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EFFECT OF CHRONIC CLOFIBRATE ADMINISTRATION ON MITOCHONDRIAL FATTY ACID OXIDATION*

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Abstract—The effect of chronic clofibrate administration on fatty acid oxidation by isolated liver and skeletal muscle mitochondria was studied to determine if the hypolipidemic action of clofibrate may be mediated by reducing levels of fatty acyl substrates via enhanced fatty acid oxidation. Oxygen consumption and CO_2 production associated with the oxidation of fatty acids were decreased 30 per cent in liver mitochondria from clofibrate-treated rats. By contrast, CO_2 production from acetate and citric acid cycle intermediates was not significantly affected. This indicates impairment of β-oxidation of fatty acids to the level of acetyl CoA, an interpretation supported by the findings of a decrease in ketone body production. In liver mitochondria, oxygen consumption associated with the oxidation of glutamate, succinate and ascorbate was depressed. The per cent decrease was comparable in the absence or presence of ADP or dinitrophenol, suggesting impairment of the respiratory chain. There was no effect on energy production or utilization, as evidence by unchanged respiratory control, ADP/O ratio, ATP-^{32}P exchange reaction, and substrate- or ATP-supported Ca^{2+} uptake. Unlike isolated liver mitochondria, there were no effects on oxygen uptake or fatty acid oxidation by muscle mitochondria. It is unlikely that the hypolipidemic effects of clofibrate are mediated by reducing fatty acyl substrate levels via enhanced fatty acid oxidation.

The ethyl ester of α-*p*-chlorophenoxyisobutyric acid (clofibrate) is widely used to lower serum levels of cholesterol and triglycerides [1, 2]. Reduction of triglyceride levels may be due to the ability of clofibrate to displace thyroxin from albumin-binding sites [3] since thyroid hormones exhibit hypocholesterolemic and hypotriglyceridemic properties. Clofibrate increases the uptake of triglycerides by adipose tissue [4] and decreases the release of fatty acids from adipose tissue [5, 6]. Turnover of low-density lipoproteins is lowered by clofibrate [7] and hepatic release of lipids or lipoproteins is depressed [8, 9]. Hence decreased triglyceride formation or release, or decreased lipoprotein formation or release, may be involved in the lipid-lowering action of clofibrate. It is possible that reduction of fatty acid levels in the liver may participate in the clofibrate effect. Depression of fatty acid or triglyceride biosynthesis is suggested by an inhibitory effect of clofibrate on acetyl CoA carboxylase [10] or on the acylation of glycerol-3-phosphate [11, 12]. Accelerated fatty acid oxidation by hepatic mitochondria may contribute to the hypolipidemic effect of clofibrate by reducing stubstrate concentrations. Indeed, it has been reported that, in man, chronic clofibrate administration increased the oxidation of palmitate to CO_2 and acetoacetate [13].

Clofibrate administration was reported to augment hepatic activities of mitochondrial α-glycerophosphate dehydrogenase, cytochrome oxidase and catalase [14], and to increase the content of mitochondria [15, 16]. Mitochondria protein synthesis *in vivo* was stimulated by clofibrate administration. However, the half-life of this organelle was unchanged [17]. Hepatic mitochondrial oxygen uptake and oxidative phosphorylation were reported not to be affected by clofibrate feeding [18], whereas the specific activities of several enzymes of the outer mitochondrial membrane were decreased 30–40 per cent [17]. *In vitro*, clofibrate was reported to have numerous effects on mitochondrial functions [19–21]. We previously reported that clofibrate, *in vitro*, inhibits mitochondrial oxygen consumption with several substrates, respiratory control, oxidative phosphorylation, energized Ca^{2+} uptake, and the transfer of reducing equivalents into the mitochondria, as catalyzed by reconstituted substrate shuttles [22]. In this report, a study was made of the effect of chronic clofibrate administration on fatty acid oxidation by hepatic and skeletal muscle mitochondria, to determine if altered fatty acid oxidation plays a role in the hypolipidemic action of clofibrate.

MATERIALS AND METHODS

Male Sprague–Dawley rats, weighing about 200 g, were injected subcutaneously with 30 mg clofibrate/100 g of body wt/per day for 14 or 18 days [23]. Controls were injected with saline. The animals were killed 16–24 hr after the last dose of clofibrate. Liver mitochondria were prepared as previously described [24], washed and suspended in 0.25 M sucrose—0.01 M Tris–HCl, pH 7.4—0.001 M EDTA. Muscle mitochondria were prepared by the method of Chappell and Perry [25]. Protein was determined according to Lowry *et al.* [26]. All radioactive counting procedures were performed in a liquid scintillation counter using either Econofluor or Aquafluor as the scintillation medium.

