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EFFECTS OF CLOFIBRATE ON MITOCHONDRIAL FUNCTION

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Abstract—The effects of ethyl and sodium clofibrate on mitochondrial function were studied. Both compounds exerted similar effects, the ethyl derivative being more potent. State 3 respiration was inhibited; the order of inhibitory effectiveness was NAD^+-dependent substrates > succinate > ascorbate. State 4 NAD^+-linked oxidation was not significantly affected, but state 4 oxidations of succinate and ascorbate were stimulated by ethyl clofibrate. Energy production was inhibited, as evidenced by the decrease in the respiratory control ratio, the P/O ratio and the ATP-^{32}P exchange reaction. Energy utilization, assessed by substrate or ATP-supported energy-linked Ca^{2+} uptake, was also inhibited. By contrast, energy-independent Ca^{2+} uptake was not affected. Clofibrate interfered with the integrity of the mitochondrial membranes, since it stimulated ATPase activity and increased the normally low permeability of intact mitochondria toward NADH. The transfer of reducing equivalents into the mitochondria, catalyzed by the α-glycerophosphate, fatty acid or malate-aspartate shuttles, was inhibited by sodium clofibrate. These results may explain our previous finding that the reconstituted α-glycerophosphate shuttle was not stimulated in rats fed clofibrate, despite an increase in the activity of mitochondrial α-glycerophosphate dehydrogenase.

ETHYL CLOFIBRATE (ethyl-4-chlorophenoxyisobutyrate), because of its hypolipemic activity, is used in the treatment of atherosclerosis. When administered orally (400 mg/kg) to rats for 2–6 weeks, ethyl clofibrate lowers total lipids and cholesterol levels in blood and liver, while increasing the content of esterified fatty acids and phospholipids in the liver and heart.[1] Ethyl clofibrate is also effective in lowering serum triglyceride and cholesterol levels in man.[2] Administration of ethyl clofibrate increases the activity of mitochondrial α-glycerophosphate dehydrogenase.[3,4] Despite this increase, the activity of the reconstituted α-glycerophosphate shuttle for the transport of reducing equivalents into mitochondria was unchanged.[4] However, injection of thyroxin, which also increased the activity of mitochondrial α-glycerophosphate dehydrogenase, did increase the activity of the reconstituted α-glycerophosphate shuttle.[4] This suggested that clofibrate may have additional effects on the mitochondria, other than stimulation of α-glycerophosphate dehydrogenase activity, e.g. an inhibitory effect on mitochondrial respiration.[5,6] We, therefore, studied the effects of ethyl clofibrate on mitochondrial function. In addition, because the ethyl ester is hydrolyzed rapidly to the active[7] salt form *in vivo*,[8] we also studied the effects of sodium clofibrate.

MATERIALS AND METHODS

Sodium clofibrate was dissolved in water and ethyl clofibrate in 95% ethanol. In all experiments, controls contained the same amount of ethanol as did the ethyl clofibrate. These amounts of ethanol had no appreciable effect on the reactions studied.

Rat liver mitochondria were prepared as previously described.[9] The mitochondria were washed and suspended in 0·25 M sucrose–10 mM Tris HCl (pH 7·4)–1 mM EDTA. For those experiments in which Ca^{2+} uptake was studied, EDTA was omitted from the final two washings and resuspensions. All radioactive counting procedures were performed in a liquid scintillation counter, using a mixture consisting of 100 ml absolute methanol (spectranalyzed), 100 ml toluene (scintanalyzed), 7 g 2,5-diphenyloxazole, 0·5 g 1,4 bis 2-(5 phenyloxazol)benzene, 100 g naphthalene and 1 litre dioxane. Protein was determined according to Lowry *et al.*[10]

Oxygen consumption. O_2 uptake was assayed at 23° in a Yellow Springs oxygen monitor, equipped with a Clark oxygen electrode, using a reaction system consisting 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 and mitochondria equivalent to 2–4 mg protein, in a final volume of 3·0 ml. Substrates included 10 mM succinate, 5 mM ascorbate–0·2 mM TMPD*, 20 mM α-ketoglutarate and 10 mM glutamate. ADP (1·5 mM) was added to initiate state 3 conditions.

