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METABOLIC CHANGES IN FED RATS CAUSED BY CHRONIC ADMINISTRATION OF ETHYL 2{5(4-CHLOROPHENYL)PENTYL}OXIRANE-2-CARBOXYLATE, A NEW HYPOGLYCAEMIC COMPOUND

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Abstract—Ethyl 2{5(4-chlorophenyl)pentyl}oxirane-2-carboxylate (POCA) is strongly hypoglycaemic in fasted normal and diabetic rats [H. P. O. Wolf, K. Eistetter and G. Ludwig, *Diabetologia* **22**, 456 (1982)]. POCA was fed for 12 weeks to rats on a standard low-fat (3%) diet at levels of 0.05% and 0.2% to give daily intakes of about 50 and 200 mg/per kg body-wt respectively. This is much more than effective hypoglycaemic doses in fasted rats (5–10 mg/kg body-wt). The animals appeared healthy but they had slightly decreased rates of weight gain compared with the controls. POCA caused a 15% increase in the weight of the myocardium and accumulation of lipid in the liver. Chronic administration of POCA did not cause any large changes in water-soluble blood metabolite concentrations, although VLDL-triacylglycerol and both VLDL and HDL cholesterol concentrations were lowered. There were only small changes in some metabolites of the glycolytic and gluconeogenic pathways and the citrate cycle in liver and skeletal muscle. ATP concentrations were maintained in all groups. There were 2- to 3-fold increases in the total content of CoA and of carnitine and their acylated forms. POCA-feeding caused small decreases in LPL activities in heart and had variable effects in adipose tissue. POCA was also fed to a few rats on a high fat (30%) diet for 4 weeks. Only small changes in blood, liver and muscle metabolite concentrations were found, except for large increases in the liver CoA and carnitine contents. It was concluded that POCA does not cause large perturbations of glucose homeostasis, or acute toxic effects, during 12 weeks administration to normal animals at high dose levels. The very-long term importance of accumulation of lipid in liver; increase in myocardial weight; and also of hepatic peroxisomal proliferation [A. J. Bone, H. S. A. Sherratt, D. M. Turnbull and H. Osmundsen, *Biochem. biophys. Res. Commun.* **104**, 708 (1982)] cannot yet be determined. The possible use of POCA and related compounds in the chemotherapy of diabetes merits further investigation.

The metabolic effects of inhibitors of long-chain fatty acid oxidation are of considerable theoretical and practical importance because of the close relationship between fatty acid oxidation, gluconeogenesis, glucose utilisation and glycaemia [1]. Such inhibitors are of potential interest for the chemotherapy of diabetes [1]. Many compounds known to inhibit fatty acid oxidation are hypoglycaemic, but they usually have multiple metabolic effects and are toxic [1]. In 1978, Tutwiler introduced methyl tetradecyloxirane-2-carboxylate (tetradecylglycidate, 2-TDGA), a metabolite of which, presumably its CoA ester, selectively inhibits the oxidation of long-chain fatty acids and which is hypoglycaemic in fasted normal and diabetic rats [2]. More recently, ethyl 5{2(4-chlorphenyl) pentyl}oxirane-2-carboxylate (POCA) and some related substituted 2-oxirane-2-carboxylates with similar properties have been described by Wolf *et al.* [3–5]. In acute experiments POCA is a powerful hypoglycaemic agent in fasted normal and diabetic, but not fed, animals of several species and is the most potent hypoketonaemic compound known [4]. It was of interest, therefore, to determine the metabolic and toxic effects of chronic administration of high concentrations of POCA to normal rats for 12 weeks. In the accompanying paper we show that POCA-CoA strongly inhibits the oxidation of long-chain fatty acids at the stage of carnitine palmitoyltransferase I (EC 2.3.1.21) [6]. Preliminary accounts of some of this work have already appeared [7–9].

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** Abbreviations: POCA, ethyl 2{5(4-chlorophenyl)pentyl}oxirane-2-carboxylate; POCA$^-$, 2{5(4-chlorophenyl)pentyl}oxirane-2-carboxylate; POCA-CoA, 2{5(4-chlorophenyl)pentyl}oxirane-2-carbonyl-CoA; 2-TDGA, methyl 2-tetradecyloxirane-2-carboxylate (tetradecylglycidate); 2-TDGA-CoA, 2-tetradecyloxirane-2-carbonyl-CoA; CoASH; coenzyme A (free form); -CoA, coenzyme A (esterified form); CoA, coenzyme A (sum of all forms); CPT I, carnitine palmitoyltransferase I; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-ethanesulphonic acid; EDTA, ethylenediamine-tetra-acetate; EGTA, ethanedioxybis-(ethylamine)-tetra-acetate; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LPL, lipoprotein lipase.

MATERIALS AND METHODS

Chemicals. POCA and POCA$^-$ (Na salt) were gifts from the BYK Gulden Lomberg Chemische Fabrik GmbH, Konstanz, F.R.G. Most enzymes and cofactors were obtained from Boehringer Corp. (London) or Sigma (London). The sources of other materials used are given elsewhere [10, 11].

Preparation of 2-oxoglutarate dehydrogenase (EC 1.2.4.2). 2-Oxoglutarate dehydrogenase was prepared from pig heart and taken to the second polyethyleneglycol precipitation by the method of Stanley and Perham [12] and stored as recommended [12]. The specific activity assayed at 20° and pH 7.4 was 18 units per mg of protein.

Determination of protein. Protein was determined by the method of Lowry *et al.* [13].

Animals. Male albino Wistar rats from a local inbred strain were used.

Chronic administration of POCA to rats. POCA was added to a standard low fat (about 3%) cube diet (Rat/Mouse Diet No. 1, expanded, Special Diet Services Ltd., Witham, Essex, U.K.) by spraying a 10% solution in acetone with vigorous shaking to give dose levels of 0.05% and of 0.2%. The POCA content of individual cubes was measured at the BYK Gulden Laboratories by HPLC. For experiments with a high fat diet (30%) diet (Special Diet Services Ltd.) liquid POCA was mixed directly with the powdered diet to give dose levels of 0.05 and 0.1%, which were stored at −20°.

