistar rats fed the 30% and 20% SFO diets were 60% and 80%, respectively, those of rats fed the 5% SFO diet ($P<.05$; Table 3). In contrast, liver TG concentrations of Wistar rats increased with increasing content of dietary SFO ($P<.05$; Table 3). The liver NEFA concentrations of rats fed the 30% SFO diet were significantly higher than those of rats fed the 5% SFO diet ($P<.05$; Table 3). Serum and liver cholesterol concentrations of Wistar rats fed the 30% and 20% SFO diets were comparable and significantly higher than those of control rats fed the 5% SFO diet ($P<.05$; Table 3). For C57BL/6J mice, serum and liver lipid concentrations were not significantly different among the three dietary groups, except for liver NEFA. The

Table 2
Body weight gain, food and fat intake, liver weight and adipose weight of rats and mice fed diets containing 5 g/100 g, 20 g/100 g or 30 g/100 g SFO for 13 weeks (Wistar rats) or 22 weeks (C57BL/6J mice)

Species	Wistar rats			C57BL/6J mice		
Test diets	5% SFO	20% SFO	30% SFO	5% SFO	20% SFO	30% SFO
n (per group)	10	10	9	10	13	10
Body weight gain (g)	234±7^{b}	238±11^{b}	273±7^{a}	15.9±1.2^{b}	20.6±1.0^{a}	16.1±1.1^{b}
Total food intake (g)	1816±44^{a}	1489±46^{b}	1433±22^{b}	662±21^{a}	522±9^{b}	410±11^{c}
Total energy intake (kcal)	7010±171	6762±208	7206±110	2554±79^{a}	2367±40^{b}	2063±54^{c}
Fat intake (g/day)	1.0±0.0^{c}	3.3±0.1^{b}	4.8±0.1^{a}	0.22±0.01^{c}	0.62±0.01^{b}	0.80±0.02^{a}
Tissue weight (g)						
Epididymal fat	14.4±1.1^{b}	17.2±1.6^{b}	21.2±1.0^{a}	1.9±0.2^{b}	2.4±0.2^{a}	2.2±0.1^{a,b}
Retroperitoneal fat	16.4±0.9^{b}	18.7±1.9^{b}	24.9±1.0^{a}	0.6±0.1^{b}	0.8±0.1^{a}	0.7±0.0^{a,b}
Liver	14.7±0.5	14.1±0.4	15.4±0.4	1.7±0.1	1.8±0.1	1.6±0.2

Values are mean±S.E.M. The significance of difference among the three groups for each species was analyzed by one-way ANOVA and Duncan's multiple range test. Values not sharing a superscript letter among the three diet groups of the same species are significantly different ($P<.05$.).

Table 3
The concentration of TG, cholesterol and NEFA in the serum and liver of rats and mice fed diets containing 5 g/100 g, 20 g/100 g or 30 g/100 g SFO for 13 weeks (Wistar rats) or 22 weeks (C57BL/6J mice)

Species	Wistar rats			C57BL/6J mice		
Test diets	5% SFO	20% SFO	30% SFO	5% SFO	20% SFO	30% SFO
n (per group)	10	10	9	10	13	10
Serum (mmol/L)						
TG	1.89±0.18[a]	1.13±0.12[b]	1.20±0.12[b]	1.02±0.07	1.12±0.06	1.05±0.06
NEFA	0.69±0.04[a]	0.56±0.06[b]	0.56±0.03[b]	1.71±0.10	1.61±0.06	1.60±0.06
Cholesterol	2.48±0.12[b]	3.09±0.20[a]	3.01±0.18[a]	3.91±0.34	4.44±0.27	4.47±0.19
Liver (μmol/g liver)						
TG	23.89±2.82[c]	34.50±2.59[b]	45.54±5.20[a]	36.98±3.23	48.61±3.96	39.37±4.62
NEFA	19.64±0.98[b]	20.69±0.85[a,b]	22.72±1.07[a]	74.33±8.07[b]	98.86±7.34[a]	101.39±9.81[a]
Cholesterol	16.26±0.51[b]	20.41±0.76[a]	20.40±0.87[a]	10.61±1.14	10.77±1.02	8.28±0.51

