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Studies with etofibrate in the rat. Part I: effects on glycerol, free fatty acid and triacylglycerol metabolism

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Etofibrate is the 1,2-ethandiol diester of clofibric acid and nicotinic acid that decreases circulating levels of triacylglycerols and cholesterol. To understand the mechanism by which the drug affects plasma triacylglycerols, normolipemic rats were treated daily with 300 mg of etofibrate/kg body weight or with the medium by a stomach tube. They were decapitated on the 10th day, and showed lower levels of plasma β-hydroxybutyrate, glycerol, free fatty acids (FFA), total triacylglycerols and cholesterol and VLDL triacylglycerols and cholesterol, whereas glucose and RIA-determined insulin levels were unmodified. Epididymal fat pad pieces from etofibrate-treated rats incubated in vitro released more glycerol but the same amount of FFA to the medium, and had greater uptake of [U-^{14}C]glycerol for [^{14}C]acylglycerol formation. In the presence of heparin, they also showed an enhanced release of lipoprotein lipase activity to the medium. The disappearance from plasma of intravenously administered [1-^{14}C]palmitate was faster in the etofibrate-treated rats, and although they showed a decrease in ^{14}C-esterified fatty acids of neutral lipids in both liver and plasma VLDL, there was an increase in liver ^{14}C-labelled water-soluble components. After intravenous [U-^{14}C]glycerol administration, there was a decrease in plasma VLDL [^{14}C]acylglycerol and [^{14}C]glucose and in liver [^{14}C]acylglycerol, but an increase in plasma [^{14}C]lactate. In the liver, etofibrate treatment heightened the cytosolic glycerol-3-phosphate dehydrogenase activity and the total carnitine concentration, whereas it reduced triacylglycerol and cholesterol concentrations. It is proposed that etofibrate enhances the reesterification of fatty acids and glycerol in adipose tissue, which, together with its augmented lipoprotein lipase activity, may facilitate the clearance of circulating triacylglycerols. These effects may act concomitantly with the decreased synthesis of triacylglycerols, secondary to the increased utilization of their precursors, acyl-CoA and glycerol-3-phosphate, in other pathways, causing the reduction of plasma VLDL triacylglycerols produced by etofibrate treatment.

Introduction

The synergistic effects of clofibrate and nicotinic acid in decreasing elevated levels of plasma lipids [1,2] motivated the development of etofibrate (Lipo-Merz, Merz & Co., Frankfurt/Main, F.R.G.), the 1,2-ethandiol diester of clofibric acid

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Abbreviations: FFA, free fatty acids; VLDL, very-low-density lipoproteins; RIA, radioimmunoassay.

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and nicotinic acid. This drug has been shown to be a potent hypolipidemic agent in animals [3,4] and humans [5,6]. It has been proposed that, due to the reduced bioavailability of both unchanged nicotinic acid and clofibric acid in comparison to when equivalent doses of these compounds are given either alone or as a mixture [7], etofibrate has fewer side-effects than its constituent moieties administered as a mixture [8], and has greater inhibitory effects on platelet aggregation [6,9]. Its hypolipidemic effect is not well understood, although its qualitative effects are reported to be like those of clofibrate, with respect to reducing serum cholesterol, triacylglycerol, phospholipid and free fatty acid (FFA) levels and liver cholesterolgenesis in both normal [3] and hyperlipidemic rats [4]. The present study was undertaken to examine the effects of a very high dose of this drug on fatty acids in adipose tissue and liver and on glycerol metabolism in the normal rat. The results obtained indicate that multiple mechanisms are involved in the hypolipidemic action of this drug, one of the most intensive being a reduction in the synthesis of VLDL triacylglycerols from the lipolytic products, FFA and glycerol, secondary to their preferential utilization for other pathways.

Materials and Methods

Animals, drug administration and collection of the samples. Male Sprague-Dawley rats weighing 180–210 g, fed ad libitum purina chow diet (Panlab, Barcelona, Spain) and subjected to a 12 h on-off light cycle were used. From days 1–9 of the experiment, food was removed from the cages at the onset of the light cycle (7.00 h), and 3 h later, either 300 mg etofibrate freshly suspended in 2% Tween-80/kg body weight or the medium (controls) was administered to the rats by stomach tube without anaesthesia. After treatment, rats were again allowed free access to food. On the 10th day, the animals were treated as above, but they were kept fasted up to killing, which was done by decapitation 3 h after drug administration. Blood was collected from the neck into heparinized tubes and liver and epididymal fat pads were immediately dissected. An aliquot of liver was placed in liquid nitrogen and another aliquot of liver and the epididymal fat pads were placed in ice-cold Krebs Ringer bicarbonate buffer (pH 7.4) [10]. When the enzyme activities were to be assayed, liver aliquots were placed in ice-cold 0.9% NaCl and processed as indicated below.

