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REDUCTION BY CLOFIBRIC ACID OF SERUM ARACHIDONIC ACID IN RATS

EFFECT ON THE ACYL COMPOSITION OF RENAL PHOSPHOLIPIDS

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Abstract—Alterations induced by *p*-chlorophenoxyisobutyric acid (clofibric acid) in the composition of phosphatidylcholine and cholesterol esters in serum and their influence on the composition of phosphatidylcholine in the kidney were studied. Rats of different ages responded differently to the drug in terms of the levels of arachidonic acid (20:4) and linoleic acid (18:2) in the phosphatidylcholine and cholesterol esters in the serum. Administration of clofibric acid to 26-week-old rats for 2 weeks caused a marked decreased in the relative level of 20:4 in phosphatidylcholine and cholesterol esters in serum, whereas similar treatment of 6-week-old rats resulted in a reduction of 18:2 and, to a lesser extent, of 20:4 in serum lipids. The decrease in phosphatidylcholine that contained 20:4 in the serum of old rats was mainly due to a decrease in the concentration of stearoyl-arachidonoyl (18:0-20:4) species. The decrease in cholesterol arachidonate in serum caused by the treatment of old rats with clofibric acid seemed to be due to a reduction in the relative level of serum phosphatidylcholine containing 20:4. The marked reduction in serum lipids that contained 20:4 caused a decrease in the relative level of 20:4 in renal phospholipids, in particular, a decrease in the proportion of palmitoyl-arachidonoyl (16:0-20:4) and 18:0-20:4 phosphatidylcholine.

The pharmacological activity of so-called peroxisome proliferators is considered to be due to their ability to stimulate the proliferation of peroxisomes and to induce peroxisomal β-oxidation of fatty acids [1]. A second characteristic of these compounds is their ability to induce many enzymes that participate in the modification of fatty acids (for example, desaturation and chain elongation) [2–6] and in the biosynthesis of phospholipids (such as glycero-3-phosphate acyltransferase and 1-acyl-glycerophosphocholine (GPC†) acyltransferase) [7–9]. In rats, as a result of the induction of these enzymes, the acyl composition of hepatic lipids changes markedly; treatment of rats with *p*-chlorophenoxyisobutyric acid (clofibric acid), a typical peroxisome proliferator, increases the proportion of oleic acid (18:1‡) and decreases the proportion of linoleic acid in hepatic glycerolipids [10]. The liver actively secretes lipids into the blood as lipoproteins and the acyl moieties of serum lipids are utilized as components of phospholipids in tissues where the levels of activity required for the modification of fatty acids are low. Moreover, clofibric acid lowers the total concentration of lipids in serum. Thus, it appears possible that the changes induced by clofibric acid in hepatic lipids might cause alterations in the acyl composition and/or concentration of lipids in the serum and, subsequently, in other tissues. However, little is known about the effects of clofibric acid on the acyl composition of lipids in tissues other than liver.

In the present study, we examined the effects of clofibric acid on the acyl composition of serum lipids and found that rats of different ages responded differently to this drug with respect to the acyl composition of phosphatidylcholine and cholesterol esters in serum. Clofibric acid reduced markedly the relative levels of phosphatidylcholine and cholesterol esters that contain 20:4 in the serum of old rats, whereas 18:2 and, to a lesser extent, 20:4 were reduced in young rats. Moreover, the reduction in the concentration of lipids that contained 20:4 in the serum led to a decrease in the proportion of phosphatidylcholine that contained 20:4 in the kidney.

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† Abbreviations: clofibric acid, *p*-chlorophenoxyisobutyric acid; GPC, glycerophosphocholine.

‡ The numerical designation of fatty acids indicates their chain lengths and numbers of double bonds: 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, 9,12-octadecadienoic acid (linoleic acid); 20:3, 8,11,14-eicosatrienoic acid; 20:4, 5,8,11,14-eicosatetraenoic acid (arachidonic acid); 22:6, 4,7,10,13,16,19-docosahexaenoic acid. The use of a hyphen between two numerical designations indicates that a particular phosphoglyceride contains both fatty acids.

MATERIALS AND METHODS

Materials. Phospholipase C (from *Bacillus cereus*) was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and dilauroyl-GPC from Avanti Polar Lipid (Pelham, AL, U.S.A.). All other chemicals were of analytical grade.