Values are mean±S.E.M. The significance of difference among the three groups for each species was analyzed by one-way ANOVA and Duncan's multiple range test. Values not sharing a superscript letter among the three diet groups of the same species are significantly different ($P<.05$).

liver NEFA concentrations of the C57BL/6J mice fed the 20% and 30% SFO diets were comparable and significantly higher than those of the control mice fed the 5% SFO diet ($P<.05$; Table 3). However, concentrations of serum TG, serum NEFA and liver TG were not significantly different among the three groups of C57BL/6J mice (Table 3). There were positive correlations between liver TG concentration and the weight of epididymal fat ($r=.39$, $P=.036$ for rats; $r=.43$, $P=.015$ for mice) and retroperitoneal fat ($r=.51$, $P=.0084$ for rats; $r=.35$, $P=.05$ for mice). Liver NEFA concentration was positively correlated to total fat intake ($r=.39$, $P=.035$ for rats; $r=.40$, $P=.02$ for mice), epididymal fat weight ($r=.38$, $P=.04$ for rats; $r=.39$, $P=.025$ for mice) and liver TG concentration ($r=.43$, $P=.019$ for rats; $r=.61$, $P=.0002$ for mice), indicating that feeding of high-SFO diets is associated with increases in hepatic lipid content and adipose mass.

3.3. Serum leptin

Wistar rats fed the 30% SFO diet had significantly higher serum leptin concentrations than the remaining two groups ($P<.05$; Fig. 1). Serum leptin concentrations of the C57BL/6J mice fed the 20% SFO diet were significantly higher than the remaining two groups ($P<.05$; Fig. 1). Serum leptin concentrations were comparable between the C57BL/6J mice fed the 30% and 5% SFO diets and between Wistar rats fed the 5% and 20% SFO diets ($P>.05$). In both Wistar rats and C57BL/6J mice, concentrations of serum leptin positively correlated to weights of epididymal fat ($r=.60$, $P=.0008$ for rats; $r=.64$, $P<.0001$ for mice) and retroperitoneal fat ($r=.89$, $P<.0001$ for rats; $r=.54$, $P=.0014$ for mice). In addition, serum leptin also positively correlated to liver TG concentrations in both rats ($r=.42$, $P=.027$) and mice ($r=.60$, $P=.0003$).

3.4. Activity and protein levels of liver ACO

Hepatic peroxisomal ACO and microsomal CYP 4A are PPARα target genes, and their expression is regarded as an indicator of the transcriptional activation of PPARα. The activity and protein expression of ACO in the liver of Wistar rats fed the 30% SFO diet were 145% those of the rats fed the 5% SFO diet ($P<.05$; Fig. 2A and C). The hepatic peroxisomal ACO activity of the C57BL/6J mice fed the 20% and 30% SFO diets was also significantly higher than that of mice fed the 5% SFO diet ($P<.05$; Fig. 2A). The hepatic peroxisomal ACO protein level in mice of the 30% SFO group was also higher than that of the 5% SFO group ($P<.05$; Fig. 2C). Hepatic peroxisomal ACO activity (nmol H_2O_2/min/liver) was positively correlated to hepatic NEFA content (μmol/liver) in both rats ($r=.49$, $P=.0072$) and mice ($r=.57$, $P=.0008$). In addition, the hepatic peroxisomal ACO activity of C57BL/6J mice also positively correlated to hepatic TG concentration ($r=.64$, $P<.0001$). These results suggested that the higher liver ACO activity was related, at least in part, to increasing hepatic NEFA concentration. Forward stepwise multiple regression was used to determine the strongest predictor for ACO activity (dependent variable) among body weight gain, serum leptin concentration, daily fat intake, liver and serum lipid concentration, and the expression of PPARα and ACO mRNA (independent variables) in Wistar rats and

