The Up-Regulation of Hepatic Acyl-CoA Oxidase and Cytochrome P_{450} 4A1 mRNA Expression by Dietary Oxidized Frying Oil Is Comparable Between Male and Female Rats

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ABSTRACT: We previously demonstrated that oxidized frying oil (OFO) activates peroxisome proliferator-activated receptor α (PPARα) and up-regulates hepatic acyl-CoA oxidase (ACO) and cytochrome P_{450} 4A1 (CYP4A1) genes in male rats. As female rats were shown to be less responsive to some peroxisome proliferators (PP), this study compared the expression of a few PPARα target genes in male and female rats fed diets containing OFO. Male and female rats were fed a diet containing 20 g/100 g OFO (O diet) or fresh soybean oil (F diet) for 6 wk. Both male and female rats fed the O diet showed significantly higher liver weight, hepatic ACO and catalase activities, CYP4A protein, and expression of ACO and CYP4A1 mRNA ($P < 0.05$) compared with their control groups. The mRNA expression of two other PPARα target genes, FA-binding protein and HMG-CoA synthase, were marginally increased by dietary OFO ($P = 0.0669$ and 0.0521, respectively). Female rats fed the O diet had significantly lower CYP4A protein than male rats fed the same diet. The remaining OFO-induced effects were not significantly different between male and female rats fed the O diet. These results indicate that dietary OFO, unlike clofibrate or other PP, had minimal sexual dimorphic effect on the induction of hepatic PPARα target gene expression.

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Peroxisome proliferator-activated receptor α (PPARα), a ligand-dependent transcription factor that belongs to the steroid hormone receptor family, plays a pivotal role in regulating liver lipid homeostasis (1–5). Peroxisome proliferators (PP), including the fibrate class of hypolipidemic drugs, FA, and eicosanoids are known ligands of PPARα (1,6). Upon activation by a ligand, PPAR heterodimerizes with retinoid X receptor and promotes the transcription of its target genes. The target genes of PPARα are mainly a homogeneous group of genes that participate in aspects of lipid catabolism such as FA uptake and binding; FA oxidation in microsomes, mitochondria, and peroxisomes; and lipoprotein assembly and transport. Studies in PPARα gene knockout mice clearly demonstrate that the pleiotropic response and enhanced FA oxidation in the liver of mice treated with PP are mediated by PPARα (7).

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Abbreviations: ACO, acyl-CoA oxidase; CFAM, cyclic FA monomer; CYP4A1, cytochrome P_{450} 4A1; FABP, fatty acid-binding protein; HS, HMG-CoA synthase; NEFA, nonesterified FA; OFO, oxidized frying oil; PFOA, perfluoro-octanoic acid; PL, phospholipids; PNS, post-nuclear supernatant; PP, peroxisome proliferators; PPARα, peroxisome proliferator-activated receptor α; RT, reverse transcription; TC, total cholesterol; TG, triacylglycerol.

A number of studies showed that there were sex-related differences in the response level to the treatment of some PP. When rodents were treated, females showed lower induction of hepatic peroxisome proliferation (8), peroxisomal β-oxidation (9,10), cytochrome P_{450} 4A (CYP4A) activity (11), protein and mRNA (11,12), acyl-CoA oxidase (ACO) activity (12), and protein levels of several PPARα target genes (8) compared with males. The difference has been attributed to the inhibition effect of estradiol and the enhancing effect of testosterone (9–11,13).

Recently, we reported that dietary oxidized frying oil (OFO) up-regulated the expression of hepatic PPARα target genes including ACO and CYP4A1 in male rats (14). In addition, we demonstrated that hydrolyzed OFO displayed a higher potency for PPARα transactivation than hydrolyzed fresh soybean oil. The results indicated that dietary OFO contained PPARα activators that exhibited a higher activating potency than the original FA such as linoleic acid or linolenic acid in fresh soybean oil.

To examine whether there is a gender difference in the response of PPARα target genes to dietary OFO, male and female rats were fed diets containing 20 g/100 g OFO or fresh soybean oil for 6 wk, and the expression of PPARα target genes including ACO and CYP4A1 in liver, was detected in this study.

