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EFFECT OF CLOFIBRATE ON THE METABOLISM OF OLEATE IN THE PERFUSED RAT LIVER

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Abstract—The effect of clofibrate on the metabolism of [1-^{14}C]- and [U-^{14}C]oleate was examined in the perfused rat liver. Clofibrate feeding severely reduced hepatic triglyceride secretion and enhanced ketone body production. The increase in the rate of incorporation of labeled tracers into perfusate oxidation products and ketone bodies due to the clofibrate treatment was demonstrated only with [U-^{14}C]oleate. Clofibrate strongly reduced the rate of incorporation of oleate into perfusate triglyceride, whereas that into the phospholipid fraction of the post-perfused liver doubled. In consequence, the sum of the radioactivities in esterified lipids in the perfusate and the post-perfused liver was not altered by clofibrate. A clofibrate-dependent increase in phospholipid synthesis may restrict the amount of exogenous fatty acid which is available for the formation of triglyceride-rich lipoproteins.

We have shown previously that clofibrate, a hypolipidemic agent, increases the production of ketone bodies and conversely decreases the rate of secretion of triglyceride and cholesterol in the perfused rat liver [1]. The decrease in the lipid secretion was found to be due to the alteration of the rate of very low density lipoprotein (VLDL) secretion. The reduction was exaggerated when the oleate-substrate was supplied to the perfusion medium. These results led us to suggest that altered hepatic metabolism of free fatty acids that enter the liver from the circulation is the primary cause for the decrease in VLDL secretion.

In this study, the metabolism of radioactive oleate in isolated perfused livers from rats fed clofibrate was examined. There is the possibility that the metabolic fate of acetyl-CoA, produced via β-oxidation of long-chain fatty acids, differs depending on the difference in its positional origin in the acyl carbon chain. In rats fed clofibrate, this possibility is particularly likely since clofibrate specifically induces the hepatic peroxisomal fatty acid β-oxidation pathway [2–6], and at least a part of the peroxisomal oxidation products is considered to be transferred to mitochondria for further oxidation [7]. In this context, we compared the fate of [U-^{14}C]- as well as [1-^{14}C]-oleate.

MATERIALS AND METHODS

Liver perfusion. Male Wistar rats (Kyudo Co., Kumamoto) weighing 200–210 g were fed powdered commercial chow (Type NMF, Oriental Yeast Co., Tokyo) with or without (control) 0.3% clofibrate (Yamanouchi Pharmaceutical Co., Tokyo) *ad lib.* for 1 week. Recirculating liver perfusion was performed under conditions described elsewhere [1, 8]. At the beginning of recirculation, 5 ml of 20 mM sodium [1-^{14}C]oleate (New England Nuclear Corp., Boston MA; specific activity was adjusted to 2 mCi/mmole) or [U-^{14}C]oleate (New England Nuclear Corp.; specific activity was adjusted to 1 mCi/mmole) was added, and the solution was continuously infused at the rate of 5 ml/hr. At 45-min intervals, approximately 20 ml of the perfusate was removed for analysis of lipids and ketone bodies. Perfusions were continued for 225 min.

Analysis of perfusate ketone bodies and its radioactivities. The liver perfusate was deproteinized with 30% perchloric acid and then neutralized with 3 M K_2CO_3 [9]. Acetoacetate [10] and β-hydroxybutyrate [11] were measured enzymatically in the deproteinized sample. Acid soluble radioactivities were also measured in the deproteinized sample after exhaustive removal with petroleum ether of any remaining lipid radioactivities [12]. Radioactivities in ketone bodies were measured as previously described [13].

Analysis of triglyceride and lipid radioactivities. Lipids in the perfusate and liver were extracted and purified [14], and triglyceride was determined as described previously [8]. Perfusate and liver lipids were fractionated by thin-layer chromatography on silica gel G using a solvent mixture of petroleum ether–diethyl ether–glacial acetic acid (82:18:1, by vol.). The lipid fractions were detected by iodine vapor, scraped into vials, and counted in the toluene based scintillation fluid [15]. Data were analyzed by Student's *t*-test.