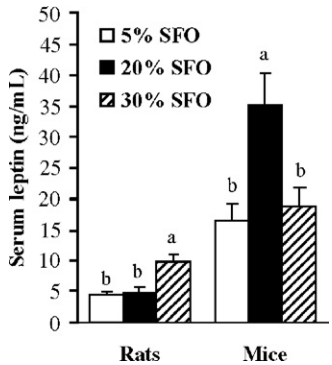

Fig. 1. Serum leptin concentration of Wistar rats and C57BL/6J mice fed diets containing 5 g/100 g (5% SFO), 20 g/100 g (20% SFO) or 30 g/100 g (30% SFO) SFO for 13 weeks (Wistar rats) or 22 weeks (C57BL/6J mice). Values are mean±S.E.M.; $n=9–10$ (rats) or 10–13 (mice). Data were analyzed by one-way ANOVA and Duncan's multiple range test for the significance of difference among the three diet groups of each species. Values not sharing a common letter among the three diet groups of the same species are significantly different ($P<.05$).

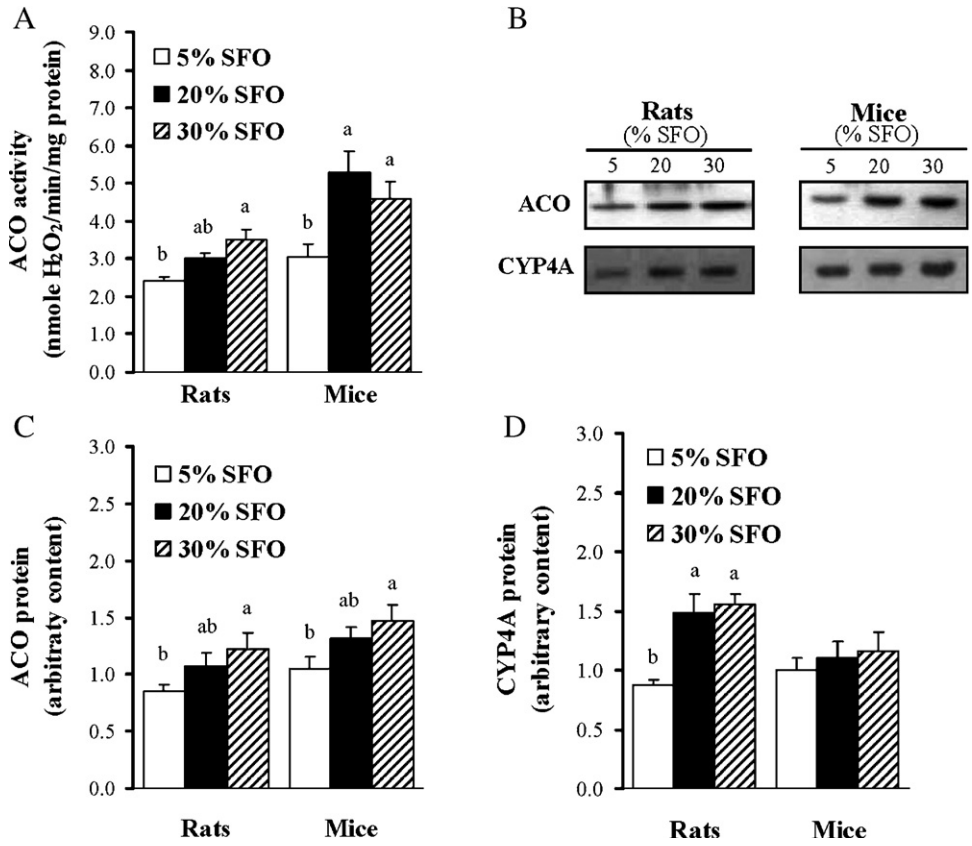

Fig. 2. The specific activities (A) and protein expression (B–D) of ACO (A–C) in liver PNS and cytochrome *P*450 4A (CYP4A) protein expression (B and D) in liver microsomes of rats and mice fed diets containing 5 g/100 g (5% SFO), 20 g/100 g (20% SFO) or 30 g/100 g (30% SFO) SFO for 13 weeks (Wistar rats) or 22 weeks (C57BL/6J mice). The protein expressions of ACO and CYCP4A were analyzed by Western blot analysis using specific antibodies (B) and quantified by image analysis (C and D). Values are mean±S.E.M.; $n=9$–10 per group (rats) or 10–13 per group (mice). The significance of difference among the three diet groups of each species was analyzed by one-way ANOVA and Duncan's multiple range test. Values not sharing a common letter among the three diet groups of the same species are significantly different ($P<.05$).

C57BL/6J mice, respectively. The result showed that the level of ACO activity was mainly affected by serum leptin concentration in both rats ($R^2=.33$, $P=.015$) and mice ($R^2=.43$, $P=.0008$).

3.5. Protein level of CYP4A

The hepatic CYP4A protein content of Wistar rats fed the 20% and 30% SFO diets was significantly higher than that of rats fed the 5% SFO diet ($P<.05$; Fig. 2D). The CYP4A protein was positively correlated to peroxisomal ACO activity ($r=.43$, $P=.021$) and protein content ($r=.48$, $P=.0087$) in Wistar rats. However, in C57BL/6J mice, the hepatic CYP4A protein content was not significantly different among the three groups ($P>.05$).