MATERIALS AND METHODS

Animals and diets. Male and female weanling Sprague-Dawley rats weighing 60–80 g were purchased from the laboratory animal center of the National Science Council (Taipei, Taiwan). Based on a 2×2 factorial design, two groups of male and two groups of female rats were respectively fed the two test diets (F or O diet). The O diet contained 20 g/100 g OFO, and the F diet (the control) contained a similar amount of fresh soybean oil. All 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 and free access to food and tap water. Body weight and food intake were recorded weekly. Animal care and handling conformed to accepted guidelines (15).

The OFO was prepared by frying wheat dough sheets in soybean oil (President, Tainan, Taiwan) at 205 ± 5°C for 24 h, as described previously (14). The composition of the test diets is shown in Table 1; the ratios of casein, vitamin, and mineral mixtures to energy were comparable to the AIN-76 diet.

Biochemical analyses. After 6 wk of feeding, rats were killed by carbon dioxide asphyxiation after overnight fasting. Blood was collected from the abdominal vena cava with EDTA-containing tubes. Liver and kidney were excised and

weighed, and a small portion of each was immediately frozen in liquid nitrogen and stored at –80°C for the analysis of mRNA expression. A second portion of liver was frozen at –20°C for the analysis of liver lipids. Remaining portions of liver were freshly homogenized for the preparation of post-nuclear supernatant (PNS) and microsome, respectively, as described (14). Plasma samples were obtained by centrifugation of blood and stored at –20°C for the analysis of lipids. For the analysis of liver lipids, frozen liver samples were thawed and extracted by the method of Folch *et al*. (16). Total lipids, triacylglycerol (TG), total cholesterol (TC), nonesterified FA (NEFA), and phospholipids (PL) in plasma and liver lipid extract were measured enzymatically by a commercial kit (Randox Lab, Crumlin, Northland, United Kingdom). The peroxisomal ACO and catalase activities in the PNS of liver were determined by the method of Lazarow (17) and Luck (18), respectively. The CYP4A protein in liver microsomal suspension was detected by Western blot analysis as previously described (14). Briefly, 5 μg liver microsomal protein was subjected to 10% SDS-PAGE, then transferred to a polyvinylidene fluoride-plus transfer membrane (NEN Life Science, Boston, MA). The blot was immunodetected with an enhanced chemiluminescence Western blotting kit (Amersham International, Amersham, United Kingdom) 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.

RNA purification and Northern blot analyses. Total RNA was extracted from the liver and kidney with trizol reagent (Life Technologies, Rockville, MD). Total RNA (20 μg) was separated by electrophoresis in denaturing formaldehyde agarose gel and then transferred to nylon membrane. The blots were prehybridized at 42°C for 3 h in the hybridization buffer containing salmon sperm DNA (20 μg/mL), then hybridized at 42°C for 12–15 h with ^{32}P-labeled cDNA probes of ACO, CYP4A1, FA binding protein (FABP), HMG-CoA synthase (HS), or β-actin sequentially after deprobing previous probe remained on membrane. All the cDNA probes were synthesized by reverse transcription (RT)-PCR to amplify encoding base pairs 74–2059 for ACO (according accession number J02752), 13–2040 for CYP4A1 (M14972), 33–405 for FABP (M35991), 385–1374 for HS (M33648), and 103–642 for β-actin (55574). To correct for possible differences in transfer and loading, β-actin was used as an internal control. After washing at the appropriate stringency, the blots were exposed to X-OMAT AR film (Kodak). Signals were quantified using the microcomputer imaging device image analysis system (Fuji, Tokyo, Japan). PPARα mRNA content was semiquantified by RT-PCR as previously described (14).

Statistical analysis. Data were expressed as mean ± SD. To test the significance of the effects of dietary fat quality (fresh soybean oil vs. OFO), gender, and their interaction, data of the four groups were analyzed by two-way ANOVA. When a significant interaction ($P < 0.05$) existed between fat quality and gender, the significance of differences among the four groups was further analyzed statistically by one-way ANOVA and Duncan's multiple range test. Data were transformed to log values for the statistical analysis 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 $P < 0.05$.