RESULTS

Consistent with the results in the previous report [1], the chemical analysis showed that clofibrate clearly increased ketone body production (Fig. 1A) and, conversely, decreased the secretion of triglyceride (Fig. 1B).

Ninety percent or more of both the [1-^{14}C]- and the [U-^{14}C]oleate that were infused was taken up by livers in each experimental period (see Fig. 2 and Table 3). As shown in Fig. 2, clofibrate feeding

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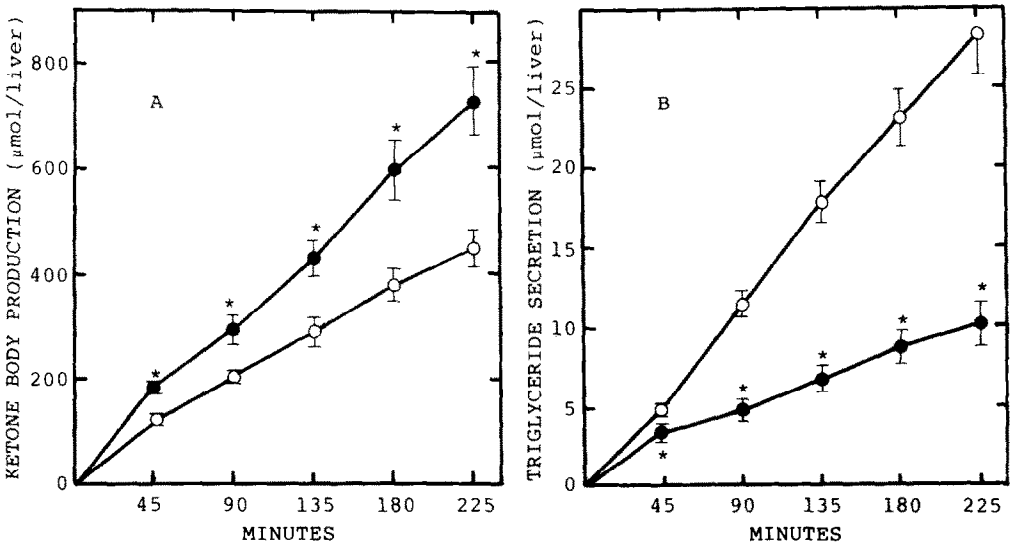

Fig. 1. Effect of clofibrate on the production of ketone bodies (A) and the secretion of triglyceride (B) in the perfused rat liver. Rats weighing 200–210 g were fed powdered commercial chow with or without 0.3% clofibrate for 1 week. Livers were isolated and perfused in the presence of the oleate-substrate. Each point represents the mean ± S.E. of nine livers. Key: (○) control; (●) clofibrate; and (*) significantly different at $P < 0.05$.

slightly but significantly increased oleate uptake in each experimental period except for one occasion (225 min of perfusion with [U-^{14}C]oleate); this clofibrate-dependent increase in the amount of oleate taken up never exceeded 3% of the exogenously provided fatty acid.

Incorporation of radioactive oleate in perfusate oxidation products and ketone bodies is shown in Fig. 3. Radioactivities found in acid soluble products and ketone bodies, especially in clofibrate treated rats, were considerably higher when [U-^{14}C]oleate was used as a tracer compared to [1-^{14}C]oleate. When [1-^{14}C]oleate was infused, no indication of augmented fatty acid oxidation was observed in livers from clofibrate fed rats. On the other hand, livers from clofibrate fed rats significantly increased the rate of incorporation of [U-^{14}C]oleate into acid soluble products and ketone bodies. Approximately 70% of the radioactivities found in acid soluble products was in ketone bodies in each experiment.

