Organic aciduria in rats made resistant to hypoglycin toxicity by pretreatment with clofibrate

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1. The lethal, hypoglycaemic and hypothermic effects of hypoglycin in fasted rats are prevented if the rats had been fed on a diet containing clofibrate (0.5% w/w). 2. Injection of hypoglycin into fasted rats maintained on a standard diet caused severe prostration, hypothermia and a massive dicarboxylic aciduria [Tanaka (1972) *J. Biol. Chem.* **247**, 7465–7478]. 3. Rats maintained on a diet containing clofibrate appeared normal after injection of hypoglycin, but had a marked dicarboxylic aciduria which was less than that induced in rats on a normal diet. 4. After administration of hypoglycin, butyryl-CoA and decanoyl-CoA, but not palmitoyl-CoA, dehydrogenase activities were strongly inhibited (80–95%) in the livers of animals on a standard diet. 5. Clofibrate feeding decreased the inhibition of these dehydrogenases to about 40–60%. 6. It was concluded that although clofibrate protects against the toxic effects of hypoglycin, some enzyme inhibitions as indicated by dicarboxylic aciduria are only partly prevented.

INTRODUCTION

Poisoning by hypoglycin {[+](2*S*:4*S*)-2-amino-4,5-methanohex-5-enoic acid}, the toxin of the unripe Jamaican ackee fruit, *Blighia sapida*, is characterized by severe metabolic disturbances, including hypoglycaemia, organic acidaemia and aciduria accompanied by prostration, hypothermia and often death (Tanaka, 1972; Sherratt, 1986). These metabolic disturbances are consequences of the inactivation of several, but not all, acyl-CoA dehydrogenases by methylenecyclopropylacetyl-CoA, a metabolite of hypoglycin. When hypoglycin was given to rats that had been fed on a diet containing the hypolipidaemic drug clofibrate (ethyl 4-chlorophenoxyisobutyrate) (0.5%, w/w) for 4 weeks, the lethal, hypothermic and hypoglycaemic effects were completely prevented (Van Hoof *et al.*, 1985). However, liver mitochondrial butyryl-CoA dehydrogenase (EC 1.3.99.2) was still partly inhibited in these animals, and the oxidation of decanoylcarnitine by liver mitochondria showed some unusual features (Van Hoof *et al.*, 1985). It was therefore decided to determine whether feeding clofibrate influenced the organic aciduria caused by administration of hypoglycin.

MATERIALS AND METHODS

Materials

Hypoglycin (85% pure) was prepared from ackee seeds as described by Billington & Sherratt (1981). AnalaR-grade diethyl ether and ethyl acetate were purchased from BDH Chemicals, and freshly distilled over copper gauze before use. Clofibrate was a gift from I.C.I., Macclesfield, Cheshire, U.K. Acyl-CoA esters and other biochemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Animals

Male Wistar rats (100–135 g) were fed on either a standard low-fat (Rat and Mouse No. 1 expanded diet; Special Diet Services Ltd., Witham, Essex, U.K.) Laboratory-rat chow, or the same diet sprayed with a 10% (v/v) solution of clofibrate in acetone to give a final content of 0.5% (w/w) clofibrate, for 26 days. The animals were maintained on a standard 12 h-dark/12 h-light cycle and weighed regularly to ensure normal weight gain. Clofibrate had no significant effect on growth, all animals weighing 200–230 g when used. On the day of use, they were placed on individual metabolism cages without food, with water *ad libitum*, at 09:00 h. At 18:00 h, hypoglycin was administered intraperitoneally (10 mg/kg body wt.) to five animals maintained on the standard diet and to three animals on clofibrate. Three animals on each diet received saline intraperitoneally as controls. Urine was collected overnight, and the following morning the animals were examined before being killed by cervical dislocation. Livers were removed, and a mitochondrial fraction was prepared as described by Turnbull *et al.* (1984) in 250 mM-sucrose/2 mM-Tris/0.1 mM-EGTA, pH 7.2, the final pellet being resuspended in this medium at 20 mg of protein/ml. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Acyl-CoA dehydrogenases

Mitochondrial fraction (1 ml) was diluted with 4 ml of 20 mM-potassium phosphate (pH 7.5) containing 0.05% Triton X-100, and centrifuged at 140 000 *g*_{av} for 45 min at 4 °C. The resulting supernatant was applied to a column of DEAE-Sephadex A-50 (2 ml bed volume) equilibrated in 20 mM-phosphate buffer. The column was then washed with 5 ml of 20 mM-phosphate buffer before

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Table 1. Acyl-CoA dehydrogenase activities assayed in extracts of liver mitochondrial fractions

Activities are expressed as nmol of dichlorophenolindophenol reduced/min per mg of mitochondrial protein extracted. Means $\pm$ S.E.M. for three animals in each group are shown, with and without 10 min preincubation in 100 μ M-FAD at 0 °C. ^a*P* < 0.05 versus controls (control diet); ^b*P* < 0.05 versus clofibrate-fed controls.

