Effects of riboflavin deficiency and clofibrate treatment on the five acyl-CoA dehydrogenases in rat liver mitochondria

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Rats were maintained on a riboflavin-deficient diet or on a diet containing clofibrate (0.5 %, w/w). The activities of the mitochondrial FAD-dependent straight-chain acyl-CoA dehydrogenases (butyryl-CoA, octanoyl-CoA and palmitoyl-CoA) and the branched-chain acyl-CoA dehydrogenases (isovaleryl-CoA and isobutyryl-CoA) involved in the degradation of branched-chain acyl-CoA esters derived from branched-chain amino acids were assayed in liver mitochondrial extracts prepared in the absence and presence of exogenous FAD. These activities were low in livers from riboflavin-deficient rats (11, 28, 16, 6 and < 2 % of controls respectively) when prepared in the absence of exogenous FAD, and were not restored to control values when prepared in 25 μ M-FAD (29, 47, 28, 7 and 17 %). Clofibrate feeding increased the activities of butyryl-CoA, octanoyl-CoA and palmitoyl-CoA dehydrogenases (by 48, 116 and 98 % of controls respectively), but not, by contrast, the activities of isovaleryl-CoA and isobutyryl-CoA dehydrogenases (62 and 102 % of controls respectively). The mitochondrial fractions from riboflavin-deficient and from clofibrate-fed rats oxidized palmitoylcarnitine in State 3 at rates of 32 and 163 % respectively of those from control rats.

INTRODUCTION

Organic acidurias occur in several clinical conditions, and they are often an indicator of the primary biochemical defect, particularly when this involves one of the mitochondrial acyl-CoA dehydrogenases (Gregersen, 1985). Some animal models with experimentally induced metabolic defects may be suitable for the study of organic acidurias (Sherratt & Veitch, 1984). In these, some unmetabolized long-chain fatty acids are ω -oxidized to dicarboxylic acids, which are activated to their CoA esters by an enzyme in the endoplasmic reticulum (Vamecq *et al.* 1985) and then β -oxidized to medium-chain dicarboxylic acids, which are excreted in the urine (see Sherratt & Veitch, 1984). A typical dicarboxylic aciduria is observed in riboflavin-deficient rats (Goodman, 1981; Gregersen & Kølvrå, 1982). Rat liver mitochondria contain five FAD-dependent acyl-CoA dehydrogenases (Ikeda *et al.* 1983), three of which are involved in β -oxidation of straight-chain fatty acids. The decrease in those three activities in riboflavin-deficient rats is associated with a concomitant decrease in the capacity for mitochondrial β -oxidation (Hoppel *et al.* 1979; Sakurai *et al.* 1982; Veitch *et al.* 1987a). These animals also excrete large amounts of the glycine conjugates of isovalerate, 2-methylbutyrate and isobutyrate, which suggests that the two FAD-dependent dehydrogenases which act on the branched-chain acyl-CoA esters formed by the metabolism of leucine, isoleucine and valine respectively, are also affected (Goodman, 1981). There has been no direct demonstration that these enzymes are affected in riboflavin-deficient rats.

A dicarboxylic aciduria and excretion of isovalerylglycine and isobutyrylglycine occurs in rats poisoned with hypoglycin [(+)-(2S:4S)-2-amino-4,5-methylenehex-5-enoic acid] (Tanaka, 1972), caused by inhibition by its active metabolite, methylene-cyclopropylacetyl-CoA, of the short-chain and medium-chain acyl-CoA dehydrogenases (Billington *et al.*, 1978; Wenz *et al.*, 1981). Excretion of isovalerylglycine and isobutyrylglycine is consistent with inhibition of isovaleryl-CoA and 2-methylbutyryl-CoA dehydrogenases (Ikeda & Tanaka, 1983a,b). Pretreatment of rats with the hypolipidaemic drug clofibrate (ethyl 4-chlorophenoxyisobutyrate) dramatically protects rats from the toxic effects of hypoglycin (Van Hoof *et al.*, 1985), although some dicarboxylic aciduria and the excretion of large amounts of isovalerylglycine and isobutyrylglycine by these animals remains, consistent with inhibition of the branched-chain acyl-CoA dehydrogenases (Veitch *et al.*, 1987b). There have been no studies on the effects of clofibrate on the branched-chain acyl-CoA dehydrogenases. We therefore determined the effects of riboflavin deficiency and of clofibrate feeding on these enzymes in rat liver mitochondria.

