Major Clofibrate Effects on Liver and Plasma Lipids Are Independent of Changes in Polyunsaturated Fatty Acid Composition Induced by Dietary Fat

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ABSTRACT: The effects of clofibrate on the content and composition of liver and plasma lipids were studied in mice fed for 4 wk on diets enriched in n-6 or n-3 polyunsaturated fatty acids (PUFA) from sunflower oil (SO) or fish oil (FO), respectively; both oils were fed at 9% of the diet (dry weight basis). Only FO was hypolipidemic. Both oil regimes led to slightly increased concentrations of phospholipids (PL) and triacylglycerols (TG) in liver as compared with a standard chow diet containing 2% fat. Clofibrate promoted hypolipidemia only in animals fed SO. Its main effect was to enlarge the liver, such growth increasing the amounts of major glycerophospholipids while depleting the TG. SO and FO consumption changed the proportion of n-6 or n-3 PUFA in liver and plasma lipids in opposite ways. After clofibrate action, the PUFA of liver PL were preserved better than in the absence of oil supplementation. However, most of the drug-induced changes (e.g., increased 18:1n-9 and 20:3n-6, decreased 22:6/20:5 ratios) occurred irrespective of lipids being rich in n-6 or n-3 PUFA. The concentration of sphingomyelin (SM), a minor liver lipid that virtually lacks PUFA, increased with the dietary oils, decreased with clofibrate, and changed its fatty acid composition in both situations. Thus, oil-increased SM had more 22:0 and 24:0 than clofibrate-decreased SM, which was significantly richer in 22:1 and 24:1.

Paper no. L8485 in *Lipids 36*, 121–127 (February 2001).

In rodents, the administration of clofibrate and of a wide range of structurally diverse compounds causes liver peroxisomes, mitochondria, and endoplasmic reticulum to proliferate in hepatic parenchymal cells (1–3). Treatment with these xenobiotics also leads to alteration in the level of expression of many enzymes, including peroxisomal and mitochondrial β-oxidation proteins (4), some enzymes involved in the microsomal synthesis of lipids (5) and fatty acids (6), and hydrolases, such as the carboxylesterases, capable of hydrolyzing lipophilic substances with ester, thioester, and amide groups (7). Most of these changes may in fact play a role in successful detoxification. Peroxisomal proliferators are known to act by binding to a receptor protein, a ligand-activated transcription factor being responsible for intensifying the transcription of specific nuclear genes (8). Sustained treatment with these drugs in high doses promotes liver hypertrophy and hyperplasia and may eventually lead to the development of tumors (9). Our previous work, focusing specifically on the lipids (10), showed that the enlargement induced in mouse liver by clofibrate was accompanied by an increase in the amount, though not in the concentration, of liver glycerophospholipids and a decrease in both the amount and the concentration of triacylglycerols (TG). Although the fatty acid composition of the newly formed phospholipids (PL) was less affected than that of TG, both had reduced percentages of polyunsaturated fatty acids (PUFA), especially those of the n-3 series, with respect to controls. The PUFA of liver TG were apparently consumed in the process of supporting the stimulated PL synthesis, but still were insufficient to provide for the increased energetic and biosynthetic demands imposed by the drug-induced cell growth and proliferation. It was suggested that the chow diet (CD), although adequate for most purposes, in this case may have been short of n-3 PUFA. In the present work we investigate the effects of clofibrate in mice whose liver and plasma lipids were considerably enriched in n-3 or n-6 PUFA.

EXPERIMENTAL PROCEDURES

Male albino mice of the Balb-C strain, weighing 32 ± 2 g, were kept under standard laboratory conditions for 4 wk, and divided into SO (sunflower oil) and FO (fish oil) groups. An additional group of animals receiving a standard rodent chow diet (CD), containing 2% fat, was used for comparison. Each group consisted of five animals, of which four were analyzed. The experimental diets consisted of 23.2% protein, 62.8% carbohydrates, 4.9% vitamin and mineral supplements, and 9.1% of the corresponding oil, on a dry weight basis. From day 15 onward, half the animals from the SO and FO groups continued with their regimes, and the other half were fed the same diet but with the addition of clofibric acid (Sigma Chemical Co., St. Louis, MO), 0.5 g per 100 g of food (10). The protocol for the study conformed to accepted standards of animal care and experimental procedures. The fatty acid

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Abbreviations: CD, chow diet; CE, cholesterol esters; DPG, diphosphatidylglycerol; FO, fish oil; GC, gas chromatography; MFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SM, sphingomyelin; SO, sunflower oil; TG, triacylglycerols; TLC, thin-layer chromatography.

composition of CD, SO, and FO, in that order, was as follows: saturated fatty acids (SFA): 19.3, 14.1, and 25.8%; monounsaturated fatty acids (MFA): 22.4, 22.0, and 29.6%; n-6 PUFA: 57.9, 63.8, and 13.7%; and n-3 PUFA: 0.4, 0.1, and 30.9%, respectively. Taking into account the amount of fat in each diet, and the above fatty acid percentages, the content (w/w) of n-3 PUFA was similar in CD and SO (~0.8%), whereas the content of n-6 PUFA was similar in CD and FO (~1.2%). At day 30 the animals were killed after having been anesthetized with acepromazine and ketamine. Blood samples were obtained by cardiac puncture, collected using heparinized material, and centrifuged to separate plasma from blood cells. The livers were removed, rinsed in saline, weighed, cut into pieces, and homogenized in the solvents used for lipid extraction.