Consistent with the chemical data (Fig. 1B), the appearance of both [1-^{14}C]- and [U-^{14}C]oleate in perfusate triglyceride was severely reduced by clofibrate feeding (Fig. 4). The extent of the reduction was appreciably greater than that observed on chemical analysis.

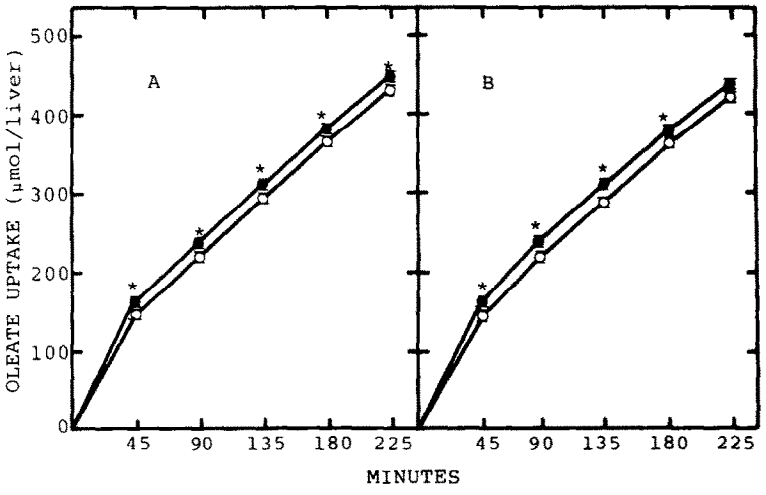

Fig. 2. Effect of clofibrate on the uptake of [1-^{14}C]oleate (A) and [U-^{14}C]oleate (B) by perfused rat liver. Five and four livers from clofibrate fed rats were perfused with [1-^{14}C]- and [U-^{14}C]oleate respectively. Numbers of control livers were four and five respectively. Each point represents the mean ± S.E. Experimental details were the same as those described in the legend to Fig. 1. Key: (○) control; (●) clofibrate; and (*) significantly different at $P < 0.05$.