MATERIALS AND METHODS

Clofibric acid, FAD and phenazine ethosulphate were from Sigma Chemical Co., St Louis, MO, U.S.A. DEAE-Sephadex A-50 was purchased from Pharmacia, Uppsala, Sweden. Acyl-CoA esters were from either Sigma or Pharmacia. All other chemicals were from either Janssen Chimica, Beerse, Belgium, or Merck, Darmstadt, Germany.

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Young male Wistar rats (approx. 80 g) were maintained on one of the following diets. Control animals were given standard laboratory chow diet (A-03; U.A.R., Epinay, France) for 6 weeks. Clofibrate-treated animals received the same diet containing 0.5 % (w/w) neutralized clofibric acid for 2 weeks. Riboflavin-deficient rats were prepared by giving them a semi-synthetic vitamin-deficient diet (ICN Pharmaceuticals, Cleveland, OH, U.S.A.) for 6 weeks (Veitch *et al.*, 1987a). All animals were fed *ad libitum* and were fasted overnight (18 h) before being killed by decapitation. Since this work was done, Ross & Hoppel (1987) have reported that the low butyryl-CoA dehydrogenase activity in the livers of riboflavin-deficient rats was approximately doubled after a 24 h fast.

Mitochondrial fractions were prepared in 0.3 M-mannitol/10 mM-Hepes/1 mM-EGTA, pH 7.4, and the mitochondrial oxidation rates measured polarographically in State 3 at 30 °C as described by Turnbull *et al.* (1984). Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Acyl-CoA dehydrogenases were extracted from mitochondrial fractions (stored at -80 °C) as previously described (Furuta *et al.*, 1981a; Veitch *et al.*, 1987b), except that 1 mM-EDTA was included in all solutions and the thawed mitochondria were first sonicated (5 s at 50 W, with a Branson sonifier model B12 with microtip). Activities were measured at 30 °C by monitoring the initial linear rate of bleaching of dichlorophenol-indophenol at 600 nm ($\epsilon = 21 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) with phenazine ethosulphate as intermediate electron acceptor in a total volume of 1 ml. Butyryl-CoA and isovaleryl-CoA dehydrogenase activities were assayed by using 1.5 mM-phenazine ethosulphate, and octanoyl-CoA, palmitoyl-CoA and isobutyryl-CoA dehydrogenases with 3 mM-phenazine ethosulphate. When the branched-chain acyl-CoA dehydrogenases were assayed, the mitochondrial extracts were preincubated for 5 min until reduction of dichlorophenol-indophenol by endogenous compounds was complete, before starting the reaction with 100 μM -isovaleryl-CoA or 100 μM -isobutyryl-CoA. Isobutyryl-CoA, which is available commercially, was used to measure 2-methylbutyryl-CoA dehydrogenase, as this enzyme is equally active with both substrates (Ikeda & Tanaka, 1983a). The straight-chain acyl-CoA dehydrogenase activities were measured with 30 μM acyl-CoA esters in the presence of 1 mM-*N*-ethylmaleimide (which inhibits the branched-chain enzymes; Ikeda *et al.*, 1983), and only 1–2 min preincubation was necessary.

Citrate synthase (Shepherd & Garland, 1969), succinate dehydrogenase (Hoppel & Cooper, 1968) and glutamate dehydrogenase (Fisher, 1985) were assayed by standard methods. One unit of enzyme is that amount required to catalyse the formation of 1 μmol of product/min. All results quoted are means $\pm$ S.E.M. for four animals in each experimental group.