TABLE 1
Composition of Test Diets Used in the Feeding Experiment[a,b]

	F diet	O diet
	(g/kg diet)	
Casein	235	235
Cornstarch	448	448
Fresh soybean oil	200	—
Oxidized frying oil[c]	—	200
Cellulose	59	59
Mineral mixture	41	41
Vitamin mixture	12	12
DL-Methionine	3	3
Choline	2	2

[a]Two test diets containing 20% fresh soybean oil (F diet) or oxidized frying oil (O diet) were given to male and female rats.
[b]Sources of ingredients: casein, AIN-76 mineral mixture, and AIN-76 vitamin mixture ICN (Aurora, OH); 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).
[c]Prepared by frying sheets of dough in soybean oil at 205 ± 5°C for 24 h.

RESULTS

Effect of OFO on growth and tissue weight of female vs. male rats. As shown in Table 2, there were significant effects of diet fat quality, gender, and the interactions of these two factors on body weight gain and feed efficiency. Both male and female rats fed the O diet showed significantly lower body weight gain and feed efficiency than rats fed the F diet ($P < 0.05$), but the differences between the O and F groups were to a greater extent in male rats than in females. Diet fat quality ($P = 0.0001$) and gender ($P = 0.0001$) significantly affected food intake without a significant interaction. Relative kidney weight was also significantly affected by the interaction between gender and fat quality ($P < 0.01$). Male rats fed the O diet showed significantly higher relative kidney weight than those fed the F diet. In contrast, there was no difference in the relative kidney weight between the F and O diet group in female rats. Both male and female rats fed the O diet showed significantly higher relative liver weight ($P = 0.0001$) than those fed the F diet, and there was neither a gender effect nor interaction ($P > 0.05$). The extent of liver enlargement induced by dietary OFO observed in female rats was comparable to males (1.68-fold in both male and female rats).

Effect of OFO on liver and plasma lipids of female vs. male rats. Not only gender and fat quality but also the interaction of these two factors had significant effects on liver total lipid, TG, and TC ($P = 0.0001$, Table 3). Rats fed the O diet showed significantly lower liver lipids except for PL than those fed the F diet. Female rats fed the F diet had significantly lower liver total lipid, TG, and TC than male rats fed the same diet. In contrast, liver total lipid, TG, and TC of female rats fed the O diet were comparable to those of male rats fed the O diet. This indi-

TABLE 2
Body Weight Gain, Food Intake, Feed Efficiency, and Relative Liver and Kidney Weight of Male and Female Rats Fed Diets Containing 20 g/100 g Fresh Soybean Oil (F diet) or Oxidized Frying Oil (O diet) for 6 wk[a]

	Body weight gain	Food intake	Feed efficiency	Relative liver weight	Relative kidney weight
	(g/d)		(g gain/g feed)	(g/100 g body)	
Male					
F diet	8.1 ± 0.9[a]	16.1 ± 1.3	0.51 ± 0.03[a]	3.5 ± 0.2	0.73 ± 0.05[b]
O diet	4.8 ± 0.6[b]	11.2 ± 1.1	0.42 ± 0.04[b]	5.9 ± 0.4	0.91 ± 0.08[a]
Female					
F diet	4.8 ± 0.8[b]	13.1 ± 1.5	0.36 ± 0.03[c]	3.4 ± 0.3	0.87 ± 0.13[a]
O diet	3.1 ± 0.4[c]	9.3 ± 1.3	0.33 ± 0.02[d]	5.7 ± 0.5	0.88 ± 0.06[a]
			P-value		
Gender	0.0001	0.0001	0.0001	0.3162	0.0613
Fat quality	0.0001	0.0001	0.0001	0.0001	0.0032
Gender × quality	0.0020	0.2587	0.0377	0.6089	0.0066

[a]Values are means ± SD, $n = 8$. *P* values for gender, fat quality, and their interaction were analyzed by two-way ANOVA. When there was a significant interaction between gender and fat quality, the significance of differences among four groups was analyzed by Duncan's multiple range test. Values not sharing a superscript letter within a column are significantly different, $P < 0.05$.

cated that the lowering effect of dietary OFO on liver lipids was greater in male than in female rats.

Rats fed the O diet had significantly lower plasma total lipid than those fed the F diet ($P < 0.05$, Table 3). The difference in plasma total lipid between rats fed O diet and F diet was similar in male and female rats. The plasma NEFA concentration was affected by gender and not by dietary fat quality. Female rats showed significantly higher plasma NEFA than male rats ($P < 0.005$). Neither gender nor fat quality significantly affected plasma TG, TC, and PL ($P > 0.05$).