Determinations in blood and liver. Plasma aliquots were kept at $-30\,^{\circ}C$ until processing for the analysis of glucose [11], glycerol [12], β-hydroxybutyrate and acetoacetate [13] in supernatants after protein precipitation [14], and the levels of triacylglycerols [15], cholesterol [16] and FFA [17] were determined, by enzymatic procedures in total plasma and insulin [18], which was determined using the radioimmunoassay (RIA) kit for rat insulin, generously provided by Novo Industri A/S (Copenhagen, Denmark). The livers were collected under liquid N_2 and kept at $-70\,^{\circ}C$ until processing. Aliquots of the frozen liver were digested in 30% KOH and used for glycogen purification [19] and glucose determination [11] after acid hydrolysis. This procedure was previously validated for glycogen determination [20]. Other frozen liver aliquots were used for determining the dry liver weight, protein [21] and DNA [22], and for lipid extraction [23] to determine phospholipid phosphorus [24], triacylglycerols [25] and cholesterol [26]. Another frozen liver aliquot was extracted with methanol and was saponified for the determination of total carnitine [27].

Adipose tissue in vitro. Lipolytic activity in in vitro adipose tissue was determined following the method already described by us [28,29], with a few modifications. After being weighed, fresh epididymal fat pads were cut into small pieces and 30–35 mg were placed in vials containing 1.0 ml of Krebs Ringer bicarbonate buffer (pH 7.4) supplemented with 5 mM glucose and fatty acid-free bovine albumin (20 mg/ml). At zero time, 1.00 ml of Krebs Ringer bicarbonate buffer containing 5 mM glucose and 1 μCi of [U-^{14}C]glycerol (from Amersham International, Amersham, U.K., spec. act. 171 mCi/mmol) with or without 1.00 μg of adrenaline bitartrate was added to each vial. Incubations were carried out for 0, 90 or 180 min as previously described [28,29] and stopped by placing the vials in an ice-bath. Tissue pieces were thoroughly rinsed with 0.9% NaCl and placed into chloroform/methanol (2 : 1, v/v) for lipid extrac-

tion [23] and fractionation [29]. An aliquot of each medium was treated with 10% $HClO_4$ for protein precipitation and the neutralized supernatants were used for glycerol determination [12]. Another aliquot of each medium was used for FFA determination [17]. An estimation of the adipose tissue glycerol utilization rate for esterification and fatty acid synthesis was done using a derivation which we have previously reported [28–30], using the following equation: rate of glycerol utilization during incubation = dpm in tissue ^{14}C-labelled lipids × (glycerol in medium at time 0 + net glycerol production during incubation time)/dpm of [^{14}C]glycerol in medium at time 0 − dpm in tissue ^{14}C-labelled lipids.

To estimate lipoprotein lipase activity, a weighed aliquot of fresh epididymal fat pad was directly used to determine the enzyme activity as indicated below. The rest of the tissue from each animal was cut in small pieces and 80–120 mg aliquots were placed in vials containing 5 ml Krebs Ringer bicarbonate (pH 7.4) supplemented with 1% bovine albumin, 5 mM glucose, 20 μl of fasted rat plasma with or without heparin (2.4 IU/ml in the final medium). Vials were incubated for 0 ('Basal') or 30 min at 37°C and processed as previously described [31] for lipoprotein lipase activity estimation using the emulsified substrate preparation described by Nilsson-Ehle and Shotz [32] and Corey and Zilversmit [33].