Substrate	Diet . . .	Activity			
		Control		Clofibrate	
		Control	+ hypoglycin	Control	+ hypoglycin
Butyryl-CoA					
No FAD		9.6 $\pm$ 0.8	1.03 $\pm$ 0.51 ^a	13.0 $\pm$ 1.2	7.6 $\pm$ 0.1 ^b
+ FAD		13.7 $\pm$ 0.5	1.67 $\pm$ 0.70 ^a	18.2 $\pm$ 0.8 ^a	11.5 $\pm$ 0.8 ^b
Decanoyl-CoA					
No FAD		4.5 $\pm$ 0.5	0.63 $\pm$ 0.27 ^a	10.3 $\pm$ 0.8 ^a	3.7 $\pm$ 0.3 ^b
+ FAD		5.3 $\pm$ 0.5	1.54 $\pm$ 0.40 ^a	10.8 $\pm$ 1.3 ^a	5.1 $\pm$ 0.5 ^b
Palmitoyl-CoA					
No FAD		2.0 $\pm$ 0.1	2.0 $\pm$ 0.6	6.1 $\pm$ 0.6 ^a	4.9 $\pm$ 1.0 ^a
+ FAD		3.0 $\pm$ 0.2	2.6 $\pm$ 0.5	6.5 $\pm$ 0.3 ^a	6.3 $\pm$ 0.7

Table 2. Major organic acids detected in the urines of the experimental animals

Values are expressed as μ g/mg of creatinine. Means $\pm$ S.E.M. for three animals in each group are shown.

Compound	Diet . . .	Content			
		Control		Clofibrate	
		Control	+ hypoglycin	Control	+ hypoglycin
Succinate		142 $\pm$ 29	329 $\pm$ 35	165 $\pm$ 54	205 $\pm$ 34
Ethylmalonate		0	163 $\pm$ 78	0	86 $\pm$ 26
Methylsuccinate		0	87 $\pm$ 36	0	1.4 $\pm$ 0.7
Glutarate		4.4 $\pm$ 2.3	4264 $\pm$ 1860	3.4 $\pm$ 0.4	271 $\pm$ 202
Isobutyrylglycine		0	76 $\pm$ 20	0	156 $\pm$ 21
Adipate		13 $\pm$ 1	576 $\pm$ 40	5 $\pm$ 1	89 $\pm$ 32
Butyrylglycine		0	629 $\pm$ 106	0	310 $\pm$ 4
Isovalerylglycine		31 $\pm$ 4	1569 $\pm$ 568	29 $\pm$ 6	2071 $\pm$ 158
Octenedioic acid		45 $\pm$ 5	352 $\pm$ 229	18 $\pm$ 7	94 $\pm$ 13
Suberate		16 $\pm$ 3	188 $\pm$ 60	0	8 $\pm$ 0.2
Citrate		141 $\pm$ 35	1191 $\pm$ 150	47 $\pm$ 15	146 $\pm$ 63
Decenedioic acid		44 $\pm$ 14	5891 $\pm$ 2412	0	53 $\pm$ 9
Sebacate		63 $\pm$ 5	871 $\pm$ 344	41 $\pm$ 11	78 $\pm$ 14
Hippurate		320 $\pm$ 41	431 $\pm$ 59	255 $\pm$ 99	199 $\pm$ 72

elution of the acyl-CoA dehydrogenase activities with 4.5 ml of 500 mM-potassium phosphate (pH 7.5).

The three straight-chain dehydrogenases were assayed at 30 °C, with 20 μ M-butyryl-CoA, -decanoyl-CoA and palmitoyl-CoA as substrates for short-, medium- and long-chain activities respectively. Phenazine ethosulphate was used as intermediate electron acceptor (0.9 mM with butyryl-CoA, 3.6 mM with decanoyl-CoA and palmitoyl-CoA) with 35 μ M-dichlorophenolindophenol as final acceptor in 50 mM-phosphate (pH 7.5), containing 1 mM-*N*-ethylmaleimide to inhibit non-specific reaction with free CoA and thioesters (Furuta *et al.*, 1981a). Assays were performed in duplicate, and after incubation of extracts with 100 μ M-FAD for 10 min at 0 °C.