Plasma lipids were determined with commercial kits and the standard techniques used in clinical settings for human plasma. Lipid extracts were prepared from liver and plasma (11), and total lipid phosphorus was determined. Neutral lipids were separated by thin-layer chromatography (TLC) on silica using hexane/ether/acetic acid (80:20:2, by vol). The PL, TG, and cholesterol ester (CE) bands were scraped off after being located with dichlorofluorescein, and eluted (12). Liver PL were resolved into classes by TLC (13) and quantified by phosphorus measurement (14). For sphingomyelin (SM) fatty acid analysis, this lipid was isolated by TLC (15), eluted (12), dried under N_2, treated with 0.5 N NaOH in methanol at 50°C for 10 min, and subjected again to TLC. This ensured that SM was free of any possible contamination with traces of glycerophospholipids.

Fatty acids of all lipids were analyzed by standard gas chromatography (GC) of their fatty acid methyl esters on packed columns (10). Before GC, the methyl esters prepared from lipids were purified by TLC using hexane/ether (95:5, vol/vol) on silica plates that had been pre-washed with methanol/ether (75:25, vol/vol), and were recovered from the support by partition between water/methanol/hexane (1:1:2, by vol). Statistical analysis of the results was performed using the two-tailed Student's *t* test.

RESULTS AND DISCUSSION

Liver and plasma lipid concentrations. Against our expectations, and despite the substantial amount of fat ingested on a long-term basis by our animals fed on both PUFA-rich diets, SO and FO, there was no proportional change in liver lipid content (Table 1). Both dietary oils resulted in unchanged body weight, and the small increase in liver weight was significant only when expressed in terms of the liver/body weight ratio. By contrast, the administration of clofibrate to animals receiving either SO or FO promoted a significant liver enlargement, similar in percentage to that previously reported in animals receiving CD, without affecting body weight (10).

TABLE 1
Liver and Plasma Lipids of Mice Fed Diets with Sunflower Oil (SO) and Fish Oil (FO) and Changes Induced by the Simultaneous Administration of Clofibrate[a]

	Diet/clofibrate (+ or –)				
	CD	SO		FO	
	–	–	+	–	+
Weights (g)					
Body	32.6 ± 1.3	29.9 ± 0.1	29.0 ± 0.9	30.0 ± 1.0	29.0 ± 1.0
Liver	1.3 ± 0.1	1.7 ± 0.1[b]	2.4 ± 0.3[d]	1.6 ± 0.1	2.5 ± 0.2[d]
Liver/body × 100	3.9 ± 0.5	5.7 ± 0.3[b]	8.4 ± 1.1[d]	5.2 ± 0.5	8.3 ± 0.7[d]
Liver lipids					
Phospholipids (mg/liver)	21.8 ± 0.3	28.5 ± 0.6[b]	48.6 ± 2.9[d]	29.8 ± 1.2	48.0 ± 2.1[d]
(mg/g)	16.1 ± 0.7	17.4 ± 0.3[b]	20.1 ± 1.2	19.5 ± 0.7	19.4 ± 0.8
Triacylglycerols (mg/liver)	6.6 ± 1.7	14.8 ± 1.3[b]	4.6 ± 1.1[d]	22.9 ± 2.4[c]	3.2 ± 1.5[d]
(mg/g)	5.1 ± 2.2	8.7 ± 0.9	1.9 ± 0.7[d]	14.3 ± 2.1[c]	1.3 ± 0.4[d]
TG/PL × 100	30.3 ± 4.5	51.9 ± 3.4[b]	9.5 ± 4.5[d]	76.8 ± 5.3[c]	6.7 ± 1.8[d]
Diacylglycerols (mg/g)	0.13 ± 0.02	0.16 ± 0.01	0.14 ± 0.05	0.11 ± 0.01	0.13 ± 0.06
Total cholesterol (mg/g)	1.20 ± 0.2	1.10 ± 0.4	1.00 ± 0.2	1.30 ± 0.1	1.00 ± 0.2
Cholesterol esters (mg/g)	0.10 ± 0.02	0.09 ± 0.04	0.05 ± 0.02	0.09 ± 0.01	0.03 ± 0.01[d]
Esterified/total cholesterol × 100	8.3 ± 2.0	7.8 ± 3.0	5.3 ± 1.5[d]	7.3 ± 0.7	2.7 ± 1.0[d]
Plasma lipids (mg/dL)					
Phospholipids	158 ± 13	154 ± 10	160 ± 10	123 ± 17[c]	117 ± 17
Triacylglycerols	135 ± 18	142 ± 12	101 ± 9[d]	71 ± 12[c]	79 ± 17
Total cholesterol	127 ± 4	107 ± 1[b]	93 ± 5[d]	48 ± 3[c]	41 ± 10
HDL-cholesterol	85 ± 1	77 ± 7	51 ± 3[d]	40 ± 3[c]	25 ± 3[d]
LDL-cholesterol	21 ± 4	15 ± 7	20 ± 5	13 ± 2[c]	16 ± 2
HDL/LDL cholesterol	4.0 ± 1.0	3.3 ± 1.4	2.7 ± 0.5	2.2 ± 0.1[c]	1.6 ± 0.4[d]

[a]Plus and minus signs indicate the presence or absence of clofibrate. Results are presented as mean values ± SD (n = 4 animals per group). CD, chow diet; SO, sunflower oil; FO, fish oil.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs FO; $P < 0.05$).
[d]Significant differences due to clofibrate (SO or FO without, vs. SO or FO with, clofibrate; $P < 0.05$).

