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EFFECT OF CLOFIBRATE FEEDING ON PALMITATE AND BRANCHED-CHAIN 2-OXO ACID OXIDATION IN RAT LIVER AND MUSCLE

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Abstract—Oxidation rates of palmitate (total and antimycin-insensitive), pyruvate, leucine, 4-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate and activities of two mitochondrial marker enzymes (citrate synthase and cytochrome *c* oxidase) were assayed in liver and muscle homogenates of fed, clofibrate-treated and 18 hr-starved rats. Significant alterations in the clofibrate-treated and the starved rats were predominantly observed in the liver. Clofibrate feeding increased antimycin-insensitive (peroxisomal) and antimycin-sensitive (mitochondrial) palmitate oxidation and 4-methyl-2-oxopentanoate and pyruvate oxidation in liver. In muscle, only the activities of citrate synthase and cytochrome *c* oxidase were slightly decreased. Short starvation increased antimycin-sensitive palmitate and 4-methyl-2-oxopentanoate oxidation in liver. The rates of pyruvate and 3-methyl-2-oxobutanoate oxidation were decreased in muscle homogenates. Results suggest that myopathic phenomena observed after chronic clofibrate administration are not related to changes in the capacity of oxidative metabolism of muscle.

Clofibrate [ethyl 2-(4-chlorophenoxy) 2-methylpropanoate] is an effective hypolipidemic and hypocholesterolemic agent in man and rat [1, 2]. Chronic administration of this drug to rodents increases the weight of the liver and its content of mitochondria [3, 4] and peroxisomes (reviewed in ref. [5]). The marked increase of the peroxisomal β-oxidation of fatty acids [6, 7] suggested a prominent role of this system in the effects of clofibrate. Both mitochondrial and peroxisomal oxidation of palmitate appear, however, to increase markedly in hepatocytes and liver homogenates of clofibrate-fed rats [8–12]. Perfused livers from such rats showed an increased oleate oxidation, but also an increased fatty acid synthesis, measured with ^3H_2O incorporation [13]. The drug induces, especially, a series of enzymes and proteins involved in hepatic fatty acid metabolism [12]. Oxidation of pyruvate and succinate was unaltered in liver homogenates [8], like that of acetate and various citric acid cycle intermediates in isolated liver mitochondria from clofibrate-fed rats [14, 15]. The oxidation of palmitoylcarnitine by isolated liver mitochondria has been reported to decrease [14], to increase [10, 16] and not to change [11], presumably due to variations in conditions of assay or in mitochondrial preparations.

Clofibrate appeared also to affect occasionally skeletal muscle in treated patients [17, 18]. This effect was partly related to an impaired fatty acid and glucose oxidation according to Paul and Adibi [19]. These investigators used, however, $^{14}CO_2$ production as a measure for fatty acid oxidation. Since $^{14}CO_2$ forms only a small fraction of the oxidation products from [^{14}C]palmitate in homogenates of liver and muscle [8, 10, 20], we re-evaluated their data by determining palmitate oxidation from the sum of $^{14}CO_2$ and ^{14}C-labeled acid-soluble products. Simultaneously, we determined the rate of antimycin-insensitive oxidation in muscle to explore possible peroxisomal contribution.

Recently, Paul and Adibi [21] reported an increase of leucine and 4-methyl-2-oxopentanoate (2-ketoisocaproate) oxidation in muscle homogenate of clofibrate-fed rats. The rate of leucine oxidation by liver homogenates was not changed in these animals. In liver, leucine oxidation is, however, limited by the low transaminase activity [22, 23]. Our oxidation rates in muscle from 18 hr-starved animals [23] appeared also to be higher than their values in fed animals [21]. Therefore, we assayed the oxidation rates of leucine, 4-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate in liver and muscle homogenates from fed, clofibrate-treated fed and 18 hr-starved rats. These activities were compared with the activities of pyruvate oxidation and of two mitochondrial marker enzymes, citrate synthase (EC 4.1.3.7) and cytochrome *c* oxidase (EC 1.9.3.1).