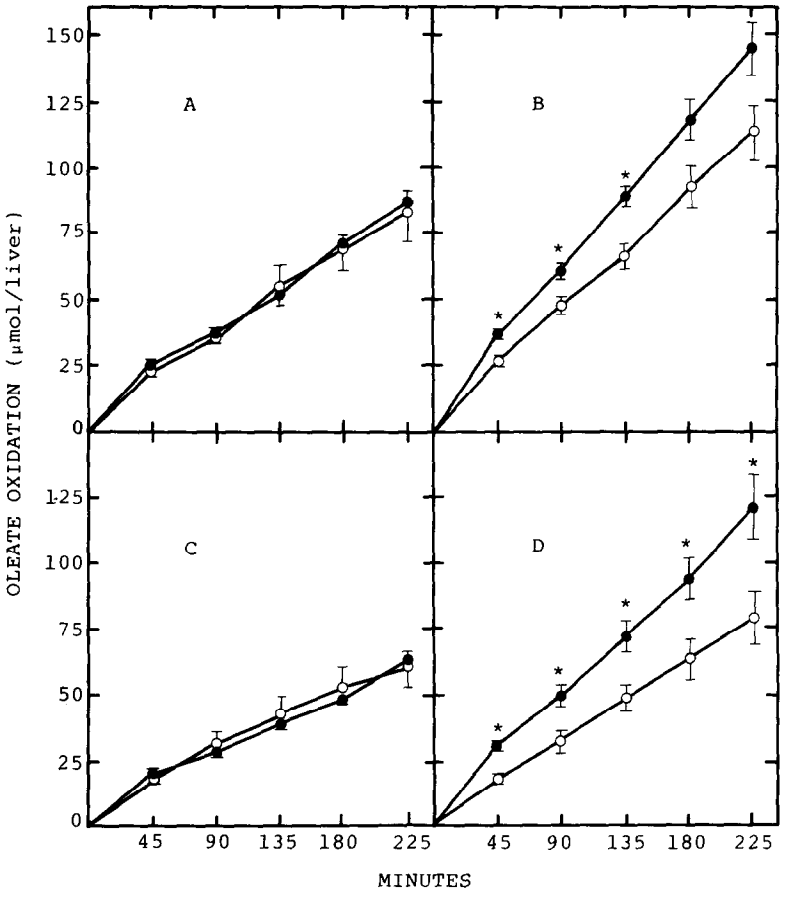

Fig. 3. Effect of clofibrate on the oxidation of oleate in perfused rat liver. Incorporation of [1-^{14}C]oleate (A) and [U-^{14}C]oleate (B) into perfusate acid soluble oxidation products and [1-^{14}C]oleate (C) and [U-^{14}C]oleate (D) into perfusate ketone bodies was determined. Each point represents the mean ± S.E. of four or five livers. Experimental details were the same as those described in the legends to Figs. 1 and 2. Key: (○) control; (●) clofibrate; and (*) significantly different at $P < 0.05$.

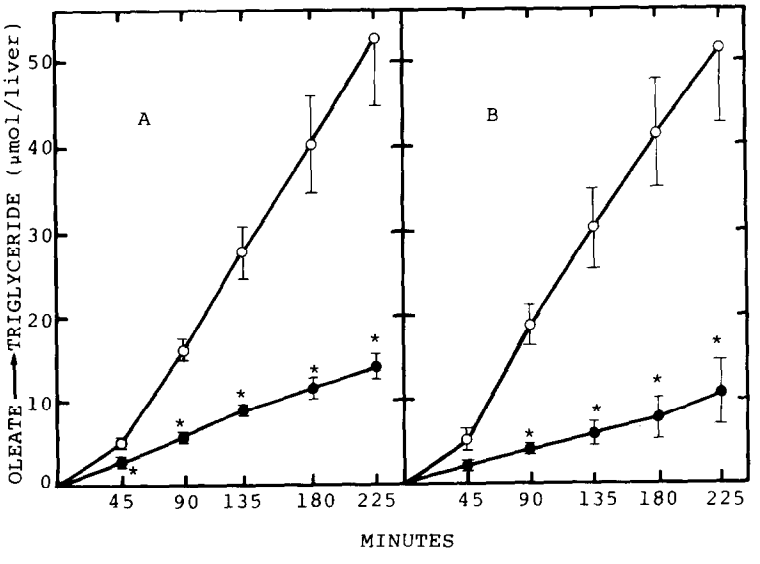

Fig. 4. Effect of clofibrate on the incorporation of [1-^{14}C]oleate (A) and [U-^{14}C]oleate (B) into perfusate triglyceride in perfused rat liver. Each point represents the mean ± S.E. of four or five livers. Experimental details were the same as those described in the legends to Figs. 1 and 2. Key: (○) control; (●) clofibrate; and (*) significantly different at $P < 0.05$.

Table 1. Percentages of perfusate ketone body carbon and triglyceride-fatty acid derived from exogeneous oleate*