Animals. Male Wistar rats of 6 weeks of age were supplied *ad lib.* with a control diet or a diet that contained 0.25% (w/w) clofibric acid for 2 or 22 weeks. Male rats of 26 weeks of age were supplied

Table 1. Changes caused by clofibric acid in the acyl composition of serum lipids

Treatment with clofibric acid	16:0	18:0	18:1 (mole %)	18:2	20:4
			Phosphatidylcholine		
Rats aged 6 weeks					
None (4)	28.0 ± 0.7	21.0 ± 0.3	7.8 ± 0.4	27.5 ± 0.6	12.6 ± 0.9
2 weeks (5)	34.0 ± 1.3*	14.0 ± 0.6*	15.3 ± 0.3*	19.6 ± 2.0*	10.1 ± 1.6‡
22 weeks (5)	32.9 ± 0.9*¶	17.4 ± 1.2†¶	12.5 ± 1.1*§	19.6 ± 1.4*	12.2 ± 1.8‖
Rats aged 26 weeks					
None (4)	25.8 ± 4.2	22.0 ± 4.1	7.0 ± 0.8	22.3 ± 3.6	18.1 ± 3.0†
2 weeks (5)	33.5 ± 0.8‖	14.8 ± 1.1‖	13.7 ± 1.5§	20.6 ± 1.7	11.3 ± 0.7‖
			Cholesterol esters		
Rats aged 6 weeks					
None (4)	15.1 ± 1.4	1.8 ± 0.2	16.5 ± 1.4	31.6 ± 1.7	29.2 ± 1.7
2 weeks (5)	16.8 ± 0.9	3.2 ± 0.5*	25.9 ± 2.8*	18.7 ± 0.8*	25.4 ± 4.4
22 weeks (5)	20.6 ± 2.1†§	4.6 ± 1.0*‖	21.3 ± 3.7‡§	19.5 ± 0.8*	26.0 ± 7.1§
Rats aged 26 weeks					
None (5)	12.5 ± 2.0	2.3 ± 0.8	6.7 ± 1.1*	21.4 ± 2.0*	50.6 ± 6.0*
2 weeks (7)	20.7 ± 3.7‖	4.0 ± 0.9‖	18.0 ± 1.8§	20.1 ± 1.1	29.1 ± 6.2§
			Triacyglycerol		
Rats aged 6 weeks					
None (4)	29.6 ± 1.6	4.7 ± 0.9	29.7 ± 1.6	31.7 ± 2.9	ND
2 weeks (4)	30.2 ± 2.4	5.1 ± 0.8	42.4 ± 2.7*	16.4 ± 5.8*	ND
22 weeks (4)	31.8 ± 1.5†‖	3.4 ± 0.7‡	41.6 ± 1.9*§	18.2 ± 2.9*§	ND
Rats aged 26 weeks					
None (4)	35.1 ± 0.6*	4.5 ± 2.0	26.0 ± 2.8‡	27.8 ± 2.2	ND
2 weeks (4)	30.0 ± 1.5§	3.8 ± 0.6	40.4 ± 2.7§	19.5 ± 3.8‖	ND

Values are means ± SD. Rats of 6 weeks of age were supplied with a control diet or a diet that contained 0.25% (w/w) clofibric acid for 2 or 22 weeks. Rats of 26 weeks of age were supplied with a control diet or a diet that contained 0.25% (w/w) clofibric acid for 2 weeks. The numbers in parentheses represent the numbers of animals used.

* Significantly different from control 6-week-old rats at $P < 0.001$.
† Significantly different from control 6-week-old rats at $P < 0.01$.
‡ Significantly different from control 6-week-old rats at $P < 0.05$.
§ Significantly different from control 26 week-old rats at $P < 0.001$.
‖ Significantly different from control 26 week-old rats at $P < 0.01$.
¶ Significantly different from control 26 week-old rats at $P < 0.05$.
ND, not determined.

with a control diet or a diet that contained 0.25% (w/w) clofibric acid for 2 weeks. In other experiments, 27-week-old male rats were supplied with a control diet or a diet that contained 0.5% (w/w) clofibric acid for 1 week. Rats were killed by decapitation. Livers and kidneys were isolated and washed with cold 0.9% NaCl. Livers were perfused with cold 0.9% NaCl to wash out traces of blood. After clotting on ice, blood was centrifuged at 1700 *g* for 15 min for preparation of serum.