The total amount of PL per liver was augmented slightly with the SO and FO diets, but increased considerably upon addition of clofibrate to the diets irrespective of oil type (Table 1). Since liver PL and liver weight increased simultaneously, the resultant PL concentration per gram of tissue was unchanged by the drug. In contrast, the amount and the concentration of TG increased after 1 mon on both PUFA-rich diets. The TG increase was modest, especially considering that irrespective of this enrichment, the TG were dramatically depleted by clofibrate treatment (Table 1). The diacylglycerols did not change in any of the experimental conditions. The CE, unaffected by dietary oils, were also reduced by clofibrate, whereas free cholesterol did not change.

In plasma, the concentrations of PL, TG, and total cholesterol, which were not affected by dietary SO, were decreased by FO supplementation (Table 1). Conversely, clofibrate did not much affect the lipids already reduced by FO, but it decreased plasma TG and cholesterol in animals receiving SO. In both diets clofibrate decreased high density lipoprotein-cholesterol.

The TG depletion induced by clofibrate may be ascribed to drug-induced changes that demand and consume fatty acids, such as peroxisomal and mitochondrial biogenesis to provide for drug substrate oxidation, or microsomal membrane formation to provide for conjugation; in turn, these processes cause hypertrophy. The latter, and the hyperplasia that ensues upon prolonged drug treatment, are processes that require PL and fatty acids. By having their fatty acids assist as substrates for oxidative and synthetic processes, liver TG may support PL formation and liver enlargement. A possible explanation for the failure of our high-fat diets to increase liver TG more than a few mg/g in the presence of unchanged body weight and unchanged or even decreased lipid plasma levels may be that the excess dietary fat may have modified the way the body metabolized fat. Even when tissue PL admit a given amount of n-6 or n-3 exogenous PUFA, the prolonged excess of these fatty acids may have resulted in the stimulated oxidation of the own fat provided. This possibility is supported by the fact that, in rats, the number of peroxisomes and mitochondria and the rates of β-oxidation increase under high-fat diets especially those containing a high n-3/n-6 PUFA ratio (16).

Fatty acids of liver and plasma lipids. The fatty acid profiles of principal lipids from liver and plasma (Table 2) were notably influenced by the type of oil given in the diet and by clofibrate. The effects of the two dietary oils tended to affect the n-6/n-3 PUFA ratios in opposite ways. The most marked qualitative changes were produced in the fatty acids of TG. In liver, SO or FO resulted in increased percentages of n-6 or n-3 PUFA, which partly replaced the major TG component, 18:1n-9. In turn, the most conspicuous outcome of clofibrate administration was that it decreased TG PUFA. Since TG levels were substantially reduced by the drug treatment (Table 1), it caused a greater decrease in the polyunsaturated than in the other acyl groups of TG. This was accompanied by a significant clofibrate-related increase in the percentage of 18:1n-9, to the point where this fatty acid again became the major TG acyl group.

The fatty acid composition of mouse liver and plasma CE (Table 2) and the changes induced by diet and clofibrate on such fatty acids are not extensively known. The content of liver CE was small, and low in PUFA; compared with the other lipids it was modified little in amount or fatty acid composition by the dietary manipulations. On the contrary, the abundant plasma CE changed from an n-6 to an n-3 fatty acid-rich lipid class as a consequence of dietary FO, the major circulating CE fatty acid becoming 20:5n-3 instead of 20:4n-6. Clofibrate tended to affect EC and its fatty acids in the same direction as those of TG.

In FO-consuming mice, while liver clofibrate-wasted TG were selectively depleted of PUFA, the major PUFA profiles of the enlarged PL fraction remained quite similar to those of their controls. This observation contrasts with previous results in which the mass of PL formed after clofibrate action was relatively impoverished in n-3 PUFA (10) and agrees with the interpretation that CD, even when not n-3 PUFA-deficient, was insufficient to provide for the stimulated PL biosynthesis imposed by the proliferator.

The increase in the percentage of 18:1n-9 in TG and in PL may be related to the fact that, like other peroxisomal proliferators, clofibrate induces hepatic stearoyl CoA desaturase (2, 16) among many other enzymes. Recent studies have shown that clofibrate increases, whereas PUFA decrease, the level of activity and the mRNA content of 9-desaturase (17). Another general change induced by clofibrate is a significant increase in the percentage of 20:3n-6 (Table 2), leading to a markedly increased 20:3n-6/20:4n-6 ratio in all lipids analyzed but especially in liver PL. With the present diets, the 22:4n-6/22:5n-6 and the 22:5n-3/22:6n-3 ratios in liver PL are increased by clofibrate just as in previous work (10), suggesting an action of the drug on PUFA desaturases. The administration of clofibrate thus resulted in qualitative changes in specific fatty acids that were independent of the type of PUFA predominating in the diet.