Analysis of urinary organic acids

Urine (2 ml) was acidified by the addition of 100 μ l of 5 M-HCl, and internal standards were added (100 μ g

each of octanoate and phenyl butyrate). Organic acids were extracted with redistilled diethyl ether and ethyl acetate. The organic extract was evaporated to dryness and treated with an excess of ethereal diazomethane and left at room temperature for at least 30 min before analysis. The methylated extract was analysed by g.l.c. by using a fused-silica column (DB-5 capillary column; J & W Scientific, Jones Chromatography, Hengoed, Mid Glamorgan, U.K.; 30 m $\times$ 0.25 mm internal diam., film thickness 0.25 μ m) in a Hewlett-Packard gas chromatograph (model 5890) fitted with an on-column injector and a flame-ionization detector. The gas flow rates were as follows; carrier gas (hydrogen) 1 ml/min, detector hydrogen 30 ml/min, air 300 ml/min, nitrogen 30 ml/min. Temperature programming was used (50–250 °C at 2 °C/min) and the detector temperature was 320 °C. Quantification was performed by reference to the phenyl butyrate internal standard, by using peak area ratios

determined with a Hewlett-Packard integrator (model 3392). Relative response ratios were assumed to be unity. Results are expressed with respect to the urinary creatinine concentration, determined by the alkaline-picric acid reaction. Urinary organic acids were identified by reference to standard compounds where available and by the analysis of representative samples by g.l.c./mass spectrometry (Finnigan model 1020B instrument).

Statistical significance of the differences between means was calculated by Student's *t* test for the enzyme activities. In view of the wide variation in the amounts of organic acids excreted, no tests of significance were performed for these values.

RESULTS

Of the five animals maintained on a normal diet and given hypoglycin, at the end of the experiment two were dead, two were comatose and severely hypothermic, and one appeared normal. This is consistent with the large variability of response to hypoglycin (Billington & Sherratt, 1981). The clofibrate-fed animals given hypoglycin appeared normal.

As shown in Table 1, the hypoglycin-treated animals on the normal diet had significantly decreased activities of short- and medium-chain dehydrogenases, long-chain activity being unaffected. As expected, the clofibrate-fed animals had higher activities (Furuta *et al.*, 1981b), which were significantly decreased by hypoglycin treatment, but only to values not significantly different from those for controls fed on normal diet. Incubation with 100 μ M-FAD caused similar increases in activity in all groups, suggesting that there was no difference in the amounts of apoenzyme present.

The massive organic aciduria in fasted rats given hypoglycin (Tanaka, 1972) was confirmed (Table 2, Fig. 1). Only traces of organic acids and glycine conjugates were found in normal controls and clofibrate-treated controls. Clofibrates were excreted by the clofibrate-fed animals, and there were compounds representing hypoglycin metabolites by the hypoglycin-treated animals. However, clofibrate-fed animals given hypoglycin still gave a pronounced organic aciduria, which was less than that in rats that had received hypoglycin alone (Table 2, Fig. 1), the most notable compounds excreted being glutaric acid and the glycine conjugates of isobutyrate, butyrate and isovalerate.

DISCUSSION

The rate of β -oxidation of saturated acylcarnitine esters by liver mitochondrial fractions from hypoglycin-treated rats is slowed and only proceeds as far as butyrate (Osmundsen & Sherratt, 1975), because of the inhibition of general acyl-CoA and butyryl-CoA dehydrogenases (Billington *et al.*, 1974; Wenz *et al.*, 1981). Dicarboxylic aciduria is thought to be due to excess fatty acids in the liver being partly converted into long-chain dicarboxylic acids in the endoplasmic reticulum, the mono-CoA esters of which are formed in the cytosol and then chain-shortened to medium-chain dicarboxylic acids in the peroxisomes (Sherratt & Veitch, 1984; Vamecq *et al.*, 1985). 4-*cis*-Decenoic acid and 4-*cis*-octenoic acid are formed specifically from linolenate (Kunau & Lauterbach, 1978) after administration of hypoglycin, possibly because of inhibition of 4-enoyl-

Fig. 1. Examples of gas-liquid chromatograms from the four treatment groups

Abbreviation: FID, flame-ionization-detector response. The major peaks of interest are identified as: S, solvent front; 1, succinate; 2, ethylmalonate; 3, methylsuccinate; 4, octanoate (internal standard); 5, glutarate; 6, isobutyrylglycine; 7, adipate; 8, butyrylglycine; 9, isovaleryl-glycine; 10, phenyl butyrate (internal standard); 11, octenedioate; 12, suberate; 13, citrate; 14, decenedioate; 15, sebacate; 16, hippurate.