Analysis of lipids. Lipids were extracted by the method of Bligh and Dyer [11]. Neutral lipids were separated by TLC on silica gel G plates, which were developed with *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v). Phospholipids were separated by TLC on silica gel H as described by Skipski *et al.* [12]. The regions on each plate that corresponded to specific lipids were scraped off and transferred to tubes. Pentadecanoic acid (15:0) was added to each tube as an internal standard for quantitation. The lipid was extracted from the silica gel twice with 5 mL of chloroform/methanol/0.1 M HCl (4:4:1, v/v). The combined extracts were washed with 3 mL of 0.1 M HCl. An aliquot of each extract was used to prepare methyl esters of fatty acids with BF_3-methanol [13]. Methyl esters were analysed by gas–liquid chromatography as described previously [10]. All sovents used for the extraction of lipids contained 0.005% (w/v) butylated hydroxytoluene.

Analysis of molecular species of diacyl-GPC. For the quantitative analysis of molecular species of diacyl-GPC, dilauroyl-GPC was added to samples as an internal standard before the extraction of lipids. Phosphatidylcholine was converted to diradylglycerol benzoates, which were separated into diacyl, alkylacyl and alkenylacyl subclasses by TLC by the method of Blank *et al.* [14]. Diacylglycerol benzoates recovered from thin-layer plates were dissolved in acetone and separated into individual molecular species by HPLC on a Model LC-6A system (Shimadzu Co., Kyoto, Japan), equipped with a reverse-phase column (4.6 mm × 25 cm; Lichrosorb RP-18; E. Merck, Darmstadt, Germany). The analytical conditions were essentially the same as described by Blank *et al.* [14]. Fractions under peaks corresponding to more than two molecular species were collected and transmethylated with 0.5 M sodium methoxide. The methyl esters formed were analysed by gas–liquid chromatography as described above. All solvents used for the extraction

of lipid contained 0.005% (w/v) butylated hydroxytoluene.

Statistical analysis. The statistical significance of the difference between two means was determined by the Student's *t*-test after an F test. When the Student's *t*-test was not suitable, Welch's test was employed.

RESULTS

Reduction in levels of arachidonic acid in serum

When rats were supplied with a diet that contained clofibric acid, the acyl composition of serum lipids changed considerably (Table 1). The treatment of 6-week-old rats with 0.25% (w/w) clofibric acid for 2 weeks decreased the levels of 18:2 and, to a lesser extent, 20:4 in phosphatidylcholine in serum. No further changes in the acyl composition of phosphatidylcholine were brought about by the prolonged administration of the drug to rats for up to 22 weeks. Upon treatment of 26-week-old rats with clofibric acid for 2 weeks, the level of 20:4 in phosphatidylcholine decreased markedly, whereas no significant changes were observed in the level of 18:2, as compared with age-matched control. The level of 20:4 in cholesterol esters in serum decreased markedly after the treatment of 26-week-old rats with clofibric acid, whereas the level of 18:2, rather than that of 20:4, was reduced in 6-week-old rats. In contrast to levels of 20:4 in phosphatidylcholine and cholesterol esters, the level of 20:4 in triacylglycerol in serum was very low (about 2%) and a considerable decrease in the level of 18:2 was observed in both young and old rats subsequent to treatment with clofibric acid.

Alterations in the composition of cholesterol esters and diacyl-GPC

Since clofibric acid effectively decreased the relative level of 20:4 in cholesterol esters and phosphatidylcholine in the serum of old rats, the effects of the drug on the composition of cholesterol esters and diacyl-GPC in the serum and liver of old rats were examined.

