Oxidized Frying Oil Up-Regulates Hepatic Acyl-CoA Oxidase and Cytochrome P_{450} 4 A1 Genes in Rats and Activates PPARα[1]

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ABSTRACT Oxidized LDL (oxLDL) and its component hydroxy fatty acids were shown to activate peroxisome proliferator-activating receptor α (PPARα) and γ (PPARγ). To test the hypothesis that lipid oxidation products in oxidized frying oil (OFO) can activate PPARα and up-regulate its target genes, a feeding experiment and a transactivation experiment were conducted. Based on a 2 $\times$ 2 factorial design, four groups of Sprague-Dawley male weanling rats were fed diets containing either high (20 g/100 g, HO and HF) or low (5 g/100 g, LO and LF) levels of oxidized frying soybean oil (HO and LO) or fresh soybean oil (HF and LF) for 6 wk. The OFO sample was prepared by frying wheat dough sheets in soybean oil at 205 $\pm$ 5°C for 24 h. OFO dose dependently and significantly increased ($P < 0.05$) mRNA of acyl-CoA oxidase (ACO) and cytochrome P_{450} 4A1(CYP4A1) in liver of rats. Dietary OFO also dose dependently increased liver microsomal CYP4A protein ($P < 0.05$). The activity of hepatic ACO of the HO group was sixfold that of the HF group ($P < 0.05$). Plasma total lipids, liver triglycerides, cholesterol and total lipids were reduced in rats fed the LO and HO diets ($P < 0.05$). Through the ligand binding domain of PPARα, the hydrolyzed OFO enhanced the expression of alkaline phosphatase (ALP) reporter gene to a significantly greater extent ($P < 0.05$) than the hydrolyzed fresh soybean oil in a transactivation assay using a clone of CHO K1 cells stably expressing Gal4-PPARα chimeric receptor and UAS_4-ALP reporter. The results support our hypothesis that dietary OFO, by activating PPARα, up-regulates the expression of PPARα downstream genes and alters lipid metabolism in rats. J. Nutr. 131: 3166–3174, 2001.

KEY WORDS: • *oxidized frying oil* • *PPARα* • *acyl-CoA oxidase* • *CYP4A1* • *rats*

Because of their crispness and aromatic properties, fried foods are immensely popular throughout the world and contribute markedly to our total energy intake. During the deep frying process, a series of degradation reactions, including autoxidation, thermal oxidation, polymerization, cyclization and fission occur in the frying fat/oil (1,2). Lipid peroxides, the primary autoxidation products, are rapidly degraded at the high temperature of frying. The nonvolatile secondary oxidation products retained in the used frying oil, comprised mainly of oxidized triglyceride (TG)[4] monomers, dimers and polymers, are of great nutritional importance because they are absorbed into the fried foods and ingested. The oxidized TG contain at least one oxygenated function in the esterified fatty acids and may be a mixture of epoxides, ketones, alcohols, as well as polyoxygenated compounds. The dimers and polymers are complex structures in which TG monomers are covalently linked through C—C or C—O—C bonds (2).

Although toxic fractions have been isolated from laboratory-abused oil (3), long-term feeding studies using fat samples oxidized under more realistic cooking practices as part of a nutritional balanced diet resulted only in mild symptoms such as less body weight gain and feed intake and enlargement of liver and kidney (4,5). Therefore, for the following reasons, the frying oil that is ingested with the fried foods is generally regarded as safe as long as the organoleptic quality of the fried foods is acceptable (3,6): *1*) with substantial accumulation of oxidation products, especially dimers and polymers, the frying operation becomes difficult due to vigorous foaming and foods fried in such an oil become unpalatable (3); *2*) digestion and absorption of the dimerized and polymerized TG are decreased (7,8); and *3*) the effective detoxifying capability of liver microsomal enzymes, including cytochrome P_{450} monoxygenase and phase II conjugation enzymes, which are significantly induced by an oxidized frying oil (OFO)-containing diet (9). Interestingly, a lowered TG in plasma and liver of rats fed OFO was repeatedly observed (9,10).

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[4] Abbreviations used: ACO, acyl-CoA oxidase; ALP, alkaline phosphatase; BIEN, bifunctional enzyme; CFAM, cyclic fatty acid monomer; CYP 4A1, cytochrome P_{450} isoform 4A1; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HETE, hydroxy eicosatetraenoic acid; HF, high fresh soybean oil (20 g/100 g diet)group; HNE, *trans*-4-hydroxy-2-nonenal; HO, high oxidized frying oil (20 g/100 g diet)group; HODE, hydroxy octadecadienoic acid; LBD, ligand binding domain; LF, low fresh soybean oil (5 g/100 g diet)group; LO, low oxidized frying oil (5 g/100 g diet)group; LPL, lipoprotein lipase; MCID, microcomputer imaging device; NEFA, nonesterified fatty acid; OFO, oxidized frying oil; oxLDL, oxidized LDL; PC, positive control group; PNS, postnuclear supernatant; PP, peroxisome proliferators; PPAR, peroxisome proliferator-activating receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; TG, triglyceride.

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Fibrates, a class of commonly used hypolipidemic drugs that effectively lower serum TG and cholesterol, trigger a coordinate transcription of genes participating in fatty acid oxidation including acyl-CoA oxidase (ACO), bifunctional enzyme (BIEN), thiolase and cytochrome P_{450} isoform 4A (CYP4A) (11). A transcription factor belonging to the nuclear receptor family (12) that can be activated by peroxisome proliferators (PP), including fibrates, was cloned and named peroxisome proliferator-activated receptor (PPAR). PPAR have a common modular structure including a DNA-binding domain and a ligand-binding domain (13,14). In addition to PP, fatty acids and eicosanoids are PPAR ligands. Upon activation by a ligand, PPAR heterodimerizes with retinoid-X receptor and promotes the transcription of its target genes through binding to a peroxisome proliferator responsive element. The target genes of PPARα are mainly a homogeneous group of genes that participate in aspects of lipid catabolism such as fatty acid uptake and binding, fatty acid oxidation (in microsomes, peroxisomes and mitochondria) and lipoprotein assembly and transport. The regulatory role of PPARα in lipid metabolism and lipoprotein assembly (14) and in the action of fibrates has been clearly demonstrated in the PPARα gene knockout mouse model (14–16). Recent observations further demonstrated a role of PPAR in regulating glucose homeostasis, cellular differentiation and apoptosis, cancer development as well as in the control of the inflammatory response (17,18).

In vitro studies showed that oxidized LDL (oxLDL) and its component hydroxy fatty acids, including hydroxy eicosatetraenoic acid (HETE) and hydroxy octadecadienoic acid (HODE), activated PPARγ (19) and α (20). Because hydroxy fatty acids are among the various oxidation products in frying oil (21) that may be absorbed after digestion, we proposed that dietary OFO can modify lipid metabolism in rats by transcriptionally regulating expression of genes through the activation of PPARα. To test this hypothesis, we examined the expression of two typical PPARα down-stream genes, i.e., ACO and CYP4A1, in rats fed two levels of OFO (5 g/100 g, the LO diet, and 20 g/100 g, the HO diet), using the same levels of fresh soybean oil as the negative control (LF and HF diet) and clofibrate as a positive control (PC). In addition, attempts were made to compare the potency of hydrolyzed fresh soybean oil and OFO in activating PPARα using a transactivation assay.