Effect of OFO on enzyme activities and protein content of female vs. male rats. In Figure 1 are shown the specific activities of ACO and catalase in the liver of four groups of tested rats. These two enzymes were markers of peroxisomal β-oxidation and peroxisome proliferation, respectively. Feeding OFO resulted in significant ($P = 0.0001$) increases of ACO and catalase activities in both male and female rats. There were neither significant gender effects nor interactions with fat quality on ACO and catalase activities. In female rats, the specific activities of ACO and catalase induced by dietary OFO were about 5.5-fold and 3.7-fold higher, respectively, which were almost equivalent to the responses observed in males (6.0-fold and 2.3-fold induction, respectively).

Western blot analysis demonstrated that the protein content of CYP4A in liver microsomes was significantly increased by the feeding of O diet (Fig. 2A). Results of two-way ANOVA showed that gender, fat quality, and the interaction exerted significant effects on the liver CYP4A protein ($P < 0.05$, Fig. 2B). In rats fed the F diet, there was no significant difference between males and females ($P > 0.05$). In contrast, male rats fed the O diet had a significantly higher CYP4A protein than female rats fed the same diet ($P < 0.05$). The induction of CYP 4A by feeding the O diet was greater in male rats (75-fold) than in females (24-fold).

Effect of OFO on mRNA content of female vs. male rats. In Figure 3 is shown the result of Northern blot analysis of the mRNA content of PPARα target genes including ACO, CYP4A1, FABP, and HS in the liver of four groups rats. Both male and female rats fed the O diet had significantly higher mRNA of ACO ($P < 0.05$), CYP4A1 ($P < 0.005$), FABP ($P =$

TABLE 3
Plasma and Liver Lipids of Male and Female Rats Fed Diets Containing 20 g/100 g Fresh Soybean Oil (F diet) or Oxidized Frying Oil (O diet) for 6 wk[a]

	Plasma lipids					Liver lipids				
	Total lipid	TG	TC	PL	NEFA	Total lipid	TG	TC	PL	NEFA
	(g/L)		(mmol/L)			(mg/g)		(μmol/g)		
Male										
F diet	4.8 ± 2.0	1.1 ± 0.4	2.8 ± 0.8	2.0 ± 0.4	0.6 ± 0.1	75 ± 9[a]	98 ± 14[a]	12.4 ± 2.1[a]	16 ± 2	25 ± 10
O diet	3.3 ± 0.9	0.8 ± 0.3	2.4 ± 0.5	2.1 ± 0.3	0.6 ± 0.1	25 ± 3[c]	28 ± 6[c]	5.6 ± 0.4[c]	16 ± 2	10 ± 4
Female										
F diet	4.4 ± 2.0	1.1 ± 0.4	2.3 ± 0.7	2.0 ± 0.5	0.8 ± 0.2	40 ± 16[b]	54 ± 19[b]	7.3 ± 1.2[b]	15 ± 2	16 ± 7
O diet	3.1 ± 0.7	1.0 ± 0.2	2.1 ± 0.4	1.8 ± 0.2	0.7 ± 0.2	24 ± 2[c]	31 ± 7[c]	5.7 ± 2[c]	18 ± 3	10 ± 3
					P-value					
Gender	0.5499	0.5486	0.0813	0.4105	0.0011	0.0001	0.0001	0.0001	0.2327	0.0349
Fat quality	0.0191	0.1352	0.2535	0.8995	0.5162	0.0001	0.0001	0.0001	0.0403	0.0001
Gender × quality	0.9013	0.4542	0.6319	0.3189	0.3141	0.0001	0.0001	0.0001	0.2042	0.0524

[a]Values are means ± SD, $n = 8$. *P*-values for gender, fat quality, and their interaction were analyzed by two-way ANOVA. When there was a significant interaction between gender and fat quality, the significance of differences among four groups was analyzed by Duncan's multiple-range test. Values not sharing a superscript letter within a column are significantly different, $P < 0.05$. TG, triacylglycerol; TC, total cholesterol; PL, phospholipids; NEFA, nonesterified FA.