Blood sampling. Blood samples were collected between 09.00 and 10.00 hr. Blood was taken routinely from the tail vein. When appropriate, blood was also obtained directly by cardiac puncture or by cannulation of the dorsal aorta of rats anaesthetised by intraperitoneal Sagatal (about 0.001 ml of a 6% (w/v) solution/g body-wt). Blood was allowed to clot to obtain serum or was collected in heparinised tubes when plasma was required.

Assay of metabolite concentrations in blood. Blood glucose, 3-hydroxybutyrate, lactate, pyruvate, alanine and glycerol concentrations were determined by automatic fluorimetric assays [14] and acetoacetate by a manual spectrophotometric assay [15]. Total cholesterol and triacylglycerol concentrations in serum were determined enzymically using kits obtained from Boehringer. Plasma free fatty acid concentrations in serum were determined by the method of Duncome [16] at the BYK Gulden Laboratories, Konstanz.

Determination of plasma enzyme activities. Plasma creatine kinase (EC. 2.7.3.2), glutamate-oxaloacetate aminotransferase (EC 2.6.1.1) and alkaline phosphatase (EC 3.1.31) activities were assayed by standard automated techniques in the Routine Laboratories, Department of Clinical Biochemistry and Metabolic Medicine, Royal Victoria Infirmary, Newcastle.

Determination of plasma lipoprotein concentrations. Rats were anaesthetised and 5 ml of blood was obtained from each rat by cannulation of the aorta. VLDL and serum minus VLDL fractions were separated by centrifugation of serum at 100,000 *g* for 16 hr at 4° [17]. It was assumed that most of the cholesterol in the lower layer represented HDL cholesterol. Triacylglycerol and cholesterol were determined enzymically using kits provided by Boehringer, and VLDL protein by the method of Lowry *et al.* [13].

Whole body lipid and protein composition. The liver was removed and weighed, and the stomach and intestines were removed and their contents washed out with 0.14 M NaCl. These organs were replaced and the incision sewn up. The carcases were dried to constant weight and then extracted with light petroleum. The petroleum was evaporated to give the total lipid content. The total nitrogen content of the lipid free carcase was determined by the Kjeldahl method after digestion with concentrated H_2SO_4. The nitrogen content was multiplied by 6.25 to give an estimate of total protein.

Assay of metabolites in freeze-clamped tissues. Livers were freeze-clamped within 10 s of death of the animal. Rat quadriceps muscle was also frozen within 10 s. The frozen tissues were extracted with 0.33 M $HClO_4$ and the pH adjusted to about 6 with 5 M KOH. Glucose, glucose-6-phosphate, fructose-1,6-biphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, lactate, pyruvate, alanine, 2-oxoglutarate, glutamate, oxaloacetate, malate, aspartate, acetoacetate, CoASH, acetyl-CoA, carnitine, acetyl-carnitine, total acid-soluble acyl-carnitine esters, ATP, ADP and AMP were estimated in the extract by standard enzymic methods [18, 19]. Long-chain acyl-carnitine esters were assayed in the residue [18, 19]. Glycogen was determined in a separate piece of tissue [18].

Determination of lipoprotein lipase activity. LPL activity was measured in 0.025 M NH_3/NH_4Cl, pH 8.1 extracts of acetone-ether powders made from pooled hearts or epididymal adipose tissue from groups of 6 rats [20].

RESULTS

Effects of POCA on whole body and organ weights

Low fat diet. All groups of rats gained weight at the same rate for the first 5 weeks of the feeding experiment. From then on the POCA-fed groups gained weight more slowly (Fig. 1). One rat in the 0.05% POCA-fed group lost weight rapidly and was killed 10 days after the start of the experiment and found to have pancreatic necrosis. None of 98 other animals used, including control and treated groups, became ill during the 12 weeks experiment and their appearances remained normal throughout. The amount of diet eaten was not altered significantly by the addition of POCA.

The effects of POCA-feeding on the weights of the liver, heart and adrenals are shown in Table 1. There was an increase of up to 17% in liver weight (to about 3.5% of the total body-wt), which was only significant with the 0.05% POCA-diet. A few livers from the 0.02% POCA-fed group appeared pale. An increased fat content in the treated groups was apparent on electron microscopy, up to about 15% of the volume fraction of the hepatocytes [9, 21], or after centrifugation of liver homogenates [6]. All livers were soft with no evidence of fibrosis. POCA-feeding caused a highly significant increase

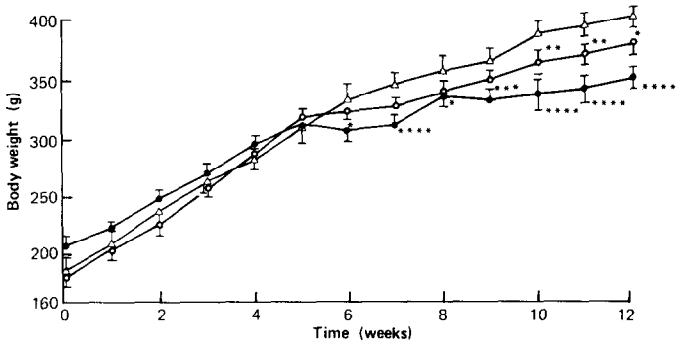

Fig. 1. Effects of POCA-feeding on the weight of rats on a standard low-fat diet. POCA was added to the diet as described in the Materials and Methods section. Controls (△), $n = 10$; diet containing 0.05% POCA (○), $n = 19$; diet containing 0.2% POCA (●), $n = 20$. Results are expressed as means ± S.E.M. Significance of differences from controls: $^{*}P < 0.05$; $^{**}P < 0.02$; $^{***}P < 0.01$; $^{****}P < 0.001$.

of about 15–17% in the wet-wt of the heart. There was no significant differences in adrenal weights between the groups.

