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IN VIVO INDUCTION OF 4-ENOYL-CoA REDUCTASE BY CLOFIBRATE IN LIVER MITOCHONDRIA AND ITS EFFECT ON PENT-4-ENOATE METABOLISM

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SUMMARY: Feeding of clofibrate to male rats leads to a 4-7 fold increase in the activity of the 4-enoyl-CoA reductase in the liver. Concomitantly the inhibition of fatty acid oxidation by pent-4-enoate is abolished, and an increased glucose formation in the presence of pent-4-enoate is observed. It is suggested that pent-4-enoate is converted to propionyl-CoA via the reaction sequence pent-4-enoyl--CoA→pent-2,4-dienoyl-CoA→pent-2-enoyl-CoA→propionyl-CoA + acetyl-CoA.

The rate of fatty acid oxidation is increased in hepatocytes isolated from clofibrate-fed rats (1). The increased oxidation can be explained by an increased rate of chain shortening of fatty acids by the peroxisomal β-oxidation system (2,3). However, induction of mitochondrial and cytosolic enzymes by clofibrate also takes place (4-6).

In an effort to find conditions under which the peroxisomal β-oxidation system can be studied separately in the intact hepatocyte we have tested the effects of different inhibitors of mitochondria fatty acid oxidation. Such experiments showed that fatty acid oxidation is not inhibited by pent-4-enoate in hepatocytes from clofibrate-treated rats. Such a protective effect of clofibrate against pentenoate, and against hypoglycine as well, has previously been observed by van Hoof et al. (7). Further studies of this phenomenon have shown that clofibrate induces an increased activity of the mitochondrial 4-enoyl-CoA reductase (or rather 2,4-dienoyl-CoA reductase),

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leading to an increased capacity to metabolize pent-4-enoate in the liver.

MATERIALS AND METHODS

Hepatocytes and mitochondria: Male Wistar rats (weight approximately 150 g) from Møllengaard Avlsstasjon, Havdrup, Denmark, were fed on a standard pelleted diet which contained 0.25% (w/w) clofibrate for about two weeks. Hepatocytes from these rats and from corresponding normal rats were prepared according to Seglen (8) except that a Krebs-Henselite bicarbonate buffer with 0.5 mM $CaCl_2$ in an atmosphere of 5% CO_2-95% O_2 was used as the suspension and incubation medium. In experiments on gluconeogenesis the rats were fasted for 24 hours before they were killed to minimize glucose formation from endogenous glycogen.

Rat liver mitochondria were prepared by standard centrifugation methods after homogenizing the liver in 10 volumes of 300 mM mannitol containing 5 mM N-2-hydroxyethylpiperazine-N'--2-ethanesulfonic acid (HEPES), pH 7.4, and 1 mM ethyleneglycole-bis (ß-aminoethyl ether) N.N'-tetraacetic acid (EGTA).

Mitochondrial respiration was measured with a Clark oxygen electrode.

Chemicals: (-)Carnitine hydrochloride was kindly donated by the Otsuka Farmaceutical Factory, Tokushima, Japan. Pent-4--enoic acid and but-3-enoic acid were obtained from Fluka, Buchs, Switzerland. Pent-4-enoylchloride was prepared by addition of a slight excess of oxalylchloride to pentenoic acid at 0^{o}. The mixture was permitted to heat up to room temperature. After gas bubling from the reaction mixture had ceased, insoluble products were removed by centrifugation, and the remaining pentenoylchloride was used without any further purification. (-)Pent-4-enoylcarnitine was prepared by treating a solution of carnitine perchlorate in acetonitril with a 5-fold excess of pentenoylchloride (9). After standing over night at room temperature most of the acetonitril was removed by vacuum destillation and the pentenoylcarnitine was precipitated as a solid with an excess of ethyl ether. The precipitate was dissolved in a small volume of methanol. Chrystallization was obtained by addition of 3-4 volumes of acetone followed by ethyl ether to starting cloudiness. The mixture was left at 0^{o} over night. The chrystalline product was washed with ether and dried. The product discoloured permanganate, showing the presence of an intact double bound. Acyl-CoA esters were prepared and characterized as described (3).

