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EFFECT OF CLOFIBRATE ON BRANCHED-CHAIN AMINO ACID METABOLISM

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Abstract—Clofibric acid inhibited the oxidative decarboxylation of 4-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate in mitochondria and homogenates of rat liver and quadriceps muscle. In rat hemidiaphragms clofibric acid inhibited the oxidative decarboxylation of 4-methyl-2-oxopentanoate and had no effect on that of 3-methyl-2-oxobutanoate. Clofibric acid displaced branched-chain 2-oxo acids from bovine serum albumin. Clofibrate-treatment of rats decreased the actual activity and activity state of the branched-chain 2-oxo acid dehydrogenase complex in quadriceps muscle, and increased the total activity in heart and liver without a change of the activity state. All interactions of clofibric acid with the metabolism of branched-chain amino acids appear to relate to its structural resemblance to the branched-chain 2-oxo acids. Both reduced plasma and muscle concentrations of branched-chain amino acids and reduced muscle oxidation may play a role in the myopathic side-effects of clofibrate-treatment.

Clofibrate has been widely used as a therapeutic agent in the treatment of patients with hyperlipidemia. Chronic treatment with this drug occasionally causes myopathy in both man [1] and rat [2]. The urinary excretion of 3-methylhistidine is higher [3] and the total muscle protein content lower [4] in clofibrate-fed than in control rats. Furthermore, the release of tyrosine is higher in muscle slices from clofibrate-fed than from control rats [3]. Together, these data indicate that muscle protein degradation is increased in clofibrate-fed rats. Clofibrate-administration decreases the concentration of all three branched-chain amino acids in rat muscle [3] and human blood [5] and that of leucine in rat plasma [3]. Since protein turnover in skeletal muscle is believed to be linked to the metabolism of the branched-chain amino acids and in particular to that of leucine (reviewed in [6]), the decrease of the branched-chain amino acid concentrations may contribute to the myopathic phenomena.

Decreased concentrations of the branched-chain amino acids may reflect increased oxidation of these compounds in one or more tissues. Most investigations to localize the site of the increased oxidation were done before much was known about the regulation of the branched-chain 2-oxo acid dehydrogenase complex by covalent modification, with activation by dephosphorylation and inactivation by phosphorylation (reviewed in [7]). Hence, it is not evident whether branched-chain 2-oxo acid dehydrogenase activities measured in tissue homogenates of clofibrate-fed and control rats (see e.g. [3] and [8]) reflect actual activities present in the tissues or whether they have been changed during preparation and incubation of the homogenates. Therefore, we have applied in the present study our assays to measure both the actual activity and the total activity (100% active state) of the branched-chain 2-oxo acid dehydrogenase [9] to homogenates of quadriceps muscle, heart and liver from clofibrate-fed rats.

The active compound which accumulates during clofibrate-treatment is clofibric acid [2-*p*-(chlorophenoxy)-2-methylpropionic acid]. Apart from being a direct (mixed or competitive) inhibitor of the branched-chain 2-oxo acid dehydrogenase complex [10, 11], clofibric acid also is a powerful inhibitor of the kinase of this enzyme complex [11]. To obtain a better understanding of the consequences of this complex dual interaction for the flux through the branched-chain 2-oxo acid dehydrogenase complex, we have also studied the effect of clofibric acid on oxidative decarboxylation rates of branched-chain 2-oxo acids in various tissue preparations. Finally, we have investigated the effect of clofibric acid on the binding of 4-methyl-2-oxopentanoate to bovine serum albumin.

MATERIALS AND METHODS

Materials. Clofibrate (Atromid S) was from ICI (Rotterdam, The Netherlands). Clofibric acid [2-*p*-(chlorophenoxy)-2-methylpropionic acid] and bovine serum albumin (fraction V) were from Sigma (St Louis, MO). Bovine serum albumin was made fatty acid-free according to Chen [12] and dialyzed. Sources of other chemicals and of radioactive substrates have been described previously [9, 13].

Animals. Hemidiaphragms were isolated from 18-hr-starved male albino Wistar rats weighing 90–125 g as described in [13]. In all other experiments male albino Wistar rats of 200–240 g were used. All rats were fed on a standard commercial stock diet (RMH-

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TM, obtained from Hope Farms BV, Woerden, The Netherlands) and received water *ad libitum*. Clofibrate-treated rats received the drug orally 50 mg/day in 1 ml 60% (v/v) glycerol solution for 7–10 days. Animals were starved for 18 hr for experiments in Table 1.