Liver phospholipid classes and their fatty acids. In plasma, dietary FO decreased total PL. Since this reduction mostly affected PC, the proportion of other circulating PL, including SM, increased with no apparent changes in concentration (data not shown). Clofibrate did not affect this situation, the plasma PL composition remaining similar to that in the corresponding SO or FO controls. In liver (Table 3), some small but significant differences in PL proportions were observed that indicated effects of dietary oils on specific lipid classes. Thus, SO and FO ingestion resulted in a different percentage of PC (48.0 ± 1.1 to 43.0 ± 1.7%, respectively) with an unchanged percentage of PE, and hence in slightly different phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratios (1.8 ± 0.1 to 1.5 ± 0.2, respectively). An interesting effect of SO and FO was that both tended to increase liver diphosphatidylglycerol (DPG) and SM relatively more than other PL, the increase in DPG being similar with both oils and that of SM being larger for FO. In the presence of clofibrate

TABLE 2
Major Fatty Acids of Phospholipids, Cholesterol Esters, and Triacylglycerols from Liver and Plasma of Mice Fed Polyunsaturated Oils and Clofibrate[a]

	Diet/clofibrate (+ or –)																			
	Phospholipids										Cholesterol esters									
	Liver					Plasma					Liver					Plasma				
	CD	SO		FO		CD	SO		FO		CD	SO		FO		CD	SO		FO	
	–	–	+	–	+	–	–	+	–	+	–	–	+	–	+	–	–	+	–	+
16:0	23.2	20.3	23.3	28.2[c]	26.6	23.7	25.2	24.7	25.1	26.7	51.2	61.8[b]	55.1[d]	60.5	66.9[d]	3.6	5.2[b]	4.3[d]	6.3[c]	6.4
17:0	0.3	0.4	0.1	0.6	0.2	0.7	0.6	0.7	1.0	0.3	0.8	1.0	0.8	1.1	1.0	0.3	0.3	0.2	0.2	0.3
18:0	15.8	21.9[b]	11.1[d]	18.9	11.4[d]	15.1	16.7	19.3	16.3	15.6	6.1	7.6	5.9[d]	8.2	6.8[d]	1.3	2.7	1.0[d]	1.7	2.2
16:1	1.0	0.7	1.6[d]	1.9[c]	1.8	3.7	3.2	2.8	4.2	5.5	6.4	3.1[b]	5.6[d]	4.8	5.3	1.7	2.0	2.2	4.8[c]	5.0
17:1	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.2	0.4	0.2	0.5	0.4	0.6	0.6	0.6	0.2	0.2	0.2	0.3	0.5
18:1	9.4	6.9[b]	15.8[d]	8.8	16.0[d]	13.7	12.3	15.1	17.6	24.4	18.4	10.0[b]	15.5[d]	14.2[c]	12.6	5.2	7.9[b]	5.9[d]	8.6[c]	12.7[d]
18:2n 6	14.2	18.4[b]	13.8[d]	7.8[c]	6.8	17.6	21.1[b]	19.0	8.2[c]	6.5[d]	8.7	10.6[b]	9.7	3.6[c]	3.0	30.1	31.6	36.1[d]	17.5[c]	18.7
18:3n-6	0.2	0.7[b]	0.2[d]	0.2[c]	0.2	0.4	0.3	0.3	0.3	0.1	0.1	0.4	0.6	—	—	0.5	1.5[b]	1.1	0.4	0.2[d]
20:3n 6	0.5	0.7[b]	5.3[d]	0.4[c]	2.1[d]	0.5	0.6	3.7[d]	0.4	1.9[d]	0.1	0.3	0.6[d]	0.2	0.2	0.3	0.3	2.3[d]	0.2	0.7[d]
20:4n 6	20.1	21.8[b]	24.4[d]	6.7[c]	5.8	12.4	12.3	9.5[d]	4.9[c]	2.3[d]	4.0	3.1	4.2	0.9[c]	0.7	51.7	44.7[b]	43.7	18.6[c]	12.1[d]
22:4n-6	0.2	0.3[b]	0.1[d]	0.3	0.1[d]	0.4	0.4	0.3[d]	0.6	1.1[d]	0.1	0.1	0.2	—	—	—	—	—	—	—
22:5n-6	1.3	3.8[b]	1.1[d]	0.4[c]	0.5	2.5	2.2	1.6[d]	2.0	0.3[d]	0.3	0.4	0.2[d]	0.2[c]	0.2	0.3	0.1	0.1	—	—
18:3n-3	0.2	0.1	0.1	0.3[c]	0.1[d]	0.5	0.2[b]	0.2	0.5[c]	0.4	0.4	—	—	0.3	0.3	—	—	—	0.1	0.2
20:5n 3	0.2	0.2	0.2	3.3[c]	5.1[d]	3.7	2.2[b]	1.6	6.0c	6.8	0.7	0.5	0.6	1.7[c]	0.9[d]	1.2	1.4	1.8	24.9[c]	29.9[d]
22:5n-3	0.3	0.1[b]	0.1	1.0[c]	1.5[d]	0.2	0.1[b]	0.1	1.0[c]	1.0[d]	—	0.1	—	0.1	0.3	—	—	—	0.5	0.3
22:6n 3	12.7	3.6[b]	2.5[d]	21.0[c]	21.8	4.5	2.4[b]	1.2	11.4[c]	6.8[d]	2.5	0.6[b]	0.5	3.5[c]	1.3[d]	3.7	2.4[b]	1.2[d]	16.0[c]	10.9[d]
SFA / MFA	3.7	5.5[b]	2.0[d]	4.3	2.1[d]	2.2	2.7[b]	2.5	1.9[c]	1.4	2.3	5.2[b]	2.9[d]	3.6[c]	4.0	0.7	0.8	0.7	0.6	0.5
n-6/n-3 PUFA	2.7	11.6[b]	16.0[d]	0.6[c]	0.5	3.8	7.5[b]	11.4[d]	0.9[c]	0.8	3.8	12.8[b]	13.7	0.9[c]	1.5[d]	17.1	20.9[b]	28.2[d]	0.9	0.8