Oxidative phosphorylation. Oxidative phosphorylation was assayed at 30° using a Gilson differential manometer, as previously described.[11] The reaction mixture consisted of the same medium used for measurement of oxygen consumption, with the addition of 10 mM ATP, 16·7 mM succinate, 25 mM dextrose and 0·5 mg hexokinase (8·5 units, Sigma, Type III). The flasks were incubated for 10 min, ATP was tapped in from the side arm, the system was closed and manometer readings were taken every 5 min for the next 0·5 hr. The reaction was terminated by the addition of trichloroacetic acid (final concentration, 10%). After centrifugation, the P_i concentration was determined by the method of Sumner.[12] Blanks contained the acid added before the preincubation period.

ATP-^{32}P exchange. The ATP-^{32}P exchange reaction was assayed at 30° as described by Pullman,[13] using $^{32}PO_4$ (20,000 cpm/μmole P_i) and mitochondrial protein equivalent to 1·2 mg, in a final volume of 1·0 ml. The labeled ATP was extracted with isobutanol-benzene as described by Pullman[13] and aliquots were counted.

Ca^{2+} uptake. The uptake of Ca^{2+} by the mitochondria was assayed at 30° in the same medium used to study oxygen consumption, but with 0·5–1·0 mg mitochondrial protein and 1·0 mM $^{45}Ca^{2+}$ (200,000 cpm/μmole). Ten mM succinate plus 5 mM ATP or ATP itself served as the energy source. In the latter system, 5 μg antimycin was added to prevent endogenous respiration. The reaction was initiated by the addition of Ca^{2+}. After 2 min, the samples were passed through 0·45 μ Millipore filters. The filters were washed three times with ice-cold buffer, dried and counted. Blanks were processed in a similar manner except for the omission of mitochondria.

Enzyme assays. ATPase activity was determined as described previously.[11] Succinic dehydrogenase activity was assayed at 23° by following the reduction of phenazine methosulfate[14] in a reaction system consisting of 50 mM potassium phosphate, pH 7·4, 1·5 mM KCN, 1·2 mM phenazine methosulfate, 50 μM dichlorophenolindolephenol and freshly prepared intact mitochondria (about 0·1 mg protein). The reaction was initiated by the addition of succinate (final concentration of 10 mM). Cytochrome oxidase activity was assayed at 23° by following the oxidation of ferrocytochrome C[15] in a reaction system consisting of 10 mM potassium phosphate, pH 7·0, 40–45 μM reduced cytochrome c and freshly prepared intact mitochondria

* The abbreviation used is: TMPD, *N,N,N',N'*-tetramethyl-P-phenylenediamine.

(about 0·1 mg protein). Cytochrome c was reduced with ascorbate, and then dialyzed overnight against three changes of 10 mM phosphate buffer, pH 7·0. NADH oxidase activity was assayed spectrophotometrically by measuring the oxidation of NADH to NAD^+.

Transport and oxidation of reducing equivalents (shuttles). The reaction of ethanol with alcohol dehydrogenase generates NADH in the cytoplasm. In view of the virtual impermeability of the mitochondria to NADH,[16] several shuttle mechanisms have been proposed for the transport of reducing equivalents into the mitochondria, including the malate-aspartate,[17] fatty acid[18] and α-glycerophosphate shuttles.[19] The equilibrium of the alcohol dehydrogenase reaction favors formation of ethanol and NAD^+ from acetaldehyde and NADH. Therefore, the rate of ethanol disappearance is low in the absence of a shuttle mechanism to remove one of the products of the reaction (NADH). Since dissociation and reoxidation of NADH bound to the enzyme probably represent the rate-limiting step in the reaction,[20,21] the rate of ethanol disappearance reflects the rate of passage of reducing equivalents into the mitochondria. The malate-aspartate, α-glycerophosphate and fatty acid shuttles for the transport of reducing equivalents into the mitochondria were reconstituted, using isolated mitochondria and the extramitochondrial components of the shuttles, as previously described.[4,9,22] An NADH-generating system was produced by the addition of 0·25 mM NAD^+, 6 mM ethanol and 16 units of alcohol dehydrogenase. The fatty acid shuttle was reconstituted by adding 1 mM ATP, 0·2 mM coenzyme A and 0·1 mM of albumin-bound fatty acid. The malate-aspartate shuttle was assembled by adding 1 mM malate plus 1 mM glutamate, 3 units of malate dehydrogenase and 3 units of glutamic oxalacetic transaminase. The α-glycerophosphate shuttle was formed by adding 10 mM α-glycerophosphate, 1 mM ATP and 3 units of α-glycerophosphate dehydrogenase. The concentration of ethanol was determined by the method of Bonnichsen.[23]