MATERIALS AND METHODS

Animals and diets. Male weaning Sprague-Dawley rats ($n = 40$; 60–80 g) were purchased from the laboratory animal center of the National Science Council (Taipei, Taiwan). The rats were housed individually in stainless steel wire cages in a room maintained at 23 ± 2°C, with a controlled 12-h light:dark cycle. The rats were fed a nonpurified diet (Laboratory Rodent Chow, Ralston Purina, St. Louis, MO) for 3 d. Then rats ($n = 32$) were randomly assigned to four groups based on a 2 × 2 factorial design. They were fed the four test diets containing a high level (20 g/100 g, HF or HO diet) or a normal level (5 g/100 g, LF or LO diet) of OFO (LO or HO diet) or fresh soybean oil (LF or HF diet). To serve as a positive control (PC), a fifth group of rats was also fed the LF diet but with a supplementation of 0.5 g/100 g clofibrate (Yamanouchi, Tokyo, Japan) for the last 7 d. All rats had free access to food and tap water. Body weight and food intake were recorded weekly. Animal care and handling conformed to accepted guidelines (22).

The OFO was prepared as described previously (9). Briefly, 9 kg of soybean oil (President, Tainan, Taiwan) was poured into a cast iron wok and heated on a gas stove that was adjusted to maintain the oil temperature at 205 ± 5°C. Wheat flour dough sheets (12 × 4.5 × 0.15 cm, ~11 g) were fried in the oil, one at a time. The frying proceeded for 6 h/d and was repeated successively for 4 d. The acid value (mg KOH/g), peroxide value (meq/kg), UV absorbance at 233nm (OD/g), polar compounds (%) and fatty acid composition were analyzed according to AOCS methods (23) and the results are shown in **Table 1**. The composition of the four test diets is shown in **Table 2**. Because the rats fed high fat diets (HF and HO) consumed less food due to the higher energy density of diets, 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 four diets.

Tissue sampling and preparation. After 6 wk of feeding, food was withheld overnight and rats were killed by carbon dioxide asphyxiation. Blood was collected from the abdominal vena cava into an EDTA-containing tube. The plasma was separated and stored at −20°C for lipid analysis. Tissues were excised and weighed. Aliquots of liver and one of the two kidneys were quick-frozen in liquid N_2 and stored at −80°C for RNA extraction. A portion of fresh liver was homogenized in 0.01 mol/L phosphate buffer (pH 7.4) for preparing microsomal pellets as described (9). Another portion of fresh liver was homogenized in 50 mmol/L Tris-HCl buffer (pH 8.3) and centrifuged at 600 × *g* for 6 min. The resulted postnuclear supernatant (PNS) was analyzed for activities of ACO and catalase.

Biochemical analyses. Plasma and liver lipids were measured by enzymatic methods using commercial kits (Randox Lab, Crumlin, Northland, UK) for cholesterol, TG, nonesterified fatty acid (NEFA), total lipid and phospholipid (Audit Diagnostic, Cork, Ireland). Liver lipids were extracted by a mixture of $CHCl_3$/MeOH (2:1, v/v) and triton X-100 was added to aid in solubilizing the extract. The peroxisomal ACO and catalase activities in PNS were determined according to Lazarow (24) and Luck (25) respectively. The cytochrome P_{450} content in liver microsomal suspensions was determined by the method of Omura and Sato (26). The microsomal CYP4A protein was detected by Western blot analysis. Liver microsomal suspension containing 5 μg protein were subjected to SDS-PAGE, then transferred to polyvinylidene fluoride-plus transfer membrane (NEN Life Science, Boston, MA). The blot was immunodetected with enhanced chemiluminescence (ECL) Western blotting kit (Amersham International, Amersham, UK) in which sheep anti-rat CYP4A was used as the primary antibody and a biotinylated donkey anti-sheep immunoglobulin G was used as the secondary antibody. This in turn was detected with streptavidin-HRP and ECL detection reagents. The emitting light was captured on a film (BioMax Light film; Kodak, Rochester, NY). The film was then subjected to the microcomputer imaging device (MCID) image analysis system [MCID; Fuji, Tokyo, Japan]. Protein was quantified by Bio-Rad protein assay dye (Bio-Rad, Hercules, CA).

Preparation of cDNA probes. CYP4A1, ACO and β-actin cDNA were synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR). Rat liver cDNA was used as the template, and the primer sequences used were as follows: 5′-ATGAGCGTCTCTGCACTGAG-3′ (forward) and 5′-TTGGAGAAAGGAG-

TABLE 1

The quality index and fatty acid composition of the fresh soybean oil and oxidized frying oil[1]

	Fresh soybean oil	Oxidized frying oil
Quality index		
Acid value, *mg KOH/g*	0.096	1.216
Peroxide value, *meq/kg*	2.85	6.34
UV_{233}, *OD/g*	407	4525
Total polar compound, *g/100 g*	1.65	54.3
Fatty acid composition, *g/100 g*		
16:0	11	15
18:0	4.7	5.5
18:1 (n − 9)	22	29
18:2 (n − 6)	53	46
18:3 (n − 3)	8.6	4

[1] Data were means of duplicate analyses.

TABLE 2

Composition of test diets used in the feeding experiment[1,2]

	LF	LO	HF	HO
		g/kg diet		
Casein	200	200	235	235
Cornstarch	650	650	448	448
Fresh soybean oil	50	—	200	—
Oxidized frying oil[3]	—	50	—	200
Cellulose	50	50	59	59
Mineral mixture	35	35	41	41
Vitamin mixture	10	10	12	12
DL-Methionine	3	3	3	3
Choline	2	2	2	2

[1] LF, low fresh soybean oil diet; LO, low oxidized frying oil diet; HF, high fresh soybean oil diet; HO, high oxidized frying oil diet.

[2] Sources of ingredients: casein, ICN (Aurora, Ohio); cornstarch, Samyang (Seoul, Korea); cellulose, J. Rettenmaier & Söhne (Holzmühle, Germany); soybean oil, President Co. (Tainan, Taiwan); methionine and choline chloride, Sigma Chemical (St. Louis, MO); AIN-76 mineral mixture and AIN-76 vitamin mixture, ICN (Aurora, Ohio).

[3] Prepared by frying dough sheet in soybean oil at 205 ± 5°C for 24 h as described (9).