High fat diet. All animals lost weight initially, presumably because the diet was not very palatable (Fig. 2). One animal on the 0.1% POCA-diet died after 6 days from unidentified causes and another had a pre-existing lung tumour. The livers from all groups of animals were 5–5.5% of the body-wt and were pale and fragile with a high lipid content.

Effects of POCA-feeding on whole body lipid and protein content

There were no significant differences between whole body composition of controls and 0.05% POCA-fed rats. However, rats fed on the 0.2% POCA-fed diet had about 20% less whole body fat (Table 2).

Effects of POCA on blood components

Low fat diet. Intermediary metabolites. The changes in water-soluble blood metabolite concentrations after feeding both POCA-containing diets for 6 and 12 weeks were small (Table 3). There was a lowering of alanine concentrations of about 25% in both groups and small decreases in glucose and lactate concentrations with the 0.2% POCA-fed diet. Free fatty acid concentrations in serum were similar in the control and 0.2% POCA-fed groups, but surprisingly were decreased by about 50% in the 0.05% POCA-fed group (Table 4). Total cholesterol and triacylglycerol concentrations were lowered in the POCA-fed groups.

Low fat diet. Lipoprotein concentrations. Analysis of plasma lipoproteins after 12 weeks of POCA-feeding showed a large decrease in the concentration of VLDL-triacylglycerol while the cholesterol concentration was lowered in both the VLDL and serum minus VLDL fractions with the 0.2% POCA diet (Table 5).

High fat diet. Blood pyruvate concentrations in all animals were higher than in animals on the low fat diet with much lower lactate/pyruvate ratios. Total ketone body concentrations were about twice as great as were found in animals on the low fat diet (cholesterol and triacylglycerol were not determined) (Table 6).

Plasma enzyme activities. No significant changes were found in the activities of creatine kinase, glutamate-oxaloacetate aminotransferase or alkaline phosphatase after 12 weeks POCA-feeding (not shown).

Lipoprotein lipase activity. LPL activities after 12 weeks were 144, 100 and 84 units/g wet-wt in heart of rats fed 0, 0.05% and 0.2% POCA in their diets respectively. Adipose tissue LPL activities in the same groups were 50, 68 and 34 units/g wet-wt respectively.

Table 1. Effects of 12 weeks POCA-feeding on total body, liver, heart and adrenal weights

Diet	Total body wt			Liver wt (g)	Heart wt (g)	Adrenal wt† (mg)
	Starting wt (g)	Final wt (g)	Weight gain (g)			
Control	194.4 ± 3.8	387.3 ± 10.7	193	11.4 ± 0.5	0.847 ± 0.23	36.3 ± 2.5
0.05% POCA-fed	191.1 ± 3.4	375.8 ± 6.7	185	13.4 ± 0.5**	0.999 ± 0.039***	39.9 ± 2.3
0.2% POCA-fed	196.9 ± 3.4	336.1 ± 15.4*	139	12.1 ± 0.5	1.086 ± 0.067**	42.6 ± 3.1

Results are given as means ± S.E.M. ($n = 9$). These animals were different from those used for the experiment shown in Fig. 1. Significance of differences from the controls:
$^{*}P < 0.015$; $^{**}P < 0.008$; $^{***}P < 0.004$.
† Weight of both adrenals.

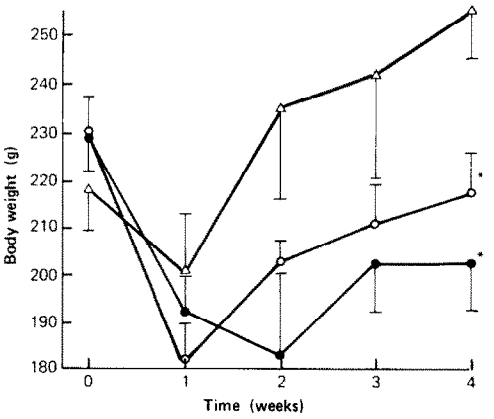

Fig. 2. Effects of POCA feeding on the weight of rats on a high fat diet. POCA was added to the diet as described in the Materials and Methods section. Controls (△), $n = 5$; diet containing 0.05% POCA (○), $n = 5$; diet containing 0.1% of POCA (●), $n = 3$. Significance of difference from controls: $^{*}P < 0.2$.

Effects of POCA-feeding on metabolite concentrations in freeze clamped tissues

Only the concentrations of a few of the metabolites determined are given in the Tables 7–9, including those which were different in POCA-fed animals compared with the controls.

Low fat diet, liver. POCA-feeding caused large increases in the concentrations of CoASH, acetyl-CoA, carnitine and acetyl-carnitine (Table 7). The concentrations of glucose-6-phosphate were lowered by 36–60%. Although the values for liver triacylglycerol concentrations for control animals were similar to those routinely obtained in this laboratory, the increases in lipid in POCA-fed animals was not reflected in enzymic assays. This suggests that the chemical nature of the lipid was changed. Indeed, lipid droplets in hepatocytes stained very strongly with OsO_4 indicating an increased extent of unsaturation [9, 21]. Preliminary examination of liver lipid by TLC suggests that it may contain large amounts of squalene (K. Bartlett, unpublished results).

Table 2. Effect of 12 weeks POCA feeding on whole body fat and protein content

	Total body-wt (g)	Percentage dry matter	Percentage of fat in dry matter	Percentage of protein in dry matter
Control	387.0 ± 7.7	38.4 ± 0.5	32.7 ± 0.6	75.1 ± 1.1
0.05% POCA-fed	341.2 ± 10.7*	38.9 ± 0.6	33.4 ± 1.9	75.6 ± 1.1
0.2% POCA-fed	299.7 ± 27.9**	37.8 ± 0.5	25.6 ± 1.1***	73.6 ± 0.7

Experimental details are given in the Materials and Methods section. Results are given as means ± S.E.M. ($n = 4$). Significant differences from the controls:
* $P < 0.02$; ** $P < 0.03$; *** $P < 0.001$.