In vivo utilization of [^{14}C]glycerol and [^{14}C]palmitate. 3 h after the last drug administration, other unanaesthetized rats were injected i.v. through a tail vein with either 20 μCi of [1-^{14}C]palmitate (sodium salt) (spec. act. 58 mCi/mmol) prepared as previously described [34] and dissolved in 0.5 ml of 8% FFA-free bovine albumin or 10 μCi of [U-^{14}C]glycerol (spec. act. 171 mCi/mmol). All radioactive material was obtained from Amersham International. Animals were killed by decapitation 15 and 30 min after the [^{14}C]palmitate injections or 30 min after the [^{14}C]glycerol injections. Blood was collected from the neck into beakers containing Na_2-EDTA, and the liver and epididymal fat pads and lumbar adipose tissue were immediately placed in liquid N_2. Aliquots of plasma were used for the analysis of triacylglycerols, cholesterol, glycerol and FFA, as indicated above. After being weighed, aliquots of each tissue and of plasma were placed in chloroform/methanol for lipid extraction [23] and fractionation, as previously described [35]. Labelled phospholipids were always removed by treatment of the lipid extract with activated silicic acid in chloroform medium. Another aliquot of plasma (1 ml) was centrifuged under 0.189 M NaCl containing 0.001 M EDTA for 18 h at 143 000 × *g* in a 40.3 rotor of a Beckman preparative ultracentrifuge. The supernatant was recovered by tube slicing and was shown to contain only VLDL, as seen in electrophoretic analysis in agarose gel and densitometry after being stained with Sudan black. The infranatant was designated 'bottom' and was shown to contain LDL, HDL and FFA. Triacylglycerols and cholesterol were determined and labelled lipids were purified and fractionated as indicated above. When [^{14}C]palmitate was the tracer administered, the rate of its incorporation into different lipidic fractions was derived with the following equation: lipid fraction formed/time (15 or 30 min) = dpm in lipid fraction per g of liver or ml of plasma/spec. act. of plasma FFA at the corresponding time (dpm/μmol). When [^{14}C]glycerol was the administered tracer, other aliquots of plasma were deproteinized [14] and supernatants were subjected to ascending chromatography on Whatman 3MM paper in the supernatant of *n*-butanol/water/methanol/formic acid (320 : 320 : 80 : 1, v/v) [36]. Unlabelled glucose, glycerol and lactate were used as carriers, and spots were identified by autoradiography and by comparison with purified standards run in parallel.

Radioactive measurements were performed in a PPO/POPOP toluene/Triton X-100-based scintillation cocktail and the samples were counted in a Beckman counter provided with an external standard device. Quenched standards were always counted with the samples to determine the channel ratio for cpm conversion to dpm.

Enzyme activities. Livers were excised and immediately placed in ice-cold 0.9% NaCl. After thorough rinsing, a weighed liver aliquot was homogenized with 3 vol. 50 mM potassium phosphate buffer (pH 7.4)/30 mM Na_2-EDTA/250 mM NaCl/1 mM dithiothreitol. The homogenized aliquot was centrifuged for 15 min at 6000 × *g* and the supernatant was centrifuged for another

15 min at 15000 × *g*. The resulting supernatant was centrifuged for 60 min at 105000 × *g*. Aliquots of the supernatant obtained in this centrifugation were used to determine the activities of cytosolic glycerol-3-phosphate dehydrogenase [37] and acetyl-CoA carboxylase [38]. The subcellular fractionation process was carried out at 4°C on the same day that the animals were killed, and purified fractions were kept at −80°C until the enzyme assays were performed, which was no more than 7 days later. Proteins were measured in all the assayed samples [21].

Expression of the results and statistical evaluation. Results are expressed as means ± S.E., and statistical comparison between the groups was with Student's *t*-test. Expression of the results from the in vitro adipose tissue experiment and their statistical evaluation was based on a procedure we have previously described [28–30].

Results

Body, adipose tissue and liver weights and liver composition

As shown in Table I, treating rats with etofibrate for 10 days did not affect their growth or epididymal fat pad weights, but did enlarge the liver by an average of 25%. Liver composition is not substantially modified by the drug treatment, as shown by the unmodified percent dry weight, and the unmodified DNA, protein, phospholipid and glycogen concentrations, although the concentration of both triacylglycerols and cholesterol was significantly reduced in the liver of the etofibrate-treated animals.

Plasma metabolites and lipoprotein

Etofibrate treatment did not modify plasma RIA-determined insulin, glucose or acetoacetate levels, but intensely affected circulating lipidic components (Table II). The drug caused significant reductions in plasma levels of β-hydroxybutyrate, glycerol, FFA, triacylglycerols and cholesterol (Table II). Etofibrate provoked a 50 and 70% reduction in VLDL triacylglycerols and cholesterol, respectively, whereas it produced a 27 and 34% reduction, respectively, in the triacylglycerols and cholesterol present in lipoproteins of higher density ($d > 1.006$), the difference with the controls being statistically significant (Table II).

Adipose tissue in vitro

To determine the role of the adipose tissue metabolism in the observed effects of etofibrate on plasma glycerol and FFA levels, the lipolysis rate was determined in vitro in epididymal fat pad pieces from treated rats and their controls. As

TABLE I

BODY, ADIPOSE TISSUE AND LIVER WEIGHTS AND LIVER COMPOSITION IN ETOFIBRATE-TREATED AND CONTROL RATS

All values are expressed as means ± S.E., corresponding to 10–16 rats/group. Concentrations are expressed on the basis of fresh tissue weights. Rats received the treatment for 10 days and were killed after 6 h of fasting, corresponding to 3 h after the last drug administration. n.s., not significant.