3.6. mRNA expression of PPARα and its target genes

Wistar rats fed the 30% SFO diet had the highest mRNA expression of liver PPARα (detected by real-time RT-PCR), mitochondria CPT1A and peroxisome ACO (detected by Northern blot analysis) among the three groups ($P<.05$; Fig. 3A and C). In contrast, the C57BL/6J mice fed the 20% SFO diet had the highest liver PPARα mRNA expression ($P<.05$; Fig. 3A). The expressions of liver ACO and FABP mRNA were also highest in the C57BL/6J mice fed the 20% SFO diet compared to those fed the 5% SFO ($P<.05$; Fig. 3D).

The PPARα mRNA expression was positively correlated to mRNA expressions of ACO ($r=.57$, $P=.008$), CYP4A ($r=.49$, $P=.023$), HMGS ($r=.53$, $P=.013$), CPT1A ($r=.66$, $P=.001$) and FABP ($r=.56$, $P=.011$) in Wistar rats. Similar correlations were also observed in mice for ACO mRNA ($r=.60$, $P=.003$) and for FABP mRNA ($r=.72$, $P=.0001$). In addition, PPARα mRNA expression level was positively correlated to fat intake ($r=.53$, $P=.01$), body weight ($r=.56$, $P=.0063$), retroperitoneal fat weight ($r=.47$, $P=.04$), liver NEFA ($r=.45$, $P=.037$) and serum leptin ($r=.50$, $P=.02$) in Wistar rats. In mice, PPARα mRNA expression level was positively correlated to body weight gain ($r=.44$, $P=.022$), retroperitoneal fat weight ($r=.46$, $P=.017$) and serum leptin ($r=.52$, $P=.0061$). These results seem to indicate that significant activation of PPARα signaling pathway is associated with elevated retroperitoneal fat weight and serum leptin. Stepwise multiple regression analysis was performed to evaluate the relative

contribution of body weight gain, serum leptin concentration, daily fat intake, liver and serum lipid concentration, and the level of PPARα mRNA expression with ACO mRNA in Wistar rats and C57BL/6J mice. The result showed that the expression of ACO mRNA was mainly influenced by the level of PPARα mRNA in both rats (R^2=.68, P=.0002) and mice (R^2=.36, P=.0038).