	Percentages				
	Minutes				
	45	90	135	180	225
Ketone bodies					
[1-^{14}C]Oleate					
Control	67.4 ± 3.0	69.1 ± 1.6	67.6 ± 2.3	62.8 ± 2.7	66.1 ± 1.7
Clofibrate	53.3 ± 3.1†	50.8 ± 1.3†	47.8 ± 1.8†	42.0 ± 1.0†	46.3 ± 1.5†
[U-^{14}C]Oleate					
Control	74.4 ± 5.5	76.9 ± 6.6	76.0 ± 7.1	71.2 ± 5.8	72.4 ± 7.0
Clofibrate	66.7 ± 3.6	59.1 ± 3.1	58.6 ± 2.2	55.2 ± 0.8	58.7 ± 1.9
Triglyceride					
[1-^{14}C]Oleate					
Control	32.2 ± 1.2	49.0 ± 3.0	52.2 ± 1.7	62.0 ± 5.1	60.5 ± 3.4
Clofibrate	27.2 ± 3.5	42.0 ± 3.9	44.9 ± 4.1	48.4 ± 3.0†	54.9 ± 3.5
[U-^{14}C]Oleate					
Control	40.5 ± 5.1	53.3 ± 3.4	58.8 ± 3.4	60.6 ± 2.6	61.8 ± 2.6
Clofibrate	27.9 ± 0.9	41.4 ± 0.7†	39.1 ± 4.8†	39.0 ± 8.1†	55.5 ± 15.6

* Experimental details were the same as those described in the legends to Figs. 1 and 2. Data represent means ± S.E. of four or five livers.
† Significantly different at $P < 0.05$.

The percentage of perfusate ketone body carbon and triglyceride-fatty acid derived from infused oleate is shown in Table 1. When [U-^{14}C]oleate was infused, the percentage of ketone bodies, but not of triglycerides, was to some extent higher. The percentage of triglyceride was initially lower and gradually increased as the perfusions continued. As with ketone bodies, the values were considerably lower in clofibrate fed rats.

The distribution of radioactivity in the lipid fractions of the liver at the termination of the perfusion is shown in Table 2. The most marked clofibrate-dependent change was observed in the phospholipid fraction; clofibrate doubled the incorporation. Changes, if any, observed in other lipid fractions were relatively moderate.

The metabolic fate of the radioactive oleate that was taken up by the liver at the termination of the perfusion is summarized in Table 3. Though livers from clofibrate fed rats took up slightly more exogenous oleate, it was too small to predict the alterations of oleate metabolism. Approximately 20% and 30% of oleate taken up was catabolized to perfusate oxidation products when [1-^{14}C]- and [U-^{14}C]oleate were used respectively. In spite of the enhancement of ketogenesis by clofibrate, the increase in the percent distribution of radioactivities in ketone bodies and acid soluble products was demonstrated only when [U-^{14}C]oleate was used as a tracer. Approximately 40% of the oleate that was taken up was incorporated into esterified lipids in all trials. Partition of the radioactivities between perfusate and liver, however, was remarkably different in both groups; clofibrate clearly depressed the value in perfusate but increased that in the liver. These changes were apparently relevant to the decreased incorporation of oleate into perfusate triglyceride (Fig. 4) and the increased incorporation of the fatty acid into hepatic phospholipid (Table 2) respectively. Though the recoveries of the radioactivities from both tracers

Table 2. Effect of clofibrate on the incorporation of radioactive oleate into hepatic lipids*

	Incorporation (μmoles/liver)			
	[1-^{14}C]Oleate		[U-^{14}C]Oleate	
	Control	Clofibrate	Control	Clofibrate
Cholesterol ester	2.7 ± 0.4	4.4 ± 0.5†	3.3 ± 0.4	5.4 ± 0.8†
Triglyceride	53.1 ± 4.7	73.8 ± 8.4	62.1 ± 11.8	65.8 ± 9.6
Free fatty acid	32.9 ± 5.0	21.4 ± 1.6	28.6 ± 4.1	21.3 ± 5.3
Diglyceride	15.5 ± 0.6	10.2 ± 0.5†	13.0 ± 1.3	9.9 ± 1.9
Phospholipid	31.6 ± 1.5	71.6 ± 4.5†	36.6 ± 3.1	76.7 ± 7.5†

* Experimental details were the same as those described in the legends to Figs. 1 and 2. Data represent means ± S.E. of four or five livers.
† Significantly different at $P < 0.05$.