RESULTS

Oxygen consumption. The effect of ethyl or sodium clofibrate on oxygen consumption depends on the substrate and the energy state of the mitochondria. Several substrates were used to supply electrons to different parts of the respiratory chain; glutamate or α-ketoglutarate, which are NAD^+-dependent; succinate, from which electrons are transferred to the cytochrome b–ubiquinone junction; and ascorbate–TMPD, which reduces cytochrome c, near the terminus of the chain. Under state 4 conditions (no ADP present), ethyl or sodium clofibrate had little effect on oxygen consumption when NAD^+-dependent substrates were used (Table 1). When ADP was added to initiate phosphorylation-linked respiration (state 3), both derivatives of clofibrate inhibited oxygen consumption, the ethyl ester being more potent. Thus, clofibrate inhibits energy-linked respiration, without any significant effect on resting respiration. Clofibrate also inhibited the oxidation of other NAD^+-dependent substrates, such as β-hydroxybutyrate and pyruvate. At ethyl clofibrate concentrations of 0·5, 1·0 and 2·0 mM, state 3 oxygen consumption was inhibited 18, 37 and 62 per cent with β-hydroxybutyrate as the substrate, and 24, 47 and 81 per cent with pyruvate as the substrate. The similarity of the effects of clofibrate on the oxidation of several NAD^+-dependent substrates suggests that the drug exerts an inhibitory effect

TABLE 1. EFFECT OF ETHYL AND SODIUM CLOFIBRATE ON MITOCHONDRIAL OXYGEN CONSUMPTION*

Addition	Concn (mM)	Oxygen consumption (n-atoms oxygen/min/mg protein) Glutamate State 4	Glutamate State 3	α-Ketoglutarate State 4	α-Ketoglutarate State 3	Succinate State 4	Succinate State 3	Ascorbate State 4	Ascorbate State 3
		9·08	49·90	9·75	56·09	20·8	84·1	64·4	100·3
Ethyl clofibrate	0·20	10·29	40·87	8·79	52·08	20·6	85·4	63·2	97·6
Ethyl clofibrate	0·50	9·32	38·43	10·19	41·07	22·5	81·3	70·5	97·6
Ethyl clofibrate	1·0	9·48	36·49	9·35	23·97	43·4	63·7	75·4	86·8
Ethyl clofibrate	2·0	8·22	21·02	8·35	12·34	48·8	55·6	72·9	85·8
Ethyl clofibrate	4·0	7·20	13·82	7·00	9·42	47·5	56·9	85·1	82·2
		10·20	51·92	8·32	59·10	24·4	84·06	70·5	104·1
Sodium clofibrate	0·20	8·80	44·92	7·48	45·35	24·4	86·80	65·7	99·7
Sodium clofibrate	0·50	8·60	40·22	7·63	44·08	27·1	84·06	67·1	99·2
Sodium clofibrate	1·0	9·90	32·29	7·81	38·41	26·6	82·0	69·5	87·5
Sodium clofibrate	2·0	9·65	28·30	7·75	29·93	29·8	70·0	68·1	85·1
Sodium clofibrate	4·0	9·50	20·13	7·10	17·03	35·3	66·9	70·5	77·8

* Oxygen uptake was assayed as described under Methods, using the indicated substrates. State 4 respiration refers to the respiratory rate in the absence of ADP, while state 3 respiration refers to the respiratory rate in the presence of 1·5 mM ADP.

directly on the respiratory chain; however, inhibition of the primary dehydrogenases cannot be ruled out since the extent of inhibition did vary among the different substrates.