GGAAGG-3′ (reverse) for CYP4A1(accession number M14972) (bases: 13–2040); 5′-ACTCGAGATGAACCCCGACCTGCGCA-3′ (forward) and 5′-TCTCGAGTCAAAGCTTGGACTGCAG-3′ (reverse) for ACO (accession number J02752)(bases: 74–2059); 5′-GTGGGCCGCTCTAGGCACCA-3′ (forward) and 5′-CTCTTTGATGTCACGCACGATTTC-3′ (reverse) for β-actin (accession number 55574)(bases:103–642). The PCR products were ligated into pGEM-T easy vector (Promega, Madison, WI) and transformed to *Escherichia coli* JM109. After a blue/white screening, the cDNA targets were cut from plasmid by appropriate restriction enzymes. All of the three cDNA targets obtained were confirmed by sequence analysis. The cDNA probes were labeled by the random primer method with a DNA labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) and [α-^{32}P]dCTP. Unincorporated nucleotides were removed by MicroSpin G-50 Micro column (Amersham Pharmacia Biotech) before hybridization.

Northern blot analyses. Total RNA was extracted from kidney with guanidium thiocyanate and from liver with trizol reagent (Life Technologies, Rockville, MD). Total RNA (20 μg) from each sample was separated by electrophoresis in denaturing formaldehyde agarose (1%) gel, transferred to nylon membrane (Gene Screen Plus; DuPont, Boston, MA) and cross-linked to membrane by UV irradiation. The blots were prehybridized at 42°C for 3 h in the hybridization buffer excluding the probe but containing salmon sperm DNA. They were then hybridized at 42°C for 12–15 h with ^{32}P-labeled cDNA probes of ACO or CYP4A1. To correct for possible difference in transfer and loading, the blots were also hybridized with labeled β-actin probe as an internal control. Afterward, the blots were washed at the appropriate stringency to remove nonspecific binding and exposed to X-OMAT AR film (Kodak). Signals were quantified using the MCID image analysis system.

Semiquantitative RT-PCR assay. PPARα mRNA in liver was measured by semiquantitative RT-PCR because of its low abundance. Total RNA (1 μg) from liver was reverse transcribed by 200 U MMLV-RT (Promega, Madison, WI). Subsequent amplifications of PPARα cDNA encoding base pairs 873-1784 were performed using different amounts of reverse transcribed products as templates to ensure quantification in the linear range. The primers used were 5′-CCTGTGAACACGATCTGAAAG-3′ (forward) and 5′-TCTGACTCGGTCTTCTTGATG-3′ (reverse) designed according to the accession number M88592. The PCR mixtures were subjected to 30 cycles of amplification by 94°C 1 min, 60°C 1 min and 72°C 1 min. As an internal standard, β-actin was also amplified. Then the products were analyzed by 1% agarose gel electrophoresis with ethidium bromide and quantified by the MCID image analysis system.

Hydrolysis and characterization of oil samples. Fresh soybean oil and OFO were hydrolyzed by a cold saponification method (27). After an overnight incubation with 10 volumes of 3.6 mol/L KOH in methanol (MeOH) at room temperature, the saponifiable fraction was extracted with water and then acidified with H_2SO_4. The fatty acids liberated were extracted with diethyl ether. The saponifiable fractions were weighed after removing solvent under a stream of N_2. Then, a stock solution in absolute ethanol was prepared and stored at −20°C. The concentration of saponified fresh oil or OFO was calculated by assuming a mean molecular weight of 280 (linoleic acid). The absorption of UV at wavelength 200–300 nm by 0.7 mmol/L hydrolyzed OFO or fresh soybean oil in *n*-hexane was measured in a spectrophotometer (Hitachi U-3400, Tokyo, Japan). The fatty acids and their oxidation products in hydrolyzed oil were separated on a reverse phase TLC (RP-18, Merck, Darmstadt, Germany) by developing with acetonitrile/MeOH/H_2O at 6:3:1 as the mobile phase and visualized with iodine vapor.

Plasmids. pBK-CMV-Gal4-rPPARα-ligand binding domain (LBD), pBK-CMV-$(UAS)_4$-tk-alkaline phosphatase (ALP), pSV_2-Neo, pKSV-Hygro were gifts from Dr. Gustaffson, Department of Medical Nutrition, Karolinska Institute. The correct in frame fusions were confirmed by sequencing.

Cell culture and stable transfection. CHO K1 cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD). For stable transfections as described previously (28), cells were seeded at a density of 2×10^8 cells/L on 6-cm round plates in 5 mL medium and grown for 24 h. Transfections were performed with lipofectamine 2000 (Gibco BRL) according to the manufacturer's instructions. Each plate received 6 μg of $(UAS)_4$-ALP and 0.6 μg of pSV_2-Neo. Transfected cells were selected in the presence of 0.8 g/L medium of G418 (neomycin). The colonies grown were pooled to form a reporter clone mix. The expression of the $(UAS)_4$-ALP in this reporter clone mix was confirmed by the induction of the ALP enzyme by Wy-14643 after transient transfection with pBK-CMV-GAL4-rPPARα. The reporter clone mix was further transfected with pBK-CMV-GAL4-rPPARα expression vectors (1 or 5 μg/plate) together with pKSV-Hygro (0.1 or 0.5 μg/plate, respectively) and selected with 0.8 g/L medium of hygromycin. Fifty clones were picked from each plate, expanded and tested for their responsiveness to 50 μmol/L Wy-14643 (Cayman Chemicals, Ann Arbor, MI), the known activator of rPPARα. The clone that showed the maximal fold induction of the reporter gene activity was used for the following experiments. The expression of rPPARα-LBD in cells of the screened clone was confirmed by RT-PCR.

Activation experiments and ALP assay. Stably transfected cells were seeded at a density of 60,000 cells/well in 96-well plates in 0.1 mL medium containing 10% FBS and grown for 24 h. Stock solutions of Wy-14643, hydrolyzed oil samples in ethanol were diluted to the desired concentrations with Ham's F-12 medium containing 10% serum replacement (TCM, Celox, St. Paul, MN). Basal levels of expression of reporter activity were obtained with medium containing vehicles [dimethyl sulfoxide (DMSO) or ethanol] at a concentration equivalent to those used in the highest concentrations of activators. Cells were incubated with these activation media at 37°C, in a 5% CO_2 incubator for 48 h. Culture supernatants were collected and assayed for ALP activity using CSPD (Tropix, Applied Biosystems, Foster City, CA) as the substrate and Sapphire-II as the enhancer. Chemiluminescence was measured in a luminometer (Wallac 1420 Victor^2 multiple label counter; Perkin Elmer, Turku, Finland). The toxicity of the treatment on cells was checked by the MTT assay (Sigma, St. Louis, MO).