	Triacylglycerols									
	Liver					Plasma				
	CD	SO		FO		CD	SO		FO	
	–	–	+	–	+	–	–	+	–	+
16:0	22.3	21.4	20.3	26.8[c]	26.4	15.6	14.6	15.6	15.5	17.2
17:0	0.3	0.2	0.1	0.1	0.4	0.4	0.4	0.4	0.4	0.5
18:0	2.3	1.8	1.2	2.8[c]	2.5	4.1	4.3	3.5	4.7	4.5
16:1	3.6	2.8	6.6	6.7[c]	6.5	1.6	2.4	3.4	2.7	3.1
17:1	0.3	0.1	0.2	0.9[c]	0.5	0.3	0.3	0.3	0.4	0.5
18:1	31.5	22.0[b]	35.5[d]	20.3[c]	35.9[d]	26.5	26.2	36.2[d]	20.9	44.0[d]
18:2n-6	29.7	41.0[b]	27.6[d]	7.4[c]	7.0	37.9	37.8	31.0	9.1[c]	7.0
18:3n-6	1.1	1.8[b]	1.0[d]	0.5[c]	0.7	0.9	1.5	0.9	0.8	0.2
20:3n-6	0.3	1.0[b]	1.5[d]	0.1[c]	0.3	1.0	0.6	1.4[d]	0.2[c]	0.2
20:4n-6	3.7	4.2[b]	3.6[d]	1.0[c]	0.8	6.6	6.8	4.4[d]	1.3[c]	0.7[d]
22:4n-6	0.5	0.9[b]	0.8	1.1[c]	0.6	0.9	0.9	0.9	0.5	1.0
22:5n-6	0.7	1.5[b]	0.3[d]	0.2[c]	0.1	2.3	1.8	0.6[d]	0.5[c]	1.0
18:3n-3	1.0	0.8	0.7	1.5	1.1	0.8	0.4[b]	0.3	1.4[c]	1.9[d]
20:5n-3	0.4	0.2[b]	0.1	7.6[c]	3.1[d]	0.1	0.2	0.2	16.0[c]	4.7[d]
22:5n-3	0.4	0.1[b]	0.1	3.8[c]	2.3[d]	0.1	0.2	0.3	3.1[c]	2.0[d]
22:6n-3	2.2	0.4[b]	0.4	19.3[c]	12.1[d]	0.9	1.5	0.8[d]	22.5[c]	11.6[d]
SFA/MFA	0.7	0.9	0.5	1.1	0.7	0.7	0.7	0.5	0.9	0.5
n-6/n-3 PUFA	9.1	34.0[b]	26.7[d]	0.3[c]	0.5	25.3	21.3	23.3	0.3[c]	0.5

[a]Values are weight percent, as mean values ± SD (n = 4 animals per group). SFA, saturated fatty acids; MFA, monoenoic fatty acids; PUFA, polyunsaturated fatty acids; for other abbreviations and for explanation of +/– signs see Table 1.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs. FO; $P < 0.05$).
[d]Significant differences due to clofibrate (SO or FO without, vs. SO or FO with, clofibrate; $P < 0.05$).

in both diets, the major hepatic glycerophospholipid classes (PC, PE, phosphatidylserine and phosphatidylinositol) increased to a similar extent in response to the growth imposed by the proliferator (Table 3), with respect to their corresponding controls. In contrast, the proportion of liver DPG, and even more markedly that of SM, tended to decrease with clofibrate, showing that the amount of these components increased less than that of other PL, or even decreased. SM was the only liver lipid whose concentration (Table 4) was significantly reduced in liver by clofibrate under both diets.

The increase in liver DPG may be correlated to the proliferation of mitochondria (16) that is induced by excessive

TABLE 3
Liver Phospholipids from Mice Fed Polyunsaturated Oils and Clofibrate[a]