	Control	*P*	Etofibrate-treated
Body weight (g)			
at day 1	232 ± 7	n.s.	233 ± 3
at day 10	277 ± 7	n.s.	274 ± 3
Epididymal fat pads weight (mg)	977 ± 61	n.s.	1147 ± 73
Liver weight (g)	10.1 ± 0.4	< 0.01	12.5 ± 0.5
Liver dry weight (%)	29.3 ± 0.3	n.s.	30.0 ± 0.2
Liver DNA (μg/mg)	1.88 ± 0.05	n.s.	1.75 ± 0.12
Liver proteins (μg/mg)	148 ± 9	n.s.	163 ± 6
Liver phospholipid P (μg/mg)	0.92 ± 0.10	n.s.	1.19 ± 0.10
Liver glycogen (mg/g)	18.1 ± 2.1	n.s.	21.6 ± 2.0
Liver triacylglycerols (μg/mg)	5.19 ± 0.30	< 0.01	3.76 ± 0.40
Liver cholesterol (μg/mg)	5.68 ± 0.21	< 0.01	4.45 ± 0.33

TABLE II

PLASMA RIA-DETERMINED INSULIN AND METABOLITE CONCENTRATIONS IN ETOFIBRATE-TREATED AND CONTROL RATS

Values are expressed as means ± S.E. of 16 rats/group. Other experimental details as indicated in legend of Table I and in the text. n.s., not significant.

	Controls	*P*	Etofibrate-treated
RIA-insulin (ng/ml)	2.15 ± 0.25	n.s.	1.59 ± 0.15
Glucose (mg/dl)	120 ± 2	n.s.	125 ± 3
Acetoacetate (μmol/l)	29.4 ± 6.6	n.s.	51.9 ± 11.2
β-hydroxybutyrate (μmol/l)	377 ± 67	< 0.01	155 ± 9
Glycerol (μmol/l)	103 ± 5	< 0.001	65 ± 7
FFA (μmol/l)	133 ± 15	< 0.001	55 ± 9
Triacylglycerols (mg/dl)	52.3 ± 3.2	< 0.001	30.0 ± 2.1
Cholesterol (mg/dl)	113 ± 3	< 0.001	62 ± 4
VLDL triacylglycerols (mg/dl)	30.0 ± 2.8	< 0.001	15.3 ± 1.4
VLDL Cholesterol (mg/dl)	11.3 ± 1.7	< 0.001	3.6 ± 0.6
Triacylglycerols in $d > 1.006$ lip. (mg/dl)	11.8 ± 1.1	< 0.001	8.7 ± 0.9
Cholesterol in $d > 1.006$ lip. (mg/dl)	94.9 ± 5.1	< 0.001	63.3 ± 4.5

shown in Fig. 1, under basal conditions, the release of glycerol and FFA to the incubation medium increased with incubation time in both groups. In tissues from etofibrate-treated rats, glycerol release in the medium was greater than in controls, whereas this difference was not found for FFA (Fig. 1). In the presence of epinephrine, the release of both glycerol and FFA to the medium greatly increased, as expected, and the observed values were similar in tissues from both etofibrate and control rats (Fig. 1). To test whether inter-

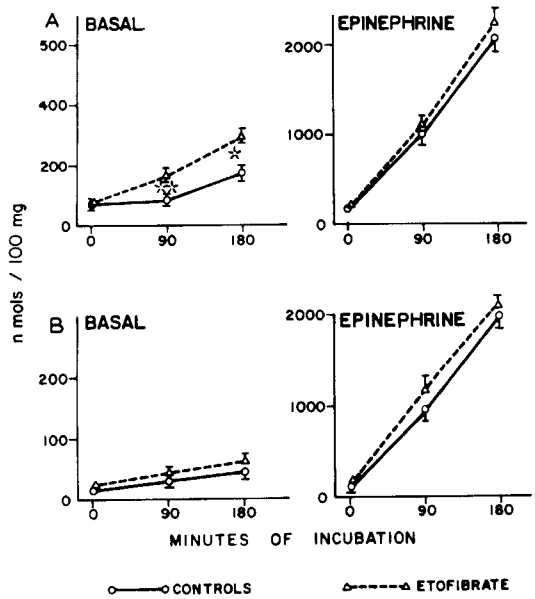

Fig. 1. Glycerol (A) and FFA (B) release by epididymal fat pieces from both experimental and control rats incubated in vitro under basal conditions or in the presence of 0.5 μg of epinephrine bitartrate/ml. Asterisks correspond to the statistical comparison between values from the etofibrate and control animals (* $P < 0.05$; ** $P < 0.01$). $n = 11$ rats/group.