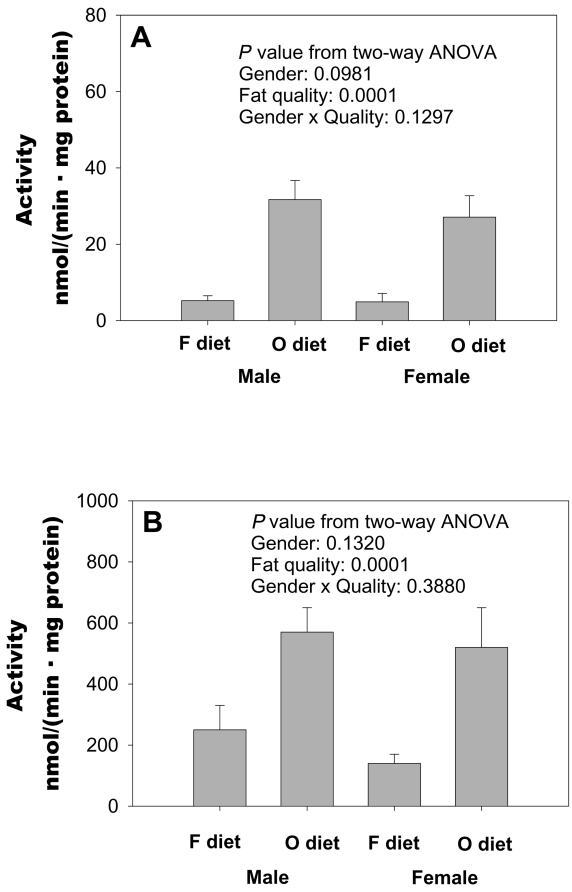

FIG. 1. The specific activities of acyl-CoA oxidase (ACO) (A) and catalase (B) in liver postnuclear supernatant (PNS) of male and female rats fed diets containing 20 g/100 g fresh soybean oil (F diet) or oxidized frying oil (O diet) for 6 wk. Values are means ± SD, $n = 8$. Two-way ANOVA was conducted for the four groups.

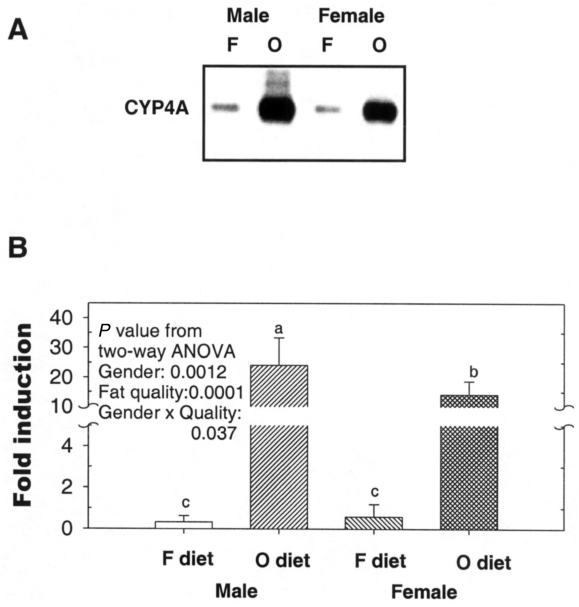

FIG. 2. Western blot analysis (A) for cytochrome P_{450} 4A (CYP4A) protein content in liver microsomes of male and female rats fed diets containing 20 g/100 g fresh soybean oil (F diet; F) or oxidized frying oil (O diet; O) for 6 wk. Signals were quantified by image analysis (B). Values are means ± SD, $n = 8$. Two-way ANOVA was conducted for the four groups. Where there was a significant interaction ($P < 0.05$), the significance of the difference was further analyzed by Duncan's multiple-range test. Values not sharing a common letter are significantly different ($P < 0.05$).

0.067), and HS ($P = 0.052$) than those fed the F diet. There were neither significant gender effects nor interactions of the two factors. In other words, a comparable induction by OFO feeding between male and female rats was observed. The liver ACO and CYP4A1 mRNA of rats fed the O diet were 1.5- and 3.1-fold those fed the F diet in males, and were 2.6- and 2.2-fold, respectively, in females. There was no significant effect of gender, fat quality, or their interaction on PPARα mRNA expression in liver, analyzed by semi-quantitative RT-PCR (data not shown).