MATERIALS AND METHODS

Materials. Clofibrate (Atromid S) was obtained from ICI, Rotterdam, The Netherlands. Cytochrome *c*, antimycin A, coenzyme A and acetyl-CoA were purchased from Boehringer-Mannheim, West Germany. L-Carnitine was a gift from Sigma-Tau, Rome, Italy. Radioactive substrates were obtained or prepared as described previously [20, 23].

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Animals. Male Wistar rats of 200–240 g were used. Animals were fed with a pelleted diet, containing 22% protein, 4.8% fat and 67% carbohydrates. Clofibrate-treated rats were given it orally 50 mg/day in 1 ml 60% (v/v) glycerol solution for 7–10 days. Rats were sacrificed by cervical dislocation.

Assays. Liver and m. quadriceps were rapidly excised and homogenized in 19 and 9 vols., respectively, of a cold (4°) buffer, containing 0.25 M sucrose, 2 mM EDTA and 10 mM Tris-HCl (pH 7.4).

The palmitate oxidation rate was measured in a total volume of 0.5 ml medium, containing 50 μl whole homogenate and 25 mM sucrose, 75 mM Tris-HCl (pH 7.4), 10 mM K_2HPO_4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM NAD^+, 5 mM ATP, 25 μM cytochrome *c*, 0.1 mM coenzyme A, 0.5 mM L-malate and 0.5 mM L-carnitine. The concentration of [1-^{14}C]- or [16-^{14}C]palmitate (bound to albumin in a molar ratio of 5 : 1) was 120 μM. In parallel incubations, 36 μM antimycin A was added to inhibit mitochondrial oxidation. After 30 min incubation at 37° the incubation was stopped by addition of 0.2 ml 3 M perchloric acid. Radioactivity of trapped CO_2 and of acid-soluble products was measured as described in ref. [20].

Oxidative decarboxylation rates of 0.5 mM [1-^{14}C]leucine, 0.1 mM 4-methyl-2-oxo[1-^{14}C]pentanoate or 0.1 mM 3-methyl-2-oxo[1-^{14}C]butanoate were determined at 37° in a medium described previously [23]. Pyruvate oxidation was assayed in the same medium with 1 mM [1-^{14}C]-pyruvate and 1 mM malate (instead of 2-oxoglutarate). Incubation occurred in a final volume of 0.5 ml containing 0.1 ml of homogenate. Substrate was added after a preincubation of 5 min at 37°. After 15 min, incubation reactions were terminated by addition of 0.2 ml 3 M perchloric acid. $^{14}CO_2$ was trapped and measured as described previously [23].

The assay procedure of cytochrome *c* oxidase was earlier described [20]. Citrate synthase activity was assayed in the 10,000 *g* supernatant from sonicated homogenates according to Shepherd and Garland [24].

All activities were calculated per g tissue (wet wt). Statistical significance was analysed with Student's *t*-test for unpaired data.

RESULTS

All assays were established to be proportional with time of incubation and amount of tissue material. The concentrations of substrates, coenzymes and cofactors were optimal [20, 23]. Only the concentrations of branched-chain 2-oxo-acids and of leucine were suboptimal, but adapted to physiological conditions. Clofibrate treatment had no effect on rat weights.

Both clofibrate feeding and short starvation increased very significantly the total palmitate oxidation capacity per g wet wt of rat liver homogenates (Table 1). Since starvation caused a decrease of liver weight by 23% (from 10.2 ± 0.4 to 7.9 ± 0.5 g, $n = 5$), the total oxidation capacity expressed per whole liver increased by about 50%. Based on cytochrome *c* oxidase and citrate synthase activity the palmitate oxidation rates increased in liver homogenates at 18 hr starvation by 38 and 41%, respectively.