TABLE III

RATE OF GLYCEROL UTILIZATION BY EPIDIDYMAL FAT PAD PIECES FROM BOTH ETOFIBRATE-TREATED AND CONTROL RATS, INCUBATED IN VITRO IN THE PRESENCE OF [^{14}C]GLYCEROL

Values are means ± S.E. of tissue pieces from 12 rats/group. They are corrected by the dilution of the tracer in the medium with the glycerol produced during incubation, as indicated in the text. n.s., not significant.

	Utilization (nmol/100 mg of fresh tissue)		
	controls	*P*	etofibrate-treated
Total lipid formation at			
90 min	9.24 ± 1.17	< 0.05	13.8 ± 1.2
180 min	24.8 ± 2.0	< 0.05	36.6 ± 4.2
Fatty acid formation at			
90 min	2.63 ± 0.35	n.s.	2.38 ± 0.38
180 min	8.39 ± 0.78	n.s.	6.59 ± 0.84
Acylglycerol formation at			
90 min	6.61 ± 0.96	< 0.01	11.4 ± 0.9
180 min	16.4 ± 1.4	< 0.01	30.1 ± 3.8

group differences in the release of glycerol and FFA by the tissues incubated under basal conditions are a consequence of modified reutilization rates, tissues from both groups were incubated in the presence of [^{14}C]glycerol to determine its incorporation into tissue [^{14}C]lipids. As shown in Table III, glycerol utilization in total lipid formation was greater in the tissues from etofibrate-treated rats than in their controls, and this effect specifically corresponded to the formation of acylglycerol, which was intensely enhanced in the former group, whereas fatty acid formation from the same substrate did not differ between either group after 90 or 180 min of incubation (Table III).

Lipoprotein lipase activity was also measured in adipose tissue from etofibrate-treated rats and their controls. Total enzyme activity in fresh unincubated tissue did not differ between either group (data not shown), but when fat pad pieces were incubated for 30 min in the presence of heparin (2.4 IU/ml), lipoprotein lipase activity in the media of tissues from etofibrate rats (39.6 ± 6.9 pkat/100 mg) was significantly greater ($P < 0.05$) than in that from controls (17.3 ± 5.5). Although this and the above findings indicate that the drug may enhance the clearance of circulating triacylglycerols by increasing the activity of adipose tissue lipoprotein lipase and by enhancing the tissular uptake of the enzyme action products, FFA and glycerol, they are not sufficient to explain the intense decrease produced in the plasma levels of both FFA and glycerol. These considerations moved us to study the fate of these metabolites after their administration in the ^{14}C form.

In vivo disappearance of [^{14}C]palmitate

The values of labelled lipids appearing in plasma after intravenous administration of [1-^{14}C]palmitate, corrected by the specific activity of circulating FFA, are summarised in Table IV. After administration, [^{14}C]palmitate disappeared from the circulation in etofibrate-treated rats faster than in controls, as shown by the lower amount of labelled FFA appearing in plasma 15 and 30 min after administration of the tracer (Table IV). A considerable amount of label appeared in plasma in the form of neutral lipid esterified fatty acids (presumably triacylglycerols). This value increased

TABLE IV

APPEARANCE OF PLASMA LIPIDS IN PLASMA AFTER THE INTRAVENOUS ADMINISTRATION OF [1-^{14}C]PALMITATE IN ETOFIBRATE-TREATED AND CONTROL RATS

Values are expressed as means ± S.E. of seven rats/group, and correspond to the lipidic fraction formed from [1-^{14}C]palmitate at each time after its intravenous administration. Values are corrected by the specific plasma FFA activity, as indicated in Materials and Methods. n.s., not significant.

