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EFFECTS OF FENOFIBRATE TREATMENT ON FATTY ACID OXIDATION IN LIVER MITOCHONDRIA OF OBESE ZUCKER RATS

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Abstract—Obese Zucker rats were dosed orally for one week with fenofibrate (100 mg/kg). Liver weights of treated rats as expressed as percent of body weight were slightly increased, while protein, DNA and lipid contents were unaffected per g of liver or increased when expressed in whole liver. Compared with the control animals, activities of fatty acid oxidase, of the peroxisomal fatty acid-oxidizing system and of catalase were markedly increased by fenofibrate both per g of liver and per total liver, while urate oxidase activity was unchanged when expressed per g of liver. The activity of monoamine oxidase and that of cytochrome *c* oxidase used as marker enzymes for mitochondria were increased only when expressed per total liver. However, fenofibrate treatment induced a pronounced increase in the activities of mitochondrial palmitoyl-CoA dehydrogenase and carnitine acyltransferases, particularly carnitine acetyltransferase. Fenofibrate also caused a significant increase of carnitine content in liver and hepatic mitochondria. The greatest observed increases were in free carnitine and in the rate of carnitine-dependent oleate oxidation, which might be favoured *in vivo* by a lesser sensitivity of CPT-I to a malonyl-CoA inhibitory effect. The present results suggest that fenofibrate treatment induces increased hepatic mitochondrial β-oxidation in obese Zucker rats.

Fenofibrate is a clofibrate-related compound which has been used in man since 1975 as a hypolipidaemic drug [1]. It has been demonstrated that fenofibrate has multiple actions: it represses *in vivo* HMG-CoA reductase [2, 3], enhances post-heparine lipase activity [4] and inhibits very low density lipoproteins (VLDL) secretion [5]. Since an enhancement of fatty acid synthesis, esterification in triacylglycerols and incorporation in VLDL is often correlated with a decreased fatty acid oxidation [6–8], stimulation of the latter pathway may explain some of the effects of fenofibrate.

Many authors have confirmed that chronic administration of clofibrate to rodents increases the weight of the liver and its content of mitochondria [9, 10] and peroxisomes [11]. As peroxisomes possess the enzymatic system for β-oxidation of fatty acids, it is suggested that increased peroxisomal β-oxidation can play an important role in the mechanism of the hypolipidaemic action of clofibrate and related compounds [12–14]. Both mitochondrial and peroxisomal oxidations of palmitate appear, however, to be markedly increased in isolated hepatocytes and liver homogenates of clofibrate-treated rats [15–19]. Perfused livers from such rats also showed an increased oleate oxidation [20]. The greatest changes in enzymatic activities are found in mitochondrial α-glycerophosphate dehydrogenase [21, 22] and in carnitine acetyl-, octanoyl- and palmitoyl-transferases [23–25].

It is well-documented that in the liver of genetically obese Zucker rats, lipogenesis and fatty acid esterification are enhanced [26–30], whereas fatty acid oxidation is depressed [31–35]. From studies on isolated hepatocytes [34], it was suggested that mitochondrial β-oxidation could be impaired in obese rats. However, using liver mitochondria, Brady and Hoppel [36] failed to demonstrate any difference in O_2 consumption required for fatty acid oxidation, and proposed that the depressed oxidation in isolated hepatocytes from obese rats could be due to a decreased mitochondrial content. On the other hand, we have previously reported that carnitine palmitoyltransferase I is much more sensitive to malonyl-CoA inhibition in mitochondria from obese than from lean Zucker rats [37]. Thus we have proposed that the decreased fatty acid oxidation by hepatocytes from obese rats could be due, in part, to a much lower entry of fatty acids into mitochondria rather than to an actual decrease in the oxidative capacity of mitochondria.

Thus, our objective in the present study was to investigate the effect of fenofibrate on mitochondrial fatty acid oxidation in liver of obese Zucker rats, by focussing on the enzymatic steps regulating entry of long-chain fatty acids into mitochondria. This mechanism has been shown to be of major importance in the control of β-oxidation [38–40].

MATERIALS AND METHODS

Chemicals. Fenofibrate was supplied by Laboratoires Fournier (Dijon, France). The chemicals

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were purchased from Prolabo (Paris) and from Merck (Darmstadt). The biochemicals were obtained from Sigma (St. Louis, MO). L- and D-carnitine were a gift from Dr C. Cavazza of Sigma-Tau (Pomezia, Italy). [1-^{14}C]oleic acid was from CEA Saclay, France; it was diluted with the unlabelled fatty acid to a specific activity of 2 Ci/mole and used as potassium salt. Oleoyl- and palmitoyl-CoA were prepared by using the method of Goldman and Vagelos [41].