Enzyme assays: NADPH-dependent 2-enoyl-CoA reductase and 4-enoyl-CoA reductase were assayed as described by Kunau and Dommes (10) in the particle-free supernatant of isolated mitochondria treated with 0.2% deoxycholate.

RESULTS AND DISCUSSION

Effects of pent-4-enoate in isolated hepatocytes: Table I shows that hepatocytes from clofibrate-fed rats have an increased capacity to oxidize palmitate compared to normal

Table I. The oxidation of [U-^{14}C]palmitate in hepatocytes isolated from normal and clofibrate-treated rats with and without pent-4-enoate, KCN, or antimycine A in the incubation medium. The results are given as palmitate recovered as acid soluble products (nmol x mg protein^{-1} x 30 min^{-1}), and (in parenthesis) as palmitate oxidized in per cent of oxidation in the absence of inhibitors. (means of 4 rats + S.E.M.).

Inhibitor	Normal rats	Clofibrate treated rats
None	32.8 ± 2.3	44.1 ± 1.2*
Pent-4-enoate, 2 mM	12.0 ± 1.4 (37 ± 3.3)	51.9 ± 3.3 (118 ± 7.5)**
KCN, 2mM	9.7 ± 1.0 (29.5 ± 2.9)	13.6 ± 1.7 (31 ± 4.2)
Antimycin A, 2 μM	9.0 ± 0.9 (28 ± 2.1)	13.8 ± 1.3 (31 ± 2.5)

* $P < 0.005$, ** $P < 0.001$ when compared with normal cells.

cells. Antimycin A and KCN are equally strong inhibitors in both types of cells, while 2 mM pentenoate inhibits in normal cells only.

Studies on the effects of pentenoate in the heart have shown that in this tissue pentenoate is only a weak inhibitor of fatty acid oxidation. However, glycolysis is inhibited, most likely by a relatively huge accumulation of citrate and other citric acid cycle intermediates. This accumulation of citric acid cycle intermediates is probably explained by the formation of propionyl-CoA from pentenoate (11,12).

In the liver pentenoate is a strong inhibitor of gluconeogenesis from lactate (Table II) (13). However, a conversion of pentenoate to propionyl-CoA also makes its conversion to glucose possible. Table II shows that this is barely detectable only with 0.5 mM pentenoate in normal hepatocytes, but in hepatocytes from the clofibrate-treated rats glucose formation is stimulated even with 5 mM pentenoate. With 1-2 mM pentenoate the stimulation is about as great as with 2 mM propionate or

Table II. Gluconeogenesis from pent-4-enoate and other precursors in hepatocytes isolated from fasted normal and clofibrate-treated rats. Hepatocytes (approximately 15 mg of protein) were incubated for 30 min in a volume of 2 ml of Krebs-Henselite bicarbonate buffer with fatty acid free bovine serum albumine, 0.54 mM; palmitate, 1 mM; glucagon, 2 x 10^{-7}M; and glucose precursors as shown. The parallels represent results obtained with hepatocytes from two different animals. The results are given as change in glucose formation compared with incubations without any glucose precursor added.

Glucose precursor	Glucose (nmol x mg protein^{-1} x 30 min^{-1})			
	Normal rats		Clofibrate treated rats	
Lactate 2 mM	104	99	111	68
Lactate 10 mM	196	239	172	73
Pentenoate 0.5 mM	10	4	18	22
Pentenoate 1 mM	-4	-5	21	14
Pentenoate 2 mM	-4	-9	14	8
Pentenoate 5 mM	-14	-10	12	6
Propionate 2 mM	20	28	29	15
Propionate 10 mM	2	11	17	15
Pentanoate 2 mM		9		15
Pentanoate 10 mM		7		11
Lactate 10 mM +pentenoate 1 mM		7		19

pentanoate. Gluconeogenesis from lactate is inhibited by pentenoate in both types of hepatocytes.