	Appearance (nmol/ml of plasma)		
	controls	*P*	etofibrate-treated
Plasma FFA at			
15 min	133 ±11	< 0.001	55 ±6
30 min	134 ±19	< 0.01	53 ±8
Plasma esterified FA of neutral lipids at			
15 min	64.0 ± 5.0	< 0.01	37.0 ± 4.3
30 min	76.6 ± 11.6	n.s.	53.2 ± 7.8
Esterified fatty acids of neutral lipids in VLDL at			
15 min	54.0 ± 6.1	< 0.001	11.4 ± 2.0
30 min	30.7 ± 5.2	< 0.001	6.6 ± 1.8
Esterified fatty acids of neutral lipids in lipoproteins of $d > 1.006$ at			
15 min	11.0 ± 1.3	n.s.	11.6 ± 2.1
30 min	18.5 ± 4.5	n.s.	13.8 ± 2.6

with time after tracer administration, and although it was always lower in etofibrate-treated rats than in controls, the difference was significant only at 15 min (Table IV). At the two time points studied, the amount of label present in VLDL triacylglycerols in etofibrate-treated rats was greatly decreased with respect to the controls. However, the amount of label present in triacylglycerols associated with higher-density lipoproteins ($d > 1.006$) did not differ between the two groups (Table IV). Neither of these intergroup differences changed when absolute counts were considered (data not shown). More than 14% of the administered label appeared in the liver of all rats. In Table V, the values of labelled components found in liver after [^{14}C]palmitate administration, also corrected by their specific activity in plasma, are shown. The amount of FFA appearing in liver from the administered tracer was greater in the etofibrate-treated animals than in controls, although the difference was only significant at 15 min, and whereas the amount of esterified fatty

TABLE V

APPEARANCE OF LIVER COMPONENTS AFTER THE INTRAVENOUS ADMINISTRATION OF [1-^{14}C]PALMITATE IN ETOFIBRATE-TREATED AND CONTROL RATS

Values are from the same animals as in Table IV, and are expressed as means ± S.E. of seven rats/group, corresponding to the lipidic fraction appearing in liver at each time point after [^{14}C]palmitate administration. Values are corrected by the specific activity of plasma FFA, as indicated in Materials and Methods. n.s., not significant.

	Appearance (nmol/g of fresh tissue)		
	control	*P*	etofibrate-treated
FFA at			
15 min	1252 ± 236	< 0.05	2335 ± 388
30 min	1755 ± 217	n.s.	2410 ± 335
Esterified FA of neutral lipid at			
15 min	901 ± 181	n.s.	938 ± 178
30 min	1231 ± 299	< 0.05	459 ± 61
Water-soluble components at			
15 min	115 ± 20	< 0.01	268 ± 38
30 min	94 ± 11	< 0.01	200 ± 29

acids was lower in the first group, the difference was only significant at 30 min (Table V). No difference between the two groups appeared in the liver phospholipids formed from the administered [^{14}C]palmitate (data not shown), but the appearance of water-soluble components was greatly and significantly enhanced in liver from the etofibrate-treated animals both 15 and 30 min after tracer administration (Table V). Although not shown, when these values are expressed per total liver weight, the augmented appearance of labelled FFA and water-soluble components in the etofibrate rats as compared to their controls was even greater than that shown in Table V, whereas the appearance of liver triacylglycerols remained significantly lower in the former at 30 min.

All these findings after [^{14}C]palmitate administration indicate that whereas drug treatment enhances the rate of fatty acid disappearance from the circulation and uptake by the liver, it inhibits FFA esterification for triacylglycerol synthesis. The fact that the drug enhances the formation of water-soluble compounds in liver from administered palmitate also indicates that it facilitates the utilization of fatty acids for β-oxidation.

In vivo utilization of [^{14}C]glycerol

As shown in Table VI, 30 min after i.v. administration of [^{14}C]glycerol to the rats, total plasma radioactivity and radioactivity present in both lipidic (mainly acylglycerol) and water-soluble components was significantly lower in the

TABLE VI

LABELLED COMPONENTS IN PLASMA 30 MIN AFTER [U-^{14}C]GLYCEROL ADMINISTRATION TO ETOFIBRATE-TREATED AND CONTROL RATS

Values are means ± S.E. of eight rats/group. Rats received the tracer intravenously and were killed 30 min thereafter. n.s., not significant.

	Controls	*P*	Etofibrate-treated
Total radioactivity (dpm/ml)	103050 ± 2856	< 0.001	66744 ± 5959
Water-soluble comp. (dpm/ml)	82190 ± 3115	< 0.01	57514 ± 5345
Glucose (% of water-soluble comp.)	85.9 ± 1.6	< 0.001	72.4 ± 1.8
Lactate (% of water-soluble comp.)	13.7 ± 1.6	< 0.001	27.6 ± 1.8
Esterified fatty acids of neutral lipids (dpm/ml)	351 ± 92	n.s.	712 ± 305
Acylglycerol (dpm/ml)	6242 ± 619	< 0.01	1872 ± 869
Esterified fatty acids of neutral lipids in VLDL (dpm/ml)	283 ± 71	n.s.	433 ± 141
Acylglycerol in VLDL (dpm/ml)	5171 ± 1011	< 0.01	1033 ± 259
Acylglycerol in lipoproteins of $d > 1.006$ (dpm/ml)	971 ± 88	< 0.05	654 ± 95