Different results were obtained when succinate was the substrate (Table 1). State 4 respiration was appreciably stimulated by ethyl clofibrate (0·5–4 mM), but only slightly by sodium clofibrate (2–4 mM). The ethyl derivative also inhibited state 3 succinate oxidation, while the sodium salt was less effective. Both derivatives of clofibrate, however, were inhibitors of succinic dehydrogenase activity (25 and 50 per cent at 2 mM sodium or ethyl clofibrate respectively). At this concentration (2 mM), state 3 succinoxidase activity was inhibited 34 and 17 per cent by ethyl and sodium clofibrate respectively. However, state 4 respiration was stimulated 133 and 22 per cent by ethyl and sodium clofibrate respectively. Presumably, under state 4 conditions, the turnover of the enzyme is sufficiently rapid, so that some other factor may be rate-limiting. This may explain why state 4 succinoxidase activity is stimulated despite inhibition of succinic dehydrogenase activity. When the respiratory chain is operating maximally (ADP present), necessitating rapid turnover of succinic dehydrogenase, inhibition of enzyme activity may result in inhibition of oxygen consumption. It has been shown that the rate-limiting step in state 4 oxygen consumption with α-glycerophosphate as the substrate is not at the level of α-glycerophosphate dehydrogenase itself, but involves electron transport components beyond the level of the flavin.[24] The ascorbate-linked system is the least sensitive to clofibrate (Table 1). Ethyl clofibrate produced some stimulation of state 4 respiration and slight inhibition of the state 3 system. The sodium salt also inhibited state 3 respiration, but did not alter state 4 respiration. Both clofibrate derivatives inhibited cytochrome oxidase activity (16 per cent at 1 mM sodium clofibrate, 30 per cent at 1 mM ethyl clofibrate). At this concentration (1 mM), state 3 oxygen consumption with ascorbate as the substrate was inhibited 13 and 16 per cent by ethyl and sodium clofibrate respectively. However, state 4 oxygen consumption was unaffected by the sodium derivative and actually stimulated by the ethyl derivative (+ 16 per cent, Table 1). Again, it would appear that the turnover of the enzyme is sufficiently rapid so that, despite 16–30 per cent inhibition of cytochrome oxidase activity, the remaining activity is sufficient to allow state 4 oxygen consumption to continue unimpaired. The rate-limiting factor here is probably either the phophorylation state of the mitochondria or the reoxidation of reduced cytochrome a_3 by molecular oxygen. When the respiratory chain is operating maximally (ADP present), and maximum activity of cytochrome oxidase is required, inhibition of enzyme activity may result in inhibition of oxygen consumption.

Energy production and utilization. The data indicate that ethyl clofibrate, and to a lesser extent, sodium clofibrate, inhibit energy-linked respiration. This is confirmed by the depression of the respiratory control ratio by both clofibrate derivatives, regardless of the substrate (Table 2). Even the slight respiratory control associated with ascorbate oxidation was abolished by clofibrate. With glutamate and α-ketoglutarate, the ratio is depressed because of the inhibition of state 3 respiration, without any effect on state 4 respiration. With succinate or ascorbate, ethyl clofibrate stimulated state 4, but depressed state 3 respiration; hence, the decrease in the respiratory control ratio. The effects of clofibrate on the P/O ratio of oxidative phosphorylation and on the ATP-^{32}P exchange reaction, a partial reaction of oxidative phosphoryla-

TABLE 2. EFFECT OF ETHYL AND SODIUM CLOFIBRATE ON THE RESPIRATORY CONTROL RATIO*

Addition	Concn (mM)	Respiratory control ratio			
		Glutamate	α-Ketoglutarate	Succinate	Ascorbate-TMPD
		5·50	5·75	4·04	1·56
Ethyl clofibrate	0·20	4·73	5·92	4·14	1·54
Ethyl clofibrate	0·50	4·12	4·03	3·61	1·38
Ethyl clofibrate	1·0	3·85	2·56	1·47	1·15
Ethyl clofibrate	2·0	2·56	1·48	1·14	1·18
Ethyl clofibrate	4·0	1·92	1·35	1·20	0·97
		5·09	7·10	3·45	1·48
Sodium clofibrate	0·20	5·10	6·06	3·56	1·52
Sodium clofibrate	0·50	4·68	5·78	3·10	1·48
Sodium clofibrate	1·0	3·26	4·92	3·08	1·26
Sodium clofibrate	2·0	2·93	3·86	2·68	1·24
Sodium clofibrate	4·0	2·12	2·40	2·04	1·10