Animals. Obese (fa/fa) male Zucker rats were bred in the Centre de Sélection et d'Elevage d'Animaux de Laboratoire, C.N.R.S., (Orléans-la-Source, France). They were 11 weeks old on arrival and were given a standard laboratory chow (AO3, UAR, 91360 Epinay-sur-Orge, France) *ad libitum*. They received by gastric intubation a daily dose of 100 mg fenofibrate emulsified in 5 ml of 3% arabic gum per kg body wt for 7 days. In each experiment, control rats received 5 ml of arabic gum mixture per kg by the same route for the same period. Animals were fasted overnight and received the last dose 20 hr before being sacrificed at 8:00 hours.

Lipid determinations. The livers of treated and untreated rats were withdrawn, quickly blotted with paper, weighed and cut into small pieces. Lipid extractions were made on 2 g samples of liver as described by Folch *et al.* [42]. Tripentadecanoin was used as an internal standard. After saponification, total fatty acids were measured as methyl esters by GLC [43], and fatty acids esterified in triacylglycerols were estimated by the same procedure after separation of the triacylglycerol fraction by TLC.

Mitochondrial fraction. The remaining liver of each animal was immediately cooled to 4° in a mixture of 0.25 M sucrose, 10 mM triethanolamine and 1 mM EDTA, at pH 7.4. It was then chopped, rinsed several times, blotted, weighed and homogenized in 15 volumes of the same mixture with two strokes of the Teflon pestle in a Potter–Elvehjem homogenizer. In order to prepare a highly purified mitochondrial fraction poor in peroxisomes, mitochondria were isolated from a postnuclear supernatant as previously described [37]. This procedure discarded the peroxisomes almost completely and left in the mitochondrial fraction a very small amount of nuclei [44], without modifying the specific activity of cytochrome *c* oxidase.

Enzyme assays. The liver mitochondrial content was estimated by the activities of monoamine oxidase for the external membrane [45] and of cytochrome *c* oxidase for the internal membrane [46]. The activity of total carnitine palmitoyl-transferase (CPT-I + CPT-II) was measured in the presence of 40 μM palmitoyl-CoA [47] except that Hepes buffer was used instead of Tris [48] and 4,4′-dithiobispyridine instead of 5,5′-dithionitrobenzoic acid [49]. The activity of the outer carnitine palmitoyltransferase I (CPT-I) alone and the sensitivity of this enzyme to malonyl-CoA were estimated at 25° according to Bremer [50] with slight modifications [37].

Peroxisomal activities of liver were measured in homogenate by using catalase [51] and urate oxidase [52]; the fatty acyl-CoA oxidase activity which is the first oxidative reaction in peroxisomes, was assessed by the palmitoyl-CoA dependent H_2O_2 generation as described in [53]; the peroxisomal fatty acid-oxidizing system reported by Lazarow and De Duve [54] was determined by CN^--insensitive palmitoyl-CoA-dependent NAD^+ reduction [55] in the presence of 75 μM palmitoyl-CoA.

Total and free carnitine contents in homogenates and mitochondria were measured in the presence of carnitine acetyltransferase by spectrophotometry [49].

Protein determination. The protein content of various preparations was determined by the biuret method [56], slightly modified for the liver homogenates which contain high triacylglycerol amounts [44].

Oxidative activities. The incubation medium for oleate oxidation was a mixture of 20 mM potassium phosphate pH 7.4, 50 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, 50 μM CoA, 1 mM L-carnitine, 0.2 mM L-malate, 50 μM [1-^{14}C]potassium oleate bound to bovine albumin (fraction V, fatty acid-free) in molar ratios from 1 to 3; carnitine was omitted for estimating the strictly carnitine-dependent oxidation. The reaction was started with 1 mg of mitochondrial protein in 2 ml of medium kept at 35° with gentle shaking. After 8 min, the reaction was stopped at 0° by adding 0.1 ml 10 N HCl and then 8 ml 10% $HClO_4$ which precipitates proteins and long-chain fatty acids still intact or bound to CoA or carnitine. Thereafter the medium was filtered on a Millipore filter (pore 0.45 μm). The radioactivity of the filtrate, which corresponds to labelled ketone bodies, acetate molecules bound to CoA or carnitine and to intermediary products of the Krebs cycle, was measured in picofluor 15 (Packard Instrument Co.) in a Packard 300C scintillation counter. The carbon dioxide production was found to be negligible. The palmitoyl-CoA dehydrogenase activity was measured according to Korsrud *et al.* [57] in a medium containing 35 μM palmitoyl-CoA with 2 mM KCN, 15 μM rotenone, 10 μM antimycin to block the respiratory chain and 0.05% Triton X-100 to obtain maximal enzymatic activity. Oxygen consumption and phosphorylation ratios were assessed polarographically (Gilson KM oxygraph) [58].