Oxygen consumption. Oxygen uptake was assayed at 30° using a Clark oxygen electrode as previously described [22, 27, 28]. The reaction system consisted of 0.3 M mannitol, 10 mM Tris–HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4), 10 mM KCl, 2.5

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mM $MgCl_2$, and mitochondrial protein equivalent to about 3–5 mg protein in a final volume of 3.0 ml. Substrates included 10 mM glutamate, 10 mM succinate, 5 mM ascorbate–0.2 mM tetramethyl-*p*-phenylenediamine, 17 μM palmitoyl-*l*-carnitine and 1 mM NADH. State 3 oxygen consumption is the rate of oxygen uptake in the presence of 1 mM ADP, whereas state 4 is the rate in the absence of ADP. When present, the final concentration of dinitrophenol* was 0.08 mM.

ATP-^{32}P exchange. ATP-^{32}P exchange was assayed as previously described [22, 27, 28], using $^{32}PO_4$ (20,000 cpm/μmole of Pi).

Ca^{2+} uptake. The uptake of Ca^{2+} by the mitochondria was measured using 1 mM $^{45}Ca^{2+}$ (60,000–80,000 cpm/μmole of Ca^{2+}) as previously described [22, 27, 28]. The source of energy was 10 mM glutamate (plus ATP), 10 mM succinate (plus ATP) or 3.3 mM ATP alone. Energy-independent Ca^{2+} uptake is defined as the uptake which occurs in the presence of 0.1 mM DNP.

ATPase activity. ATPase activity was determined as previously described [22, 27, 28] in the absence or presence of 3 mM Mg^{2+} or 0.1 mM DNP.

CO_2 production from fatty acids and citric acid cycle intermediates. CO_2 production was assayed at 30° in center-well flasks containing 0.3 ml hydroxide of hyamine in the center well [29, 30]. The reaction system consisted of 0.3 M mannitol, 10 mM Tris·HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4), 2.5 mM $MgCl_2$, 10 mM KCl, 3.3 mM ADP and about 2 mg of mitochondrial protein in a final volume of 3.0 ml. Substrates included 67 μM sodium palmitate[1-^{14}C], 67 μM sodium octanoate[1-^{14}C], 1 mM sodium acetate[1-^{14}C], 6.7 mM sodium α-ketoglutarate[1-^{14}C], 6.7 mM sodium succinate[1,4-^{14}C], 6.7 mM sodium citrate[6-^{14}C], or 5.4 mM sodium malate (uniformly labeled). When fatty acids were the substrates, 3 mM ATP plus 3 mM carnitine and 0.2 mM sodium malate was also added, and the palmitate was bound to albumin. The reaction was terminated after 15 min by the addition of 1 ml of 2 N HCl. After a 60-min equilibration period, the contents of the center well were collected, the center wells were washed three times with Econofluor, and the samples counted.

Ketone body production. Ketogenesis was assayed at 25° as previously described [20, 29], using an incubation mixture of 100 mM KCl, 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 0.1 mM sodium malate, 6 mg of fatty acid-free bovine serum albumin, 1 mM ADP and about 5 mg of mitochondrial protein in a final volume of 3.0 ml. The reaction was initiated by the addition of 0.1 mM palmitoyl-*l*-carnitine. After 20 min the contents of the flasks were poured into centrifuge tubes containing 0.3 ml ice-cold $HClO_4$ (60% w/v). After centrifugation and neutralization, the concentrations of acetoacetate and β-hydroxybutyrate were determined enzymatically [31] in 1.5-ml aliquots of the supernatant.

Source of materials. Palmitoyl-*l*-carnitine was a gift of Otsuka Pharmaceuticals, Tokoshima, Japan. Clofibrate was obtained from Ayerst Laboratories, Inc., New York. Hydroxide of hyamine-10X, Econofluor and Aquafluor were from Packard Instruments, Downers Grove, Ill. Sodium octanoate[1-^{14}C] (sp. act. 20 mCi/m-mole), palmitic acid[1-^{14}C] (56.8 mCi/m-mole), $^{45}CaCl_2$ (12.8 mCi/mg), $NaH_2\ ^{32}PO_4$ (500 mCi/m-mole), α-ketoglutaric acid[1-^{14}C] (5 mCi/m-mole), citric acid[6-^{14}C] (5 mCi/m-mole) and succinic acid[1,4-^{14}C] (5 mCi/m-mole) were obtained from New England Nuclear Corp., Boston, Mass. L-Malic acid[^{14}C] (U) (35 mCi/m-mole) was obtained from Amersham Searle, N.Y.

Statistics. All values refer to mean ± S.E.M. The number of pairs is indicated in parentheses or stated in the legends. Statistical analysis was made by Student's *t*-test.

*The abbreviation used is: DNP, 2,4-dinitrophenol.

RESULTS

The ratio of liver to body weight was increased from 3.8 per cent in controls to 4.8–5.2 per cent in rats treated with clofibrate for 14 or 18 days. This increase of 29 per cent is similar to that found by others [16, 18, 32]. Mitochondrial α-glycerophosphate dehydrogenase activity increased 3 to 4-fold (11–15 nmoles cytochrome *c* reduced/min/mg of protein). The recovery of mitochondria increased by about 30–40 per cent after clofibrate administration.