* Data were obtained from the state 4 and state 3 respiratory rates given in Table 1.

tion, were also measured. Both derivatives of clofibrate inhibited oxidative phosphorylation and the exchange reaction, the ethyl ester being somewhat more potent than the sodium salt (Table 3).

The uptake of Ca^{2+} by the mitochondria is an important energy-utilizing reaction which may be driven by ATP or by energy derived from substrate oxidation. The latter system is more efficient.[25] Ethyl clofibrate inhibited Ca^{2+} uptake by the mitochondria when energized by either system (Table 4). Sodium clofibrate inhibited to a lesser extent. In the absence of an energy source, the uptake of Ca^{2+} was depressed by about 95 per cent. Clofibrate had no effect on this energy-independent uptake of Ca^{2+} by the mitochondria.

TABLE 3. EFFECT OF ETHYL AND SODIUM CLOFIBRATE ON THE P/O RATIO OF OXIDATIVE PHOSPHORYLATION AND THE ATP-^{32}P EXCHANGE REACTION*

Addition	Concn (mM)	P/O ratio	ATP-^{32}P exchange (nmoles ATP-^{32}P/min/mg protein)	Inhibition (%) P/O	Inhibition (%) ATP-^{32}P
		1·87	108		
Ethyl clofibrate	0·20	1·75	100	6	7
Ethyl clofibrate	0·50	1·56	73	17	32
Ethyl clofibrate	1·0	0·97	35	48	67
Ethyl clofibrate	2·0	0·78	16	58	85
Ethyl clofibrate	4·0	0·27	0	86	100
		1·72	141		
Sodium clofibrate	0·20	1·70	133	1	6
Sodium clofibrate	0·50	1·60	109	7	22
Sodium clofibrate	1·0	1·22	85	29	40
Sodium clofibrate	2·0	0·95	57	45	60
Sodium clofibrate	4·0	0·33	22	81	84

* Reactions were assayed as described under Methods. Succinate was used as the substrate in determining the P/O ratio.

TABLE 4. EFFECT OF ETHYL AND SODIUM CLOFIBRATE ON ENERGIZED Ca^{2+} UPTAKE*

Addition	Concn (mM)	Ca^{2+} uptake (nmoles/mg protein) Succinate + ATP	ATP	Inhibition (%) Succinate + ATP	ATP
		424	137		
Ethyl clofibrate	0·20	408	116	4	15
Ethyl clofibrate	0·50	329	105	23	23
Ethyl clofibrate	1·0	237	80	44	42
Ethyl clofibrate	2·0	134	17	69	88
Ethyl clofibrate	4·0	21	10	95	93
		466	151		
Sodium clofibrate	0·20	398	146	15	3
Sodium clofibrate	0·50	352	135	25	11
Sodium clofibrate	1·0	295	121	37	20
Sodium clofibrate	2·0	286	99	39	34
Sodium clofibrate	4·0	266	73	43	52

* Reactions were assayed as described under Methods, using either 10 mM succinate plus 5 mM ATP or 5 mM ATP alone as the energy source.

Mitochondrial integrity. The inhibition of energy production and utilization by clofibrate suggests that this compound may affect the integrity of the mitochondria and act as an uncoupler of oxidative phosphorylation. Both ethyl and sodium clofibrate increased ATPase activity (Fig. 1). However, the extent of stimulation was not as great as that observed with dinitrophenol. When ATPase activity was stimulated by dinitrophenol, clofibrate did not further augment this activity. Because ethyl clofibrate is a more potent inhibitor of energy-linked reactions than sodium clofibrate,