Effects of pent-4-enoylcarnitine in isolated mitochondria: Fig. 1 shows that pentenoyl-carnitine is a strong inhibitor of palmitylcarnitine oxidation in normal mitochondria (measured as rate of O_2 consumption). This strong and permanent effect was obtained when the mitochondria were preincubated for a short time (1 min) with pentenoylcarnitine. In mitochondria from clofibrate-treated rats the inhibition was much weaker.

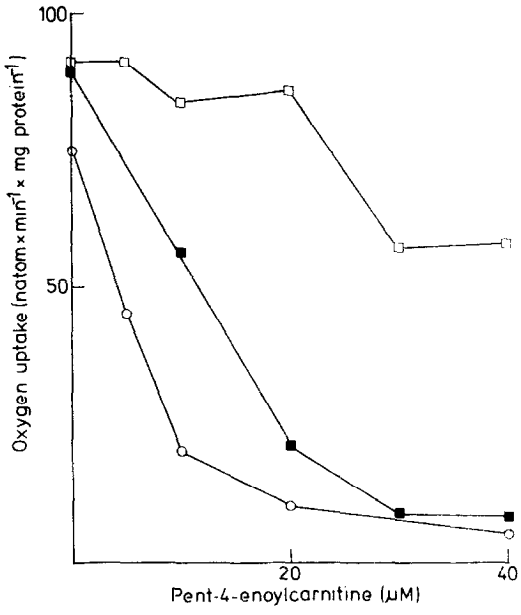


Fig. 1. The effect of pent-4-enoylcarnitine on the oxidation of palmitylcarnitine in liver mitochondria isolated from normal and clofibrate-treated rats. Palmitoylcarnitine (40 μM) was added to mitochondria (about 2 mg of protein/ml) which had been preincubated for 1 min with pent-4-enoylcarnitine as shown. Other additions: KCl, 130 mM; HEPES buffer (pH 7.2), 10 mM; ADP, 3 mM; Phosphate, 2 mM; $MgCl_2$, 1 mM; malonate, 10 mM; EGTA, 0.1 mM; defatted bovine serum albumin, 2 mg/ml; and, where indicated, FCCP, 4 μM (added before the pentenoylcarnitine). The temperature was 30°. With mitochondria isolated from clofibrate-treated rats and preincubated with pentenoylcarnitine, a lag period was observed between the addition of palmitoylcarnitine and the start of the respiration. The lag increased (up to 3-4 min) with the higher concentrations of pentenoylcarnitine. The figure shows the final, stable respiration rates.
○, normal mitochondria; □, mitochondria from a clofibrate treated rat; ■, mitochondria from a clofibrate-treated rat in the presence of FCCP.

However, a strong, temporary inhibition of respiration after the addition of palmitylcarnitine was observed when high concentrations of pentenoylcarnitine was used (up to 4 min with 40 μM pentenoylcarnitine). Fig. 1 shows the final, maximum rates obtained.

It is striking that uncoupling of the mitochondria increased the sensitivity to pentenoylcarnitine, and made the mitochondria from clofibrate-treated rats almost as sensitive as

Table III. The activities of enoyl-CoA reductases in liver mitochondria isolated from normal and clofibrate-treated rats. The results are given as nmol NADPH oxidized x min^{-1} x mg soluble mitochondrial $protein^{-1}$ (means ± S.E.M).

Substrate	Normal rats	Clofibrate treated rats
Sorboyl-CoA (0.4 mM) (n = 5)	28.8 ± 2.2	125 ± 18
Pentenoyl-CoA (0.4 mM) (n = 5)	1.15 ± 0.19	2.11 ± 0.11**
Crotonyl-CoA (0.5 mM) (n = 3)	2.35 ± 0.11	2.43 ± 0.10
But-3-enoyl-CoA (0.3 mM) (n = 3)	2.02 ± 0.13	2.45 ± 0.28

*$P < 0.001$, **$P < 0.005$.

normal mitochondria. These results suggest that the metabolism of pentenoylcarnitine is energy dependent, most likely because the process requires transfer of reducing equivalents from an oxidizable substrate via one of the NADPH-dependent enoyl-CoA reductases to a pentenoate metabolite.