4. Discussion

Significantly higher body and adipose weights indicated obesity in rats fed the 30% SFO diet, but not in those fed the 20% SFO diet. However, in mice, signs of obesity were observed in the group fed 20% SFO, but not in the group fed 30% SFO, indicating that Wistar rats and C57BL/6J mice

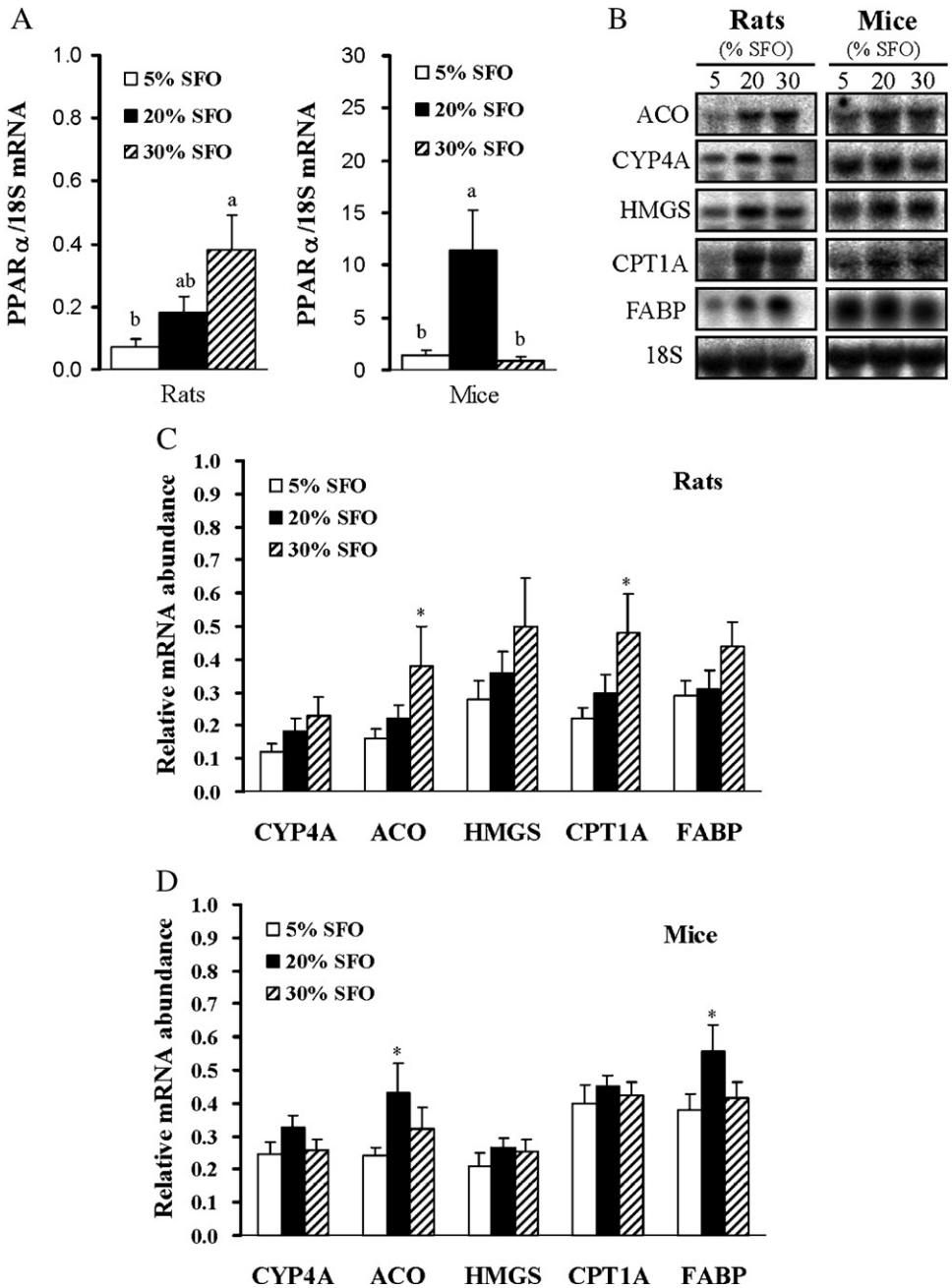

Fig. 3. The mRNA expression of PPARα and some of its target genes in the liver of rats and mice fed diets containing 5 g/100 g (5% SFO), 20 g/100 g (20% SFO) or 30 g/100 g (30%) SFO for 13 weeks (Wistar rats) or 22 weeks (C57BL/6J mice). PPARα mRNA (A) was detected by real-time RT-PCR, as described in Section 2. The result of each sample was expressed as relative cycle threshold (C_t) standard method and was normalized by 18*S* expression. The significance of difference among the three diet groups of each species was analyzed by one-way ANOVA and Duncan's multiple range test. Values not sharing a common letter among the three diet groups in the same species are significantly different (P<.05). The mRNA expressions of CYP4A, ACO, HMGS, CPT1A and FABP were analyzed by Northern blot analysis (B), as described in Section 2. Signals were quantitated by image analysis (C and D, rats and mice, respectively). Each value was normalized for loading with the 18*S* signal. Values are mean±S.E.M.; n=9–10 per group (rats) or 10–13 per group (mice). *Significantly different from animals fed the 5% SFO diet (Student's t test; P<.05).

have different susceptibilities to high-SFO diets. Both rats and mice consumed less food in response to high-SFO diets. The reduction in food intake was to a lesser extent in rats (which resulted in comparable energy intakes among the three groups) and to a higher extent in mice (which resulted in significantly lower energy intakes in groups fed high-fat diets) (Table 2). Therefore, the metabolic changes induced by a high-fat diet should be related to a difference in the level of fat consumption and not to excessive energy intake. Obesity seems to develop as a result of inadequate reduction of food intake in response to a high-fat diet.

It is well known that leptin has an important function in the control of food intake. In this study, the serum leptin level was elevated only in the rats fed the 30% SFO diet and mice fed the 20% SFO diet (i.e., obese animals), suggesting that a higher serum leptin level might not directly contribute to lowered food intake in rats and mice fed high-fat diets. These results support the notion that serum leptin is closely correlated to body fat mass and may be an important signal of fat storage [32].