The difference due to fenofibrate-treatment was assessed by the Student's *t*-test.

RESULTS

Table 1 shows that after one week of treatment with the chosen dose of 100 mg/kg of fenofibrate, the body weight of the rats was not changed. However, the liver weight, expressed in g/100 g body weight, was significantly increased (+20%). Hepatic protein and DNA contents were unaffected when expressed per mg/g tissue, but increased in the whole organ because of the liver hypertrophy. Fenofibrate did not alter total lipid and triacylglycerol contents per g of liver.

The content in organelles involved in fatty acid oxidation was estimated by measuring the activities of the specific enzymes in liver homogenates (Table 2). The activity of monoamine oxidase, a marker enzyme of mitochondria, was slightly diminished and no significant modification of cytochrome *c* oxidase activity was observed when expressed per g of tissue;

Table 1. Effects of fenofibrate treatment on body and liver weights, protein, DNA and lipid contents of liver from obese Zucker rats

	Control	Fenofibrate-treated
Body weight (g)	392 ± 7	396 ± 9 (NS)
Liver weight		
(g)	15.1 ± 0.7	18.2 ± 0.8*
(g · 100 g body wt^{-1})	3.83 ± 0.12	4.58 ± 0.14*
Protein (mg · g $liver^{-1}$)	209 ± 3	213 ± 2 (NS)
DNA (mg · g $liver^{-1}$)	5.19 ± 0.18	5.21 ± 0.16 (NS)
Liver lipids		
Total fatty acids (mg · g $liver^{-1}$)	67.0 ± 2.6	68.3 ± 2.6 (NS)
Triacylglycerols (mg fatty acids · g $liver^{-1}$)	35.2 ± 2.6	37.7 ± 2.7 (NS)

Five 12-week-old Zucker rats were given a daily dose of 100 mg fenofibrate per kg for 7 days by gastric intubation. Five control rats were given the excipient only. Results are means ± SEM. (NS): non-significant; *$P < 0.05$ (Student's *t*-test).

Table 2. Enzymatic activities in liver homogenates of obese Zucker rats after treatment by fenofibrate

	Control	Fenofibrate-treated
Monoamine oxidase (μmol · min^{-1} · g $liver^{-1}$)	0.542 ± 0.016	0.500 ± 0.006*
Cytochrome *c* oxidase (μmol · min^{-1} · g $liver^{-1}$)	51.7 ± 7.1	59.0 ± 4.4 (NS)
Urate oxidase (μmol · min^{-1} · g $liver^{-1}$)	3.10 ± 0.15	3.23 ± 0.17 (NS)
Catalase (10^{-3} μmol · min^{-1} · g $liver^{-1}$)	48.6 ± 4.5	69.0 ± 3.8†
Fatty acid oxidase (μmol · min^{-1} · g $liver^{-1}$)	0.75 ± 0.08	4.05 ± 0.22‡
Peroxisomal fatty acid-oxidizing system (μmol · min^{-1} · g $liver^{-1}$)	1.06 ± 0.05	6.69 ± 0.41‡

Five 12-week-old Zucker rats were given a daily dose of 100 mg fenofibrate per kg for 7 days by gastric intubation. Five controls received only the gum water-excipient. Results are means ± SEM. (NS): non-significant; *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ (Student's *t*-test).

Table 3. Effects of fenofibrate treatment on the mitochondrial carnitine transferase activities and carnitine contents in the liver from obese Zucker rats

	Control	Fenofibrate-treated
Carnitine trasferase activity (nmol · min^{-1} · mg mitochondrial $protein^{-1}$)		
Total carnitine palmitoyltransferase (CPTI + II)	12.6 ± 0.8	24.1 ± 0.2‡
Carnitine palmitoyltransferase I (CPTI)	2.87 ± 0.08	3.47 ± 0.12*
Carnitine octanoyltransferase	29.3 ± 2.1	59.1 ± 4.4‡
Carnitine acetyltransferase	1.45 ± 0.11	25.2 ± 2.0‡
Carnitine contentin		
Liver mitochondria (nmol · g $protein^{-1}$)		
free form	34 ± 3	266 ± 8‡
total	69 ± 7	377 ± 11‡
Liver (nmol · g wet $tissue^{-1}$)		
free form	140 ± 4	653 ± 22‡
total	238 ± 7	883 ± 42‡