$^{14}CO_2$ production was only about 6, 4 and 1% of the total oxidation rate of [1-^{14}C]palmitate with liver homogenates from fed, clofibrate-fed and 18 hr-starved rats, respectively. The rate of $^{14}CO_2$ production was, therefore, also higher in liver homogenates with clofibrate-fed rats than with fed animals, but lower with 18 hr-starved animals.

Antimycin completely inhibited $^{14}CO_2$ production from [1-^{14}C]- and [16-^{14}C]palmitate and reduced the acid-soluble products from [16-^{14}C]palmitate to about 10%. The latter values were applied to correct the rate of [1-^{14}C]palmitate oxidation in the presence of antimycin for mitochondrial contribution. Starvation increased only the antimycin-sensitive, or mitochondrial, oxidation, while clofibrate treatment increased also the antimycin- insensitive oxidation in liver homogenates (Table 1).

In muscle homogenates, $^{14}CO_2$ was in all three conditions about 4—6% of the sum of $^{14}CO_2$ and radioactive acid-soluble products. $^{14}CO_2$ production rates were lower with clofibrate-fed rats than with fed animals [4.1 ± 1.0 (10) vs 7.5 ± 2.6 (9) nmoles/min per g wet wt]. The latter observations are similar to those of Paul and Adibi [19]. When the palmitate oxidation rates were, however, calculated from the

Table 1. Effect of clofibrate treatment and short starvation on total and antimycin-insensitive palmitate oxidation in liver and muscle homogenates

		Palmitate oxidation		
Tissue	Condition	Total (nmoles/min per g wet wt)	Antimycin-insensitive (nmoles/min per g wet wt)	(% of total)
Liver	Fed (5)	366 ± 28	142 ± 17	39 ± 5
	Clofibrate-fed (10)	978 ± 255*	390 ± 74*	37 ± 5
	Starved 18 hr (8)	713 ± 92*	165 ± 16	23 ± 2*
Muscle	Fed (10)	130 ± 24	15 ± 2	11 ± 2
	Clofibrate-fed (10)	125 ± 27	15 ± 2	11 ± 1
	Starved 18 hr (11)	115 ± 19	16 ± 5	14 ± 3

Values are means ± S.D. of the number of animals given within parentheses. Total palmitate oxidation was determined from $^{14}CO_2$ and ^{14}C-labeled acid-soluble products formed with [1-^{14}C]palmitate as substrate. Antimycin-insensitive palmitate oxidation was calculated from the difference in oxidation rates of [1-^{14}C]- and [16-^{14}C]palmitate in the presence of 36 μM antimycin A. P vs fed animals * < 0.001.

Table 2. Effect of clofibrate treatment and short starvation on leucine and branched-chain 2-oxo-acid oxidation in liver and muscle homogenates

Tissue	Condition	Oxidation rate (nmoles/min per g wet wt)		
		Leucine	4-Methyl-2-oxopentanoate	3-Methyl-2-oxobutanoate
Liver	Fed	10.4 ± 1.4 (5)	128 ± 25 (7)	259 ± 27 (5)
	Clofibrate-fed	11.9 ± 1.9 (5)	322 ± 34* (11)	294 ± 46 (5)
	Starved 18 hr	10.6 ± 0.4 (4)	233 ± 44* (12)	288 ± 55 (11)
Muscle	Fed	27.8 ± 5.6 (5)	29.6 ± 6.6 (5)	77.4 ± 19.6 (5)
	Clofibrate-fed	23.6 ± 6.5 (4)	23.6 ± 3.9 (6)	60.9 ± 9.9 (4)
	Starved 18 hr	22.2 ± 7.4 (5)	27.6 ± 7.9 (13)	58.5 ± 16.4 (13)

Values are means ± S.D. of the number of animals given within parentheses. P vs fed animals * <0.001.

sum of $^{14}CO_2$ and ^{14}C-labeled acid-soluble products, no differences were observed between the muscle homogenates from rats in all three conditions (Table 1). The contribution of the antimycin-insensitive palmitate oxidation was also not influenced in muscle homogenates by the condition of the rat.