	Diet/clofibrate (+ or –)				
	CD	SO		FO	
Phospholipid	–	–	+	–	+
	mg PL/liver (%)				
CGP	10.1 ± 0.3 (46.5)	13.7 ± 0.3 (48.0)	23.2 ± 0.8[d] (47.7)	12.9 ± 0.5 (43.4)[c]	22.5 ± 1.1[d] (46.8)
EGP	6.2 ± 0.2 (28.4)	7.8 ± 0.4 (27.2)	13.3 ± 0.6[d] (27.4)	8.5 ± 0.4 (28.4)	13.7 ± 0.3[d] (28.6)
LysoPC	0.7 ± 0.2 (3.0)	0.5 ± 0.1 (1.8)	1.1 ± 0.1[d] (2.3)	0.9 ± 0.2 (3.0)	1.7 ± 0.5[d] (3.5)
PS	0.9 ± 0.1 (3.9)	1.1 ± 0.2 (3.8)	2.0 ± 0.2[d] (4.2)	1.2 ± 0.1 (3.9)	1.8 ± 0.2[d] (3.7)
PI	2.2 ± 0.2 (10.1)	2.4 ± 0.2 (8.3)	5.0 ± 0.1[d] (10.2)	2.7 ± 0.2 (9.1)	4.4 ± 0.2[d] (9.1)
DPG	1.1 ± 0.02 (4.9)	2.0 ± 0.2[b] (7.1)	2.9 ± 0.2[d] (5.9)	2.1 ± 0.2[b] (7.2)[b]	2.7 ± 0.5[d] (5.6)
SM	0.7 ± 0.1 (3.2)	1.1 ± 0.1[b] (3.8)	1.1 ± 0.2 (2.3)[d]	1.5 ± 0.1[b,c] (5.0)[c]	1.2 ± 0.2 (2.6)[d]

[a]Phospholipid classes were determined by phosphorus analysis after thin-layer chromatography. Values are means ± SD (n = 4 animals per group). CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; SM, sphingomyelin; for other abbreviations and for explanation of ± see Table 1.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs. FO; $P < 0.05$).
[d]Significant differences due to clofibrate ($P < 0.05$).

TABLE 4
Fatty Acids of Sphingomyelin from Livers of Mice Fed Polyunsaturated Oils and Clofibrate[a]

	Diet/clofibrate (+ or –)				
	CD	SO		FO	
Fatty acid	–	–	+	–	+
16:0	6.3 ± 0.52	6.7 ± 0.86	7.1 ± 1.51	5.7 ± 1.13	7.5 ± 1.44
18:0	4.6 ± 0.52	3.7 ± 0.41	2.9 ± 0.48	2.4 ± 0.16[b]	3.1 ± 0.15[d]
20:0	3.5 ± 0.76	3.3 ± 0.42	2.8 ± 0.24	3.0 ± 0.28	3.4 ± 0.49
22:0	28.4 ± 0.95	38.9 ± 0.44[b]	19.1 ± 1.61[d]	38.0 ± 2.24[b]	24.5 ± 1.61[d]
24:0	16.5 ± 0.57	17.4 ± 2.58	13.9 ± 2.31	17.6 ± 1.85	11.2 ± 2.35[d]
SFA, even chain	59.3 ± 2.47	70.7 ± 1.42[b]	45.6 ± 2.36[d]	66.4 ± 2.89[b]	49.1 ± 3.42[d]
17:0	0.2 ± 0.03	0.2 ± 0.13	0.2 ± 0.05	0.1 ± 0.04[b]	0.1 ± 0.04
19:0	0.03 ± 0.01	0.1 ± 0.05	0.04 ± 0.04	0.07 ± 0.06	0.08 ± 0.01
21:0	0.1 ± 0.04	0.2 ± 0.10	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.03
23:0	6.3 ± 0.72	5.2 ± 0.18	5.0 ± 0.37	5.9 ± 0.23	3.7 ± 0.45[d]
SFA, odd chain	6.7 ± 0.74	5.6 ± 0.09	5.3 ± 0.37	6.2 ± 0.27	4.0 ± 0.35[d]
16:1	0.6 ± 0.20	0.3 ± 0.06[b]	0.3 ± 0.13	0.1 ± 0.11[b]	0.2 ± 0.13
18:1	3.0 ± 0.90	1.0 ± 0.27[b]	1.0 ± 0.48	0.7 ± 0.11[b]	1.0 ± 0.34
20:1	0.4 ± 0.21	0.4 ± 0.02	0.2 ± 0.05[d]	0.4 ± 0.03	0.2 ± 0.03[d]
22:1	2.4 ± 0.27	2.0 ± 0.33	4.8 ± 0.76	2.2 ± 0.16	5.0 ± 0.89[d]
24:1	22.2 ± 0.47	14.5 ± 1.10[b]	37.4 ± 0.94[d]	21.5 ± 2.90[c]	37.9 ± 1.86[d]
MFA, even chain	28.5 ± 0.63	18.3 ± 1.72[b]	43.7 ± 0.94[d]	24.9 ± 3.09[c]	44.1 ± 2.23[d]
17:1	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.08 ± 0.07
19:1	1.0 ± 0.44	0.4 ± 0.21	0.4 ± 0.39	0.2 ± 0.09	0.2 ± 0.13
23:1	1.9 ± 0.19	1.7 ± 0.31	1.1 ± 0.13[d]	1.4 ± 0.06	1.3 ± 0.09
MFA, odd chain	2.9 ± 0.56	2.2 ± 0.47	1.5 ± 0.39[d]	1.6 ± 0.07	1.6 ± 0.22
18:2	1.0 ± 0.21	1.4 ± 0.17	1.0 ± 0.01[d]	0.3 ± 0.01[b,c]	0.4 ± 0.02[d]
24:2	1.6 ± 0.16	1.8 ± 0.15	2.9 ± 0.27[d]	0.6 ± 0.16[b,c]	0.8 ± 0.22
Dienes	2.6 ± 0.20	3.2 ± 0.30[b]	3.9 ± 0.30	0.9 ± 0.20[b,c]	1.2 ± 0.20
Total SFA	67.7 ± 1.6	75.3 ± 1.4[b]	51.0 ± 2.4[d]	72.8 ± 3.1	53.4 ± 2.7[d]
Total MFA	33.3 ± 0.8	20.5 ± 2.2[b]	44.9 ± 1.0[d]	26.5 ± 3.1[b]	45.2 ± 2.2[d]
SFA/MFA	2.0 ± 0.1	3.7 ± 0.4[b]	1.1 ± 0.1[d]	2.8 ± 0.5[b]	1.2 ± 0.1[d]
μmol SM/g	0.64 ± 0.02	0.82 ± 0.01[b]	0.57 ± 0.04[d]	1.22 ± 0.05[b,c]	0.62 ± 0.02[d]

