Clofibrate elevates enzyme activities of the tricarboxylic acid cycle in rat liver

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Abstract Activities of the tricarboxylic acid cycle enzymes were measured in subcellular fractions of liver from rats that had been fed clofibrate for 3 weeks. Large changes in these activities per gram tissue were found in the large particle fraction, which also showed an increase in total protein concentration of 76% under clofibrate treatment. The three regulatory enzymes of the cycle, namely citrate synthase, NAD^+-linked isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase were significantly enhanced by 24% ($P < 0.02$), 54% ($P < 0.02$), and 153% ($P < 0.005$), respectively. Fumarase and malate dehydrogenase rose by 71% ($P < 0.005$) and 95% ($P < 0.02$), whereas succinate dehydrogenase remained unchanged. Enhancement of the citrate synthase, NAD-isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase may play a role in decreasing intracellular availability of acetyl-CoA for lipid metabolism.—**Prager, C., H. J. Schön, M. Nikfardjam, D. Schmid, M. Untersalmberger, K. Kremser, and R. Kramar.** Clofibrate elevates enzyme activities of the tricarboxylic acid cycle in rat liver. *J. Lipid Res.* 1993. **34:** 359–364.

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Patients on a therapeutic regimen with clofibrate (ethyl 2-(4-chlorophenoxy)-2-methylpropionate) show an increase of liver mitochondrial and peroxisomal volumes by 34% and 23% (1). Rodents treated with high doses of clofibrate show an increase of peroxisomal volume by 450% (2), whereas the mitochondrial volume rises to a similar extent as in humans treated with therapeutic doses (3). This enhancement in mitochondrial volume is accompanied by increased matrical density (4). Activities of glycerol-3-phosphate dehydrogenase (G3-PDH) (5), which controls the dihydroxyacetone phosphate/glycerol phosphate ratio as well as the regulatory enzyme NAD-isocitrate dehydrogenase (NAD-ICDH) (6) are induced by clofibrate.

Certain systemic effects of clofibrate and related fibrates are presumably relevant to their pharmacological action, as they enhance lipoprotein lipase activity in blood (7), skeletal muscle (8), and in adipose tissue (9). This effect leads to increased clearance of triglycerides from plasma. In addition clofibrate lowers lipoprotein release from the liver (10).

Clofibrate-induced changes in hepatic lipid metabolism are also observed. The activity levels of carnitine palmitoyl transferase (11) and carnitine acetyl transferase (12) are elevated by clofibrate. While cholesterol biosynthesis is inhibited by clofibrate in liver slices (13), fatty acid β-oxidation is stimulated in mitochondria as well as in peroxisomes (14) thereby raising the acetyl-CoA production in liver. The increased amount of acetyl-CoA groups is presumably distributed to a similar extent over ketogenesis and the tricarboxylic acid cycle (TCAC). Clofibrate has been demonstrated to augment ketogenesis from fatty acids in rat liver mitochondria (15). But the CO_2 formation is also nearly doubled, which shows the importance of TCAC in dealing with the higher acetyl supply (16).

The respiratory capacity of clofibrate-treated mitochondria can keep pace with an increased acetyl-CoA utilization in TCAC. When TCAC substrates are used, the active (state 4) respiration expressed as oxygen uptake per mg mitochondrial protein in the presence of ADP does not change. The respiration rises proportionally to the mitochondrial protein (17).

The TCAC plays a crucial role not only in the degradation of acetyl-CoA but also in providing precursors and/or activators for lipid synthesis, gluconeogenesis, and amino acid synthesis. The flux through the cycle metabolon (18, 19) is adjusted to respiratory and metabolic needs by the redox potential of the NAD^+/NADH couple, the concen-

Abbreviations: PNS, postnuclear supernatant; LP, large particle; S, supernatant; CS, citrate synthase; NAD^+-ICDH, NAD^+-linked isocitrate dehydrogenase; 2-OGDH, NAD^+-dependent 2-oxoglutarate dehydrogenase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; G3-PDH, glycerol-3-phosphate dehydrogenase; INT, 2-(*p*-iodophenyl)-3-*p*-(*p*-nitrophenyl)-5-phenyl-tetrazoliumchloride; TCAC, tricarboxylic acid cycle.