Fatty acid oxidation. The oxidation of palmitoyl-*l*-carnitine by isolated liver mitochondria was studied after 14 and 18 days of clofibrate administration. Oxygen consumption associated with the oxidation of palmitoyl-*l*-carnitine was decreased under state 4 (41 per cent) and state 3 (32 per cent) conditions (Fig. 1) after 14 days of clofibrate administration. However, oxidative phosphorylation was not impaired, as evidenced by the comparable respiratory control and ADP/O ratios in mitochondria isolated from controls and from clofibrate-treated rats. Similar results were obtained after 18 days of clofibrate treatment; oxygen uptake associated with palmitoyl-*l*-carnitine oxidation was decreased in the absence of ADP, from 13.6 ± 1.4 natoms/min/mg of protein to 8.5 ± 0.6 after clofibrate administration (−38 per cent, N = 6, P = 0.01) and was decreased in the presence of ADP from 56.9 ± 6

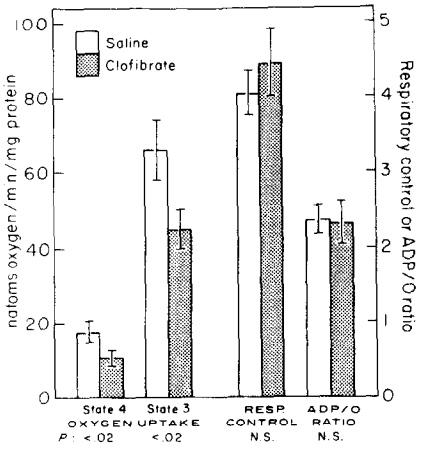

Fig. 1. Effect of chronic clofibrate administration on oxygen consumption associated with palmitoyl-*l*-carnitine as substrate. Oxygen uptake was assayed as described in Materials and Methods after 14 days of clofibrate administration. The concentration of palmitoyl-*l*-carnitine was 17 μM. Results are from seven pairs of animals.

Table 1. Effect of clofibrate administration on CO_2 production from labeled fatty acids*

Substrate	No. of pairs	CO_2 production (cpm/mg protein) Control	Clofibrate	Effect (%)	P
A. Palmitate[1-^{14}C]	13	5998 ± 617	4253 ± 543	−29	0.02
Octanoate[1-^{14}C]	13	4610 ± 497	3247 ± 716	−30	<0.05
B. Palmitate[1-^{14}C]	7	4326 ± 722	2594 ± 490	−40	<0.02
Octanoate[1-^{14}C]	7	5778 ± 566	3930 ± 800	−32	<0.05

* CO_2 was collected and counted as described in Materials and Methods. Experiments A and B refer to 14 and 18 days of clofibrate administration respectively.

natoms/min/mg of protein to 40 ± 3 (30 per cent, N = 6, P = 0.02). Respiratory control and ADP/O ratios were not affected. In these experiments palmitoyl-*l*-carnitine was used as the substrate so that the fatty acid was already available as the carnitine ester, which is the activated, easily penetrable, form. Clofibrate administration was reported to increase the activities of long-chain acyl CoA synthetase and carnitine palmitoyl transferase [33]. Consequently, we studied the effects of clofibrate administration on the oxidation of the albumin-bound free fatty acid, a system which requires the participation of these enzymes. Palmitate oxidation was also decreased under state 4 (27 per cent) and state 3 (37 per cent) conditions after clofibrate treatment. The respiratory control and ADP/O ratios were not affected.

CO_2 production from fatty acids and citric acid cycle intermediates. In man, CO_2 production from palmitate was increased by clofibrate ingestion [13]. We, therefore, investigated the effect of chronic clofibrate administration on CO_2 production from labeled palmitate and octanoate by isolated rat liver mitochondria. CO_2 production from both palmitate and octanoate was decreased 30 per cent after 14 days of clofibrate administration (Table 1), a decrease comparable to that of state 3 oxygen consumption associated with fatty acids as substrate (32 per cent). After 18 days of clofibrate administration, CO_2 production from palmitate was decreased 40 per cent, while that from octanoate was decreased 32 per cent (Table 1). Since activation and translocation of fatty acids are enhanced by clofibrate feeding [33], the decrease in CO_2 production by isolated mitochondria may reflect impaired β-oxidation of the fatty acids to the level of acetyl CoA or decreased citric acid cycle activity. To distinguish between these two possibilities, the effect of clofibrate administration on CO_2 production from acetate and several citric acid cycle intermediates was studied.

The use of acetate[1-^{14}C] allows assay of the complete citric acid cycle, since neither of the CO_2 molecules produced is derived from acetate on the first turn of the cycle. Upon subsequent revolutions, labeled acetyl carbon is eliminated as radioactive CO_2. The use of citrate[6-^{14}C] allows measurement of the formation of labeled CO_2 produced in the citrate-α-ketoglutarate span of the cycle, while use of α-ketoglutarate[1-^{14}C] permits measurement of the formation of labeled CO_2 produced in the α-ketoglutarate-succinate span of the cycle [34]. The use of succinate[1,4-^{14}C], or uniformly labeled malate, coupled with the information derived from experiments with citrate[6-^{14}C] and α-ketoglutarate[1-^{14}C], provides information concerning the spans succinate → citrate or malate → citrate respectively [34]. CO_2 production from acetate was not affected by 14 or 18 days of clofibrate administration (Table 2), suggesting that the overall activity of the citric acid cycle was not impaired. This may correlate with the lack of effect of clofibrate administration on CO_2 production from citrate[6-^{14}C] (Table 2). The slight decreases in CO_2 production observed with α-ketoglutarate, succinate and malate were not statistically significant (Table 2).