Although feeding of 27-week-old rats with a diet that contained 0.5% (w/w) clofibric acid for 1 week resulted in a considerable increase in the hepatic content of phosphatidylethanolamine, no significant changes were observed in the hepatic contents of phosphatidylcholine, cholesterol esters and triacylglycerol. By contrast, the concentrations of these three lipids in the serum were reduced markedly (Table 2). In contrast to the high proportions of 18:2 and 20:4 in serum cholesterol esters, the levels of 18:2 and 20:4 in hepatic cholesterol esters of 28-week-old rats were only 4.3% and 3.0%, respectively. Moreover, treatment of 27-week-old rats with clofibric acid for 1 week did not have much effect on either the relative proportions or the contents of the various molecular species of cholesterol esters in the liver (unpublished data). By contrast, treatment of the rats with this drug reduced the level in serum of cholesterol arachidonate from 0.246 to 0.049 μmol/mL. The composition of diacyl-GPC in the serum of control 28-week-old rats was similar to that in the liver, with the exception of higher proportions of 16:0-18:2 and 18:0-18:2 species (Fig. 1A and B). The administration of clofibric acid to 27-week-old rats for 1 week caused a conspicuous increase in the proportion of 16:0-18:1 species and a marked decrease in the proportion of 18:0-20:4 species of diacyl-GPC in both the liver and serum (Fig. 1A and B). The administration of the drug greatly reduced the concentration in the serum of all molecular species of diacyl-GPC and the decrease in the concentration of 18:0-20:4 species was particularly striking (Fig. 1C).

Changes in the composition of diacyl-GPC in the kidney

Although renal contents of phospholipids were not affected by the feeding of 27-week-old rats with a diet that contained 0.5% (w/w) clofibric acid for 1 week, this drug significantly reduced the relative proportions of 20:4 in phospholipids. Among the phospholipids, the greatest changes were associated with phosphatidylcholine (Table 3). In contrast to

Table 2. Effects of clofibric acid on the fatty acid content in various classes of lipid from liver and serum of old rats

Lipids	Liver		Serum	
	Control (Fatty acid, μmol/g liver)	Clofibric acid fed (Fatty acid, μmol/g liver)	Control (Fatty acid, μmol/mL serum)	Clofibric acid fed (Fatty acid, μmol/mL serum)
Phosphatidylcholine	23.57 ± 4.88	30.07 ± 3.31	3.09 ± 0.24	1.01 ± 0.84*
Phosphatidylethanolamine	9.64 ± 2.77	16.42 ± 2.41†	ND	ND
Phosphatidylinositol plus phosphatidylserine	4.95 ± 0.88	6.27 ± 2.41†	ND	ND
Cholesterol esters	2.72 ± 0.77	2.52 ± 0.33	0.47 ± 0.09	0.17 ± 0.05*
Triacyglycerol	14.72 ± 1.23	13.24 ± 4.68	4.68 ± 0.80	1.30 ± 0.31*

Values are means ± SD of the results from four animals. Rats of 27 weeks of age were supplied with a control diet or a diet that contained 0.5% (w/w) clofibric acid for 1 week.

* Significantly different from control at $P < 0.001$.

† Significantly different from control at $P < 0.05$.

ND, not determined.