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tration of oxaloacetate, the regulatory enzyme activities, and substrate channelling (19). Here we test the hypothesis that the enzyme activities of the TCAC, which obtain acetyl-CoA in liver mainly through fatty acid degradation, may contribute to the hypolipidemic action of clofibrate.

MATERIAL AND METHODS

Animals

Male Sprague Dawley rats (140–190 g) were fed a standard diet containing 0.75% clofibrate. The food intake and gain in body weight were recorded three times a week. The average daily intake dose of clofibrate was 248 ± 47 mg per rat, calculated on the basis of an average food intake of 33 ± 6 g (mean ± SD) per day. After 3 weeks the animals were anesthetized and killed by cervical dislocation.

Subcellular preparation

A large particle (LP) fraction (20, 21), essentially consisting of mitochondria, lysosomes, and peroxisomes, was pelleted from a postnuclear supernatant (PNS) from rat liver homogenates at 20,000 *g*. The supernatant (S) of the LP fraction was also saved. All steps were carried out at 4°C. Fractions were kept frozen at −70°C until enzyme activities were determined.

Analytical procedures

Enzyme assays in all three cell fractions were performed with equal preincubation times and concentrations of Triton X-100 (1 g/l). Conditions of freezing and thawing of the enzyme samples were strictly standardized. Citrate synthase (CS) and NAD^+-ICDH activity were determined by measuring the CoASH formed with Ellman's reagent (22) and NADH formation (23). Determination of 2-oxoglutarate dehydrogenase (2-OGDH) activity was based on the overall 2-oxoglutarate oxidation leading to NADH formation (24). SDH and G3-PDH activity were both assayed with 2-(*p*-iodophenyl)-3-*p*-(*p*-nitrophenyl)-5-phenyl-tetrazoliumchloride (INT) as electron acceptor under test conditions described previously (25). The fumarase (FUM) and malate dehydrogenase (MDH) activities were also determined photometrically (26, 27). Protein concentration, serum total triglycerides, and cholesterol concentration were measured by standard procedures (28–31).

TABLE 1. Effect of clofibrate feeding on body weight, liver weight/body weight, LP protein, and LP G3-PDH activity/g liver

Group	Body Weight	Liver Wt/Body Wt	LP Protein	G3-PDH
	g	*g/100 g*	*mg/g tissue*	*U/g*
Control	306 ± 23	5.0 ± 0.5	35.7 ± 7.8	0.35 ± 0.10
CPIB	286 ± 21[a]	6.5 ± 0.8[b]	62.8 ± 13.4[b]	1.65 ± 0.63[b]

Each group represents 14 animals. Specific activity of G3PDH (mU/mg protein) is given in μmol INT reduced per min (U). Values represent means ± SD.

[a] $P < 0.05$; [b] $P < 0.02$: significantly different from the control as indicated (unpaired Student's *t*-test).

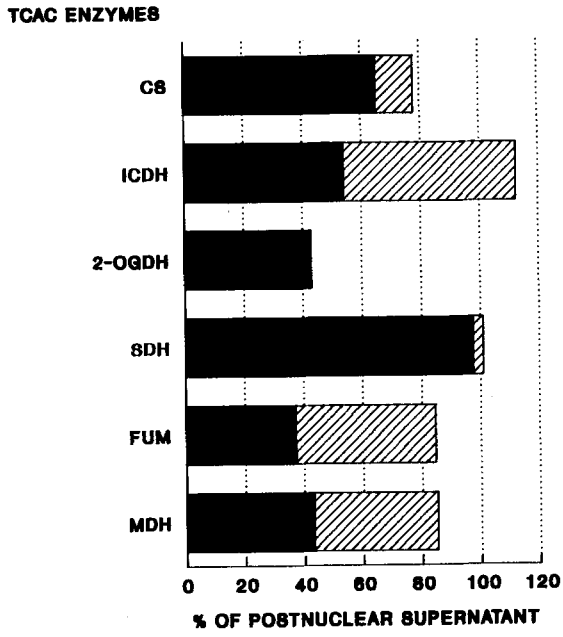

Fig. 1. TCAC enzyme recovery in subcellular fractions of rat liver. The sum of the activities in the large particle (■) and supernatant (▨) fractions is shown in relation to the postnuclear supernatant (PNS) fraction (100%) for each of the measured cycle enzymes.