The CYP4A1 mRNA expression in kidney was also measured by Northern blot analysis. The CYP4A1 mRNA in kidney was significantly lower in female than in male rats (gender effect, $P < 0.05$). There was neither a significant effect of fat quality nor an interaction with gender on kidney CYP4A1 mRNA expression (data not shown).

DISCUSSION

A 2 × 2 factorial design was used in the present study to observe the effects of OFO and gender on the expression of some PPARα target genes in rats. A significant interaction between OFO (fat quality) and gender, shown in the results of the two-way ANOVA statistical analysis, indicated that the response to OFO was different between male and female rats, or the gender difference was not similar between rats fed the O diet and the control F diet. Without a significant interaction, a significant gender effect indicated that the gender difference in rats fed the F diet was similar to those in rats fed the O diet.

Data from this study indicated that in female rats, dietary OFO also induced liver enlargement and up-regulated the expression of hepatic PPARα target genes ACO and CYP4A1, as in male rats. There were no significant interactions between gender and fat quality on relative liver weight, enzyme activities of ACO and catalase, and mRNA content of ACO, CYP4A1, FABP, and HS. However, there was a significant interaction ($P < 0.05$) in the hepatic CYP4A protein expression between gender and quality (Fig. 2). Female rats fed the O diet had significantly lower CYP4A protein than male rats fed the same diet.

It has been reported that hepatic responses to the treatment of several PP were less pronounced in female than in male rodents. For example, feeding a 0.02% perfluoro-octanoic acid (PFOA) diet for 1 wk increases peroxisomal β-oxidation and liver weight in male rats, but not in females (9). On intraperitoneal administration of 40 mg/kg clofibrate (ethyl ester) for 3 d, Sundseth and Waxman (12) found a 26-fold vs. 6-fold increase in ACO activity and 39-fold vs. 3-fold increase in CYP4A1 mRNA in male vs. female rats. Sugiyama *et al.* (10) also reported a significantly higher increase in ACO activity in male rats (12-fold) than in females (4-fold) after they had been

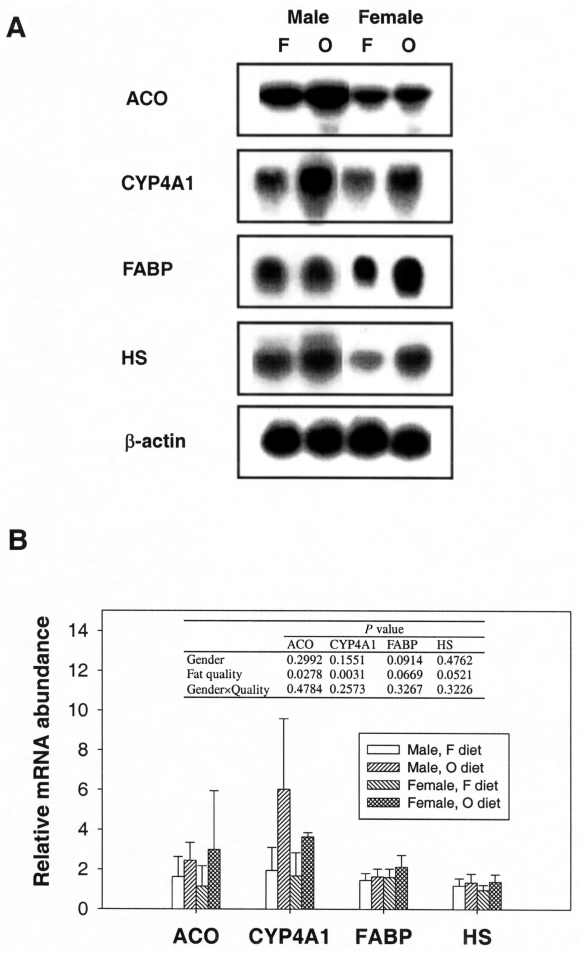

FIG. 3. Northern blot analysis (A) for mRNA of liver ACO, CYP4A1, FA-binding protein (FABP), and HMG-CoA synthase (HS) of male and female rats fed diets containing 20 g/100 g fresh soybean oil (F diet; F) or oxidized frying oil (O diet; O) for 6 wk. Signals were quantified by image analysis (B). Each value was normalized by β-actin. Values are means ± SD, n = 6. Two-way ANOVA was conducted for the four groups and results were shown in the inset table. For other abbreviations see Figures 1 and 2.

orally administered with 300 mg/kg of clofibric acid for 3 d. Hiratsuka *et al*. (11) showed that the increased activity, protein, and mRNA content of lauric acid ω-hydroxylase (CYP4A) in rats intraperitoneally treated with 250 mg/kg clofibrate for 3 d were to a significantly lower extent in female than in male rats.