Table 3. 12 Weeks POCA-feeding experiment, blood metabolite concentrations

	0 Weeks feeding		
Metabolite concentration (mM)	0	0.1% POCA-fed	0.2% POCA-fed
Glucose	6.17 ± 0.23	5.96 ± 0.12	5.86 ± 0.19
Lactate	2.17 ± 0.21	2.41 ± 0.27	2.22 ± 0.13
Pyruvate	0.19 ± 0.01	0.140 ± 0.01	0.15 ± 0.01
Lactate/pyruvate ratio †	14.0 ± 1.0	17.2 ± 1.8	14.8 ± 0.7
3-Hydroxybutyrate	0.059 ± 0.017	0.051 ± 0.008	0.064 ± 0.007
Acetoacetate	0.063 ± 0.008	0.057 ± 0.010	0.065 ± 0.016
3-Hydroxybutyrate/acetoacetate ratio †	0.86 ± 0.35	1.25 ± 0.32	1.46 ± 0.44
Alanine	0.43 ± 0.03	0.45 ± 0.03	0.50 ± 0.04
Glycerol	0.19 ± 0.02	0.15 ± 0.01	0.16 ± 0.01

Table 3 continued.

	6 Weeks feeding		
Metabolite concentration (mM)	0	0.1% POCA-fed	0.2% POCA-fed
Glucose	7.82 ± 0.25	6.85 ± 0.13*	6.53 ± 0.34*
Lactate	2.00 ± 0.16	1.82 ± 0.16	1.84 ± 0.16
Pyruvate	0.19 ± 0.02	0.15 ± 0.01	0.14 ± 0.01
Lactate/pyruvate ratio†	10.8 ± 0.7	12.1 ± 0.9	13.5 ± 1.0
3-Hydroxybutyrate	0.058 ± 0.005	0.051 ± 0.007	0.056 ± 0.005
Acetoacetate	0.069 ± 0.006	0.050 ± 0.005	0.073 ± 0.016
3-Hydroxybutyrate/acetoacetate ratio †	0.86 ± 0.06	1.13 ± 0.21	0.86 ± 0.11
Alanine	0.43 ± 0.02	0.31 ± 0.03***	0.29 ± 0.02***
Glycerol	0.17 ± 0.01	0.15 ± 0.01	0.15 ± 0.03

Table 3 continued.

Metabolite concentration (mM)	12 Weeks feeding		
	0	0.1% POCA-fed	0.2% POCA-fed
Glucose	6.11 ± 0.17	5.48 ± 0.13*	5.47 ± 0.06*
Lactate	1.87 ± 0.23	1.45 ± 0.09*	1.42 ± 0.15**
Pyruvate	0.15 ± 0.01	0.14 ± 0.004	0.13 ± 0.01
Lactate/pyruvate ratio†	12.7 ± 0.8	10.2 ± 0.5	11.1 ± 0.8
3-Hydroxybutyrate	0.055 ± 0.007	0.054 ± 0.008	0.047 ± 0.003
Acetoacetate	0.060 ± 0.009	0.054 ± 0.010	0.046 ± 0.003
3-Hydroxybutyrate/acetoacetate ratio†	0.97 ± 0.07	1.10 ± 0.15	1.06 ± 0.08
Alanine	0.40 ± 0.03	0.37 ± 0.01	0.35 ± 0.02**
Glycerol	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.02

Male albino rats (Table 1) were used and experimental details are given in the Materials and Methods section. Each value is mean ± S.E.M. for 9 animals. Significance of differences from the controls: * $P < 0.05$; ** $P < 0.002$; ***$P < 0.002$; **** $P < 0.001$. † Ratio of means.

Table 4. The effects of POCA-feeding on the concentrations of triacylglycerols, cholesterol and free fatty acids in serum

Metabolite concentration (mM)	6 Weeks POCA feeding			12 Weeks POCA feeding		
	0	0.05% POCA-fed	0.2% POCA-fed	0	0.05% POCA-fed	0.2% POCA-fed
Triacylglycerols	2.61 ± 0.25	1.85 ± 0.26	1.53 ± 0.10***	2.53 ± 0.19	1.32 ± 0.30**	1.20 ± 0.22**
Cholesterol	1.66 ± 0.31 ($n = 4$)	1.29 ± 0.08	1.23 ± 0.05*	1.27 ± 0.08	1.00 ± 0.15	0.91 ± 0.13*
Free fatty acids				0.78 ± 0.12 ($n = 5$)	0.35 ± 0.08 ($n = 6$)	0.76 ± 0.07 ($n = 4$)

Experimental details are given in the Materials and Methods section. Results are given as means ± S.E.M. for 7 observations except where indicated. Significance of differences from the controls: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

Low fat diet, muscle. There were few changes in metabolites compared with the controls, although glycerol-3-phosphate, dihydroxyacetone phosphate and CoASH concentrations were increased (Table 8).

High fat diets. Only a few determinations were made. In all groups pyruvate and malate concentrations were lower (Table 9) than in the livers of rats on a low fat diet (0.015 and 0.144 μmol/g wet-wt compared with 0.055 ± 0.017 and 0.228 ± 0.040 μmol/g wet-wt, respectively), and glucose 6-phosphate was not detected. Glycogen and glycerol-3-phosphate concentrations were lower in the POCA-fed animals. There were large increases in CoASH and carnitine concentrations, and of their acylated forms, in the livers of POCA-fed animals, with the control values also being higher than in the livers of rats fed a low fat diet (Table 9). There were few changes in muscle metabolites in POCA-fed animals compared with the controls except for 40–50% increases in glycerol-3-phosphate and 2–5 fold increases in CoASH concentrations (not shown).