Activities of branched-chain 2-oxo-acid and leucine oxidation are maximal at the used substrate concentrations. The short preincubation of 5 min at 37° assured maximal activity. The amounts of endogenous substrates are negligible in comparison to the added ^{14}C-labeled substrates (<1%). Since addition of the transaminase inhibitor, amino-oxyacetate (1 mM), did not affect the oxidation rates measured for the branched-chain 2-oxo-acids (data not shown), the endogenous production of 2-oxo acids during the assay can be neglected.

Leucine oxidation by rat liver homogenates was not dependent on the condition of the rat, in contrast to the oxidative decarboxylation of its 2-oxo-acid derivative, 4-methyl-2-oxopentanoate, which was markedly increased by clofibrate feeding and starvation (Table 2). The oxidation rate of 3-methyl-2-oxobutanoate, the 2-oxo-acid derived from valine, did not change in liver homogenates in both conditions. Just like palmitate oxidation, leucine and branched-chain 2-oxo acid oxidation were not markedly changed in skeletal muscle homogenates by clofibrate feeding or starvation (Table 2). Clofibrate treatment did also not affect the oxidation of 4-methyl-2-oxopentanoate by intact rat hemidiaphragm. Rates at a 0.1 mM concentration of this 2-oxo-acid were 3.8 ± 0.4 and 2.8 ± 0.7 nmoles/min per g muscle (means ± S.D. for six fed and three clofibrate-fed animals, respectively).

Clofibrate treatment increased pyruvate oxidation in liver homogenates, but did not change the activities of citrate synthase or cytochrome *c* oxidase (Table 3). The only significant effect of clofibrate on muscle was a decrease of the activities of these mito-

Table 3. Effect of clofibrate treatment and short starvation on pyruvate oxidation and activities of citrate synthase and cytochrome *c* oxidase in liver and muscle homogenates

Tissue	Condition	Pyruvate oxidation rate	Citrate synthase	Cytochrome *c* oxidase
		(μmoles/min per g wet wt)		
Liver	Fed	2.55 ± 0.84 (8)	11.7 ± 1.2 (10)	286 ± 41 (7)
	Clofibrate-fed	4.32 ± 0.50** (7)	13.8 ± 1.8 (10)	355 ± 38 (10)
	Starved 18 hr	2.84 ± 0.64 (13)	15.4 ± 2.0** (41)	317 ± 98 (30)
Muscle	Fed	1.96 ± 0.35 (5)	11.9 ± 2.6 (12)	81 ± 12 (9)
	Clofibrate-fed	1.76 ± 0.30 (6)	8.5 ± 1.0** (10)	67 ± 7* (10)
	Starved 18 hr	1.35 ± 0.30* (14)	13.5 ± 2.6 (56)	68 ± 20 (41)

Values are means ± S.D. of the number of animals given within parentheses. P vs fed animals * <0.01; ** <0.001.

chondrial marker enzymes. Short starvation increased liver citrate synthase activity and decreased the pyruvate oxidation in muscle homogenates.

DISCUSSION

A simultaneous estimation of the effect of clofibrate feeding on the oxidative capacity for various substrates in liver and muscle homogenates was not earlier reported. Together with palmitate oxidation, both mitochondrial and peroxisomal [6–9, 12] (Table 1), also pyruvate and 4-methyl-2-oxopentanoate oxidation increased in liver homogenates, but 3-methyl-2-oxobutanoate oxidation and the activities of citrate synthase and cytochrome *c* oxidase were not changed. The latter observations establish that not all mitochondrial activities increase, as was earlier also found with isolated liver mitochondria [14, 15]. The unchanged cytochrome *c* oxidase activity was also reported by Mannaerts *et al.* [8] in combination with an increased glutamate dehydrogenase activity. Lazarow [6, 7] noted a variability in the effects of clofibrate on cytochrome *c* oxidase activity. The lower concentration of cytochrome aa_3 in liver mitochondria from clofibrate-treated rats [5] is in accordance with the simultaneous increase of mitochondrial protein [5] and the unchanged cytochrome *c* oxidase activity.