Leptin is an adipocyte-derived hormone that is known to regulate body weight by reducing food intake and increasing energy expenditure [33]. Circulating leptin can be transported to the cerebrospinal fluid (CSF), bind to specific receptors on hypothalamic neurons and activate them to regulate energy homeostasis. In rats, it has been reported that there was a high degree of correlation between plasma and CSF leptin levels when plasma leptin level was below 5 ng/ml. However, when plasma leptin level exceeded 15 ng/ml, no correlation between plasma and CSF leptin was observed in hyperleptinemia models. It was suggested that leptin transported across the blood–brain barrier was saturated when plasma leptin level was between 5 and 15 ng/ml [34]. In this study, the serum leptin concentration of all three groups of C57BL/6J mice all exceeded 15 ng/ml. As for Wistar rats, the serum leptin concentration of the 5% and 20% SFO diet groups (4.5±1.46 and 4.9±2.46 ng/ml, respectively) was approximately 5 ng/ml, and that of the 30% SFO diet group (9.9± 3.28 ng/ml) exceeded 5 ng/ml.

PPARα is known to regulate lipid metabolism through a ligand-dependent transcriptional activation of the expression of genes involved in the fatty acid oxidation pathway. The activity of the PPARα signaling pathway presumably depends on available ligand concentration and on the expressed receptor level in the cell and is manifested in the expression of PPARα target genes [10]. It has been shown in in vitro experiments that common long-chain fatty acids not only activate PPARα but also bind to PPAR [3,5–7,35,36]. Palmitic acid, oleic acid, linoleic acid and arachidonic acid were identified as endogenous activators of PPARα [37]. The relative potency of individual fatty acid varies with types of measurements as well as species of PPAR derived. In experiments using rat, mouse and human PPARα, the potency of binding and the activation of oleic acid were comparable to those of linoleic acid [3,7,36]. High-oleic-acid SFO was therefore used in this study because PUFAs were reported to suppress lipogenesis, besides activating PPARα [38].