Statistical analysis. Data are expressed as means ± SD. To test the significance of the effects of fat quality, fat quantity and their interaction, data from the LF, LO, HF and HO groups were analyzed by two-way ANOVA. When a significant interaction ($P < 0.05$) existed between fat quality and fat quantity, the significance of differences among the four groups (LF, LO, HF, HO) was further analyzed statistically by Duncan's multiple range test. Significance of differences between LF and PC groups was analyzed by Student's *t* test. For data obtained in the transactivation assay, the difference between the activation effect of different concentrations of hydro-

lyzed oil samples and vehicle, and between saponified fresh soybean oil and OFO at the same concentration was analyzed by Student's *t* test taking the fold activation of the ALP activity of the vehicle (DMSO or ethanol) treatment as 1. For all of the statistical analyses, data were transformed to log values before the statistical analysis was performed if the variances were not homogeneous. The SAS System (SAS Institute, Cary, NC) was employed for the statistical analysis and differences were considered significant at $P < 0.05$.

RESULTS

Body weight gain, food intake and relative tissue weight. There were significant interactions ($P < 0.0005$) between the effects of dietary fat quantity and quality on body weight gain, food intake, relative liver and kidney weight (**Table 3**). Dietary OFO significantly reduced body weight gain and food intake, increased relative liver and kidney weight ($P < 0.05$) at a high dietary level (20 g/100 g, HF vs. HO), but not at the normal dietary fat level (LF vs. LO). After 6 wk of feeding, rats fed the HO diet had a significantly lower body weight gain, food intake and higher relative liver and kidney weights than the other three groups ($P < 0.05$; Table 3). The body weight gain of rats in the HO group was only about half that of those in the HF group. In contrast, the LO group did not show growth depression, liver and kidney enlargement ($P < 0.05$) compared with the LF group. Interestingly, rats fed the LO diet had a significantly greater ($P < 0.05$) food intake than the other three groups, yet the body weight gain was not different from that of the LF control group. The feed efficiency was significantly reduced by dietary OFO ($P < 0.0005$) and increased by a high fat diet ($P < 0.0005$), indicating a negative effect of OFO on the energy and/or nutrient utilization.

As expected, feeding a 0.5 g/100 g clofibrate-containing diet for 1 wk resulted in a significantly lowered body weight gain and feed efficiency, as well as enlarged liver and kidney (PC vs. LF, $P < 0.05$)(Table 3).

Plasma and liver lipids. Neither dietary fat quantity nor fat quality significantly affected plasma cholesterol, phospholipid or NEFA (**Fig. 1**A). A high fat diet, however, significantly decreased plasma TG ($P < 0.01$) and total lipid ($P < 0.05$) concentration (Fig. 1A). There were significant interactions ($P < 0.05$) between dietary fat quantity and fat quality on liver TG, cholesterol, NEFA and total lipid. In rats fed control diets, the HF group had significantly higher liver

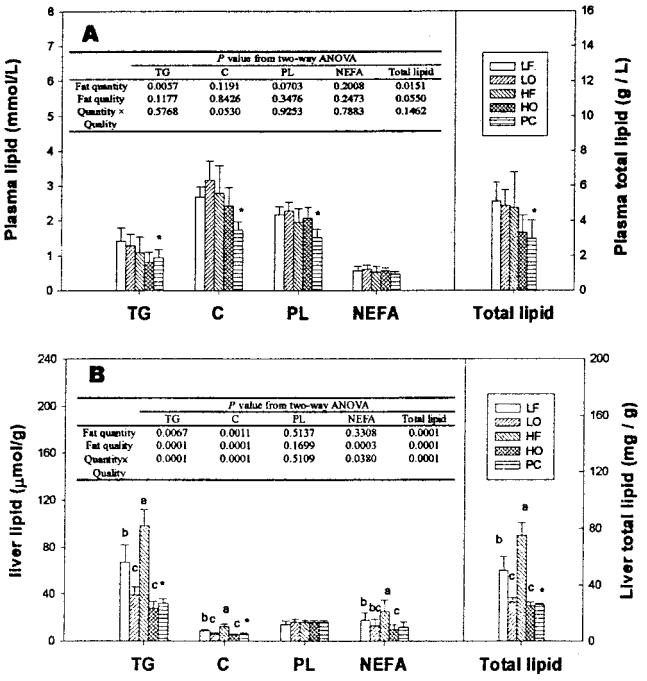

FIGURE 1 Triglyceride (TG), cholesterol (C), phospholipid (PL), nonesterified fatty acids (NEFA) and total lipid in plasma (*A*) and liver (*B*) of rats fed diets containing 5 g/100 g (LF and LO) or 20 g/100 g (HF and HO) fresh soybean oil (LF and HF) or oxidized frying oil (LO and HO) for 6 wk. PC is a group of positive control rats that was fed the LF diet but supplemented with 0.5 g/100 g clofibrate in the diet for the last week. Values are means ± SD, $n = 8$. Two-way ANOVA was conducted for LF, LO, HF and HO groups, and results are shown in the inset table. Where there was a significant interaction ($P < 0.05$), data were further analyzed by Duncan's multiple range test. Values not sharing a common letter are significantly different ($P < 0.05$). *Significantly different from LF, $P < 0.05$ (Student's *t* test).

TABLE 3

Body weight gain, food intake, feed efficiency and relative liver and kidney weight of rats fed diets containing 5 g/100 g (LF and LO) or 20 g/100 g (HF and HO) fresh soybean oil (LF and HF) or oxidized frying oil (LO and HO) for 6 wk[1]

	Body weight gain	Food intake	Feed efficiency	Relative liver weight	Relative kidney weight
	g/d		*g gain/g feed*	*g/100 g body*	
LF	7.59 ± 0.40^{ab}	17.80 ± 1.08^{b}	0.43 ± 0.03	3.7 ± 0.3^{b}	0.77 ± 0.03^{bc}
LO	7.37 ± 0.78^{b}	20.12 ± 1.20^{a}	0.37 ± 0.04	3.6 ± 0.2^{b}	0.79 ± 0.04^{b}
HF	8.13 ± 0.85^{a}	16.01 ± 1.31^{c}	0.51 ± 0.03	3.5 ± 0.2^{b}	0.73 ± 0.05^{c}
HO	4.75 ± 0.58^{c}	11.17 ± 1.08^{d}	0.42 ± 0.04	5.9 ± 0.4^{a}	0.91 ± 0.08^{a}
PC[2]	6.34 ± 0.62*	17.22 ± 1.06	0.37 ± 0.03*	5.1 ± 0.3*	0.94 ± 0.04*
			P-value from two-way ANOVA[3,4]		
Fat quantity	0.0002	0.0001	0.0001	0.0001	0.0496
Fat quality	0.0001	0.0049	0.0001	0.0001	0.0001
Quantity × Quality	0.0001	0.0001	0.4404	0.0001	0.0002

[1] Values are means ± SD, $n = 8$.
[2] PC is a group of positive control rats that was fed the LF diet but supplemented with 0.5 g/100 g clofibrate in the diet for the last week.
[3] *P*-values for fat quantity, fat quality and their interaction were analyzed by two-way ANOVA among LF, LO, HF and HO groups.
[4] When there was a significant interaction between quantity and quality, the significance of differences among LF, LO, HF and HO groups were analyzed by Duncan's Multiple Range Test. Values not sharing a superscript letter are significantly different, $P < 0.05$.
* Significantly different between PC and LF groups, $P < 0.05$ (Student's *t* test).