Ketone body production. In rat liver mitochondria, clofibrate administration depressed CO_2 production from fatty acids, without any effect on the overall activity of the citric acid cycle. It is possible that clofibrate acts by inhibiting β-oxidation of fatty acids to

Table 2. Effect of clofibrate administration on CO_2 production from citric acid cycle intermediates*

Substrate	No. of pairs	CO_2 production (cpm/mg protein) Control	Clofibrate	Effect (%)
A. Acetate[1-^{14}C]	13	8913 ± 515	8734 ± 414	−2
Citrate[6-^{14}C]	8	5527 ± 402	5328 ± 647	−4
α-Ketoglutarate[1-^{14}C]	8	9478 ± 890	8328 ± 1085	−12
Malate[^{14}C] (U)	8	2411 ± 301	2025 ± 404	−16
B. Acetate[1-^{14}C]	5	4192 ± 411	4676 ± 510	+10
Citrate[6-^{14}C]	5	4788 ± 838	5078 ± 1030	+6
α-Ketoglutarate[1-^{14}C]	5	5991 ± 1025	5241 ± 1054	−13
Succinate[1,4-^{14}C]	5	1648 ± 314	1284 ± 324	−22

* CO_2 was collected and counted as described in Materials and Methods. Experiments A and B refer to 14 and 18 days of clofibrate administration respectively. In all cases there were no statistically significant differences between controls and rats given clofibrate.

the level of acetyl CoA. The effect of clofibrate on ketone body production from palmitoyl-*l*-carnitine was therefore studied, because this system is dependent upon the formation of acetyl CoA. The endogenous rate of ketone body formation (in the absence of added fatty acid) was not significantly affected by clofibrate administration (Fig. 2). When 100 μM palmitoyl-*l*-carnitine was added, ketone body production was increased in mitochondria from controls and from clofibrate-treated rats. Both the total (24 per cent) and the net (28 per cent) rates of ketone body production were decreased after clofibrate administration (Fig. 2). The extent of decrease was comparable to that in CO_2 production (29–30 per cent) and the oxygen uptake (32 per cent) associated with the oxidation of fatty acids. Thus, the decreased formation of the end products of fatty acid oxidation appears to result either from an inhibition of β-oxidation to acetyl CoA or from an effect on the respiratory chain. The latter would decrease the rate of regeneration of NAD^+ and FAD, cofactors which participate in the process of β-oxidation.

Oxygen consumption by isolated liver mitochondria. To determine if clofibrate administration altered β-oxidation of fatty acids by affecting the respiratory chain, the effect of clofibrate administration on oxygen consumption was studied. Several substrates were used to supply electrons to different parts of the respiratory chain *in vitro*: glutamate, an NAD^+-dependent substrate, which donates electrons to NADH dehydrogenase; succinate, which reduces ubiquinone; and ascorbate, which reduces cytochrome *c*, near the terminus of the respiratory chain. After 14 days of clofibrate administration, a modest decrease of 15–25 per cent in mitochondrial oxygen consumption was observed with all substrates tested (Table 3). The extent of decrease was comparable under state 4 and state 3 conditions, suggesting that clofibrate administration affected the respiratory chain, rather than the energy coupling sequence. As a consequence, the respiratory control ratio (an index of mitochondrial integrity) was not altered by clofibrate (Table 3). If clofibrate affected the respiratory chain itself, the rapid rate of oxygen uptake in the presence of an uncoupling agent would also be decreased. Indeed, the rate of oxygen uptake in the presence of DNP was also decreased by clofibrate (Table 3). The extent of the decrease was comparable to that in state 4 and state 3 oxygen consumption. Clofibrate administration had no effect on the ratio of oxygen uptake in the presence of DNP to that in the absence of DNP. Thus, it appears that chronic administration of clofibrate has an inhibitory effect on the respiratory chain, but not on the phosphorylation sequence. These effects contrast with those of clofibrate *in vitro* [22], which are dependent on the substrate and the energy state of the mitochondria. After 18 days of clofibrate administration, oxygen uptake associated with the oxidation of all substrates tested was again depressed, with results similar to those observed after 14 days of clofibrate administration (Table 3). The respiratory control ratio was, however, not affected by prolonged clofibrate administration.