Table 3. Fate of radioactive oleate in perfused rat liver*

	Hepatic uptake (μmoles)	Percent distribution						
		Perfusate oxidation products		Esterified lipids			Hepatic FFA†	Recovery
		Total	Ketone bodies	Total	Perfusate	Liver		
[1-^{14}C]Oleate								
Control	431.9 ± 1.6 (90.9 ± 0.3)‡	19.1 ± 2.5	14.3 ± 2.1	36.1 ± 2.8	13.1 ± 1.8	23.0 ± 1.2	7.6 ± 1.2	62.8 ± 2.2
Clofibrate	446.5 ± 2.7§ (94.0 ± 0.6§)	20.2 ± 0.4	14.1 ± 0.7	40.4 ± 1.9	3.9 ± 0.3§	36.8 ± 2.2§	4.9 ± 0.5	65.8 ± 2.4
[U-^{14}C]Oleate								
Control	419.8 ± 6.4 (85.9 ± 0.9)	27.9 ± 2.4	18.9 ± 2.6	40.8 ± 6.4	13.2 ± 2.0	27.2 ± 3.1	6.8 ± 1.0	75.2 ± 2.8
Clofibrate	434.8 ± 7.7 (91.6 ± 1.6)	33.2 ± 2.7	28.1 ± 2.8§	40.3 ± 6.5	3.2 ± 0.9§	37.0 ± 6.1	5.5 ± 1.5	78.8 ± 7.0

* Experimental details were the same as those described in the legends to Figs. 1 and 2. Data represent means ± S.E. of four or five livers.
† FFA, free fatty acid.
‡ Percentages of oleate which was taken up by the liver are in parentheses.
§ Significantly different at $P < 0.05$.

were comparable in the control and clofibrate fed rats, the value with [U-^{14}C]oleate was considerably higher than that with [1-^{14}C]oleate. This was mostly attributable to the increase in the amount of radioactivities in perfusate oxidation products.

DISCUSSION

This study clearly demonstrated impaired export as acyl moieties of perfusate triglycerides of exogenous oleate by the liver from clofibrate fed rats. Clofibrate fed rats, compared to control rats, secreted 18 μmoles less triglyceride at the termination of the perfusions (Fig. 1B) whereas the difference in the amount of exogenous oleate that was found in perfusate triglyceride was approximately 43 μmoles (Fig. 4). Thus, 80% of the difference was attributable to the impairment of the incorporation of exogenous oleate into perfusate triglyceride.

Livers from clofibrate fed rats took up infused oleate slightly more than did those from control rats (Fig. 2). Enhanced fatty acid uptake by clofibrate also has been demonstrated in non-recirculating perfused rat liver [16] as well as in isolated hepatocytes [12, 17]. The liver has a very high capacity for taking up circulating fatty acids and, in a recirculating perfused rat liver, fatty acid uptake is virtually quantitative over a wide range of fatty acid supply [18]. In this study, approximately 90% of the oleate that was supplied to the perfusion medium was taken up by the liver (Fig. 2 and Table 3). The observed differences in oleate uptake between control and clofibrate fed rats (approximately 15 μmoles in 225 min) were relatively small compared to the total amount of oleate taken up. It is thus unlikely that under these experimental conditions the difference in oleate uptake was a predominant factor in modifying the subsequent hepatic metabolism of the fatty acid.