TG, cholesterol, NEFA and total lipid than the LF group (Fig. 1*B*). However, these liver lipid concentrations did not differ between the HO and LO groups and they were all markedly lower than those of LF and HF groups ($P < 0.05$). The liver TG of the LO and HO groups were only 58 and 28% that of the LF and HF groups, respectively. Dietary OFO effectively lowered liver lipids even at the 5 g/100 g dietary level. The reductions in liver lipid concentrations were not a result of liver enlargement because differences remained when liver lipid was expressed as total lipid content per liver (data not shown).

As expected, supplementation of 0.5 g/100 g clofibrate to rats fed the LF diet for the final week of the experiment significantly reduced plasma total lipid, TG, cholesterol and phospholipid concentrations compared with the unsupplemented LF group ($P < 0.05$). The liver total lipid, TG and cholesterol concentrations were also significantly reduced ($P < 0.05$).

Peroxisomal ACO and catalase activity. Because induction of peroxisomal β-oxidation is a general response to PPARα activation, the activities of ACO, which catalyzes the conversion of acyl-CoA to Δ^2-*trans*-enoyl-CoA in the peroxisomal β-oxidation and catalase, the marker enzyme of peroxisome, were analyzed. There were significant interactions between dietary fat quantity and quality on ACO (**Fig. 2***A*) and catalase (Fig. 2*B*) activities. The HF and LF groups did not differ in the activities of these two enzymes, whereas the HO group had significantly higher activities of ACO and catalase than the LO group ($P < 0.05$). The ACO activity in the liver of the HO group was sixfold that of the HF group ($P < 0.05$). The ACO activity of the LO group was slightly but not significantly higher than that of the LF group. Liver catalase activities in rats fed the OFO-containing diets (HO and LO groups) were greater than ($P < 0.05$) those in rats fed the control diets (HF and LF groups, respectively). The specific activities of ACO and catalase were approximately four- and twofold greater, respectively, than those of the LF group in rats fed the LF diet supplemented with 0.5% clofibrate (PC). Although ACO and catalase are both peroxisomal enzymes, catalase is not regulated by PPARα as is ACO. The elevated catalase activity may be an indication of peroxisome proliferation.

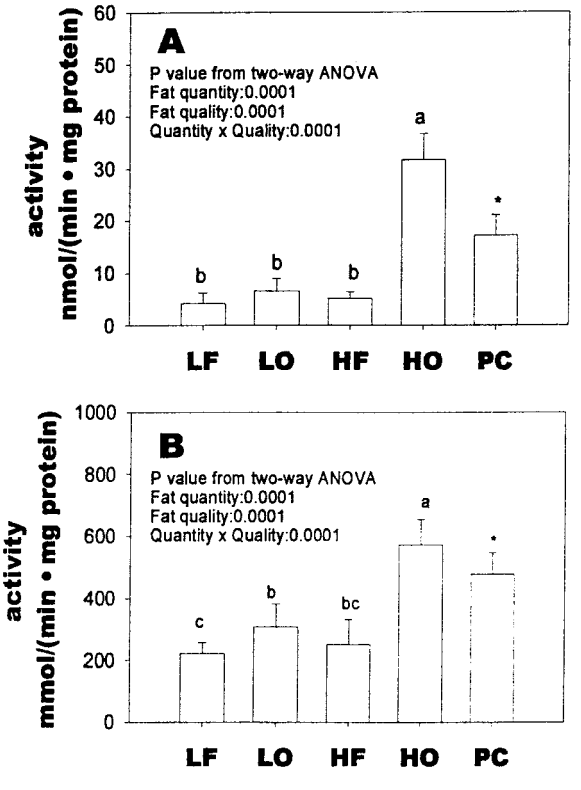

FIGURE 2 The specific activities of acyl-CoA oxidase (ACO) (*A*) and catalase (*B*) in liver postnuclear supernatant (PNS) of rats fed diets containing 5 g/100 g (LF and LO) or 20 g/100 g (HF and HO) fresh soybean oil (LF and HF) or oxidized frying oil (LO and HO) for 6 wk. PC is a group of positive control rats that was fed the LF diet but supplemented with 0.5 g/100 g clofibrate in the diet for the last week. Values are means ± SD, $n = 8$. Two-way ANOVA was conducted for LF, LO, HF and HO groups. Where there was a significant interaction ($P < 0.05$), significance of the difference was further analyzed by Duncan's multiple range test. Values not sharing a common letter are significantly different ($P < 0.05$). *Significantly different from LF, $P < 0.05$ (Student's *t* test)*.

Microsomal total cytochrome P_{450} and CYP4A protein. The drug-metabolizing enzymes cytochrome P_{450} are increased by PP treatment, and the isoform CYP4A contributes to the increment. Microsomal total cytochrome P_{450} content was increased by OFO feeding ($P < 0.0005$) (**Fig. 3***A*), consistent with results of our previous studies (9). Moreover, the CYP4A protein content in liver microsomes, detected by Western blot analysis, was dramatically increased in rats fed OFO-containing diets ($P < 0.0005$) (Fig. 3*B*). The increase in the CYP4A protein was more prominent in the HO group than in the LO group ($P < 0.05$). In contrast, the CYP4A protein content of the HF group was even lower than that of the LF group ($P < 0.05$), hence a significant interaction ($P < 0.0005$) between dietary fat quantity and fat quality was observed. Because the antibody against rat CYP4A used in this study recognized all three CYP4A forms (CYP4A1, CYP4A2 and CYP4A3) in rat liver, the band immunodetected was the sum of these three isoforms unseparated in the electrophoresis conditions used. Supplementation of 0.5 g/100 g clofibrate to the LF diet resulted in the induction of CYP4A protein as expected (Fig. 3*B*).

ACO, CYP4A1 and PPARα mRNA. To investigate whether liver ACO and CYP4A1 genes were transcriptionally activated by OFO, we measured the ACO and CYP4A1 mRNA in liver by Northern blot analysis. As shown in **Figure 4**, feeding a high fat or an OFO-containing diet significantly increased liver ACO and CYP4A1 mRNA ($P < 0.05$). There was no significant interaction between these two dietary factors; thus, rats fed the HO diet had the highest expression of ACO and CYP4A1 mRNA in livers as a result of the additive effect of the two dietary factors. Expression of CYP4A1 mRNA in kidney was also measured, but the induction effect by OFO was marginal ($P = 0.0565$, Fig. 4).

Having observed that the expression of PPARα downstream genes was enhanced, we wanted to determine whether the transcriptional activation was due to increased PPARα expression or an activation of PPARα by specific ligands. To this end, PPARα mRNA was detected by a semiquantitative RT-PCR and was shown to not differ among the four groups (data not shown).

As expected, rats fed the clofibrate-supplemented diet had significantly higher liver CYP4A1, ACO and kidney CYP4A1 mRNA than rats of the LF control group (Fig. 4), whereas the PPARα mRNA was not changed by clofibrate treatment.