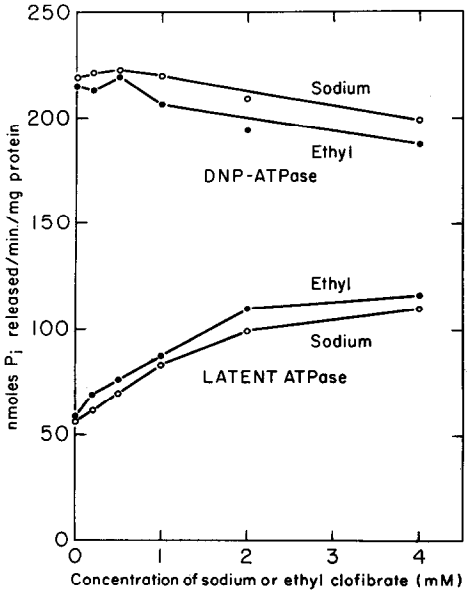

FIG. 1. Effect of ethyl and sodium clofibrate on mitochondrial ATPase activity. The reactions were assayed as described under Methods using 1–2 mg mitochondrial protein. The final concentration of dinitrophenol was 0·1 mM.

it seemed reasonable that the ethyl ester would compromise the integrity of the mitochondria to a greater extent than the sodium salt. Since the stimulation of ATPase activities by the two derivatives was comparable, we examined another index of mitochondrial integrity. The normal impermeability of mitochondria to pyridine nucleotides[16] allows the oxidation of NADH to be used as a marker of mitochondrial damage. Table 5 shows that whereas sodium clofibrate displayed minimal enhancement of NADH oxidation, ethyl clofibrate caused a significant increase at all concentrations. Thus, the greater inhibitory action of ethyl clofibrate may be related to its more pronounced effects on the stability of the mitochondria.

TABLE 5. EFFECT OF ETHYL AND SODIUM CLOFIBRATE ON THE PERMEABILITY OF MITOCHONDRIA TO NADH*

Addition	Concn (mM)	NADH oxidase (nmoles NADH oxidized/min/mg protein)	Effect (%)
		2·95	
Ethyl clofibrate	0·20	3·35	+14
Ethyl clofibrate	0·50	3·50	+19
Ethyl clofibrate	1·0	4·30	+46
Ethyl clofibrate	2·0	4·80	+63
Ethyl clofibrate	4·0	6·60	+106
		2·65	
Sodium clofibrate	0·20	2·80	+6
Sodium clofibrate	0·50	3·00	+13
Sodium clofibrate	1·0	2·90	+9
Sodium clofibrate	2·0	2·85	+7
Sodium clofibrate	4·0	2·70	+2

* NADH oxidation was assayed spectrophotometrically as described under Methods.

Transport of reducing equivalents into mitochondria. Our previous finding that clofibrate administration for 2 weeks failed to stimulate the activity of the α-glycerophosphate shuttle for the transport of reducing equivalents into the mitochondria, despite an increase in the activity of mitochondrial α-glycerophosphate dehydrogenase,[4] led us to investigate the effects of clofibrate *in vitro* on reconstituted shuttles. To assay the shuttles, we used ethanol, NAD^+ and alcohol dehydrogenase as a source of NADH, and measured ethanol disappearance. In attempting to dissolve the ethyl clofibrate in solvents other than ethanol, we found that ethyl clofibrate is hydrolyzed to ethanol and chlorophenoxyisobutyric acid. Therefore, using this system, we could not evaluate the effects of ethyl clofibrate on the shuttles. In the absence of the reconstituted shuttles, the rate of ethanol oxidation was 2·65 nmoles /min/mg of mitochondrial protein. This rate reflects the slight permeability of the mitochondria to NADH, and is consistent with the rates of NADH oxidation observed spectrophotometrically (Table 5). Upon reconstitution of the shuttles with α-glycerophosphate (α-glycerophosphate shuttle), palmitate (fatty acid shuttle) or glutamate plus malate (malate-aspartate shuttle), the rate increased 4- to 6-fold (Table 6). Sodium clofibrate inhibited all three reconstituted shuttles (Table 6). The fatty acid shuttle was somewhat more sensitive than the malate-aspartate and α-glycerophosphate shuttles.