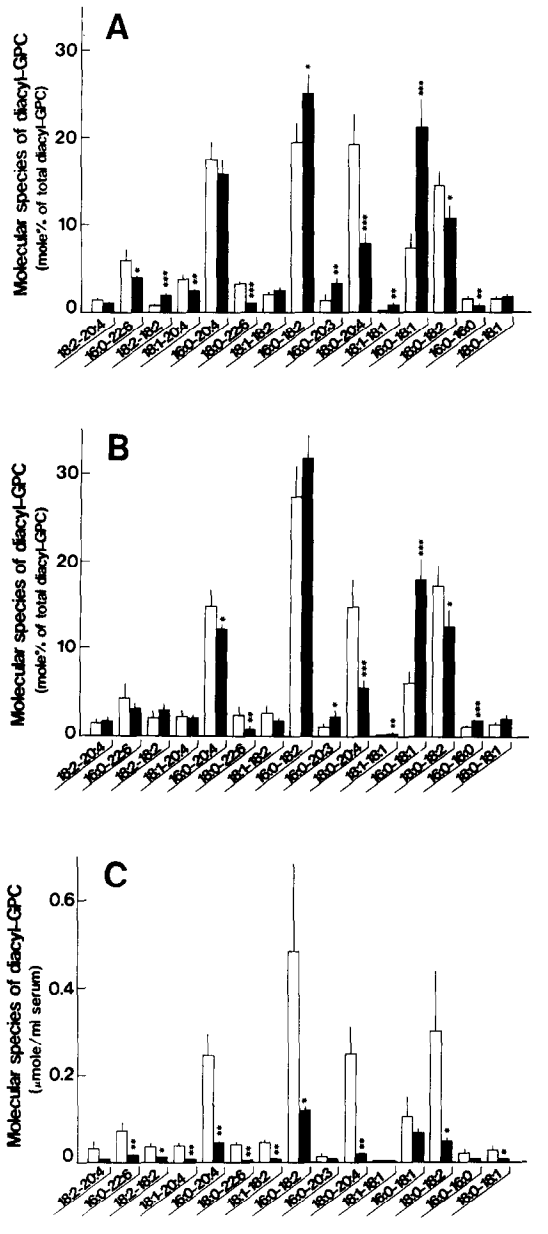

Fig. 1. Effects of clofibric acid on the composition of diacyl-GPC in the liver and serum of old rats. Values are means ± SD of results from four animals. Rats of 27 weeks of age were supplied with a control diet or a diet that contained 0.5% (w/w) clofibric acid for 7 days. (A) Liver, (B and C) serum. (□) Control, (■) rats fed clofibric acid. * Significantly different from control at $P < 0.05$, ** significantly different from control at $P < 0.01$, *** significantly different from control at $P < 0.001$.

the changes observed in the diacyl-GPC of the liver and serum, the administration of clofibric acid decreased markedly the proportions of both 16:0-20:4 and 18:0-20:4 species of diacyl-GPC in the kidney; the proportion of 16:0-18:1 species was unchanged, whereas the proportions of both 16:0–18:2 and 18:0-18:2 species were increased (Fig. 2).

Table 3. Effects of clofibric acid on the acyl composition of renal phospholipids

Lipids	Clofibric acid	(mole %) 16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:6	Total fatty acids (μmol/g kidney)
Phosphatidyl-choline	−	37.9 ± 0.8	1.3 ± 0.4	15.0 ± 0.3	10.9 ± 0.6	12.6 ± 0.5	1.0 ± 0.1	19.0 ± 2.3	1.7 ± 0.2	17.63 ± 1.13
	+	34.6 ± 1.9‡	1.5 ± 0.2	13.8 ± 0.9	14.1 ± 2.3‡	18.9 ± 3.9‡	1.4 ± 0.2‡	12.9 ± 3.9‡	1.6 ± 0.2	18.25 ± 1.31
Phosphatidyl-ethanolamine	−	12.5 ± 0.4	0.6 ± 0.2	25.2 ± 1.0	8.4 ± 0.7	6.1 ± 1.0	0.5 ± 0.1	42.3 ± 1.3	2.3 ± 0.2	14.98 ± 1.23
	+	11.4 ± 0.4†	0.7 ± 0.1	25.8 ± 2.1	12.6 ± 0.4*	9.8 ± 0.9†	0.7 ± 0.1†	35.6 ± 1.8†	1.7 ± 0.3‡	15.54 ± 1.54
Phosphatidyl-serine plus phosphatidyl-inositol	−	8.7 ± 0.3	0.3 ± 0.2	44.0 ± 1.2	5.9 ± 0.4	3.2 ± 0.5	1.6 ± 0.2	33.8 ± 0.1	0.8 ± 0.04	7.20 ± 0.40
	+	9.3 ± 0.8	0.4 ± 0.1	46.8 ± 4.4	7.6 ± 0.8†	4.5 ± 0.5†	2.3 ± 0.4‡	27.1 ± 4.6‡	0.8 ± 0.1	6.48 ± 0.75

Values are means ± SD of results from four animals. Rats of 27 weeks of age were supplied with a control diet or a diet that contained 0.5% (w/w) clofibric acid for 1 week.
* Significantly different from control at $P < 0.001$.
† Significantly different from control at $P < 0.01$.
‡ Significantly different from control at $P < 0.05$.

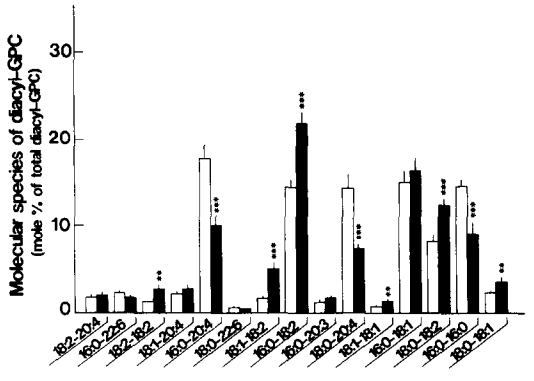

Fig. 2. Effects of clofibric acid on the composition of diacyl-GPC in the kidneys of old rats. Values are means ± SD of results from four animals. Rats of 27 weeks of age were supplied with a control diet or a diet that contained 0.5% (w/w) clofibric acid for 7 days. (□) Control, (■) rats fed clofibric acid. ** Significantly different from control at $P < 0.01$, *** significantly different from control at $P < 0.001$.