Transactivation of PPARα via the ligand-binding domain by hydrolyzed OFO. To compare the activation potency of hydrolyzed OFO and fresh soybean oil on PPARα, a stable transfection experiment was carried out. A cell clone (CHO K1) stably expressing a chimeric receptor in which the PPARα

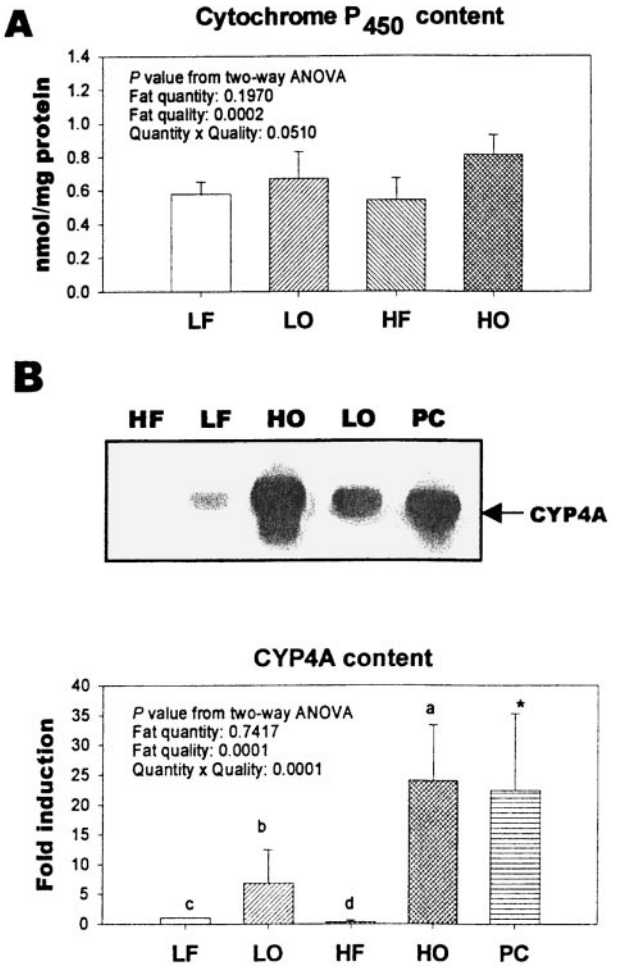

FIGURE 3 The cytochrome P_{450} content (*A*) and the isoform CYP4A protein (*B*) in liver microsomes of rats fed diets containing 5 g/100 g (LF and LO) or 20 g/100 g (HF and HO) fresh soybean oil (LF and HF) or oxidized frying oil (LO and HO) for 6 wk. PC is a group of positive control rats that was fed the LF diet but supplemented with 0.5 g/100 g clofibrate in the diet for the last week. The *upper panel* in (*B*) is the result of Western blot analysis of CYP4A protein and the *lower panel* is the result of image analysis in which the value of the LF group was taken as 1. Values are means ± SD, $n = 8$. Two way ANOVA was conducted for LF, LO, HF and HO groups. Where there was a significant interaction ($P < 0.05$), the significance of difference was further analyzed by Duncan's multiple range test. Values not sharing a common letter are significantly different ($P < 0.05$). *Significantly different from LF, $P < 0.05$ (Student's *t* test).

ligand-binding domain was fused to a DNA-binding domain of the yeast transcription factor GAL4 and an ALP reporter gene fused to $(UAS)_4$ was established and used for the transactivation experiment. This cell clone expressed the ALP reporter gene in response to WY-14643, a known PPARα ligand, in a dose-dependent manner as shown in **Figure 5***A*.

After cold saponification, the yield of hydrolyzed fresh soybean oil and OFO ranged from 75 to 82%. There was a significant absorption peak at 233 nm for hydrolyzed OFO, but not for fresh soybean oil. The UV absorption at 233 nm was not changed after saponification (hydrolyzed fresh oil, 370 absorbance/g; hydrolyzed OFO, 4785 absorbance/g compared with data in Table 1).

Both hydrolyzed OFO and fresh soybean oil activated the expression of the reporter gene in a concentration-dependent manner (Fig. 5*B*). The activities of the ALP reporter gene activated by 50 and 100 μmol/L hydrolyzed fresh soybean oil were significantly greater than the vehicle control ($P < 0.05$). Similarly, the activities of the ALP reporter gene activated by 10, 50 and 100 μmol/L hydrolyzed OFO were significantly greater than the vehicle control ($P < 0.05$). On the other hand, hydrolyzed OFO displayed a significantly higher potency for transactivation than hydrolyzed fresh soybean oil at concentrations of 50 and 100 μmol/L ($P < 0.005$). A cytotoxic effect was observed at a concentration ≥150 μmol/L.

The hydrolyzed OFO and fresh soybean oil samples were separated by a reverse phase TLC. In addition to spots corresponding to major constituent fatty acids (oleic acid, linoleic acid and linolenic acid) of the hydrolyzed fresh soybean oil, several spots relatively near the solvent front were observed with the hydrolyzed OFO sample. In this reverse-phase TLC system in which a relatively polar developing solvent mixture was used, spots moving faster than common fatty acids should be more polar compounds. This was verified by an equivalent mobilization of two hydroxy fatty acids (recinoleic acid and 13-HODE)(data not shown).

The transactivation of the expression of the reporter gene by hydrolyzed fresh soybean oil should be from its major