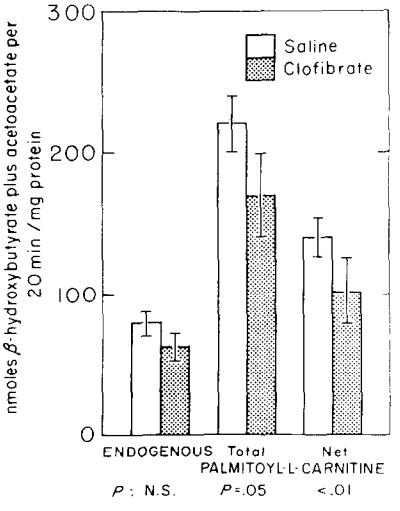

Fig. 2. Effect of chronic clofibrate administration on ketone body production. Ketogenesis was assayed as described in Materials and Methods in the absence (endogenous) or presence of 100 μM palmitoyl-*l*-carnitine. Results are from seven pairs of animals.

Clofibrate administration for 18 days depressed the activity of succinate-cytochrome *c* reductase 23 per cent (72.4 $\pm$ 7.4 nmoles cytochrome *c* reduced/min/mg of protein for controls, 55.6 $\pm$ 10.6 for rats treated with clofibrate), while succinate-dichlorophenol indophenol reductase activity was decreased 26 per cent. This suggests the possibility that one inhibitory effect of clofibrate administration may be in the succinate-ubiquinone oxidoreductase segment of the respiratory chain (complex II). The general extent of decrease of these partial electron transport reactions is similar to the decrease in succinate-linked oxygen uptake.

Energy production and utilization. Oxidative phosphorylation, as reflected in the ADP/O ratio, was not affected after 14 days of clofibrate administration (Table 4). The ATP-^{32}P exchange reaction, a partial reaction of oxidative phosphorylation, was slightly, but not significantly, decreased by clofibrate administration. The uptake of Ca^{2+} by the mitochondria may be energized by ATP itself or by energy derived from substrate oxidation [35]. Neither substrate-supported (glutamate or succinate) nor ATP-supported Ca^{2+} uptake was affected by clofibrate administration (Table 4). Energy-independent Ca^{2+} binding was also not affected by clofibrate. Again, these results contrast with the effects of clofibrate *in vitro* [22], in which case respiratory control, oxidative phosphorylation and Ca^{2+} uptake are depressed by clofibrate.

Mitochondrial integrity. The effects of clofibrate administration on mitochondrial fatty acid oxidation might reflect nonspecific effects on mitochondrial integrity or a greater sensitivity of mitochondria from rats treated with this drug to damage by the preparation techniques. Therefore, the effects of clofibrate administration on several reactions characteristic of mitochondrial damage were studied. Intact mitochondria are normally impermeable to NADH. Therefore, oxidation of NADH is often used as an index of mitochondrial damage [36]. The normally low permeability of intact liver mitochondria toward NADH was maintained in mitochondria from rats treated with clofibrate (1.82 $\pm$ 0.20 natoms oxygen consumed/min/mg of protein for controls, with 1 mM

Table 3. Effect of clofibrate administration on mitochondrial oxygen consumption*

	Substrate	Reaction	Activity: Control (natoms oxygen/min/mg) (or ratio)	Activity: Clofibrate (natoms oxygen/min/mg) (or ratio)	Effect (%)	P
A.	Glutamate (20)	State 4	19.7 ± 2.2	14.6 ± 1.0	−26	< 0.01
		State 3	95.7 ± 6.9	73.7 ± 4.4	−23	< 0.01
		Uncoupled	123.7 ± 9.9	91.4 ± 10.1	−26	< 0.05
		Respiratory control ratio	5.38 ± 0.37	6.11 ± 0.32	+14	NS
		Uncoupler control ratio	5.40 ± 0.25	6.15 ± 0.52	+14	NS
	Succinate (20)	State 4	30.9 ± 2.1	25.1 ± 1.2	−19	< 0.05
		State 3	150.2 ± 5.8	123 ± 5.1	−18	0.001
		Uncoupled	196.5 ± 13.7	152.1 ± 14.4	−23	< 0.05
		Respiratory control ratio	5.18 ± 0.3	4.82 ± 0.21	−7	NS
		Uncoupler control ratio	5.77 ± 0.37	4.99 ± 0.38	−13	NS
	Ascorbate (20)	State 4	85.0 ± 4.5	72.5 ± 4.0	−15	< 0.05
		State 3	152.6 ± 6.0	122.8 ± 5.9	−20	< 0.001
		Respiratory control ratio	1.64 ± 0.07	1.54 ± 0.06	−6	NS
B.	Glutamate (6)	State 4	12.8 ± 2.4	9.9 ± 1.8	−23	< 0.05
		State 3	73.7 ± 10.0	63.7 ± 4.3	−14	0.10 < P < 0.05
		Respiratory control ratio	5.81 ± 0.95	6.67 ± 0.78	+15	NS
	Succinate (6)	State 4	27.4 ± 2.1	22.4 ± 1.6	−18	< 0.01
		State 3	139.9 ± 9	102.7 ± 4.8	−27	< 0.01
		Respiratory control ratio	5.37 ± 0.70	4.73 ± 0.47	−12	NS
	Ascorbate (6)	State 4	70.7 ± 8.0	57.1 ± 4.4	−19	< 0.05
		State 3	93.2 ± 8.6	71.2 ± 4.4	−24	< 0.05
		Respiratory control ratio	1.40 ± 0.20	1.32 ± 0.18	−6	NS