DISCUSSION

The present study revealed age-related differences in the effects of clofibric acid on the acyl composition of phosphatidylcholine and cholesterol esters in serum. The administration of this drug to 6-week-old rats reduced the level in the serum of phosphatidylcholine that contained 18:2, whereas the level in the serum of phosphatidylcholine that contained 20:4, especially the 18:0-20:4 species, was reduced by the treatment of 27-week-old rats. Although mammalian liver actively produces phosphatidylcholine and supplies it to the blood as a component of lipoproteins, some selectivity in terms of molecular species of diacyl-GPC has been demonstrated during the process of secretion of lipoproteins from the liver [15, 16]. In fact, levels of 16:0-20:4 and 18:0-20:4 species as compared with those of 16:0-18:2 and 18:0-18:2 species of diacyl-GPC in the serum of old control rats were much lower than in hepatic diacyl-GPC. The combined effects of the lower efficiency of secretion of diacyl-GPC that contains 20:4 and the decrease caused by clofibric acid in the amounts of phosphatidylcholine secreted from the liver seem to be responsible for the reduction in the level in serum of phosphatidylcholine that contains 20:4, and of 18:0-20:4 species in particular. Cholesterol esters in serum are considered to be formed by the action of plasma lecithin:cholesterol acyltransferase from free cholesterol and phosphatidylcholine in the serum, and the acyl composition of cholesterol esters in serum is very different from that of the hepatic esters [17]. The higher proportion of cholesterol arachidonate in the serum of old control rats seems to be due to the higher proportion of 20:4 in the phosphatidylcholine in the serum, as compared with that in the serum of young control rats. Little information is, however, available about the mechanism by which old rats maintain a higher proportion in the serum of phosphatidylcholine that contains 20:4, as compared with young rats. The reduction by clofibric acid in the serum level of phosphatidylcholine that contains 20:4 in old rats may be responsible for the dramatic decrease in the level of cholesterol arachidonate in the serum of old rats.

It is of interest to determine whether a drastic reduction in the concentration in the blood of lipids that contain 20:4 causes a decrease in the proportion of 20:4 in phospholipids in tissues that have only a limited ability to produce 20:4. In the present study, the effects of clofibric acid on renal phospholipids were examined because the activity of 1-acyl-GPC acyltransferase in the kidney is high [18, 19], whereas the ability of this organ to produce 20:4 from 18:2 is absent or extremely limited [20–22]. These findings suggested the possibility that the blood serves to supply the kidney with the 20:4 that is used for the formation of phosphatidylcholine for incorporation into its membranes. As anticipated from the analysis of serum lipids, the treatment of rats with clofibric acid caused a considerable decrease in the proportion of 20:4 in renal phosphatidylcholine. In contrast to the diacyl-GPC in the liver and the serum, in which the proportion of only 18:0-20:4 species was decreased, clofibric acid reduced the proportions of both 16:0-20:4 and 18:0-20:4 species of diacyl-GPC in the kidney. These results suggest that the kidney incorporates 20:4 into diacyl-GPC via reacylation with 20:4 that is generated by deacylation of lipid(s) whose origin is in the blood. Our previous finding related to the induction by clofibric acid of renal 1-acyl-GPC acyltransferase [18, 19] tends to support this hypothesis. Consequently, it is evident from the present study that the reduction caused by clofibric acid in the proportion of diacyl-GPC that contains 20:4 in the kidney is due to the decrease in the concentration in the serum of 20:4 subsequent to the administration of the drug. However, these results are very different from the findings of Lefkowith *et al.* [23] who found that the kidney preserves 20:4 in mice that are deficient in the essential fatty acids despite hepatic depletion of 20:4. Since the kidney actively produces eicosanoids from the 20:4 of phosphaticylcholine, the reduction by clofibric acid in the level of phosphatidylcholine that contains 20:4 may represent a new pharmacological property of this drug which is known, at present, only for its role in stimulating peroxisome proliferation and its hypolipidaemic effects.

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