etofibrate-treated rats than in their controls. Labelled glucose and lactate accounted for all the water-soluble components in the plasma, whereas labelled glycerol was practically undetectable. Etofibrate reduced the proportional amount of radioactivity appearing in plasma as glucose, whereas it enhanced the appearance of labelled lactate after [U-^{14}C]glycerol administration (Table VI). More than 85% of the labelled lipids appearing in plasma after [^{14}C]glycerol administration correspond to those present in VLDL in the form of acylglycerol, and this parameter was significantly reduced in the etofibrate-treated animals as compared to controls (Table VI). A milder but still significant reduction in the appearance of [^{14}C]acylglycerol was also found in lipoproteins of density > 1.006 in the etofibrate-treated rats as compared to controls (Table VI). Practically no radioactivity was present in any of the other lipidic fractions (data not shown).

Although not shown, the radioactivity found in the water-soluble liver components was lower in etofibrate-treated rats than in controls ($P < 0.05$), whereas no difference in any of the labelled lipidic components was found between the two groups.

Although there was no possibility of correcting the data by the isotopic dilution of the administered [U-^{14}C]glycerol, due to its rapid disappearance from plasma, the lower glycerol concentration in etofibrate-treated rats as compared to controls indicates that the tracer was 'less diluted' in the former group. Present findings therefore indicate that whereas etofibrate enhances the conversion of administered labelled glycerol into lactic acid, it decreases its conversion into VLDL triacylglycerol.

As shown in Table VII, liver from etofibrate-treated rats has enhanced cytosolic glycerol-3-phosphate dehydrogenase activity, unchanged acetyl-CoA carboxylase activity and enhanced carnitine concentration as compared to controls.

Discussion

Before starting the present study, we had established that etofibrate showed a clear dose-response relationship with regard to plasma lipids and lipoproteins and was already effective at a dose of about 50 mg/kg. Nevertheless, we decided to do this study with a high dose of the drug in order to be able to detect any effect which could give us some insight in the mechanism of action. Indeed, present results show that multiple mechanisms are involved in the hypolipidemic effect of etofibrate, and that although most of them mimic those previously reported to be exerted by one or two of its constituent synthetic moieties, clofibric acid and nicotinic acid, others seem to be specific for this drug. Among the different actions found herein, we feel that the most important is the inhibition in liver of VLDL triacylglycerol synthesis and secretion as a result of decreased hepatic synthesis of triacylglycerols, from both FFA and glycerol. The effects of etofibrate decreasing the plasma FFA and glycerol levels found here agree with those of Priego et al. [3] on reductions of FFA after shorter treatment times. However, they cannot be associated with decreased lipolytic activity in adipose tissue, but may rather be due to their augmented clearance from the circulation. Although decreased adipose tissue lipolysis has been reported in rats treated with either clofibrate

TABLE VII

EFFECT OF ETOFIBRATE TREATMENT ON LIVER ENZYME ACTIVITIES AND TOTAL L-CARNITINE CONCENTRATION IN THE RAT

Values are expressed as means ± S.E. of 9–11 rats/group.

	Controls	P	Etofibrate-treated
Cytosolic glycerol-3-phosphate dehydrogenase (μmol/mg prot per min)	0.68 ± 0.02	< 0.001	1.06 ± 0.03
Cytosolic acetyl-CoA carboxylase (nmol/mg prot per min)	6.60 ± 0.26	n.s.	6.19 ± 0.36
Total L-carnitine concentration (nmol/g fresh liver)	281 ± 32	< 0.01	657 ± 33

[39,40] or nicotinic acid [39,41,42], we were unable to detect such a change in the tissue from the etofibrate-treated rats under basal or epinephrine-stimulated conditions. A dissociation between the plasma lipid-lowering effect and its inhibitory action on FFA mobilization has also been proposed for nicotinic acid in humans [43]. Although the ability of adipose tissue to utilize glycerol has negligible implications in the uptake of circulating glycerol as compared to glucose utilization [44], it may have a role in tissue utilization of the hydrolytic products of lipoprotein lipase action on circulating VLDL [31,45]. In adipose tissue from our etofibrate-treated rats, both the in vitro utilization of glycerol and the heparin-released lipoprotein lipase activity were augmented, which would indicate an increased ability to utilize circulating VLDL triacylglycerols. This effect agrees with reported increments in lipoprotein lipase activity in adipose tissue from etofibrate-treated rats [46], and may be similar to that which has been proposed for clofibrate in rats [47] and humans [48], that is, plasma triacylglycerol clearance may be improved with a consequent contribution to the hypotriglyceridemic action of the drug.