* Oxygen consumption was assayed as described in Materials and Methods. State 4 respiration is the respiratory rate in the absence of ADP, state 3 respiration is the respiratory rate in the presence of 1.0 mM ADP, and uncoupled respiration is the respiratory rate in the presence of 0.08 mM DNP. Respiratory control is the state 3/state 4 ratio, while the analogous ratio in the presence of an uncoupler is the uncoupler control ratio (uncoupled respiration/state 4 respiration). Experiments A and B refer to 14 and 18 days of clofibrate administration respectively. In experiment A, results are from 20 pairs of animals, except for the uncoupler experiments, in which 8 pairs of animals were used.

NADH as substrate, 1.45 ± 0.20 for mitochondria from rats treated with clofibrate). This agrees with the previous finding that the endogenous rates of the reconstituted substrate shuttles (a measure of NADH penetration into mitochondria) were not altered by clofibrate treatment [23].

Disruption of mitochondrial integrity results in an elevated latent or Mg^{2+}-stimulated ATPase activity. Clofibrate, *in vitro*, slightly stimulated ATPase activity [22]. However, the extent of stimulation was far less than that observed with DNP. Chronic administration of clofibrate did not increase the activities

Table 4. Effect of clofibrate administration on mitochondrial energy production and utilization*

Reaction	No. of pairs	Substrate	Activity or ratio: Control	Activity or ratio: Clofibrate	Effect (%)
ADP/O ratio	5	Glutamate	2.71 ± 0.14	2.88 ± 0.18	+6
		Succinate	1.83 ± 0.12	1.84 ± 0.13	+1
		Ascorbate	0.89 ± 0.13	0.91 ± 0.12	+2
ATP-^{32}P exchange	5		258 ± 36	210 ± 45	−19
Energized Ca^{2+} uptake	13	Glutamate + ATP	364 ± 43.4	420 ± 48.9	+15
		Succinate + ATP	413 ± 51.8	473 ± 51.7	+15
		ATP	216 ± 26.9	229 ± 37.6	+6
		None + DNP	54 ± 8.2	57 ± 9.6	+6

* Reactions were assayed as described in Materials and Methods. Specific activity for the ATP-^{32}P exchange reaction refers to nmoles ATP-^{32}P formed/min/mg of protein. Specific activity for energized Ca^{2+} uptake refers to nmoles Ca^{2+} bound/mg of protein. The ADP/O ratio was calculated from additional oxygen consumed upon the addition of 1200 nmoles ADP. There were no statistical differences between mitochondria from clofibrate-treated rats and those of controls.

Table 5. Effect of clofibrate administration on oxygen consumption by muscle mitochondria*

Substrate	No. of pairs	Reaction	Oxygen consumption		Effect (%)
			Control (natoms oxygen/min/mg protein)	Clofibrate	
Glutamate	5	State 4	34.5 ± 4.0	25.6 ± 2.3	−26
		State 3	262.5 ± 28.3	215.0 ± 28	−18
		Respiratory control	7.78 ± 0.90	8.55 ± 1.30	+10
		ADP/O ratio	2.77 ± 0.24	2.86 ± 0.19	+4
Succinate	5	State 4	58.4 ± 2.0	57.8 ± 4.7	−1
		State 3	193.9 ± 19.1	176.1 ± 27.7	−9
		Respiratory control	3.31 ± 0.28	3.01 ± 0.32	−9
		ADP/O ratio	1.75 ± 0.12	1.71 ± 0.25	−2
Ascorbate	3	State 4	143.3 ± 14.8	131.0 ± 12.5	−9
		State 3	264.2 ± 1.60	227.3 ± 2.3	−14
		Respiratory control	1.85 ± 0.20	1.73 ± 0.05	−7
		ADP/O ratio	1.01 ± 0.17	1.01 ± 0.23	0
Palmitoyl-*l*-carnitine	3	State 4	26.4 ± 2.7	24.5 ± 0.8	−7
		State 3	147.3 ± 22.3	132.5 ± 11.1	−10
		Respiratory control	5.51 ± 0.30	5.87 ± 0.67	+7
		ADP/O ratio	2.67 ± 0.18	2.29 ± 0.20	−14

* Oxygen uptake was assayed as described in the legend to Table 3. In all cases, there were no statistically significant differences between controls and rats given clofibrate.

of latent, Mg^{2+}-stimulated and DNP-stimulated ATPase. In fact, these activities were slightly decreased (20–30 per cent).

Mitochondria isolated from rats treated with clofibrate underwent swelling in isotonic solutions of ammonium phosphate and malate but not in isotonic solutions of potassium phosphate or malate. Therefore, clofibrate administration did not alter the normally low permeability of intact mitochondria to K^+ (in the absence of an ionophore). In view of the maintenance of low permeability toward NADH and K^+, the lack of elevated ATPase activity and state 4 oxygen consumption, and the lack of effect on mitochondrial energy transduction and utilization, the effects of clofibrate administration on mitochondrial functions cannot be attributed to non-specific impairment of mitochondrial integrity.