[a]Fatty acid composition is given as weight percent fatty acid, as mean values ± SD (n = 4 animals per group). For abbreviations and explanation of ± see Tables 1 and 2.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs. FO; $P < 0.05$).
[d]Significant effects of clofibrate ($P < 0.05$).

PUFA consumption, since this glycerophospholipid is a well-known component of the inner mitochondrial membrane. Our DPG, which was exceedingly rich in 18:2n-6, admitted even more n-6 and a considerable amount of n-3 PUFA with the intake of SO or FO diets, respectively, while reducing the percentage of its monounsaturated fatty acids, mainly 18:1 and 16:1 (not shown). These diet-induced compositional changes could influence mitochondrial enzyme activity. Clofibrate affected the fatty acid composition of DPG as in other lipids, decreasing the percentage of PUFA and increasing that of 18:1n-9.

The fatty acid profile of liver SM (Table 4) was significantly affected by the consumption of both PUFA-rich diets, which resulted in an increase in SM very long chain SFA such as 22:0 and 24:0. By contrast, in clofibrate-treated mice there was a marked decrease in the percentage (and hence in the concentration) of the major SFA of SM; instead, very long chain MFA such as 22:1 and 24:1 became principal acyl chains.

The selective reduction of SM with clofibrate had also been observed in the absence of oil supplementation (10). More recently, several peroxisomal proliferators including clofibrate have been shown to increase liver PC and PE, while decreasing SM in rat liver (18) in agreement with our results. To our knowledge, the only previous report showing that the intake of PUFA can affect the fatty acids of liver SM is that by Bettger *et al.* (19). The mechanism(s) involved and the possible physiological effects of such diet-induced SM modification are still unknown. The fact that clofibrate affects the ratios between very long chain saturated and monoenoic very long chain fatty acids of SM had also been observed in animals fed the CD (10). Thus, this effect of clofibrate does not depend on the amount or type of fatty acids provided in the diet.

The diet-induced SM increase may arise from stimulated synthesis or inhibited degradation of this phospholipid, and conversely, the clofibrate-induced SM decrease may be the result of inhibited synthesis or activated degradation. The possibility that clofibrate affects enzymes involved in SM degradation is exciting, since ceramide and sphingosine are well-known regulators of protein kinase C and therefore mediators of important cell functions including proliferation, differentiation, and death (20). Clofibrate has been shown to inhibit apoptosis in liver (21). A potential consequence of the clofibrate-induced decrease in SM could be the generation of SM metabolites that could be responsible for the inordinate cell proliferation induced by the drug.

Since SM is a ubiquitous lipid, the cellular and subcellular locations of the contrasting effects of dietary oils and clofibrate need to be established. A possible relationship between SM changes and nuclear PL is worth investigating, since SM is an important lipid component of liver nuclear chromatin (22) and nuclear membrane (23). Neutral sphingomyelinase has also been characterized in liver hepatic chromatin fractions and nuclear membranes, and shown to be stimulated after the typical hepatocyte regeneration/proliferation induced by hepatectomy (24,25), a process that is comparable to the clofibrate-induced liver mass expansion seen in the present work.

ACKNOWLEDGMENTS

We wish to express our appreciation to Dr. Héctor Corsetti, from Raffo Laboratories, S.A., Argentina, who kindly provided the salmon liver oil used in this study. This work was supported by grants from CONICET, SGCyT-UNS, and FONCYT, Argentina. E.N.M. is a research fellow from the Colegio de Veterinarios, Provincia de Buenos Aires, Argentina.