RESULTS

The efficacy of the clofibrate treatment was shown by a 369% increase of mitochondrial G3-PDH (**Table 1**) in the large particle fraction. This finding is in agreement with a previous report (5). The drug action was also reflected by the observed hepatomegaly and the 76% increased yield of protein in the LP-fraction (Table 1). Serum total cholesterol and triglyceride were lowered from 2.7 ± 0.1 mmol/l (means ± SD) to 1.7 ± 0.1 and 2.5 ± 0.2 to 1.4 ± 0.1 mmol/l, respectively, in agreement with previous data (14).

Enzyme activities from the LP and S fractions are expressed as percent of the total activity in the PNS fraction (**Fig. 1**). For this study the LP fraction, which contains mostly mitochondria, was considered to be suitable for the examination of clofibrate-induced changes of mitochondrial enzymes (3, 5, 32, 33). When prepared according to our protocol, the LP fraction contained largely all mitochondria of the PNS as indicated by the distribution of SDH activity. SDH, which is firmly bound to the mitochondrial membrane, was recovered quantitatively in LP (Fig. 1). SDH increased its specific activity between

PNS and LP by 3.1 in control and by 2.4 in clofibrate liver. CS, NAD^+-ICDH, 2-OGDH, FUM, but not MDH are located exclusively within the mitochondria (for a review see ref. 34) so that contamination by non-mitochondrial sources of these activities can be excluded. In the LP and S fractions together, matrix enzyme activities of CS, NAD^+-ICDH, FUM, MDH were recovered at levels of 78–115% (Fig. 1). For these enzymes the LP/PNS ratios were smaller for clofibrate livers than for controls. This might be due to some structural inferiority of the mitochondrial membrane as it has been reported for other intracellular membranes in clofibrate livers (35). Only little 2-OGDH activity was detectable in the S fraction. This leads to a 40–50% recovery rate for this labile multienzyme complex. High losses during the preparation of 2-OGDH leading to poor recovery of homogenate activities in the particle fraction have been described elsewhere (24).

The CS activity per gram liver rose by 24 and 25% in the LP and PNS fraction, respectively. The NAD^+-ICDH activity per gram liver increased similarly by 27% in the PNS and by 54% in the LP fraction as published in detail elsewhere (6). Due to the pronounced enhancement of mitochondrial protein by 76%, this increase in activity was not due to a rise of specific activity. The activity per gram of the third regulatory enzyme of the cycle, 2-OGDH, was found to be elevated by 153% ($P < 0.005$) and 122% ($P < 0.005$), respectively, as compared with the control in the LP and PNS fractions (**Table 2**). The specific activity (mU/mg protein) of this enzyme was significantly increased by 142% ($P < 0.005$) in the PNS and by 69% ($P < 0.005$) in the LP fraction. The activity of FUM per gram tissue was enhanced by 22% in the PNS and about 71% in the LP fraction. The MDH activity per gram tissue was enhanced by 26% in the PNS and 95% in the LP fraction (**Table 3**). The activity per gram tissue of SDH, another non-equilibrium enzyme, did not show any clofibrate dependence in the LP or PNS fraction (Table 2). This is in agreement with previous work (36). The specific activities demonstrate that there is no stimulation of SDH, FUM, and MDH activity compared to the enhancement of total mitochondrial protein (Tables 2 and 3).