Effect of clofibrate administration on muscle mitochondria. The effects of chronic clofibrate administration on oxygen consumption by isolated muscle mitochondria are shown in Table 5. Unlike the results with liver mitochondria, there was no effect of clofibrate administration on state 4 or state 3 oxygen uptake with succinate, ascorbate or palmitoyl-*l*-carnitine as substrates. Glutamate oxidation was decreased to a somewhat greater extent than that of other substrates, but this difference did not reach statistical significance ($0.10 > P > 0.05$, Table 5). As has been found with liver mitochondria, there was no effect of clofibrate on the respiratory control or ADP/O ratios with any of the substrates. CO_2 production was slightly increased from palmitate (+24 per cent), octanoate (+19 per cent) and acetate (+15 per cent) (means of three experiments). It appears that muscle mitochondria are more resistant to the effects of clofibrate than those of the liver.

DISCUSSION

Chronic administration of clofibrate produces changes in mitochondrial functions which persist in the absence of the drug. Some of the effects reported for clofibrate *in vitro* may not occur *in vivo*. *In vitro*, i.e. in the presence of the drug, clofibrate inhibits NAD^+-dependent oxygen consumption [19–22], oxidative phosphorylation [19–22], the ATP-^{32}P exchange reaction [22] and energized Ca^{2+} uptake [22], and stimulates ATPase activity [22]. After clofibrate administration, oxygen consumption and fatty acid oxidation are depressed in isolated hepatic mitochondria, whereas the other functions are not affected. These changes probably reflect chronic alterations of the mitochondria, because clofibrate is not present in the incubation medium, the mitochondria are washed, and clofibrate is not concentrated in the liver [37]. Hepatic levels of *p*-chlorophenoxyisobutyric acid, the active component produced upon hydrolysis of clofibrate, are low (at least in monkey liver) [38]. Several investigators [32, 39] have shown that, 12–24 hr after the last dose of clofibrate, liver size and triglyceride concentration revert to normal. Therefore, the alterations reported here represent a persistent effect produced by chronic administration of clofibrate.

It is possible that accelerated oxidation of fatty acids may contribute to the hypolipidemic action of clofibrate by reducing fatty acyl substrate levels. After 21 days of clofibrate treatment, palmitate oxidation to CO_2 and acetoacetate was increased [13]. The activities of long-chain acyl CoA synthetase and carnitine palmitoyl-transferase were doubled in the liver after 10 days of clofibrate feeding [33]. It was, therefore, suggested that after clofibrate feeding, there is increased fatty acid oxidation, with a greater part of

the activated fatty acid undergoing oxidation rather than glyceride formation [33]. However, the activities of the enzymes which activate or transport fatty acids into the mitochondria may not be rate limiting in fatty acid oxidation [29, 30]. Consequently, an effect on the enzymes which activate or translocate fatty acids into the mitochondria does not imply a similar effect on the actual oxidation of the fatty acid. A study was made, therefore, of the effects of chronic clofibrate administration on fatty acid oxidation by hepatic mitochondria. Oxygen consumption, CO_2 production and ketone body production associated with the oxidation of fatty acids are depressed about 30 per cent in isolated hepatic mitochondria. Thus, it is unlikely that the hypolipidemic action of clofibrate is mediated via accelerated oxidation of fatty acids by mitochondria. Since the activity of the citric acid cycle is not affected, inhibition of β-oxidation or the respiratory chain by clofibrate administration may be responsible for the decrease in β-oxidation of fatty acids. Pande [40] has suggested that oxidation of fatty acids by liver mitochondria is limited by the capacity of the electron transport–phosphorylation chain. Since skeletal muscle utilizes fatty acids and ketone bodies as a major fuel, we also studied the effects of chronic clofibrate administration on fatty acid oxidation by skeletal muscle mitochondria. The functions of muscle mitochondria, including fatty acid oxidation, were not affected by clofibrate administration. That clofibrate administration can have different effects in liver and skeletal muscle is not surprising, since it has been reported that the effects of clofibrate treatment on rat liver mitochondria are different from those on rat kidney mitochondria [41]. In the latter case, clofibrate feeding (0.5 per cent in diet) increased state 3 oxygen uptake with glutamate plus malate 40–60 per cent, whereas there was no significant effect with liver mitochondria [41].