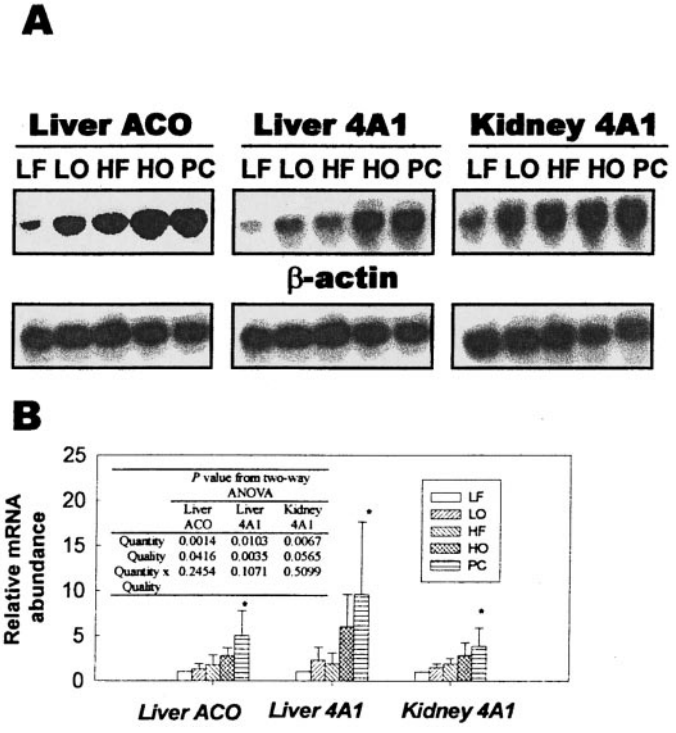

FIGURE 4 Northern blot analysis (*A*) for mRNA of liver ACO, CYP4A1 and kidney CYP4A1 of rats fed diets containing 5 g/100 g (LF and LO) or 20 g/100 g (HF and HO) fresh soybean oil (LF and HF) or oxidized frying oil (LO and HO) for 6 wk. PC is a group of positive control rats that was fed the LF diet but supplemented with 0.5 g/100 g clofibrate in the diet for the last week. Total RNA (20 μg) isolated from liver or kidney was separated by electrophoresis and transferred to nylon membranes. The blot was probed sequentially with ACO, CYP4A1 (*upper panel*) and β-actin (*lower panel*) cDNA probes, which were prepared as described in Materials and Methods. Signals were quantitated by image analysis (*B*). Each value was normalized by β-actin. The fold induction was then calculated by taking the normalized value of the LF group as 1. Values are means ± SD, $n = 6$. Two-way ANOVA was conducted for LF, LO, HF and HO groups and results are shown in the inset table. *Significantly different from LF, $P < 0.05$ (Student's *t* test).

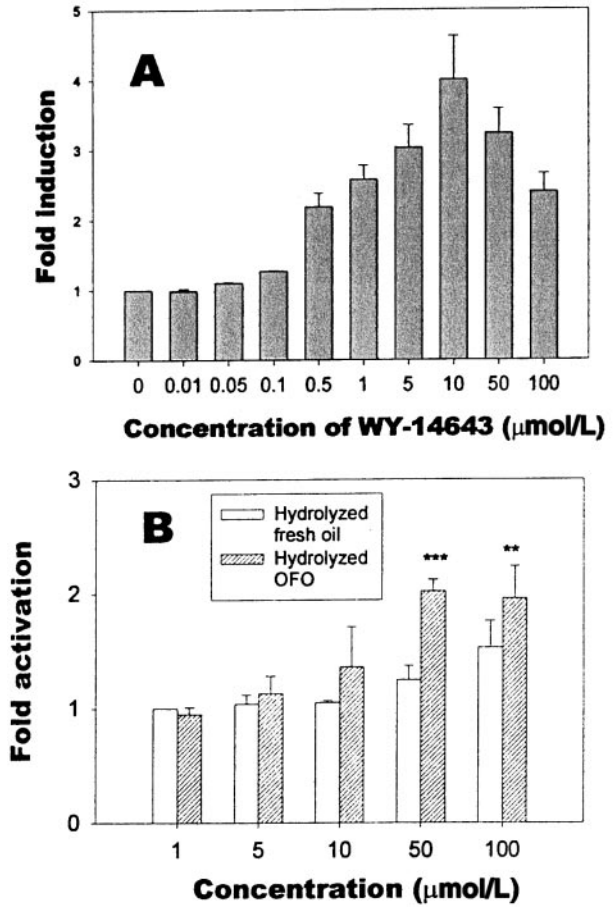

FIGURE 5 Transactivation of PPARα by WY-14643 (*A*) and hydrolyzed fresh soybean oil and OFO (*B*). A clone of CHO K1 cells stably expressing GAL4-PPARα LBD chimeric receptor and $(UAS)_4$-alkaline phosphatase (ALP) reporter were treated with various concentrations of WY-14643 or various concentrations of hydrolyzed fresh soybean oil or OFO. After 48 h, media were analyzed for the ALP activity. The fold activation was calculated by taking the ALP activity of the vehicle (dimethyl sulfoxide or ethanol) treatment as 1. Data are means ± SD, n= 3–9. The activation by 50 and 100 μmol/L hydrolyzed fresh soybean oil, and by 10, 50 and 100 μmol/L hydrolyzed OFO was significant ($P < 0.05$) compared with the vehicle control. The significance of differences between hydrolyzed fresh soybean oil and OFO treatments at the same concentration were analyzed by Student's t test. $^{*}P < 0.05$,$^{**}P < 0.005$,$^{***}P < 0.0005$.

constituent fatty acids, namely, linolenic acid, linoleic acid and oleic acid, which are PPARα ligands. Because the content of linolenic and linoleic acids was significantly reduced in the OFO (Table 1), the higher activating potency of OFO should be from the components produced in the frying process. This result suggested that there were some modified lipid components in the OFO that exhibited a higher potency in activating PPARα than the original fatty acids such as linoleic acid or linolenic acid in fresh soybean oil.

DISCUSSION

The present study demonstrated that dietary OFO significantly increased liver ACO activity and mRNA, CYP4A protein and CYP4A1 mRNA in rats, resembling the effect of feeding a 0.5 g/100 g clofibrate-containing diet for 7 d. The expression of these genes is up-regulated by PPARα. Because PPARα mRNA was not affected by feeding OFO, the increased expression of PPARα downstream genes was not due to a higher PPARα expression. Hence, the up-regulation of ACO and CYP4A1 genes should be the result of the activation of PPARα by its agonist. The result that hydrolyzed OFO activated the expression of the reporter gene to a greater extent than the hydrolyzed fresh oil through the ligand binding domain of rat PPARα in the transactivation assay supports the hypothesis that dietary OFO, through an activation of the PPARα signaling pathway, may up-regulate its target genes.

In addition to the synthetic PP such as fibrates, a wide range of natural compounds, including long-chain unsaturated fatty acids, phytanic acid, pristanic acids, conjugated linoleic acids, oxidized fatty acids such as 8S-HETE and 9- and 13-HODE, have been identified as PPARα ligands (14). The common features of this heterogeneous group of compounds include a relatively hydrophobic nature and a carboxylic acid functional group. The low specificity of the PPARα ligand binding is quite distinct from the other known members of the hormone nuclear receptor family. The previously reported oxidized fatty acids including 8S-HETE, 9- and 13-HODE that activated PPAR were mainly from oxLDL and were thought to be produced endogenously by enzymatic oxidation through the catalysis by, for example, position-specific lipoxygenases. Kamal-Eldin et al. (29) fractionated and analyzed commercial frying oils after saponification and found the major altered fatty acids to be oxidized monomers, which were a complex mixture of monomeric fatty acids with at least one oxygenated function, e.g., epoxy, keto or hydroxy. Data in Figure 5 suggest that nonenzymatically oxidized products in OFO activate PPARα.

The oxidation products in used frying oil, namely, the polar compound fraction, are composed mainly of polymeric, dimeric and oxidized TG monomers (2). The hydrolysis rate of oxidized TG monomers by pancreatic lipase in vitro was high, whereas those of TG dimers and TG polymers were moderate and low, respectively (7). A similar trend was found in a short-term in vivo digestibility study (8) that demonstrated that although the digestibility of the polar fraction was lower than an unchanged TG, there was still a large proportion of the polar fraction digested and absorbed by the body. It is conceivable that after ingestion, some of the esterified oxidized fatty acids in the frying oil were hydrolyzed in the digestive tract and absorbed and transported to the liver where they exerted the PPARα activating effect.