Table 5. Effects of 12 weeks POCA-feeding on plasma lipoprotein concentrations

Plasma component	Control	0.05% POCA-fed	0.2% POCA-fed
Total triacylglycerol (mM)	1.76 ± 0.14	1.33 ± 0.14	0.57 ± 0.10***
Total cholesterol (mM)	1.69 ± 0.14	1.46 ± 0.13	1.21 ± 0.08*
VLDL protein (mg/ml)	0.40 ± 0.02	0.42 ± 0.02	0.39 ± 0.02
Lipoprotein triacylglycerol (mM)			
Serum minus VLDL	0.23 ± 0.03	0.21 ± 0.02	0.19 ± 0.03**
VLDL	1.25 ± 0.11	0.90 ± 0.14	0.35 ± 0.08**
Lipoprotein cholesterol (mM)			
Serum minus VLDL	1.43 ± 0.13	1.46 ± 0.17	1.14 ± 0.04*
VLDL	0.17 ± 0.03	0.14 ± 0.02	0.04 ± 0.01*

Rats different from those for the experiment described in Table 4 were used. There were 6 animals in each group and the blood from 2 animals was pooled. Significance of differences from the controls: * $P < 0.02$; ** $P < 0.01$; *** $P < 0.005$.

Table 6. Blood metabolite concentrations in rats on a high fat diet fed POCA for 4 weeks

Metabolite	Concentration (mM)		
	Control	0.05% POCA-fed	0.2% POCA-fed
Glucose	6.43 ± 0.47	6.09 ± 0.24	6.48 ± 0.14
Lactate	2.25 ± 0.64	2.64 ± 0.58	4.55 ± 0.82
Pyruvate	0.96 ± 0.21	0.92 ± 0.07	1.00 ± 0.07
Lactate/pyruvate ratio†	2.4	2.9	4.5
3-Hydroxybutyrate	0.25 ± 0.02	0.16 ± 0.01	0.21 ± 0.01
Acetoacetate	0.54 ± 0.04	0.50 ± 0.07	0.69 ± 0.02
3-Hydroxybutyrate/acetoacetate ratio†	0.46	0.32	0.30
Alanine	0.54 ± 0.10	0.45 ± 0.01	0.39 ± 0.03
Glycerol	0.26 ± 0.14	0.20 ± 0.05	0.36 ± 0.16

Experimental details are given in the Materials and Methods section. The results are expressed as means ± S.E.M. for 4–6 animals.

† Ratios of means.

DISCUSSION

Metabolic effects of POCA

Effects of POCA on blood metabolite concentrations. The amount of POCA ingested daily by our rats was approx. 50 and 200 mg/kg body-wt with the 0.05 and 0.2% POCA-containing diets respectively. However, the only significant changes in blood metabolite concentrations caused by POCA in fed rats were small decreases in glucose, alanine and lactate concentrations in some groups (Tables 3 and 6), and large decreases in cholesterol and triacylglycerol concentrations (Tables 4 and 5). This is in marked contrast with the powerful hypoketonaemic and hypoglycaemic effects of single acute doses of as little as 5 mg/kg body-wt in fasted animals [4].

Hypoglycaemia caused by POCA or by 2-TDGA in fasted animals is associated with low total ketone body concentrations and low 3-hydroxybutyrate-acetoacetate ratios in blood [2, 4] which indicate a decreased rate of generation of reducing equivalents by β-oxidation. In fed rats the lack of effect of POCA on blood ketone bodies, lactate or pyruvate concentrations (Tables 3 and 6) indicated that the redox states of the matrix and cytosolic compartments of hepatocytes were within the normal range [1].

There were some differences between the acute and chronic effects of POCA. There was an elevation of free fatty acid, triacylglycerol and cholesterol concentrations after acute administration [4], which was not found in this study with fed rats after 6 and 12 weeks chronic administration (Table 4).

Effects of POCA on metabolite concentrations in liver and muscle. ATP concentrations were maintained in liver and muscle in all animals consistent with the lack of effect of POCA-feeding on oxidative

Table 7. The effects of 12 weeks POCA-feeding on the concentrations of metabolites in liver with a low-fat diet

Metabolite / Diet:	Concentration (μmol/g wet-wt)		
	Control	0.05% POCA-fed	0.2% POCA-fed
Glycogen†	230 ± 12	157 ± 42	172 ± 33
Glucose	5.65 ± 0.35	6.00 ± 0.20	5.65 ± 0.44
Glucose-6-phosphate	0.307 ± 0.037	0.229 ± 0.041	0.165 ± 0.032**
Glycerol-3-phosphate	0.146 ± 0.022	0.230 ± 0.039*	0.220 ± 0.035*
CoASH	0.052 ± 0.010	0.121 ± 0.007****	0.180 ± 0.041**
Acetyl-CoA	0.026 ± 0.006	0.045 ± 0.007*	0.049 ± 0.008*
Carnitine	0.134 ± 0.020	0.225 ± 0.012***	0.252 ± 0.024***
Acetyl-carnitine	0.029 ± 0.008	0.091 ± 0.008****	0.105 ± 0.014****
Total acid-soluble carnitine‡	0.251 ± 0.052	0.340 ± 0.026	0.420 ± 0.036*
Long-chain acylcarnitine§	0.059 ± 0.007	0.109 ± 0.015**	0.107 ± 0.021*
ATP	5.02 ± 0.18	5.52 ± 0.14*	5.41 ± 0.19
ADP	0.646 ± 0.026	0.741 ± 0.060	0.722 ± 0.024*
AMP	0.079 ± 0.136	0.079 ± 0.011	0.081 ± 0.008
Total adenine nucleotide	5.59 ± 0.14	6.34 ± 0.14***	6.21 ± 0.20*

Metabolite concentrations were determined in freeze-clamped livers as described in the Materials and Methods section and are expressed as means ± S.E.M. ($n = 6$). Significance of differences from the controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

† Expressed as nmol glucose obtained after hydrolysis with amylase.

‡ Total acid-soluble carnitine refers to the sum of free carnitine and its esters not precipitated by 0.33 M $HClO_4$.

§ Carnitine esters precipitated by 0.33 M $HClO_4$.