Oxidation assays. Rats were killed by cervical dislocation and tissues were rapidly excised. Homogenates were prepared as described in [9] and mitochondria as described in [14]. Oxidative decarboxylation rates of 4-methyl-2-oxo-[1-^{14}C] pentanoate and 3-methyl-2-oxo[1-^{14}C]butanoate (0.1 mM) were measured in these preparations with the basic assay for the total activity (Table 1). Actual and total activities of the branched-chain 2-oxo acid dehydrogenase complex were assayed in homogenates (Table 2) in paired experiments with 4-methyl-2-oxo[1-^{14}C]pentanoate (0.1 mM) as substrate to determine the proportion of enzyme present in the active state. Both assays have been described in detail [9].

Hemidiaphragms were incubated at 37° for 90 min with 1-^{14}C-labelled branched-chain 2-oxo acids (0.1 mM) in 2.0 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) gassed with O_2–CO_2 (95:5) and containing 4 mM glucose. Oxidative decarboxylation rates were assayed as described in [13].

Binding assay. The following procedure was modified from Livesey and Lund [15]. A Biogel P-6DG column (10 × 2.5 cm; 80–170 mesh) was equilibrated at 20–22° in Krebs–Henseleit phosphate buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 16.6 mM Na_2HPO_4; pH 7.4) containing 10 μM 4-methyl-2-oxo[1-^{14}C]pentanoate. A sample of 1 ml 4% (w/v) fatty acid free bovine serum albumin, equilibrated with the same 2-oxo acid-containing buffer, was added to the column and eluted at a flow rate of 0.5 ml/min with the same 2-oxo acid-containing buffer. Fractions of 3 ml were collected and assayed for 2-oxo acid (scintillation counting of radioactivity) and albumin (A_{280}). When the effect of clofibric acid was tested, it was added to the equilibration buffer (used both for column and albumin sample) and the elution buffer.

Statistics. Results are given as means ± S.D., with the number of experiments (N) in parentheses. Student's paired (Table 1) and unpaired (Table 2) *t*-tests were used for the statistical analyses.

RESULTS

The active compound which accumulates during clofibrate-treatment is clofibric acid [16]. This compound has two opposing influences on the flux through the branched-chain 2-oxo acid dehydrogenase complex, since it is an inhibitor of the enzyme itself [10, 11] and an inhibitor of the kinase of the enzyme [11], which inactivates the enzyme by phosphorylation. Clofibric acid inhibited the oxidative decarboxylation of the 2-oxo acids from leucine and valine in mitochondria and homogenates of rat liver and quadriceps muscle (Table 1). Since the enzyme complex is fully active under the assay conditions used [9], only the direct inhibitory effect of clofibric acid is noticed.

In rat hemidiaphragms clofibric acid inhibited the oxidative decarboxylation of 4-methyl-2-oxopentanoate and had no effect on that of 3-methyl-2-oxobutanoate (Table 1). This difference between the respective 2-oxo acids may relate to the larger flux through the branched-chain 2-oxo acid dehydrogenase complex of 4-methyl-2-oxopentanoate than of 3-methyl-2-oxobutanoate [13]. Another study [17] previously showed that clofibric acid inhibits the oxidative decarboxylation of leucine but not that of valine in rat hemidiaphragms. During incubation of rat hemidiaphragms the activity state increases from about 12 to 50% or more [18]. Therefore the direct inhibitory effect of clofibric acid appears also to be dominant in the partially activated state, especially with 4-methyl-2-oxopentanoate as substrate. Clofibric acid also inhibited leucine oxidation in cultured rat skeletal muscle cells [19, 20].

As compared to controls the activity state and the actual activity of the branched-chain 2-oxo acid dehydrogenase complex decreased in skeletal muscle of clofibrate-treated rats (Table 2). The total activity did not change in skeletal muscle. Total activities of the dehydrogenase increased slightly in heart and considerably in liver, but the activity states did not change in these organs.

Table 1. Effect of clofibric acid on oxidative decarboxylation of branched-chain 2-oxo acids in various tissue preparations

	Oxidation rate controls		Relative oxidation rate	
	(nmoles/min)		(%)	
Tissue preparation	4-Methyl-2-oxopentanoate	3-Methyl-2-oxobutanoate	4-Methyl-2-oxopentanoate	3-Methyl-2-oxobutanoate
Liver homogenate	233 ± 44 (12)	288 ± 55 (11)	82 ± 9 (3)	ND
mitochondria	2.7 ± 0.7 (24)	4.0 ± 0.9 (34)	76 ± 16 (6)[c]	82 ± 20 (6)
Quadriceps m. homogenate	28 ± 7.9 (13)	59 ± 16 (13)	67 ± 3 (4)[a]	59 ± 2 (4)[a]
mitochondria	1.5 ± 0.5 (44)	3.7 ± 0.9 (50)	65 ± 9 (4)[b]	50 ± 12 (4)[b]
Hemidiaphragm	5.7 ± 1.2 (100)	4.0 ± 1.3 (90)	49 ± 14 (3)[c]	97 ± 11 (3)