TABLE 6. EFFECT OF SODIUM CLOFIBRATE ON THE ACTIVITIES OF THE α-GLYCEROPHOSPHATE, FATTY ACID AND MALATE-ASPARTATE SHUTTLES FOR THE TRANSPORT OF REDUCING EQUIVALENTS INTO MITOCHONDRIA*

Shuttle	Sodium clofibrate concn (mM)	Activity (nmoles ethanol oxidized/min/mg protein)	Inhibition (%)
α-Glycerophosphate		14·43	
	0·07	14·14	2
	0·20	11·92	17
	0·50	9·60	33
	1·0	8·49	41
	2·0	6·90	52
Fatty acid		10·01	
	0·07	8·41	16
	0·20	7·22	28
	0·50	5·34	47
	1·0	5·00	50
	2·0	4·79	52
Malate-aspartate		14·69	
	0·07	14·22	3
	0·20	12·26	17
	0·50	10·95	25
	1·0	10·05	32
	2·0	9·32	37

* Shuttles were reconstituted as described under Methods. The endogenous rate (the rate of ethanol disappearance in the absence of the shuttles) was 2·65 nmoles/min/mg protein.

The malate-aspartate shuttle can also be reconstituted by adding α-ketoglutarate plus aspartate, instead of glutamate and malate. Under these conditions, ethanol is oxidized even in the absence of mitochondria, since the oxalacetate generated from aspartate (via transamination) will, in the presence of malate dehydrogenase, oxidize the NADH formed from the alcohol dehydrogenase reaction.[4] Sodium clofibrate had no significant effect on this extramitochondrial system (Table 7), at concentrations which inhibited the mitochondrial system reconstituted with glutamate and malate. Thus, inhibition of shuttles by clofibrate is due to interaction of the drug with the mitochondria. Clofibrate had no effect on the activities of glutamic-oxalacetic transaminase, malate dehydrogenase or α-glycerophosphate dehydrogenase.

TABLE 7. EFFECT OF SODIUM CLOFIBRATE ON A MITOCHONDRIAL-INDEPENDENT SYSTEM CAPABLE OF ETHANOL OXIDATION*

Addition	Concn (mM)	Specific activity (nmoles ethanol oxidized/min)	Effect (%)
		75·4	
Sodium clofibrate	0·20	77·0	+2
Sodium clofibrate	0·50	72·8	−3
Sodium clofibrate	1·0	71·8	−5
Sodium clofibrate	2·0	68·6	−9
Sodium clofibrate	4·0	66·6	−12

* The malate-aspartate shuttle was reconstituted as described under Methods, except that 1·33 mM α-ketoglutarate plus 5 mM aspartate was added instead of glutamate, malate and mitochondria.

DISCUSSION

Clofibrate inhibits numerous mitochondrial functions, including energy production and utilization, oxygen consumption and the transport of reducing equivalents into the mitochondria. Several effects are similar to those attributed to uncouplers of oxidative phosphorylation, e.g. stimulation of ATPase activity and inhibition of the P/O ratio and energized Ca^{2+} uptake. Clofibrate is similar to the classical uncouplers, such as 2,4-dinitrophenol, in possessing an ionizable hydrogen. This ionizable ion is believed to play an important role in the uncoupling action.[26] The more potent inhibitory effect of the ethyl ester, compared to the sodium salt, may be due to its hydrophobic character, which might lead to greater access to or reaction with the inner membrane. Most of the mitochondrial functions measured in this study occur in the lipid-rich inner membrane. It has been reported that feeding 0·25% clofibrate for 14 days to rats caused no change in hepatic ATP levels.[27] This may reflect low hepatic levels of clofibrate.[8] In addition, hydrolysis of the ethyl ester to the sodium salt may reduce any potential toxicity of the drug, since the latter form is less inhibitory toward several mitochondrial functions than the ethyl ester. Nevertheless, the metabolic alterations,[28,29] as well as the structural changes[30] produced by clofibrate administration, indicate some interaction of the drug with the liver. In addition, Thorp[31] previously indicated that the respiratory quotient and oxygen consumption are decreased by administration of clofibrate to rats.