Table 8. The effects of 12 weeks POCA-feeding on the concentrations of metabolites in muscle, with a low-fat diet

Metabolite / Diet:	Concentration (μmol/g wet-wt)		
	Control	0.05% POCA-fed	0.2% POCA-fed
Glycogen	21.9 ± 14.6	15.4 ± 2.0	16.2 ± 2.9
Glycerol-3-phosphate	0.612 ± 0.068	0.835 ± 0.110	0.894 ± 0.075***
Dihydroxyacetone phosphate	0.083 ± 0.006	0.138 ± 0.019**	0.120 ± 0.016*
CoASH	0.032 ± 0.004	0.045 ± 0.008	0.050 ± 0.007*
Acetyl-CoA	0.013 ± 0.003	0.011 ± 0.005	0.021 ± 0.008
Carnitine	0.922 ± 0.026	1.00 ± 0.05	1.04 ± 0.07
Acetyl-carnitine	0.017 ± 0.005	0.016 ± 0.007	0.020 ± 0.013
Total acid-soluble carnitine	1.07 ± 0.03	1.21 ± 0.06*	1.14 ± 0.06
Long-chain acylcarnitine	0.047 ± 0.005	0.048 ± 0.008	0.054 ± 0.014
ATP	5.02 ± 0.18	5.52 ± 0.14*	5.41 ± 0.19
ADP	0.646 ± 0.026	0.741 ± 0.060	0.722 ± 0.024*
AMP	0.079 ± 0.011	0.079 ± 0.011	0.081 ± 0.008
Total adenine nucleotides	5.59 ± 0.14	6.34 ± 0.14***	6.21 ± 0.20*

Metabolite concentrations were determined in freeze-clamped muscle as described in the Materials and Methods section and are expressed as means ± S.E.M. ($n = 5$). Significance of differences from the controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

phosphorylation (6). The most marked changes in liver were 2- to 3-fold increases in the concentrations of CoASH, acetyl-CoA, carnitine and acetylcarnitine (Tables 7–9). Chronic administration of 2-TGDA also increased the concentration of carnitine in the livers of suckling rats [22]. POCA-feeding lowered the concentration of glucose 6-phosphate suggesting an impaired rate of gluconeogenesis (Table 7). There were only minor changes in other metabolites (Tables 7–9). In muscle the concentrations of glycerol 3-phosphate and dihydroxyacetone phosphate were increased (Table 8).

Effects of POCA on fatty acid oxidation. Following administration of POCA long-chain fatty acid oxidation is strongly inhibited at the Stage of CPT I in liver and skeletal muscle mitochondria [6]. It is probable that the inhibitory species formed from POCA *in vivo* is POCA-CoA. CPT I in liver mitochondria from fed animals is inhibited more strongly *in vitro* by POCA-CoA than in mitochondria from fasted animals [23], paralleling inhibition of β-oxidation [6]. By contrast, CPT I is inhibited equally strongly in skeletal muscle mitochondria from fed or fasted animals [23]. In these respects, the pattern of inhibition by POCA-CoA resembles that by malonyl-CoA [24, 25] except that POCA-CoA is much more potent in liver [23]. POCA-CoA and malonyl-CoA each inhibit CPT I more strongly in liver mitochondria from fed normal and fed diabetic rats than in mitochondria from fasted normal rats [23, 24]. This suggests that the decreased sensitivity of CPT I in fasted rats to inhibition is not simply due to changes in the insulin/glucagon ratio [24].

Effects of POCA on carbohydrate metabolism. Although administration of POCA only caused small changes in metabolite concentrations in blood, liver and muscle, large changes in glucose turnover in fed animals are not necessarily excluded since the relation between flux and metabolite concentration is complex. Experiments with ^{14}C- and ^{3}H-labelled glucose are necessary to estimate any effect of POCA on glucose turnover [1].

Hypoglycaemia caused by POCA or by 2-TDGA in fasted rats is probably due to decreased gluconeogenesis [7, 26], and to increased glucose oxidation in most tissues secondary to activation of pyruvate dehydrogenase, both as a consequence of impaired fatty acid oxidation [27, 28], so that glucose

Table 9. The effects of 12 weeks POCA-feeding on the concentrations of metabolites in liver, with a high-fat diet

Metabolite / Diet:	Concentration (μmol/g wet-wt)		
	Control	0.1% POCA-fed	0.2% POCA-fed
Glycogen	220	19	11
Glycerol-3-phosphate	0.308	0.147	0.123
Pyruvate	0.105	0.021	0.024
Malate	0.144	0.193	0.112
CoASH	0.158	0.626	0.543
Acetyl-CoA	0.032	0.092	0.080
Carnitine	0.187	0.431	0.424
Acetyl-carnitine	0.067	0.125	0.146
Total acid-soluble carnitine	0.316	0.589	0.570
Long-chain acyl-carnitine	0.082	0.133	0.127

Metabolite concentrations were determined in freeze-clamped livers as described in the Materials and Methods section and are expressed as the means of two observations.

is used more quickly than it is replaced. In normal animals fed a POCA-containing diet blood glucose concentrations are maintained by dietary intake, and by glycogenolysis during short-term fasting (there is no reason to suppose that glycogen metabolism is impaired), since insulin secretion permits normoglycaemia by facilitating glucose uptake by cells and storage of excess as glycogen. Blood glucose concentrations are decreased in fasting diabetic animals (but not necessarily to normal values) where there is severe insulin deficiency and otherwise unrestrained gluconeogenesis will be decreased following administration of POCA [4].

Fatty acid oxidation stimulates gluconeogenesis by supplying NADH, ATP and acetyl-CoA [29]. Acetyl-CoA is required in the mitochondrial matrix as an allosteric activator of pyruvate carboxylase (EC 6.4.1.1), a key enzyme in gluconeogenesis. Inhibition of β-oxidation may decrease acetyl-CoA concentrations [1], although acetyl-CoA is also formed from pyruvate and several amino acids. However, the overall concentration of acetyl-CoA in livers from POCA-fed rats was 50% greater than in control livers from fed rats (Tables 7 and 9). In normal hepatocytes about 90% of the total CoA content occurs in the mitochondrial matrix [30], although its subcellular distribution after POCA-feeding is not known. Since POCA is hypoglycaemic in fasted animals where CPT I is at least sensitive to inhibition by POCA-CoA [4, 23], it must be assumed that sufficient POCA-CoA is formed in hepatocytes to inhibit fatty acid oxidation strongly.