Homogenates, mitochondria and hemidiaphragms were prepared from 18-hr-starved animals and oxidation rates assayed with 0.1 mM [1-^{14}C]branched-chain 2-oxo acid as described in the Materials and Methods section. Clofibric acid concentration amounted to 0.5 mM with homogenates and mitochondria and to 1 mM with hemidiaphragm. Oxidation rates (in nmoles/min per g tissue or per mg mitochondrial protein) and relative oxidation rates (in % of the control without clofibric acid) are given as means ± S.D. for the number of experiments in parentheses. [a,b,c] Significantly different from the control at $P < 0.001$, <0.01 and <0.05, respectively.

ND, not determined.

Table 2. Effect of clofibrate treatment on the actual and total activity of the branched-chain 2-oxo acid dehydrogenase complex in rat tissues

	Activity (nmol/min per g of tissue)			
	Control		Clofibrate-treated	
Tissue	Actual	Total	Actual	Total
Quadriceps muscle	1.8 ± 0.3	21.7 ± 3.9 (8.2 ± 1.5)	0.71 ± 0.11[a]	23.6 ± 4.5 (3.1 ± 1.0[a])
Heart	23.4 ± 1.5	301 ± 23 (7.8 ± 0.8)	27.2 ± 4.5	396 ± 38[b] (6.9 ± 1.5)
Liver	132 ± 13	136 ± 11 (97 ± 11)	392 ± 25[a]	387 ± 34[a] (102 ± 9.2)

Values are means ± S.D. for 5–6 fed and 5 clofibrate-treated animals. Actual and total activities of the branched-chain 2-oxo acid dehydrogenase complex were measured as described in the Materials and Methods section with 0.1 mM 4-methyl-2-oxo-[1-^{14}C] pentanoate as substrate. The activity state (% of enzyme present in the active state) is given in parentheses. Control data are taken from [33]. [a,b] Significantly different from the controls at $P < 0.001$ and <0.01, respectively.

In a previous paper [8] we reported branched-chain 2-oxo acid dehydrogenase activities measured with the basic assay, in liver and muscles of control and clofibrate-treated rats. These activities were shown to reflect total activities in a succeeding paper [9]. Total activities only increased in liver of clofibrate-treated rats when they were measured with 4-methyl-2-oxopentanoate, but not with 3-methyl-2-oxobutanoate [8]. This led us to the speculation that the cytosolic branched-chain 2-oxo acid oxygenase [21], which converts only the 2-oxo acid from leucine, was induced by the clofibrate-treatment. With our basic assay the activity present in 70,000 *g* supernatants of liver homogenates from control rats was only 2.7 ± 0.3% (N = 3) of that present in the homogenate. With clofibrate-treated rats this figure was 2.3 ± 0.2% (N = 3). Therefore, we conclude that the activity of the cytosolic oxygenase does not interfere with our assay and that the activity of the branched-chain 2-oxo acid dehydrogenase may be differentially changed by clofibrate-treatment for the 2-oxo acid analogues of leucine and valine.

Since clofibric acid competes with the branched-chain 2-oxo acids both for binding to the dehydrogenase complex [10, 11] and to the kinase of the complex [11, 22, 23], the action of clofibric acid may relate to its structural resemblance to the 2-oxo acids. If this hypothesis is correct, clofibric acid may also interfere with other known binding sites of the branched-chain 2-oxo acids. Clofibric acid (2 mM) indeed almost completely inhibited the binding of 4-methyl-2-oxopentanoate to bovine serum albumin (Fig. 1). Equilibrium dialysis experiments showed that the binding constants to albumin are higher for clofibric acid [24] than for branched-chain 2-oxo acids [15]. Data about a possible interference of clofibric acid with transport of branched-chain 2-oxo acids over the plasma and the mitochondrial membrane has to await future research.