RESULTS

Acyl-CoA dehydrogenase activities were linear with respect to enzyme concentration up to 2.5 munits/ml. Recoveries of the enzyme activities and protein were proportional to the amount of mitochondrial protein extracted up to 30 mg. Inclusion of FAD in extraction media increased recovery during purification of the

Fig. 1. Effects of the addition of 25 μM -FAD to the extraction and assay media on the percentage recoveries of acyl-CoA dehydrogenase activities from untreated rat liver mitochondrial preparations

Extracts were prepared and assayed in the absence of FAD (□), assayed in 25 μM -FAD (■), or prepared with 25 μM -FAD in the extraction media (△). The 25 μM -FAD in the extraction buffer gave concentrations in the assay cuvette of 0.5–0.75 μM , 1 μM and 2 μM for straight-chain acyl-CoA, isovaleryl-CoA and isobutyryl-CoA dehydrogenase activities respectively, with the volumes of enzyme solution used. Each point represents the mean of four preparations (bars indicate S.E.M.); 100 % recovery was taken as that obtained when extracts were prepared and assayed in 25 μM -FAD.

enzymes (Ikeda *et al.*, 1983), so the effects of adding 25 μM -FAD were examined in control preparations. Isovaleryl-CoA and isobutyryl-CoA dehydrogenases were virtually undetectable when FAD was omitted, and were only restored to 54 % and 30 % of their maximum activities by addition of FAD to the assay cuvette (Fig. 1). Straight-chain activities were less affected, except for octanoyl-CoA dehydrogenase activity, which was 43 % lower when FAD was omitted during extraction and assay (Fig. 1). Inclusion of FAD in the extraction media did not influence recovery of total protein, except in preparations from riboflavin-deficient animals, when the recovery was 12 % greater. Therefore 25 μM -FAD was included routinely in all extraction and assay media.

The riboflavin-deficient diet caused deficiency in the test animals, as assessed by the ratios of the activities of erythrocyte glutathione reductase in the presence and absence of added FAD (controls, 1.2 ± 0.02 ; deficient diet, 2.2 ± 0.1 ; $P < 0.001$) (Tillotson & Sauberlich, 1971) and the characteristic dicarboxylic aciduria (Gegersen, 1985; Veitch *et al.*, 1987a). The body weights of control animals were 281 ± 4 g and those of deficient animals were 114 ± 7 g, but the liver weights were proportionately larger than in controls (3.0 ± 0.1 % and 5.0 ± 0.4 % of body wt. in controls and tests respectively). Clofibrate feeding also causes hepatomegaly (liver wt. 6.1 ± 0.5 % of body wt.), with a massive proliferation of peroxisomes and an increased mitochondrial content (Gear *et al.*, 1974; Van Hoof *et al.*, 1985). The specific activities of the mitochondrial marker enzymes, succinate dehydrogenase, glutamate dehydrogenase and citrate synthase, in the liver mitochondrial fraction from clofibrate-fed rats

Table 1. Activities of mitochondrial marker enzymes and rates of mitochondrial oxidations in State 3 in liver mitochondria from untreated, riboflavin-deficient and clofibrate-fed rats

Succinate (10 mM) and palmitoylcarnitine (6.7 μ M) were used as substrates for the oxidation rates. It was confirmed that palmitoylcarnitine oxidation was inhibited by 1 mM-KCN. Activities are expressed as munits of enzyme activity per mg of mitochondrial protein, or as ng-atoms of O/min per unit of citrate synthase. Means $\pm$ S.E.M. for four animals in each group are shown, with the percentages of control values in parentheses. Significance of differences from controls: * P < 0.05, ** P < 0.01, *** P < 0.001. Other details are given in the Materials and methods section.

Activity	Rats...	Activity or rate		
		Control	Riboflavin-deficient	Clofibrate-fed
Succinate dehydrogenase		51 $\pm$ 2	23 $\pm$ 3*** (45)	40 $\pm$ 3* (78)
Glutamate dehydrogenase		191 $\pm$ 13	380 $\pm$ 34** (199)	148 $\pm$ 4* (77)
Citrate synthase		225 $\pm$ 14	220 $\pm$ 21 (98)	170 $\pm$ 3* (76)
Succinate oxidation		768 $\pm$ 33	642 $\pm$ 62 (84)	818 $\pm$ 58 (107)
Palmitoylcarnitine oxidation		360 $\pm$ 26	114 $\pm$ 14*** (32)	586 $\pm$ 30** (163)

were about 77 % of those in control fractions (Table 1), because of contamination with peroxisomes. The specific activities of mitochondrial marker enzymes are not affected by clofibrate feeding (Gear *et al.*, 1974), but riboflavin deficiency causes a decrease in succinate dehydrogenase activity and an increase in glutamate dehydrogenase activity (Table 1). The activities of the acyl-CoA dehydrogenases were therefore related to that of citrate synthase, which is not affected by riboflavin deficiency. Clofibrate feeding had no effect on the rate of succinate oxidation by liver mitochondria (107 % of controls), but increased the rate of cyanide-sensitive palmitoylcarnitine oxidation (163 %), whereas both activities were decreased by riboflavin deficiency (to 84 % and 32 % of controls respectively; Table 1).