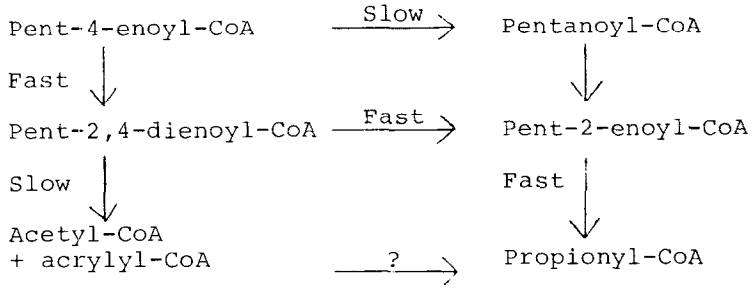
Activity of the mitochondrial enoyl-CoA reductases: Two NADPH-dependent enoyl-CoA reductases are known, the 2-enoyl-CoA reductase and the 4-enoyl-CoA reductase (10). The enhancing effect of uncoupling on the pentenoylcarnitine dependent inhibition of palmityl carnitine oxidation suggests that the energy-dependent NADH-NADP transhydrogenase, and one or both of the enoyl-CoA reductases, are involved in the metabolism of pentenoate. The reduction of the double bound of pentenoate can theoretically take place before or after β-oxidation. Thus, three possible enoyl-CoA metabolites of pentenoate can be reduced:

Pent-4-enoyl-CoA, Pent-2,4-dienoyl-CoA, and acrylyl-CoA.

Table III shows the activities of the two enoyl-CoA reductases assayed with sorboyl-CoA (hex-2,4-dienoyl-CoA), pent-4-enoyl-CoA, but-3-enoyl-CoA, and crotonyl-CoA (the pent-2,4-dienoyl-CoA and acrylyl-CoA we did not have available). The activity of the 4-enoyl-CoA reductase assayed with sorboyl-CoA showed

the highest activity with a four-fold increase in the mitochondria isolated from clofibrate-treated rats. On a cellular protein basis the increase was 7-fold because of the higher mitochondrial content in the liver of clofibrate-treated rats. The activity with pent-4-enoyl-CoA was only 1/25 - 1/50 of that with sorboyl-CoA, but also with this substrate a significantly increased activity was found after feeding clofibrate. The activities with crotonyl-CoA and with butenoyl-CoA also were much lower than with sorboyl-CoA, and the activities pr mg of mitochondrial protein was not increased in the mitochondria from clofibrate-treated rats. These results show that clofibrate induces a relatively selective increase in the activity of the 4-enoyl-CoA reductase. Kunau and Dommes (10) have shown that this enzyme is 20 times more active with deca-2,4-dienoyl-CoA than with deca-4-enoyl-CoA. Thus, it is in agreement with their results when sorboyl-CoA (hexa-2,4-dienoyl-CoA) is a much better substrate than is pent-4-enoyl-CoA, and it suggests that pent-2,4-enoyl-CoA also will be rapidly reduced.

In their studies on pentenoate metabolism, Holland et al. (14) found that pent-4-enoyl-CoA is rapidly oxidized to pent-2,4-dienoyl-CoA by butyryl-CoA dehydrogenase, while pent-2,4-dienoyl-CoA is only slowly hydrated by enoyl-CoA hydratase. Thus the following scheme can be formulated for the metabolism of pent-4-enoyl-CoA:



Altogether, our results indicate that the 4-enoyl-CoA reductase (or rather the 2,4-dienoyl-CoA reductase) is the rate limiting enzyme in the metabolism of pentenoate and that the inhibitory effects of pentenoate is connected with the accumulation of pent-2,4-dienoyl-CoA or a subsequent β-oxidation metabolite in the mitochondria (15). Clofibrate seems to decrease this accumulation by inducing an increased activity of the 4-enoyl-CoA reductase.

It has been reported that clofibrate has a protective effect also towards inhibition of fatty acid oxidation by hypoglycine (7). The toxic metabolite of hypoglycine (methylenecyclopropylacetyl-CoA) (15) posesses a 4-double bound. The 4-enoyl-CoA reductase may therefore be active in further metabolism of this metabolite as well.

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