Our observations on the effect of clofibrate feeding on fatty acid oxidation in liver are in accordance with earlier results [8–12]. With our assay of $^{14}CO_2$ and ^{14}C-labeled acid-soluble products we did not find a decrease of palmitate oxidation in muscle homogenates, as deduced by Paul and Adibi [19] from $^{14}CO_2$ production. No effect of clofibrate administration was also observed on the oxygen consumption of muscle mitochondria with palmitoyl-L-carnitine as substrate [14]. In perfused rat heart and isolated rat heart mitochondria [25] clofibrate feeding appeared to increase palmitate oxidation by about 25 and 48%, respectively.

Starvation for only 15–24 hr increases fatty acid oxidation in liver homogenates, when it is based on wet wt [26] (Table 1). In contrast to ref. [26], we found also an increase of oxidation capacity for whole liver. In hepatocytes this process is also increased [27, 28]. No increase was observed with liver mitochondria [29–31], muscle homogenates [33] (Table 1) and mitochondria [32] or intact muscle preparations [33]. The mitochondrial content of liver from starved rats shows only a small increase, when based on citrate synthase (Table 3), in contrast to the reported 60% increase, based on glutamate dehydrogenase activity [26].

Leucine oxidation in liver homogenates is not affected by clofibrate administration [21] (Table 2), due to the low transaminase activity [22, 23], since the increase of the oxidation rate of its derived 2-oxo-acid indicates an elevated branched-chain 2-oxo-acid dehydrogenase activity. This is, however, not found with the 2-oxo-acid derived from valine, These observations suggest that additional activity of the cytosolic branched-chain 2-oxo-acid oxidase, which converts only the oxo-acid derived from leucine [34], is induced by clofibrate feeding, probably in response to the inhibition of the mitochondrial branched-chain 2-oxo-acid dehydrogenase activity by clofibric acid [35].

In muscle, oxidation of leucine and branched-chain 2-oxo-acids were not increased by clofibrate, in contrast to the observations of Paul and Adibi [21]. Our activities in muscle are higher than those reported by others [21, 22], presumably due to a more gentle homogenization procedure [23]. The unchanged pyruvate oxidation in muscle homogenates from clofibrate-treated rats is in contrast with the impaired glucose oxidation reported [19]. We observed only a slight decrease of the activities of citrate synthase and cytochrome *c* oxidase, but these enzymes are not rate-limiting in oxidative metabolism.

Our results do not sustain the conclusions of Paul and Adibi [19, 21] that fatty acid and glucose oxidation are impaired and leucine and branched-chain 2-oxo-acid oxidation are increased in muscle of clofibrate-fed rats. The effects of clofibrate feeding on liver metabolism can, however, indirectly influence muscle metabolism *in vivo*. Clofibric acid [2-(4-chlorophenoxy)2-methylpropanoate], which is present in a high concentration in the plasma during clofibrate treatment [13], may also influence the uptake of metabolites, their oxidative metabolism in muscle and/or the membrane characteristics of muscle and induce in this way myopathic phenomena. Addition of clofibric acid inhibits leucine oxidation in incubations of rat diaphragm preparations and of rat cultured skeletal muscle cells [36]. Clofibric acid may displace other organic ligands from plasma albumin into tissues, as suggested already by Thorp in 1963 [37]. It interferes with the binding of fatty acids [38]. We found recently that also the binding of 4-methyl-2-oxopentanoate to albumin [39] was inhibited by this derivative of clofibrate.

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