DISCUSSION

Paxton and Harris [11] found a 13-fold increase by clofibric acid (0.1 mM) in the flux through the branched-chain 2-oxo acid dehydrogenase complex in rat hearts perfused with glucose. Rat hearts maintain at glucose perfusion a low activity state of the dehydrogenase [23, 25], but clofibric acid (0.1 mM) increased the activity state from about 4 to about 50% [11]. At incubation of hemidiaphragm the activity state increases markedly [18], but clofibric acid causes inhibition of the oxidation (Table 1). When the enzyme complex in mitochondria and homogenates is fully activated before the assay, clofibric acid has also an inhibitory effect. These data indicate that in conditions with a low activity state the inhibitory effect of clofibric acid on the kinase dominates and clofibric acid indirectly activates the

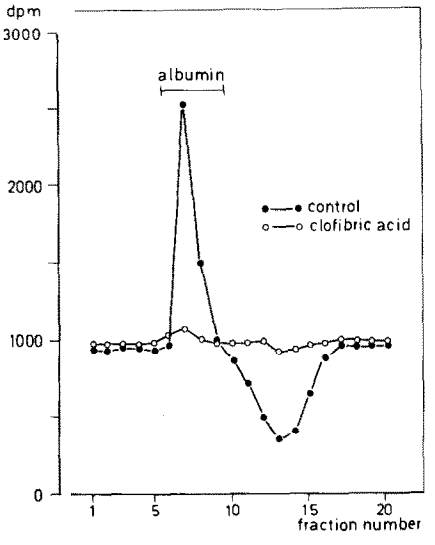

Fig. 1. Biogel P-6DG chromatography for the detection of binding of 10 μM 4-methyl-2-oxo [1-^{14}C] pentanoate to bovine serum albumin in the absence and presence of 2 mM clofibric acid. Conditions for chromatography are described in the Materials and Methods section.

dehydrogenase, whereas in conditions with a high activity state the direct inhibitory effect on the dehydrogenase prevails.

Since heart and skeletal muscle in control rats have only a low proportion of the dehydrogenase complex in the active state (Table 2), clofibrate-treatment was expected to induce conversion into active complex in these tissues [11]. However, this was not found (Table 2). Since the concentration of branched-chain amino acids is lower in muscles of clofibrate-treated rats [3], the concentration of the corresponding 2-oxo acids probably will also be decreased. The reduced inhibition of the dehydrogenase kinase by the lower concentration of 2-oxo acids may mask inhibition of the kinase by clofibric acid. Alternatively a very active, thusfar unknown, mechanism may operate in heart and skeletal muscle *in vivo* and maintain the activity state at a low level.

The specific increase of the total activity of the dehydrogenase in the liver only when measured with 4-methyl-2-oxopentanoate and not with 3-methyl-2-oxobutanoate is remarkable, since one dehydrogenase complex catalyses the oxidative decarboxylation of the 2-oxo acids from leucine, valine and isoleucine (reviewed in [7]). This finding may relate to the observation that only the concentration of leucine is significantly reduced in plasma of clofibrate-treated rats [3].

The rise *in vivo* in the flux through the dehydrogenase complex in the liver is probably smaller than the activities given in Table 2 suggest, since the direct inhibitory effect of clofibric acid will be operative in intact livers of clofibrate-treated rats. On the other hand, clofibrate-feeding also causes significant hepatomegaly [26] and therefore will increase the activity of whole liver.

Several studies reported that the activity of the dehydrogenase complex in the liver increases in rats fed diets containing large amounts of individual branched-chain amino acids or branched-chain 2-oxo acids [27–30]. Since liver of rats fed a standard diet has already 100% of its dehydrogenase in the active state [9], it appears probable that additional enzyme activity is induced by the amino acids and/or 2-oxo acids. The displacement of branched-chain 2-oxo acids from plasma albumin by clofibric acid will increase the concentration of free circulating 2-oxo acids and may indirectly induce the liver activity. In isolated rat hepatocytes the uptake and oxidation of branched-chain 2-oxo acids was limited by their binding to albumin [31]. On the other hand, the interference of clofibric acid with the metabolism of branched-chain amino acids appears to relate to its structural resemblance to the 2-oxo acids. Therefore, if high concentrations of branched-chain 2-oxo acids are able to induce dehydrogenase activity in the liver, clofibric acid may have the same direct effect.

We conclude from our data that the oxidation of branched-chain 2-oxo acids is increased in livers of clofibrate-treated rats and not in skeletal muscle or heart as was suggested by previous studies [3, 11]. The increased liver oxidation indirectly decreases concentrations of branched-chain amino acids [3] and possibly of 2-oxo acids in plasma and muscle. The flux through the dehydrogenase complex in skeletal muscle of clofibrate-treated rats will be even lower than the actual activity given in Table 2 suggests, because of the directly inhibiting effect of clofibric acid on the enzyme complex. In muscle *in vitro* stimulation of protein synthesis depends on leucine itself, whereas inhibition of protein degradation is mediated by an intermediate of leucine metabolism [32]. Therefore, both the decreased concentration of branched-chain amino acids in plasma and muscle and the reduced flux through the branched-chain 2-oxo acid dehydrogenase in muscle may play a role in the myopathic side-effects of clofibrate-treatment.

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