The high-SFO diets used in this study presumably provide more unsaturated fatty acids as endogenous ligands for PPARα. Indeed, liver NEFA concentration is positively correlated to dietary fat intake in both mice and rats ($P<.05$). In rats, the activity, protein and mRNA of ACO, CYP4A protein, PPARα and CPT1A mRNA expressions were positively correlated with dietary fat intake. In mice, only ACO activity and protein were positively correlated to fat intake. However, the activity of ACO, ACO mRNA, and FABP mRNA correlated positively to liver NEFA concentration. These data implied that an increased supply of fatty acids as endogenous ligand might contribute, to a certain extent, to increased PPARα signaling. On the other hand, the mRNA expressions of all PPARα target genes were highly correlated to the mRNA expression level of PPARα, suggesting that receptor availability seems to be a more important determinant for the mRNA expression of PPARα target genes.

The results of this study demonstrated that the mRNA expression of PPARα in the liver tended to increase with dietary fat intake in Wistar rats; in C57BL/6J mice, the expression of this gene was, nevertheless, significantly increased in the 20% SFO group, but not in the 30% SFO group (Fig. 3A), despite a higher total fat intake in this latter group. Taken together, the significant up-regulation of liver PPARα mRNA expression coincided with signs of obesity and hyperleptinemia (i.e., in rats fed the 30% SFO diet and in mice fed the 20% SFO diet). This is paradoxical to the proposed role of PPARα in the regulation of fatty acid oxidation, and an increased PPARα signaling should lead to decreased fat accumulation.

In a recent study, it was observed that approximately 50% of a group of C57BL/6J mice became obese and diabetic, but ~12% remained lean and nondiabetic after feeding on a high-fat diet (72% energy from fat) for 9 months [27]. Using a microarray analysis to compare liver mRNA expression, the expression of PPARα and fatty acid β-oxidation enzymes was found to be significantly increased in the obese/diabetic, but not in the lean, mice [27]. Compared to the lean mice, the obese mice showed significantly elevated serum leptin, hyperglycemia, hyperlipidemia and glucose intolerance [27]. A proteomics study on hepatic protein expression also demonstrated that PPARα-regulated fatty acid oxidation enzyme protein levels were higher in *ob/ob* mice than in lean controls [26]. Hepatic PPARα mRNA expression levels of *ob/ob* and *5-HT2cR* mutant mice were comparable to wild-type animals before they become obese, but were markedly increased after they become obese [24]. Our results in the present study agree with these reports that obese rodents have an elevated PPARα signaling pathway.

It has been suggested that leptin plays an important role in cellular liporegulation [39,40]. Leptin was shown to

display antisteatotic and lipopenic action through enhancement of fatty acid oxidation and suppression of lipogenesis [25,41]. Hyperleptinemia in Sprague–Dawley rats fed a 60% (wt/wt) fat diet were shown to be associated with increased PPARα protein, carnitine-palmitoyl transferase 1 mRNA and fatty acid oxidation in the liver [25]. Compared to adipose tissue, relatively limited TGs were accumulated in nonadipose tissues such as the liver, pancreatic islets, heart and muscles in these rats [25]. In contrast, rodents deficient in leptin or leptin receptor accumulated remarkably high amounts of TGs in these nonadipose tissues [25]. These same groups of authors subsequently reported that this lipopenic action of hyperleptinemia in white adipose tissues and the liver is dependent on PPARα, since these effects of hyperleptinemia could not be observed in PPARα-null mice [41]. Based on this suggested role of leptin, it seems plausible to speculate that, while a high-fat intake induced excessive adipose tissue accumulation in genetically normal animals, the resulting hyperleptinemia may then up-regulate PPARα expression in the liver. This speculation is currently under investigation in our laboratory, and preliminary results appear to be supportive. As for *ob/ob* or *db/db* obese mice, the elevated hepatic PPARα mRNA observed [24] might be mediated by factors other than leptin, such as glucocorticoids. It has been reported that circulating, hepatic, mesenteric and epididymal adipose tissue glucocorticoid concentrations were low in the normal C57BL/6J mouse, but were very high in the *ob/ob* mice [42]. Glucocorticoid has been known to up-regulate liver PPARα mRNA [11,12].