Five 12-week-old Zucker rats were given 100 mg fenofibrate emulsified in gum water per kg for 7 days by gastric intubation. Five controls received an equivalent volume of gum water only. Values are means ± SEM. *$P < 0.01$; ‡$P < 0.001$ (Student's *t*-test).

however, calculations of these activities for total liver should be increased by 20% owing to the hypertrophy. Urate oxidase and catalase are classical markers for peroxisomes. When expressed per g of liver, the activity of the former was not modified, while catalase activity was found to be enhanced by the treatment, but both were more elevated per whole liver. Table 2 also shows that the activities of the peroxisomal fatty acid oxidase and of the CN^--insensitive palmitoyl-CoA-dependent NAD^+ reduction were increased by fenofibrate per g of liver, values still amplified by the gain in liver weight.

As shown in Table 3, the enzymes implicated in the transfer of fatty acids through the mitochondrial

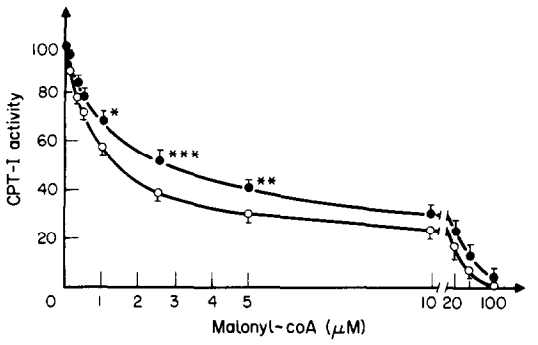

Fig. 1. Effect of increasing malonyl-CoA concentrations on the activity of CPT-I in mitochondria isolated from livers of obese Zucker rats treated by fenofibrate (●) or vehicle only (○). Results are expressed as % of CPT-I activity, estimated in the absence of malonyl-CoA. Each point represents the mean of 5 duplicated determinations; each determination corresponds to one mitochondrial preparation from one rat. T-bars show SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's t-test).

inner membrane were all stimulated by the fenofibrate treatment. The enzymes allowing the transfer of long-chain and medium-chain fatty acids were found to be twice as active, whereas the transfer of acetyl-CoA was 17-fold more elevated after treatment. Moreover Table 3 shows that the carnitine content dramatically increased in liver tissue and in mitochondria after fenofibrate treatment. Malonyl-CoA is well known to inhibit CPT-I activity [59–61]. Our results (Fig. 1) show that the mitochondria of the obese Zucker rats were rendered less sensitive to malonyl-CoA by fenofibrate treatment.

As shown in Table 4, the complete oxidation of oleic acid was 1.5-fold higher and the activity of palmitoyl-CoA dehydrogenase doubled after the treatment. The oxygen consumption and the phosphorylation rate were increased either in the presence of succinate or in the presence of the glutamate–malate mixture, but the specific activity of cytochrome c oxidase was not modified by the fenofibrate treatment. This indicates that the mitochondrial fractions exhibited a very similar purity in treated and control rats, since the enzyme activity remains about the same, when expressed per mg of mitochondrial protein.

DISCUSSION

Our results showed that the active mass of total liver in obese Zucker rats treated with fenofibrate was significantly increased as also shown in normal Wistar rats [62], while protein, DNA and lipids were unaffected per g of liver (Table 1). Clofibrate treatment is also known to produce an hepatomegalic effect in rats [21]. Lazarow [13] had observed a marked increase in palmitoyl-CoA oxidation by liver homogenates from rats treated with clofibrate. It was suggested that the enhanced oxidation might be due to an increase in peroxisomal oxidation and is concomitant with peroxisome proliferation. Peroxisomal palmitoyl-CoA oxidation requires NAD^+ and oxygen, but is cyanide-insensitive [54]. In the present study, fenofibrate enhanced peroxisomal fatty acid oxidizing system per g and per total liver. Catalase activity was also induced by the treatment. However, urate oxidase activity was not modified per g of tissue, suggesting that peroxisome proliferation may not be the only explanation of the increased peroxisomal oxidation or that newly-formed peroxisomes are different in their enzymatic activity.