A few underlying mechanisms for the lower responsiveness to PP induction in female rats have been proposed. In using hormone manipulations *in vivo* (9,10) or *in vitro* (19), it was observed that the PP responses induced by clofibrate or PFOA were stimulated by testosterone and inhibited by estradiol. In addition to a sex hormone, a pituitary-dependent factor such as a growth hormone, which has a characteristic continuous profile in adult female rats, played a suppressive role (10,12,19). An alteration in the pharmacokinetics of PP elimination by the sex hormone was also suggested. Since the serum clofibrate level was found to be lower in castrated rats than intact or testosterone-treated castrated rats (13), the metabolism of clofibrate may be accelerated by androgen deficiency.

In this study, however, we did not observe sex-related differences in mRNA expression of PPARα target genes and ACO activity in response to dietary OFO. It appears unlikely that the longer treatment period (6 wk) in this study than in the above-mentioned studies (a few days or weeks) could account for the difference. It is more likely that, unlike clofibrate, there may not be a sex-related difference in the catabolism or elimination of OFO. The sex-dependent regulation by trichloroethylene through PPARα also has been attributed to a difference in the ability to produce the metabolite trichloroacetic acid, which is the ultimate PPARα activator (8). Similar to our results, no sex differences in the extent of induction by WY-14643, a potent activator of PPARα, were mentioned in the paper of Nakajima *et al*. (8) in which the unpublished data of Aoyama *et al*. were cited.

In this study, the OFO-induced CYP4A protein in liver is significantly lower in female rats than in males. Since the anti-CYP4A antibody we used can detect CYP4A1, -4A2, and -4A3, the CYP4A protein immuno-detected in this study was the sum of three isoforms. It had been reported that CYP4A2 is not expressed in female liver (12,20,21). Hence, the significantly lower liver CYP4A protein levels in OFO-fed female rats could be due to the absence of male-specific CYP4A2 expression and induction. Although the mRNA detected by full-length CYP4A1 cDNA probe in liver tended to be lower in female than male rats, the difference did not reach statistical significance. Because the cDNA of CYP4A2 and -4A3 has 60–65% nucleotide sequence homology to that of CYP4A1, the full-length CYP4A1 cDNA probe we used may have detected the mRNA of CYP4A2 and -4A3 as well.

The ACO, CYP4A1, FABP, and HS mRNA measured in this study were all target genes regulated by PPARα (22–24). We have observed that the mRNA expression of all four of these genes was significantly induced in the liver of male rats fed a diet containing 0.5% clofibrate for 1 wk (data not shown). In contrast, the OFO-induced ACO and CYP4A1 mRNA expressions were significant, but those of FABP and HS were marginal. It seemed that FABP and HS were less responsive to OFO up-regulation than to a typical PP, such as clofibrate.

Hydroxy FA produced presumably by lipoxygenase reaction (such as 9- and 13-hydroxyoctadecadienoate, isolated from oxidized LDL) and CLA have been reported to activate PPAR (25–28). Results of a rat study showed that cyclic FA monomer (CFAM) significantly increased the activities of enzymes that were PPARα target gene products, hence implying the CFAM that may exist in frying oil in minute amounts may also activate PPARα (29). Kamal-Eldin *et al.* (30) fractionated and analyzed commercial frying oils after saponification and found the major altered FA were oxidized monomers, representing complex mixture of monomeric FA with at least one oxygenated function, e.g., epoxy, keto, or hydroxy. It is speculated that these monomeric FA with an oxygenated function may be responsible for PPARα activation.

In conclusion, results of this study showed that PPARα activators in dietary OFO, unlike clofibrate or other PP, induced ACO and CYP4A1 mRNA expression to a similar extent in female and male rats.

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