It has been reported that incorporation of 0·25% ethyl clofibrate in the diet (200 mg/kg/day) to rats, or oral administration (500 mg/day) to dogs for 2 weeks, results in plasma clofibrate concentrations of 0–250 mg/ml (0–1 mM).[7] *In vitro*, addition of 250–1000 mg/ml (1–4 mM) ethyl clofibrate inhibited fatty acid release from epididymal pads incubated in plasma.[32] These concentrations were two to ten times greater than effective plasma levels of clofibrate *in vivo* (0·4–2 mM).[32] *In vitro*, 0·5 and 5 mM ethyl clofibrate inhibited the activity of glycerol 3-phosphate acyltransferase 11 and 66 per cent respectively.[33] Serum clofibrate levels in that study ranged from 0·5 to 1·5 mM after feeding rats 0·25% clofibrate for 14 days, concentrations comparable to those employed here. Clofibrate inhibited state 3 oxidation, the order of inhibitory effectiveness being NAD^+-dependent substrates > succinate > ascorbate. Since coupling sites are located in complexes I, III and IV of the respiratory chain, the order of inhibitory effectiveness shown by clofibrate may be related to the number of coupling sites involved in the oxidation of each substrate, i.e. three for NAD^+-linked substrates, two for succinate and one for ascorbate. Thus, a clofibrate-sensitive site apparently is located in the NADH-ubiquinone oxidoreductase complex (complex I), while another site may involve succinic dehydrogenase, or reside in complex III (reduced ubiquinone–cytochrome c oxidoreductase). We find that clofibrate can inhibit succinic dehydrogenase activity. The inhibition of ascorbate-linked oxygen consumption may be related to the inhibition of cytochrome oxidase activity. Ethyl clofibrate stimulates state 4 oxygen consumption when succinate or ascorbate serve as substrates, but in contrast to the results of Mackerer *et al.*[5] has no effect with NAD^+-linked substrates. However the increase in state 4 β-hydroxybutyrate oxidation found by those authors was small relative to the increases in state 4 succinate or ascorbate oxidation. Katyal *et al.*[6] also found that ethyl clofibrate had no effect on state 4 oxidation with NAD^+-dependent substrates. Since succinic dehydrogenase and cytochrome oxidase are inhibited by ethyl clofibrate, the

observed increase in state 4 oxidation with succinate and ascorbate–TMPD may be related to a permeability factor. It has been suggested that the rate of succinate oxidation is regulated by the uptake of succinate into the mitochondria.[34,35] Therefore, ethyl clofibrate may enhance uptake or penetration of the substrates to their sites of metabolism. Preliminary experiments, using the qualitative approach of mitochondrial swelling in isotonic solutions of their ammonium salts,[36,37] indicate that ethyl clofibrate causes a slight increase in the rate and extent of swelling of mitochondria in ammonium malate (a substrate for the dicarboxylate carrier which also transports succinate into the mitochondria,[36,37]) which suggests an increased uptake of malate. A permeability factor has been invoked to explain the effects of ethyl clofibrate on ascorbate-linked oxygen consumption, because the inhibition of state 3 oxidation by clofibrate in intact mitochondria was not seen with sonicated mitochondrial fragments.[5]

We have previously found that the fatty acid, α-glycerophosphate and malate-aspartate shuttles were all NAD^+-dependent, and operated most efficiently under energy-linked conditions.[4] Therefore, the inhibition of NAD^+-dependent state 3 oxygen consumption by sodium clofibrate, as well as its effect on energy production and utilization by the mitochondria (Tables 2–4), suggest that this drug may interfere with the operation of the shuttles. Indeed, sodium clofibrate was inhibitory toward all three reconstituted shuttles. That the drug inhibits the shuttles by reacting with the mitochondria is supported by the fact that the activity of extramitochondrial component of the malate-aspartate shuttle, which is also capable of oxidizing ethanol,[4] was not significantly affected by sodium clofibrate. The inhibition of the shuttles by clofibrate may explain our previous finding that the α-glycerophosphate shuttle was not stimulated by administration of ethyl clofibrate *in vivo* for 2 weeks, despite an increase in the activity of mitochondrial α-glycerophosphate dehydrogenase.[4]

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