DISCUSSION

Our data provide evidence that the enzymes of the TCAC are selectively induced by clofibrate. While the total mitochondrial protein increased by 76%, the three regulatory enzymes of the cycle, namely CS, NAD^+-ICDH, and 2-OGDH, increased by 24%, 54%, and 153%, respectively, when given as activities per gram tissue, which is appropriate for considering their metabolic availability. The NAD^+-linked dehydrogenases ICDH, 2-OGDH, and MDH showed the most pronounced response. Corresponding to their activities per g tissue,

TABLE 2. Activity of non-equilibrium tricarboxylic acid cycle enzymes per gram liver wet weight and per mg protein in rat liver after clofibrate feeding

Group	Fraction	CS		NAD^+-ICDH	
		U/g	mU/mg	mU/g	mU/mg
Control	PNS	6.3 ± 0.4	53.8 ± 3.4	722 ± 135	5.7 ± 0.9
	LP	4.1 ± 0.9	103.3 ± 13.6	389 ± 157	9.5 ± 3.3
	S	0.8 ± 0.2	7.3 ± 0.6	421 ± 80	5.7 ± 0.9
CPIB	PNS	7.9 ± 0.8[a]	52.1 ± 1.6	917 ± 142[b]	6.0 ± 0.5
	LP	5.1 ± 1.5[b]	77.0 ± 16.2[a]	599 ± 190[b]	9.1 ± 1.7
	S	0.7 + 0.1	7.8 ± 0.5	506 ± 102[c]	6.2 ± 0.7
		2-OGDH		SDH	
		mU/g	U/mg	U/g	mU/mg
Control	PNS	515 ± 91	3.8 ± 0.5	1.19 ± 0.19	9.5 ± 1.6
	LP	219 ± 40	5.2 ± 0.8	1.16 ± 0.29	29.3 ± 6.5
	S	<2	<0.1	<0.04	<0.6
CPIB	PNS	1144 ± 206[a]	9.2 ± 1.1[a]	1.21 ± 0.12	8.1 ± 1.1[c]
	LP	554 ± 87[a]	8.8 ± 1.2[a]	1.32 ± 0.41	19.1 ± 4.7[a]
	S	<24	<0.3	<0.03	<0.4

Effect of clofibrate feeding on CS, NAD^+-ICDH, 2-OGDH, and SDH activities. Rats were fed for 3 weeks with clofibrate 0.75% in standard food. PNS (n = 7), LP (n = 15), and S (n = 7) fractions were prepared from each rat liver as described in Material and Methods. Enzyme activities (U/g liver wet weight and U/mg protein) are given as μmol CoASH formed (CS), NAD^+ (NAD^+-ICDH, 2-OGDH), and INT (SDH) reduced per min. The enzyme assays were performed in duplicate.

[a] $P < 0.005$; [b] $P < 0.02$; [c] $P < 0.05$: significantly different from the control (unpaired Student's *t*-test).

TABLE 3. Activity of fumarase and malate dehydrogenase per gram liver wet weight and per mg protein in rat liver after clofibrate feeding

Group	Fraction	FUM		MDH	
		U/g	mU/mg	U/g	U/mg
Control	PNS	20.2 ± 1.5	158.9 ± 12.3	102.2 ± 7.3	0.80 ± 0.03
	LP	7.5 ± 2.5	185.6 ± 52.9	44.0 ± 15.8	1.09 ± 0.29
	S	9.6 ± 1.2	124.8 ± 10.4	42.9 ± 2.5	0.56 ± 0.07
CPIB	PNS	24.6 ± 3.4[a]	160.5 ± 14.9	128.7 ± 13.4[b]	0.83 ± 0.04
	LP	12.8 ± 5.2[a]	186.6 ± 52.9	85.7 ± 33.1[a]	1.29 ± 0.42
	S	8.6 ± 1.9	100.2 ± 22.1[c]	39.7 ± 4.5	0.49 ± 0.05[c]

Enzyme activities (U/g liver wet weight and U/mg protein) are given in μmol fumarate formed (FUM) and NAD reduced (MDH) per min. For details, see Table 2.
[a] $P < 0.02$; [b] $P < 0.005$; [c] $P < 0.05$: significantly different from the control (unpaired Student's *t*-test).