A distinction should be made between the effects of clofibrate treatment on specific activities and on the functions of the total liver. Since the hepatic mitochondrial content of the rat increases 40–50 per cent, while fatty acid oxidation (30 per cent) or oxygen uptake (15–25 per cent) is depressed 30 and 15–25 per cent/mg of mitochondrial protein, respectively, the capacity of the whole liver to oxidize various substrates might actually increase. Westerfield *et al.* [42] found that the endogenous QO_2 for liver slices increased 30 per cent after feeding clofibrate for 3-5 weeks, while a 17 per cent increase was found after giving clofibrate (300 mg/kg) orally for 1 week [43]. These increases probably reflect the increase in mitochondrial content per g of liver. DNP-stimulated succinoxidase activity increased 41 per cent (expressed per mg liver protein) after 15 days of clofibrate feeding, but there was no significant effect (decrease of 12 per cent) when expressed per mg of mitochondrial protein [18]. The oxidation of cholesterol to CO_2 was increased 35–65 per cent after 3 weeks of clofibrate administration [32]. This increase was due to the increased liver weight, since there was no increase when the data were expressed per mg of nitrogen (protein) [32]. Therefore, the effect of clofibrate on the specific activities of various functions catalysed by isolated mitochondria, where activities are calculated per mg of mitochondrial protein, may differ from its effect on the total activity of the liver. Hence, inhibitions observed with isolated mitochondria may be overcome by the increase in total mitochondrial mass of 40–50 per cent. Thus, fatty acid oxidation may be elevated in the whole liver or animal, but not when expressed per mg of mitochondria protein. Indeed, Kritchevsky *et al.* [32] found that, after 3 weeks of administering clofibrate to rats, oxidation of octanoate by rat liver preparations was not altered. However, octanoate oxidation was decreased about 25 per cent when the data were expressed per mg of nitrogen (84 ± 5.7 to 63.2 ± 2.4 per cent, Table 5, Ref. 32). The lack of effect on skeletal muscle mitochondria, plus the possibility that the increased hepatic mitochondrial content compensates for the decrease in fatty acid oxidation observed per mg of mitochondrial protein, suggests that the hypolipidemic effect of clofibrate is not mediated via effects on mitochondrial fatty acid oxidation.

Chronic administration of clofibrate reduced oxygen consumption with various substrates in resting, energized and uncoupled hepatic mitochondria, but did not cause biochemical disruption of the mitochondrial membranes. This suggests that the drug inhibits the respiratory chain. Ascorbate oxidation may be impaired because of an effect on the activity of cytochrome oxidase. We previously found that clofibrate, *in vitro*, inhibits cytochrome oxidase activity [22]. Oxygen uptake with succinate as the substrate may be impaired because of damage to complex II. After 18 days of clofibrate administration, succinate-cytochrome and succinate-dichlorophenol indolephenol reductase activities were depressed by 25 per cent and CO_2 production from succinate was depressed by 22 per cent (Table 1). Our previous studies of clofibrate, *in vitro*, suggested that a clofibrate-sensitive site may reside in the NADH-ubiquinone oxidoreductase complex, whereas another site may involve succinic dehydrogenase or the reduced ubiquinone-cytochrome *c* oxidoreductase complex [22]. Mackerer *et al.* [20] also suggested that there are at least two distinct sites at which clofibrate *in vitro* can inhibit respiration, one in the NADH-ubiquinone oxidoreductase segment of the respiratory chain, the other between succinate dehydrogenase and cytochrome *c*. In agreement with the data of Kurup *et al.* [18], respiratory control and ADP/O ratios were not affected by clofibrate administration. In addition, we find no effect on ATP-^{32}P exchange or energized Ca^{2+} uptake. However, Kurup *et al.* [18] also reported that, after 15 days of clofibrate feeding, oxygen uptake with succinate or glutamate was not significantly affected. More recently, the administration of clofibrate to rats (100 and 300 mg/kg orally/day for 1 week) had no effect on oxygen uptake by isolated mitochondria with succinate or β-hydroxybutyrate as the substrates [43]. The addition of a very large amount of clofibrate (100 mg/kg/day via stomach tube) produced a decrease in succinate oxidase activity 6–12 hr after treatment, but no effect was observed after 7 days of treatment [44]. Thus, our findings that respiration is depressed 15–25 per cent after clofibrate administration may reflect somewhat different experimental conditions than those used by others, e.g. mode of administration (subcutaneous as opposed to oral administration

[18, 43, 44] or feeding experiments) [18], time interval after the last treatment with clofibrate and killing of the rat (e.g. 16 hr in our experiment, 1 hr in the experiments of Mackerer and Haettinger [43]) length of clofibrate administration (14 and 18 days vs 7) [43, 44], fasting [44] or not fasting the rats during the 16 hr prior to sacrifice. It is also possible that these differences may be more apparent than real, since the decrease in respiration observed here is only 15–25 per cent. Furthermore, oral, but not subcutaneous, administration of clofibrate would lead to rapid hydrolysis to the sodium salt, and the sodium derivative *in vitro* was a less potent inhibitor of mitochondrial functions than the ethyl ester [22].

Some hypocholesterolemic drugs can uncouple oxidative phosphorylation; it has been suggested that these drugs can affect hepatic ATP levels [21, 45]. ATP is required in the synthesis of cholesterol. However, in rats, clofibrate administration did not uncouple oxidative phosphorylation [18, 43] (Table 4) and 0.25 per cent clofibrate feeding for 14 days did not alter hepatic ATP levels [46]. Furthermore, the increased hepatic mitochondrial mass more than compensates for any decrease in ATP synthesis which could result from the slightly lower rate of respiration observed here after chronic clofibrate administration.

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