CoA reductase necessary for the degradation of some polyunsaturated fatty acids (Osmundsen *et al.*, 1982). Glutaric aciduria is apparently due to inhibition of glutaryl-CoA dehydrogenase (Tanaka, 1972; Hine & Tanaka, 1984). Inhibition of isovaleryl-CoA dehydrogenase and 2-methylbutyryl-CoA dehydrogenase (Tanaka, 1972; Billington *et al.*, 1978) leads to accumulation of isovaleryl-CoA and 2-methylbutyryl-CoA, derived from the metabolism of leucine and isoleucine respectively, and these esters are deacylated to isovalerate and 2-methylbutyrate or conjugated with glycine.

The complex metabolic disturbances, including inhibition of gluconeogenesis (Osmundsen *et al.*, 1978; Sherratt, 1981), are partly caused by limitation of fatty acid oxidation and by high steady-state concentrations of isovaleryl-CoA, 2-methylbutyryl-CoA and butyryl-CoA in the mitochondrial matrix in liver (Billington *et al.*, 1978; Stirk *et al.*, 1983). The effects of feeding with clofibrate on rat liver are complex, including a 6–10-fold increase in peroxisomal β -oxidation, a 2–3-fold increase in mitochondrial β -oxidation, a 2-fold increase in the fatty-acid-binding Z-protein (Renaud *et al.*, 1978) and a 3-fold increase in total contents of CoASH and carnitine (Mannaerts *et al.*, 1978). Van Hoof *et al.* (1985) showed that the virtually complete inactivation of butyryl-CoA dehydrogenase activity caused by administration of hypoglycin in untreated rats was partly prevented by

pretreatment with clofibrate. In agreement with this, the apparent activities of short- and medium-chain acyl-CoA dehydrogenases were very strongly inhibited (up to 95%) in rats given hypoglycin that had been maintained on a normal diet, with no significant effects on palmitoyl-CoA dehydrogenase activity (Table 1). The three dehydrogenase activities, particularly medium- and long-chain acyl-CoA dehydrogenases, were markedly increased by clofibrate feeding, and the short- and medium-chain activities were only partly inhibited after administration and hypoglycin (40–60%) (Table 1). Incubation with FAD before assay increased both control and inhibited rates, indicating loss of some enzyme-bound FAD during preparation. The clofibrate-induced hepatomegaly, which is also associated with a proliferation of mitochondria (Kurup *et al.*, 1970), was observed, liver weights being 50% greater in the clofibrate-fed animals. Thus the total remaining butyryl-CoA dehydrogenase activity in the livers of clofibrate-fed animals given hypoglycin was greater than in the livers of control rats fed on a normal diet.

The significant organic aciduria in clofibrate-fed rats given hypoglycin indicates that the metabolic disturbances were only partly prevented, but, in this mode, those remaining were not enough to cause hypoglycaemia, hypothermia or death (see Van Hoof *et al.*, 1985). This protection may be due to both a decrease in the extent of the inhibitions after administration of hypoglycin and an increased resistance to these inhibitions by clofibrate. The actual nature of this protective property of clofibrate has not been determined. As it was apparent that some inhibition of enzyme activities still occurred in the clofibrate-fed animals, it remains to be seen whether this protection was due to increased removal or 'detoxification' of hypoglycin, for example by increased glycine N-acylase activity (Bartlett & Gompertz, 1974). Indeed, Al-Bassam & Sherratt (1981) found that administration of large amounts of glycine protected rats from the toxic effects of hypoglycin, and decreased, but did not prevent, branched-chain organic acidemia. Another possibility is that the induced mitochondrial fatty acyl-CoA dehydrogenases are less susceptible to hypoglycin inhibition, as suggested by Van Hoof *et al.* (1985). In this respect it is interesting to note that large amounts of isovaleryl-glycine and isobutyryl-glycine, substrates for isovaleryl-CoA dehydrogenase and 2-methylbutyryl-CoA dehydrogenase respectively, are excreted by clofibrate-fed animals after treatment with hypoglycin, indicating that these activities are still severely affected in these animals. Abnormal amounts of glutarate (70 times control values) are also excreted by these animals, but to a lesser extent than by poisoned control-fed animals (1000 times), suggesting that the glutaryl-CoA dehydrogenase activity is less susceptible to hypoglycin inhibition in clofibrate-fed animals, but is still affected to some extent.

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