The activities of the three straight-chain acyl-CoA dehydrogenases were lower in the livers of riboflavin-deficient animals than in the controls (Table 2). Isovaleryl-

CoA and isobutyryl-CoA dehydrogenase activities were also very low (7 % and 17 % of control values respectively). These results were not qualitatively different when FAD was omitted from the extraction media, although the increase in recovery in the presence of FAD was greater from riboflavin-deficient livers (Table 2). These results differ from those of Sakurai *et al.* (1982), who found that preincubation of riboflavin-deficient mitochondrial preparations with 100 μ M-FAD fully restored the activities of medium-chain and long-chain acyl-CoA dehydrogenases to control values, whereas our results agree with those of Ross & Hoppel (1987), who found that all three straight-chain activities remained low in riboflavin-deficient mitochondria even when assayed in 100 μ M-FAD. Conversely, the activities of the three straight-chain acyl-CoA dehydrogenases were increased after feeding clofibrate, as reported previously (Furata *et al.*, 1981b). However, isobutyryl-CoA dehydro-

Table 2. Acyl-CoA dehydrogenase activities in extracts of liver mitochondria from untreated, riboflavin-deficient and clofibrate-fed rats

Mitochondrial fractions were divided into two samples, and each was extracted in the absence or presence of 25 μ M-FAD. The acyl-CoA dehydrogenases were assayed in the presence of 25 μ M-FAD; activities are expressed as munits/unit of citrate synthase. The results are means $\pm$ S.E.M. for four preparations in each group, and the values as percentages of the appropriate controls are given in parentheses. Significance of differences from the controls: ** P < 0.01, *** P < 0.001.

Substrate	Activity		
	Control	Riboflavin-deficient	Clofibrate-fed
(a) No FAD during extraction: 25 μ M in assay cuvette			
Butyryl-CoA	157 $\pm$ 5	17 $\pm$ 3*** (11)	250 $\pm$ 12*** (159)
Octanoyl-CoA	102 $\pm$ 4	29 $\pm$ 4*** (28)	237 $\pm$ 20*** (232)
Palmitoyl-CoA	114 $\pm$ 4	18 $\pm$ 2*** (16)	205 $\pm$ 16** (180)
Isovaleryl-CoA	34 $\pm$ 2	2.0 $\pm$ 0.3*** (6)	29 $\pm$ 2 (85)
Isobutyryl-CoA	1.8 $\pm$ 0.13	< 0.1	4.0 $\pm$ 0.22*** (222)
(b) 25 μ M-FAD in extraction media and assay cuvette			
Butyryl-CoA	180 $\pm$ 4	53 $\pm$ 6*** (29)	266 $\pm$ 10*** (148)
Octanoyl-CoA	131 $\pm$ 6	62 $\pm$ 4*** (47)	283 $\pm$ 17*** (216)
Palmitoyl-CoA	121 $\pm$ 4	34 $\pm$ 3*** (28)	239 $\pm$ 10*** (198)
Isovaleryl-CoA	65 $\pm$ 3	4.8 $\pm$ 1.2*** (7)	40 $\pm$ 2*** (62)
Isobutyryl-CoA	9.0 $\pm$ 1.1	1.5 $\pm$ 0.4** (17)	9.2 $\pm$ 0.7 (102)

genase activity was unchanged (102 % of control values), whereas isovaleryl-CoA dehydrogenase activity was significantly lower (62 % of controls; $P < 0.001$) (Table 2). However, the total hepatic isovaleryl-CoA dehydrogenase activity per rat was not decreased, since clofibrate feeding increased both the size of the livers and their content of mitochondria per gram.