Carnitine acyltransferases are present both in mitochondria and peroxisomes [24, 25, 63, 64]. Our results show that the activity of carnitine acyltransferases is increased in isolated mitochondria from livers of obese Zucker rats treated with fenofibrate. This effect was the most marked for carnitine acetyltransferase and was less with longer acyl chains. Similar results have been described after chronic clofibrate treatment of rats [23, 25, 65, 66].

Table 4. Effects of fenofibrate treatment on several oxidative activities in liver mitochondria of obese Zucker rats

	Control	Fenofibrate-treated
Carnitine-dependent oleate oxidation ($nmol \cdot min^{-1} \cdot mg\ protein^{-1}$)		
Molar ratio oleate/albumin: 1	1.48 ± 0.11	2.38 ± 0.17‡
2	2.27 ± 0.13	3.41 ± 0.16‡
3	1.68 ± 0.13	2.72 ± 0.18‡
Palmitoyl-CoA dehydrogenase activity ($nmol \cdot min^{-1} \cdot mg\ protein^{-1}$)	11.55 ± 0.72	24.94 ± 0.11‡
Oxygen consumption with succinate ($\mu mol \cdot hr^{-1} \cdot mg\ protein^{-1}$)	3.0 ± 0.1	4.1 ± 0.2†
Phosphorylation		
ratio P/O with succinate	1.3 ± 0.1	1.8 ± 0.1†
with glutamate–malate	2.7 ± 0.1	3.1 ± 0.1*
Cytochrome c oxidase ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$)	0.687 ± 0.028	0.710 ± 0.040 (NS)

Five 12-week-old Zucker rats were given a daily dose of 100 mg fenofibrate per kg for 7 days by gastric intubation. Five controls were given the excipient only. Results are means ± SEM. (NS): non significant; *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ (Student's t-test).

Overall carnitine-dependent oleate oxidation, together with palmitoyl-CoA dehydrogenase activity, the first enzymatic step after fatty acid crossing through the mitochondrial membrane, together with oxygen consumption and P/O ratio, were also increased, indicating that the accelerated oxidation of fatty acids was due to a fenofibrate-induced increase in the activity of the reactions responsible for the passage of fatty acids across inner mitochondrial membranes, and of the real mitochondrial β-oxidative route.

As already demonstrated for clofibrate [16, 17], fenofibrate treatment increased hepatic carnitine and mitochondrial carnitine. The greatest increase was in free carnitine. The role of carnitine in shuttling long-chain fatty acids across the mitochondrial inner membrane is well-documented [67–69]. Although not firmly established, several other functions have been proposed for carnitine. These include shuttling acetyl moieties between different cell compartments [68] and buffering the acetyl-CoA : CoA-SH ratio by forming acetylcarnitine which can serve as a high-energy acetyl reservoir [70, 71]. Besides Brady *et al.* [72] recently demonstrated that administration of L-carnitine to obese Zucker rats had no effect on carnitine palmitoyltransferase kinetics, mitochondrial or peroxisomal oxidative capacity and lipoprotein lipase activity. However, L-carnitine did inhibit triacylglycerol synthesis and/or secretion by the liver. Therefore the increased carnitine level induced by fenofibrate may have not only some repercussion on β-oxidation, but could also explain the decrease of triacylglycerol synthesis already described [5].

Increased mitochondrial oxidation may arise from the decreased sensitivity of carnitine palmitoyltransferase I (CPT-I) to malonyl-CoA as demonstrated herein. The concentration of malonyl-CoA that depressed CPT-I activity by 50%, was estimated to be 2.90 ± 0.16 and 1.43 ± 0.10 μM in fenofibrate-treated and control obese Zucker rats respectively ($P < 0.001$). However, this effect of fenofibrate was relatively small as compared to the difference in malonyl-CoA inhibition between lean and obese Zucker rats [37]. A malonyl-CoA-dependent effect of fenofibrate on mitochondrial β-oxidation cannot be excluded on this basis. Clofibrate has been shown to inhibit acetyl-CoA carboxylase, thereby decreasing malonyl-CoA level [73]. Investigations on this point are on the way with fenofibrate. This is of importance as it has been reported that the level of hepatic malonyl-CoA is directly related to the mechanism of the regulation of hepatic fatty acid oxidation and fatty acid synthesis [38, 40, 74].

Our present experiments indicated that beside increasing peroxisomal β-oxidation, fenofibrate potentiates mitochondrial β-oxidation in the liver of obese Zucker rats. Such effects may explain the decreased triacylglycerol synthesis already observed in rats and may be carnitine- and malonyl-CoA-mediated.

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