In spite of the clear-cut stimulative effect of clofibrate on hepatic ketogenesis (Fig. 1A), we could demonstrate a moderate enhancement, due to drug treatment, of the incorporation of radioactive fatty acid into perfusate acid soluble oxidation products and ketone bodies only when [U-^{14}C]oleate was used as a tracer. With [1-^{14}C]oleate, clofibrate did not have an effect on these variables (Fig. 3). Though reported data of the effect of clofibrate on hepatic mitochondrial fatty acid oxidation are quite variable [5, 19–21], a consistent observation is that the drug markedly enhances fatty acid oxidation in hepatic peroxisomes [2–6]. The peroxisomal pathway of fatty acid β-oxidation is distinct from that in mitochondria in its substrate specificity and end-products [7]. Enzymes for peroxisomal fatty acid oxidation are rather specific to long-chain fatty acids and virtually inactive toward fatty acids with carbon chain lengths of less than 8. In addition, when palmitoyl-CoA is used as a substrate for peroxisomal oxidation, at the most only 5 cycles of β-oxidation sequence are attained [4]. Accordingly, the end-products of β-oxidation in peroxisomes are acetyl-CoA and medium-chain fatty acyl-CoA. No enzyme system to metabolize these products has, hitherto, been reported in peroxisomes, and it is thus suggested that the acetyl-CoA formed is metabolized anaboli-

cally or catabolically in other cell compartments, while medium-chain fatty acyl-CoA is presumed to be further oxidized in mitochondria. Thus, when [1-^{14}C]oleate is used as a tracer, a part of the radioactive acetyl-CoA formed via β-oxidation presumably may not be handled in mitochondria. Moreover, the specific radioactivity of mitochondrial acetyl-CoA will be diluted by incoming non-radioactive carnitine derivatives of the medium-chain fatty acids generated via peroxisomal β-oxidation of the long-chain fatty acids. In this case, enhanced hepatic ketogenesis from exogenous fatty acids may not necessarily accompany the increase in incorporation of the tracer into ketone bodies as observed in the present study. We predicted that the use of [U-^{14}C]oleate would overcome this dilution phenomenon. As expected, there was a significant enhancement of incorporation of radioactivities from [U-^{14}C]oleate into these products in clofibrate fed rats. Further, this tracer, compared to [1-^{14}C]oleate, increased the apparent percent contribution of exogenous fatty acid to perfusate ketone body carbon, but not to perfusate triglyceride-fatty acid, in both groups of rats. The considerable decrease in percent contribution of exogenous fatty acid to perfusate triglyceride-fatty acid due to the drug feeding was also observed in experiments with both tracers, suggesting that the hepatic endogenous pool of fatty acid, accessible for esterification and oxidation, is enlarged in clofibrate fed rats.

Previously, we have demonstrated that the rate of hepatic fatty acid oxidation is a critical determinant in the regulation of the rate of fatty acid esterification and the subsequent assembly and secretion of triglyceride-rich lipoproteins in the rat liver [8]. We formerly suggested that the clofibrate-dependent decrease in hepatic triglyceride secretion is also mediated by the change in the rate of hepatic fatty acid oxidation [1]. The increase in hepatic fatty acid oxidation due to the drug feeding will, in turn, decrease the amount of fatty acid that is available for esterification to form VLDL-triglyceride and hence for secretion. Indeed, the amount of radioactivity in the perfusate oxidation products in drug-treated animals was increased when perfused with [U-^{14}C]oleate, but no difference was found in the sum of the radioactivities from the perfusate and liver esterified lipids. The drug treatment, however, drastically shifted the partitioning of the radioactivity between the perfusate and the liver. The decrease in the perfusate radioactivity approximately matched the increase in the hepatic radioactivity. The changes in the radioactivities were primarily due to those in triglyceride for the perfusate and in phospholipid for the liver respectively. Thus, the enhanced incorporation of exogeneous oleate into hepatic phospholipid rather than the increase in fatty acid oxidation seems to have been responsible for the impaired hepatic triglyceride secretion in the clofibrate fed rats. The enhancement of radioactive oleate incorporation into phospholipid seems to represent an augmented increase in hepatic phospholipid synthesis in the clofibrate-treated rats. Clofibrate is known to cause hepatomegaly and proliferation of peroxisomes [2–7]. Thus, enhanced phospholipid synthesis should be required to maintain the construction of the organelles. Mannaerts *et al.* [12] observed an increase in the rate of incorporation of oleate into phospholipid in isolated hepatocytes from clofibrate fed rats.

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