Mechanism of the hypolipidaemic effects of POCA. Both the triacylglycerol and cholesterol content of the VLDL fraction, but not its protein content, were decreased suggesting that VLDL may be secreted with a decreased lipid load. Hypolipidaemia cannot be due to retention of lipid in the liver as a result of inhibition of fatty acid oxidation, since the 2–3 g of lipid that accumulated after feeding POCA for 12 weeks would be much less than the total amount normally secreted during this time. The unusual nature of the hepatic lipid could be in part due to accumulation of precursors of cholesterol synthesis if this is inhibited. From the observed changes in heart and adipose tissue LPL activities it is not possible to judge whether POCA-feeding causes an increase in the total body activity of the enzyme. Reciprocal changes in heart and adipose LPL activity are usual [31]. In experiments in which rats were fed 0.2% POCA for 3 days, adipose tissue LPL activity increased above that found in controls while the heart activity decreased (M. P. Rogers and K. Harmes, unpublished results).

The highest level of POCA fed (0.2%) caused a 20% decrease in the percentage of total body fat (Table 1). POCA may also impair absorption of dietary fat or decrease *de novo* synthesis of fatty acids from glucose. 2-TDGA inhibits lipogenesis in isolated rat hepatocytes, although at 10-fold higher concentrations than inhibit β-oxidation [32]. 20 μM $POCA^-$ inhibited fatty acid biosynthesis by 50% in isolated hepatocytes from fed rats, measured by incorporation of ^3H from ^3H_2O (L. Agius, unpublished results).

Cardiac hypertrophy caused by feeding POCA

The 15–20% increase in the weight of the myocardium may indicate toxicity of POCA. Both POCA and 2-TDGA inhibit long-chain fatty acid oxidation in the perfused rat heart when added to the perfusion medium [27, 28]. In the presence of glucose 2-TDGA had little effect on cardiac function in a perfused working heart preparation with moderate loads, but the maximum possible work was decreased because the maximum rate of glucose oxidation was not enough to support the maximum rate of work which also requires some fatty acid oxidation [33]. Cardiac hypertrophy, therefore, in POCA-fed rats may be an adaptation to an impaired ability of the muscle to utilise long-chain fatty acids. This interpretation is supported by the failure to detect any histological or ultrastructural abnormalities in POCA-fed rats, either on the low or high fat diets (M. A. Johnson and M. J. Cullen, unpublished work). There is evidence that high concentrations of free long-chain fatty acids are harmful to the partly anoxic heart by increasing the demand for O_2 [33]. Some protection against damage by fatty acids in partial anoxia was provided by 2-TDGA which was thought to act by decreasing O_2 consumption [33]. POCA may also be expected to protect the myocardium similarly. Another compound, *p*-hydroxyphenylglyoxalate formed *in vivo* from *p*-hydroxyphenylglycine (Oxfenacine), which has been reported to inhibit cardiac CPT I selectively, also protects the anoxic heart against damage by fatty acids [34, 35].

Peroxisomal proliferation caused by chronic administration of POCA

Feeding rats a diet containing 0.2% POCA for 4 weeks causes a 3-fold increase in total CoA and carnitine concentrations in the liver (Table 7), and in the number of peroxisomes with a 3-fold increase in the capacity for peroxisomal β-oxidation [36], similar to that caused by feeding diets to rats containing large amounts of triacylglycerols containing $C_{22:1}$ fatty acids [37]. The increased number of peroxisomes may be secondary to accumulation of lipid rather than to a direct effect of POCA. Chronic administration of drugs related to clofibrate to rodents, but not to other species, causes a 6–10 fold increase in the number of peroxisomes and in their capacity for β-oxidation, a 3-fold increase in the total CoA and carnitine concentrations in the liver, an increase in the capacity for mitochondrial β-oxidation and a decrease in the volume fraction of hepatocytes occupied by lipid droplets [21, 38]. It might be thought that an increased capacity for peroxisomal β-oxidation would partly overcome the block in β-oxidation at CPT I caused by POCA-CoA. However, preliminary experiments have shown that acyl-CoA oxidase, the first enzyme of the peroxisomal β-oxidation sequence, is reversibly inhibited by about 50% by 10 μM PCOA-CoA with either 20 μM palmitoyl-CoA or 20 μM decanoyl-CoA as substrate.

Toxicity of POCA

POCA is much less toxic when high doses are administered chronically to rats than might have been expected from its inhibitory effects on long-

chain fatty acid oxidation *in vitro*. No evidence was found for any mutagenic effects of POCA in mammalian or bacterial cells [5]. The growth of human fibroblasts is only impaired when they are cultured in the presence of concentrations of $POCA^-$ above 50 μM. Further, there was no effect on the incorporation of {U-^{3}H}leucine or of {U-^{3}H}thymidine into protein or DNA when subconfluent fibroblasts were incubated for 2 hr in the presence of 5, 10, 50 or 500 μM $POCA^-$ (S. I. M. Younan and K. Bartlett, unpublished work). Three chronic effects of POCA have been found in rats with the doses fed for 12 weeks; accumulation of lipid and limited proliferation of peroxisomes in liver and mild cardiac hypertrophy. The very long-term importance of these remains to be established. The activities of glutamate-oxaloacetate aminotransferase, creatine kinase and alkaline phosphatase in plasma were unchanged after POCA feeding, indicating that there is no general tissue damage. The rats used in our study were kept in standard sized cages and were relatively inactive. Experiments on the effects of POCA in animals which exercise regularly are required because of the importance of fatty acid oxidation for sustained exercise [39].