The synthetic ligands of PPARα, such as fibrates, are known to significantly up-regulate the PPARα signaling pathway, to efficiently reduce lipid levels in the body and to prevent obesity [43,44]. Providing more common fatty acids such as endogenous ligands by feeding a high-fat diet, nevertheless, did not lead to similar results, as shown in the present study. This may be due, at least in part, to the fact that common fatty acids are low-affinity ligands [5,36]. Ligand-specific recruitment of coactivators/corepressors might also contribute to some extent [6] to the discrepancy between fibrate treatment and high-fat feeding. In addition, the liver is very active in the metabolism of fatty acids. Depending on the nutritional/physiological status, free fatty acids in the liver may be efficiently channeled into various metabolic pathways, such as mitochondria β-oxidation and biosynthesis of TG. This may limit the amount of free fatty acids that are available as ligands for PPARα. Furthermore, common fatty acids are endogenous ligands of not only PPARα but also PPARγ and PPARβ/δ [6,45,46]. PPARγ is known to be a regulator of differentiation of preadipocytes to adipocytes in vitro [47,48] and in vivo [49]. High-fat diets induced obesity, adipocyte hypertrophy and insulin resistance in wild-type mice, but not in heterozygous (±)PPARγ-deficient mice [50]. This is due to increase in leptin expression, increase in fatty acid combustion and decrease in lipogenesis in heterozygous PPARγ-deficient mice [51]. Based on these results, PPARγ is regarded to mediate obesity induced by high-fat diets [50].

The study of serum leptin levels in relation to several measures of adiposity demonstrates that obesity in humans is not characterized by leptin deficiency but rather by hyperleptinemia; in fact, leptin levels have been found to be elevated in obese patients. Diet-induced obesity rodents are considered as a better model of human obesity with leptin resistance than are *ob/ob* mice with leptin deficiency. Species difference in the susceptibility to obesity in response to the high-SFO diets observed in this study suggested that the adjustment of food intake in response to high-fat diets (i.e., the extent of the reduction of food intake) has a great impact on the genesis of obesity.

The expression level of PPARα in humans has been reported to be low relative to that in rodents [52]. However, the fact that clinically used fibrate drugs efficiently improve hyperlipidemia suggests that this receptor signaling is present and operative in humans. The basic concept of PPARα signaling established in molecular biology studies have led to the speculation that high-fat diets presumably provide more fatty acids as ligands for PPARs and, therefore, should be able to up-regulate those PPARα target genes and to enhance fatty acid metabolism through the PPARα signaling pathway. The results of this study, however, did not fully support this speculation. The capacity of the PPARα signaling pathway to protect from obesity induced by a high-fat diet might be limited.

In this study, mice fed the 30% SFO diet showed significantly higher ACO activity and immunoreactive protein than control mice fed the 5% SFO diet. But the mRNA expression of ACO was not significantly different between these two groups. The exact cause of the discrepancy remains unknown. Whether the peroxisomal ACO protein/enzyme activity could further be stabilized/influenced by fatty acids or fatty acyl-CoA (its substrate) needs to be studied further.

In conclusion, up-regulation in the mRNA expression of PPARα and some of its target genes in the liver in response to high-SFO feeding was associated with obesity and hyperleptinemia in both rats and mice. For those animals that did not develop obesity despite a high intake of dietary SFO, changes in liver PPARα signaling were only minimal. These results raised the possibility that obesity-associated hyperleptinemia may have a role in the induction of liver PPARα gene expression.

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