three groups of enzymes may be distinguished. *a)* 2-OGDH is more than doubled and its specific activity is significantly stimulated. This regulatory enzyme is selectively induced beyond the mitochondrial proliferation. *b)* CS, NAD⁺-ICDH, FUM, and MDH rise by more than 20% in PNS and, except for CS, to a greater extent in LP. No increase of the specific activity is observed, as the activities follow the augmented mitochondrial compartment (3, 32, 33). *c)* SDH remains unchanged and as a consequence its specific activity decreases. The enzyme does not keep pace with the mitochondrial proliferation (Table 2). The enhanced activities of the non-equilibrium enzymes CS, NAD⁺-ICDH, and 2-OGDH are likely to accommodate higher fluxes through the citrate, isocitrate, and 2-oxoglutarate pools under the conditions of a low NADH/NAD⁺ ratio when these enzymes are largely activated (for a review see ref. 37). Under clofibrate treatment the NADH/NAD⁺ redox couple is shifted to the oxidized form (38). While direct measurements of intermediate concentrations showed no significant changes by clofibrate (38, 39) except for oxaloacetate (40), the activities of those enzymes, which are involved in the regulation of substrate flux through the cycle (**Fig. 2**), are increased. Changes in the flux-generating enzymes CS and 2-OGDH (41) are of special interest. Thus, in spite of the lowered oxaloacetate concentration, higher flux through the TCAC may result.

Clofibrate-induced changes of FUM, which catalyzes a near-equilibrium reaction, or of MDH (Table 3), which catalyzes an endergonic reaction (42), appear to be of minor importance for the flux through the cycle. Under energized conditions (high ATP/ADP and citrate/oxaloacetate ratios) the apparent K_m of CS for oxaloacetate is increased (37). In addition, the TCAC metabolon is broken

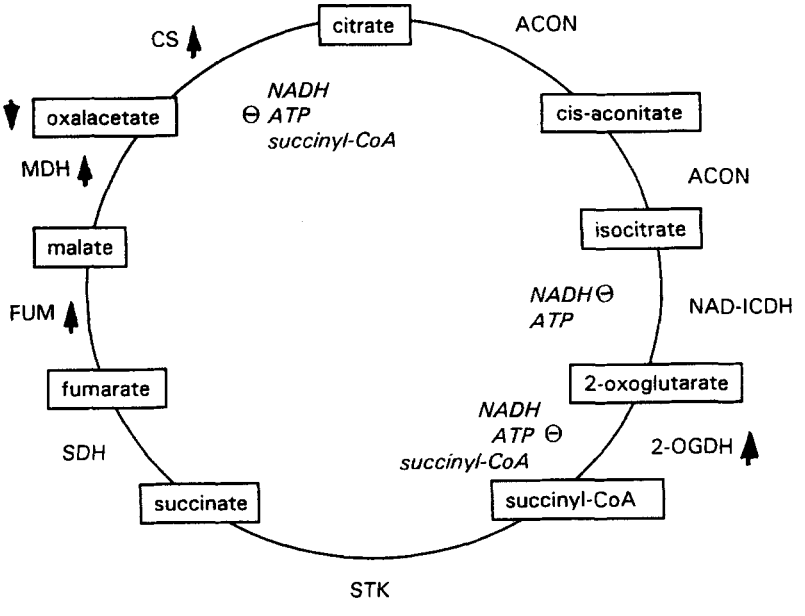

Fig. 2. Tricarboxylic acid cycle. Arrows indicate significant increase (↑) or decrease (↓) of enzyme activity or intermediate concentration under clofibrate treatment; (Θ) shows inhibition.

between MDH and FUM by distance regulation, so that accumulation of cytosolic malate is furthered by retrograde flux through the TCAC. The enhanced activity of MDH under clofibrate might contribute to this effect, which leads to gluconeogenesis. In the orthograde direction of the TCAC, the stimulated NAD^+-ICDH activity may likewise favor the formation of malate.

Our data provide evidence that all three regulatory enzyme activities of the TCAC, especially the NAD^+-linked dehydrogenases, are stimulated by clofibrate. The enzyme activities increase together with CO_2 production and ketogenesis from fatty acids (15, 16, 40), suggesting higher orthograde flux through TCAC. This clofibrate-induced increase of acetyl- CoA degradation may restrict the availability of acetyl groups for lipid synthesis and contribute to the hypolipidemic action of the drug.

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