CONCLUSIONS

POCA and 2-TDGA appear to be the only non-hormal hypoglycaemic compounds that are effective in fasted insulin-dependent diabetic animals [2, 4]. Indeed, POCA is one of the most potent hypoketonaemic and hypoglycaemic compounds known in fasted animals, although, by contrast, it has apparently relatively small effects on intermediary metabolism in fed normal rats. Its value as a potential therapeutic agent for the treatment of diabetes merits further investigation, although the effects of chronic administration of putative "therapeutic" doses have not been studied in normal or in diabetic animals. However, it is clear that POCA (which is a mixture of stereoisomers since C_2 is asymmetric) has complex metabolic effects and that it is not a pure inhibitor of mitochondrial β-oxidation of long-chain fatty acids.

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REFERENCES

1. H. S. A. Sherratt, in *Short-Term Regulation of Liver Metabolism* (Eds. L. Hue and G. van der Werve) p. 799. Elsevier/North-Holland Biomedical Press, Amsterdam (1981).
2. G. F. Tutwiler, T. Kirsh, R. H. Mohrbacher and W. Ho, *Metabolism* **27**, 1537 (1978).
3. H. P. O. Wolf, K. Eistetter and G. Ludwig, *Diabetologia* **21**, 344 (1981).
4. H. P. O. Wolf, K. Eistetter and G. Ludwig, *Diabetologia* **22**, 456 (1982).
5. H. P. O. Wolf and K. Eistetter, *J. med. Chem.* **25**, 109 (1982).
6. D. M. Turnbull, K. Bartlett, S. I. M. Younan and H. S. A. Sherratt, *Biochem. Pharmac.*, **33**, 475 (1984).
7. A. J. Bone, D. M. Turnbull, P. P. Koundakjian and H. S. A. Sherratt, *Diabetologia* **21**, 112 (1981).
8. P. P. Koundakjian, K. Bartlett, A. J. Bone, L. Golightly, D. M. Turnbull, S. I. M. Younan and H. S. A. Sherratt, *Biochem. Soc. Trans.* **9**, 573 (1981).
9. F. Van Hoof, K. Bartlett, A. J. Bone, P. P. Koundakjian, D. M. Turnbull and H. S. A. Sherratt, *Biochem. Soc. Trans.* **9**, 574 (1981).
10. D. Billington, H. Osmundsen and H. S. A. Sherratt, *Biochem. Pharmac.* **27**, 2879 (1978).
11. D. Billington, H. Osmundsen and H. S. A. Sherratt, *Biochem. Pharmac.* **27**, 2891 (1978).
12. C. J. Stanley and R. Perham, *Biochem. J.* **191**, 147 (1980).
13. O. H. Lowry, N. F. Rosebrough, A. L. Farr and R. Randall, *J. biol. Chem.* **193**, 265 (1961).
14. B. Lloyd, J. Burrin, P. Smythe and K. G. M. M. Alberti, *Clin. Chem.* **24**, 1724 (1978).
15. D. H. Williamson, J. H. Mellanby and H. A. Krebs, *Biochem. J.* **82**, 1633 (1962).
16. W. G. Duncome, *Clin. Chem. Acta* **9**, 122 (1964).
17. R. J. Havel, H. A. Eder and J. H. Bragdon, *J. clin. Invest.* **34**, 1345 (1955).
18. H. U. Bergmeyer and K. Gawelin, in *Methods of Enzymatic Analysis*, 2nd edn. Academic Press, New York (1974).
19. J. R. Williamson, S. G. Rostand and M. J. Peterson, *J. biol. Chem.* **245**, 3242 (1970).
20. S. E. Riley and D. S. Robinson, *Biochim. biophys. Acta* **369**, 371 (1974).
21. H. S. A. Sherratt, K. Bartlett, A. J. Bone, P. P. Koundakjian, D. M. Turnbull, H. Osmundsen and F. Van Hoof, *Ann. N.Y. Acad. Sci.* **249**, 312 (1982).
22. S. C. Frost and M. A. Wells, *Biochem. J.* **204**, 861 (1982).
23. D. M. Turnbull, K. Bartlett, E. Meredith and H. S. A. Sherratt, in preparation.
24. E. D. Saggerson and C. A. Carpenter, *FEBS Lett.* **129**, 225 (1981).
25. J. H. Veerkamp and H. T. B. Van Moerkerk, *Biochim. biophys. Acta* **710**, 252 (1982).
26. G. F. Tutwiler and P. Dellevigne, *J. biol. Chem.* **254**, 2935 (1978).
27. P. Rösen and H. Reinaur, *Akt. Endokr. Stoffw.* **2**, 96 (1982).
28. I. Catterson, S. J. Fuller and P. J. Randle, *Biochem. J.* **208**, 53 (1982).
29. J. H. Exton, *Metabolism* **21**, 945 (1972).
30. E. A. Sies, D. G. Brooks and O Wieland, *FEBS Lett.* **69**, 265 (1976).
31. D. S. Robinson, *Comp. Biochem.* **18**, 55 (1970).
32. S. A. McCune, T. Namura and R. A. Harris, *Lipids* **14**, 880 (1979).
33. F. J. Pearce, J. Forster, G. DeLeuw, J. R. Williamson and G. F. Tutwiler, *J. mol. cell. Cardiol.* **11**, 893 (1979).
34. A. J. Higgins, M. Morville, R. A. Burges and K. J. Blackburn, *Biochem. biophys. res. Commun.* **100**, 291 (1981).
35. R. A. Burges, D. G. Gardiner and A. J. Higgins, *Life Sci.* **29**, 1847 (1981).
36. A. J. Bone, H. S. A. Sherratt, D. M. Turnbull and H. Osmundsen, *Biochem. biophys. res. Commun.* **104**, 708 (1982).
37. C. E. Neat, M. S. Thomassen and H. Osmundsen, *Biochem. J.* **196**, 149 (1981).
38. P. B. Lazarow, H. Shio and M. A. Leroy-Houyet, *J. Lipid Res.* **23**, 317 (1982).
39. P. Felig and J. Warren, *New Engl. J. Med.* **293**, 1078 (1975).