Although present at a low level (0.01 to 0.66 g/100 g of total fatty acids in commercial frying oil), cyclic fatty acid monomers (CFAM) have been considered as the most toxic among compounds generated in deep fat frying because they are readily absorbed by the intestinal mucosa (30). Using purified CFAM prepared from heated linseed oil, Lamboni et al. (31) found increased total lipids, decreased palmitoyltransferase-I, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase activities in liver of rats fed CFAM at 0.15 g/100 g for 10 wk. However, their data also indicated that the lipogenic ability was enhanced in liver by CFAM and led to fatty liver. Subsequently, Martin et al. (32) fed rats TG containing purified CFAM generated from linseed oil and observed a peroxisomal proliferator-like response including hepatomegaly, elevated activities of peroxisomal ACO, microsomal laurate ω- and (ω-1) hydroxylase and carnitine palmitoyl transferase-1, and a coordinated decrease in some lipogenic enzymes. They speculated that the response might be regulated through the activation of PPARα although liver lipids were unaffected. The content of CFAM in the OFO used in this study was not determined and the possibility that CFAM in OFO may be one of the candidates that activate

PPARα cannot be excluded. Characterization of the molecular species in OFO that show high PPARα activating potency merits further study.

Elevated liver ACO activity and CYP 4A protein imply an enhanced fatty acid oxidation in peroxisomes and microsomes of rats fed an oxidized frying oil diet. The significant reduction of liver lipids by feeding oxidized oil was repeatedly observed in our studies (9,10) and those of others (33,34). Although reductions in the lipogenic activity (35) and in dietary lipid absorption (9) have been suggested as causes of the reduced liver lipid content, an increased capacity of peroxisomal β-oxidation (ACO) and microsomal ω-oxidation (CYP4A) of fatty acids could also be contributing factors. Many crucial enzymes involved in fatty acid metabolism in mitochondria, peroxisomes and endoplasmic reticulum are up-regulated by PPARα (11,12). Mice deficient in PPARα develop hepatic steatosis in long-term feeding or after food deprivation (36). Thus, PPARα-dependent increases in fatty acid oxidation appear to be important in efficiently metabolizing free fatty acids entering liver. Compared with mitochondrial β-oxidation, fatty acid oxidation in peroxisomes and in microsomes is generally a minor pathway. However, it becomes increasingly important during periods of increasing influx of fatty acids into liver or during PP treatment. Through the regulation of PPARα, the fatty acid metabolism may be shifted from TG synthesis to catabolism which accounts for the liver lipid-lowering effect of PP. Therefore, increased liver ACO and CYP 4A1 expression in rats fed OFO may contribute to the remarkably lowered liver TG.

Dietary OFO significantly reduced feed efficiency (Table 2). The relative epididymal fat pad weight of the HO group was also significantly reduced (data not shown). Mice deficient in PPARα show a spontaneous, late onset obesity and a substantial increase in adipose tissue after long-term feeding (37), indicating a role of PPARα in the regulation of energy homeostasis. In addition to reducing serum TG, PPARα activators were shown to reduce body weight in db/db mice and obese Zucker rats (38), high fat diet–induced obese C57BL/6 mice and obese Zucker rats (39). More interestingly, abnormal serum glucose and insulin were also improved (38–40) and adipose tissue weight reduced (39). Because the reaction catalyzed by ACO, unlike the mitochondrial acyl CoA dehydrogenase, is not coupled to the generation of ATP, a lowered energy efficiency may result when increased amounts of fatty acids are oxidized through peroxisomal β-oxidation. Moreover, some members of the uncoupling protein family may also be involved (41).

CYP4A catalyzes the ω- or (ω-1) hydroxylation of fatty acids and eicosanoids. We observed significantly increased liver CYP4A protein and CYP4A1 mRNA in rats fed OFO. Although the three isoforms were not specifically detected in the Western blot analysis in this study, it has been reported that CYP4A1, CYP4A2 and CYP4A3 in rat liver were all induced by clofibrate (42). Only CYP4A1 and CYP4A3 were induced in rat kidney because CYP4A2 was constitutively expressed to high levels in this tissue. Using PPARα-activated (fibrate treated wild-type mice) or deficient (PPARα knockout mice) models, Gueraud et al. (43) demonstrated that the metabolism of *trans*-4-hydroxy-2-nonenal (HNE), a degradation product of (n-6) polyunsaturated fatty acid hydroperoxide, is through the ω-hydroxylation of the CYP4A enzymes that are PPARα-dependent. As a result, the highly polar hydroxylated metabolites of HNE could be excreted in urine. The increased hepatic expression of CYP4A1 gene may also play some role in the metabolism of the absorbed oxidized products in OFO.

Although the level of the OFO given in the HO diet (20 g/100 g, equivalent to 40% dietary energy from frying oil) may be too high and nonphysiological, data from rats fed this diet clearly showed the up-regulation of the expression of ACO and CYP 4A1 genes and the altered liver lipid content. In contrast, the response of rats to the LO diet (5 g/100 g OFO, equivalent to 12% dietary energy from OFO), which is more realistic, is especially noteworthy. Rats fed the LO diet consumed 13.5% more food than the LF group, yet gained comparable weight and had a lower feed efficiency. They did not have liver and kidney enlargement as the HO group but had a comparably low level of liver TG and cholesterol. The increases in the liver ACO and CYP 4A1 mRNA were also significant but to a lesser extent than in the HO group. The quality of the OFO sample used in this experiment was in the range of those obtained from street vendors in the Taipei area (44) and was referred as "mildly oxidized oil" in the review article of Kubow (4). It would be of interest to further investigate the effect of frying fat/oil prepared from different types of fat/oil and with different frying times in the deep-frying process. Whether it is possible to develop a frying process that yields some beneficial effect from PPARα activation also merits further investigation.

There are species differences in the PP-induced responses. In humans, peroxisome proliferation and hepatocarcinoma are not observed, whereas the lowering of serum TG and cholesterol lowering by fibrates are present. One explanation is that PPARα is less abundant in human than in rodent liver (45). Furthermore, although the lipid-lowering action of fibrates is PPARα dependent (16), a similar action of fish oil is not regulated solely by PPARα (46). Therefore, the relevance to humans of the observations made in this study awaits further study.

In conclusion, feeding rats a diet containing oxidized frying oil led to dose-dependent increases in hepatic ACO activity and mRNA as well as CYP4A1 protein and mRNA, both PPARα downstream genes. An up-regulation of these genes by the activation of PPARα is supported by a significantly higher activation potency of the hydrolyzed OFO than the hydrolyzed fresh soybean oil in the transactivation assay. The alterations were accompanied by a marked decrease in rat liver TG concentration. These results support the contention that OFO, by activating PPARα, can up-regulate the expression of PPARα downstream genes, which may lead to enhanced hepatic fatty acid oxidation and reduced TG content.

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