REFERENCES

1. Bremer, J., Osmundsen, H., Christiansen, R., and Borrebaek, B. (1981) Clofibrate, *Methods Enzymol. 72*, 506–519.
2. van den Bosch, H., Schutgens, R.B.H., Wanders, R.J.A., and Tager, J.M. (1992) Biochemistry of Peroxisomes, *Annu. Rev. Biochem. 61*, 157–197.
3. Moody, D.E., Gibson, G.G., Grant, D.F., Magdalou, J., and Rao, M.S. (1992) Peroxisome Proliferators, a Unique Set of Drug-Metabolizing Enzyme Inducers, *Drug Metab. Dispos. 20*, 779–791.
4. Kunau, W.H., Dommes, V., and Schultz, H. (1995) β-Oxidation of Fatty Acids in Mitochondria, Peroxisomes and Bacteria: A Century of Continued Progress, *Prog. Lipid Res. 34*, 267–342.
5. Kawashima, Y., Horii, S., Matsunaga, T., Hirose, A., Adachi, T., and Kozuka, H. (1989) Co-induction by Peroxisome Proliferators of Microsomal 1-Acylglycerophosphocholine Acyltransferase with Peroxisomal β-Oxidation in Rat Liver, *Biochim. Biophys. Acta 1005*, 123–129.
6. Kawashima, Y., Musoh, K., and Kozuka, H. (1990) Peroxisome Proliferators Enhance Linoleic Acid Metabolism in Rat Liver. Increased Biosynthesis of Omega-6 Polyunsaturated Fatty Acids, *J. Biol. Chem. 256*, 9170–9175.
7. Parker, A.G., Pinot, F., Grant, D.F., Spearow, J., and Hammock, B.D. (1996) Regulation of Mouse Liver Microsomal Esterases by Clofibrate and Sexual Hormones, *Biochem. Pharmacol. 51*, 677–685.
8. Wahli, W., Devchand, P.R., Ijpenberg, A., and Desvergne, B. (1999) Fatty Acids, Eicosanoids, and Hypolipidemic Agents Regulate Gene Expression Through Direct Binding to Peroxisome Proliferator Activated Receptor, *Adv. Exp. Med. Biol. 447*, 199–209.
9. Rao, M.S., and Reddy, J.K. (1991) An Overview of Peroxisome Proliferator-Induced Hepatocarcinogenesis, *Environ. Health Perspect. 93*, 205–209.
10. Pennacchiotti, G.L., Rotstein, N.P., and Aveldaño, M.I. (1996) Effects of Clofibrate on Lipids and Fatty Acids of Mouse Liver, *Lipids 31*, 179–185.
11. Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37*, 911–917.
12. Arvidson, G.A.E. (1968) Structural and Metabolic Heterogeneity of Rat Liver Glycerophosphatides, *Eur. J. Biochem. 4*, 478–486.
13. Holub, B.J., and Skeaff, C.M. (1987) Nutritional Regulation of Cellular Phosphatidylinositol, *Method Enzymol. 141*, 234–422.
14. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Two Dimensional Thin Layer Chromatographic Separation of Polar Lipids and Determination of Phospholipids by Phosphorus Analysis of Spots, *Lipids 5*, 494–496.
15. Brown, E., and Subbaiah, P. (1994) Differential Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Human Skin Fibroblasts, *Lipids 29*, 825–829.
16. Vameq, J., Valee, L., de la Porte, P.L., Fontaine, M., de Craemer, D., van den Branden, C., Lafont, H., Grataroli, R., and Nalbone, G. (1993) Effect of Various n-3/n-6 Fatty Acid Ratio Contents of High Fat Diets on Rat Liver and Heart Peroxisomal and

Mitochontrial Beta-Oxidation, *Biochim. Biophys. Acta 1170*, 151–156.
17. Løchsen, T., Ormstad, H., Braud, H., Brodal, B., Christiansen, E.N., and Osmundsen, H. (1999) Effects of Fish Oil and n-3 Fatty Acids on the Regulation of Δ^9-Fatty Acid Desaturase mRNA and -Activity in Rat Liver, *Lipids 34 (Suppl.),* S221–S222.
18. Adinehzadeh, M., and Reo, N.V. (1998) Effects of Peroxisome Proliferators on Rat Liver Phospholipids: Sphingomyelin Degradation May Be Involved in Hepatotoxic Mechanism of Perfluorodecanoic Acid, *Chem. Res. Toxicol. 11*, 428–440.
19. Bettger, W.J., and Blackadar, C.B. (1997) Dietary Very Long Chain Fatty Acids Directly Influence the Ratio of Tetracosenoic (24:1) to Tetracosanoic (24:0) Acids of Sphingomyelin in Rat Liver, *Lipids 32*, 51–55.
20. Alessenko, A.V. (1998) Functions of Sphingosine in Cell Proliferation and Death, *Biokhimiya (Moscow) 62*, 62–68.
21. Diez-Fernández, C., Sanz, N., Alvarez, A.M., Wolf, A., and Cascales, M. (1998) The Effect of Non-Genotoxic Carcinogens Phenobarbital and Clofibrate on the Relationship Between Reactive Oxygen Species, Antioxidant Enzyme Expression and Apoptosis, *Carcinogenesis 19*, 1715–1722.
22. Albi, E., Mersel, M., Leray, C., Tomassoni, M.L., and Viola-Magni, M.P. (1994) Rat Liver Chromatin Phospholipids, *Lipids 29*, 715–719.
23. Tomassoni, M.L., Albi, E., and Viola-Magni, M.P. (1999). Changes of Nuclear Membrane Fluidity During Rat Liver Regeneration, *Biochem. Mol. Biol. Int. 47*, 1049–1059.
24. Alessenko, A., and Chatterjee, S. (1995) Neutral Sphingomyelinase: Localization in Rat Liver Nuclei and Involvement in Regeneration/Proliferation, *Mol. Cell. Biochem. 143*, 169–174.
25. Albi, E., and Viola-Magni, M.P. (1997) Chromatin Neutral Sphingomyelinase and Its Role in Hepatic Regeneration, *Biochem. Biophys. Res. Commun. 236*, 29–33.

[Received March 14, 2000, and in final revised form December 5, 2000; revision accepted January 15, 2001]