DISCUSSION

We have confirmed that the activities of the three FAD-dependent straight-chain acyl-CoA dehydrogenases are low in liver mitochondria from riboflavin-deficient rats (Hoppel *et al.*, 1979; Sakurai *et al.*, 1982; Veitch *et al.*, 1987a; Ross & Hoppel, 1987), and have shown for the first time that the activities of isovaleryl-CoA and 2-methylbutyryl-CoA dehydrogenases are also very low. It has been suggested that the excretion of isovalerylglycine and isobutyrylglycine by riboflavin-deficient rats is due to low activities of isovaleryl-CoA and 2-methylbutyryl-CoA dehydrogenases (Goodman, 1981). The accumulation of [$U-^{14}C$]2-methylbutyryl-CoA in liver mitochondria from riboflavin-deficient rats when incubated with [$U-^{14}C$]3-methyl-2-oxopentanoate, the first product in the degradation of isoleucine, also indicated a low activity of 2-methylbutyryl-CoA dehydrogenase in these animals (Causey *et al.*, 1986).

Clofibrate feeding increases the ability of the rat liver to oxidize long-chain fatty acids, with co-ordinated increases in both the capacity for mitochondrial β -oxidation, associated with increased activities of the straight-chain acyl-CoA dehydrogenases (Table 2) (Furuta *et al.*, 1981b), and for peroxisomal β -oxidation (Lazarow, 1978; Van Hoof *et al.*, 1985). There were good correlations between the rate of palmitoylcarnitine oxidation by mitochondria in Staté 3 and the activities of the three straight-chain acyl-CoA dehydrogenases in mitochondrial extracts from control, riboflavin-deficient and clofibrate-fed rat livers (Fig. 2). By contrast, clofibrate feeding had no effect on the activities of the branched-chain acyl-CoA dehydrogenases involved in leucine, isoleucine and valine degradation (Table 2), suggesting that the synthesis of the enzyme systems involved in the catabolism of straight-chain fatty acids is regulated differently from those involved in amino acid metabolism.

Poisoning rats with hypoglycin causes inhibition of short-chain and medium-chain acyl-CoA dehydrogenases (Billington *et al.*, 1978; Van Hoof *et al.*, 1985). Clofibrate feeding prevents the toxic effects of hypoglycin, with about 60 % and 50 % respectively of butyryl-CoA and decanoyl-CoA dehydrogenases remaining uninhibited (Van Hoof *et al.*, 1985; Veitch *et al.*, 1987b). It was suggested that clofibrate induces the formation of novel enzymes with straight-chain acyl-CoA dehydrogenase activities resistant to inhibition, which could partly explain resistance of hypoglycin poisoning (Van Hoof *et al.*, 1985). It is known that isovaleryl-CoA dehydrogenase is inactivated by hypoglycin metabolites (Billington *et al.*, 1978). The branched-chain acyl-CoA dehydrogenases are strongly inhibited in both control rats and clofibrate-fed rats given hypoglycin, as judged by the organic aciduria and excretion of isovalerylglycine and isobutyrylglycine that is induced (Veitch *et al.*, 1987b). This agrees with the lack of effect of clofibrate feeding on the activities of these enzymes (Table 2).

Fig. 2. Correlation between the rates of oxidation of palmitoylcarnitine by mitochondria and straight-chain acyl-CoA dehydrogenase activities in mitochondrial extracts from untreated, riboflavin-deficient and clofibrate-fed rats

Palmitoylcarnitine oxidation (ng-atoms of O/min per unit of citrate synthase) and acyl-CoA dehydrogenase activities (munits/unit of citrate synthase) extracted and assayed in the presence of 25 μ M-FAD as described in the Materials and methods section. Values for individual rats are shown: control ($\square$), clofibrate-fed ($\blacktriangle$) and riboflavin-deficient ($\blacksquare$). The equations for the calculated lines are:

$$\text{Butyryl-CoA dehydrogenase, } y = 0.43x + 17.5 \text{ (} r = 0.95, P < 0.001 \text{)}$$

$$\text{Octanoyl-CoA dehydrogenase, } y = 0.45x + 0.85 \text{ (} r = 0.95, P < 0.001 \text{)}$$

$$\text{Palmitoyl-CoA dehydrogenase, } y = 0.42x - 14.9 \text{ (} r = 0.98, P < 0.001 \text{)}$$

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