The rate of [^{14}C]glycerol and [^{14}C]palmitate incorporation into liver triacylglycerols and circulating VLDL triacylglycerols was used to determine the effect of etofibrate on VLDL synthesis and secretion. In spite of the enhanced specific activity in plasma of both substrates due to their lower endogenous levels in the treated rats, their respective incorporation into plasma VLDL triacylglycerols was greatly reduced, indicating the inhibitory action of the drug on their synthesis from both FFA and glycerol. In the case of [^{14}C]glycerol, observed effects were similar to those previously reported for clofibrate [49], and although this drug did not modify or even increase the liver triacylglycerol concentration [4,50], etofibrate caused a significant decrease in the triacylglycerol concentration, which indicates a greater effect of this drug in inhibiting liver triacylglycerol synthesis. The increased activity of cytosolic glycerol-3-phosphate dehydrogenase in the etofibrate rats may indicate preferential α-glycerol phosphate oxidation to dihydroxyacetone phosphate for other pathways, such as glycolysis and/or gluconeogenesis, decreasing its availability for triacylglycerol biosynthesis. Glycolytic utilization of glycerol is increased by the drug, as indicated by the enhanced appearance in plasma of labelled lactic acid after [U-^{14}C]glycerol administration in the etofibrate-treated rats. A similar mechanism has also been proposed for clofibrate [51,52], even though the mitochondrial and not the cytosolic enzyme was measured, in spite of the fact that both triacylglycerol biosynthesis and the other pathways using dihydroxyacetone phosphate are extramitochondrial.

In the case of palmitic acid, present data indicate that the drug decreases the transformation of free fatty acids into triacylglycerol synthesis and subsequent formation and secretion of triacylglycerol-rich lipoproteins in the liver. This effect is similar to that already proposed for clofibrate [53]. In spite of its reduced utilization for triacylglycerol synthesis, administered labelled palmitate disappeared faster from the circulation in the etofibrate-treated rats. This effect may be related to an enhancement in fatty acid oxidation, as indicated by the increase in labelled water-soluble liver components. Liver carnitine was enhanced by etofibrate, and although this would facilitate mitochondrial fatty acid oxidation, as was also proposed for clofibrate [54,55], circulating ketone bodies were, however, decreased. This indicates that intramitochondrial fatty acid oxidation was not really enhanced. It is therefore proposed that peroxisomal rather than mitochondrial fatty acid oxidation is enhanced in our etofibrate-treated rats. Although this pathway is not carnitine dependent, carnitine is also known to be present in these organelles [56], and augmented peroxisomal fatty acid oxidation has also been reported to be enhanced by other clofibric acid derivatives [57–59]. On the other hand, the moderate liver weight increase after treatment with the very high dose of etofibrate indicates that the peroxisomal pathway is not as involved as in clofibric acid derivatives.

Another difference in response between treatment with etofibrate and clofibrate in the rat is that the latter produces both hyperglycemia [53] and reductions in liver glycogen concentration [55]. We were unable to observe either diabetogenic alterations in our etofibrate-treated rats, indicating that neither of the observed changes would

be a consequence of some secondary effect of the drug on carbohydrate metabolism.

In summary, although more experiments are required to complete our knowledge of the mechanism of etofibrate action on lipid metabolism, present findings show that this drug reduces plasma VLDL triacylglycerols by the juxtapositioning of two main effects. First, it enhances reesterification of fatty acids and glycerol in adipose tissue, which, together with the augmented lipoprotein lipase activity, would accelerate the clearance of circulating triacylglycerol-rich lipoproteins. Secondly, etofibrate decreases triacylglycerol synthesis in the liver secondary to enhanced utilization of triacylglycerol precursors for other pathways: acyl-CoA for peroxisomal oxidation and α-glycerol phosphate for conversion into dihydroxyacetone phosphate, which preferentially follows the glycolytic pathway, to eventually form lactic acid. These latter changes also seem to be responsible for the rapid disappearance of FFA and glycerol from the circulation, and may also be facilitated by the known capacity of the drug to inhibit cholesterol synthesis [3], thus causing the intense reduction in plasma VLDL levels. A reduced availability of these LDL precursors may be the major mechanism by which the drug decreases circulating LDL levels in humans [60] and rats [3], and activates the LDL receptor pathway in humans [61], thereby creating the basis for its beneficial use as an active hypolipidemic agent.

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