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EFFECTS OF 2[5(4-CHLORPHENYL)PENTYL]OXIRANE-2-CARBOXYLATE ON FATTY ACID SYNTHESIS AND FATTY ACID OXIDATION IN ISOLATED RAT HEPATOCYTES

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Abstract—The effects of the hypoketonaemic and hypoglycaemic compound 2[5(4-chlorophenyl)pentyl] oxirane-2-carboxylate (POCA) on fatty acid synthesis and fatty acid oxidation in rat hepatocytes were examined. Two μM-POCA caused a small stimulation of fatty acid synthesis which might be due to an increased flux through pyruvate dehydrogenase. Ten to one hundred μM-POCA inhibited (40–70%) fatty acid synthesis. At low concentrations (≤ 5 μM) POCA was a more powerful inhibitor of fatty acid oxidation than of synthesis, but at higher concentrations (10–100 μM) the inhibition of synthesis and oxidation was similar. One hundred μM POCA-CoA inhibited acetyl-CoA carboxylase by about 22% and 100 μM-palmitoyl-CoA by about 33%. Since POCA was a more potent inhibitor of fatty acid synthesis than palmitate, but POCA-CoA did not inhibit acetyl-CoA carboxylase more strongly than palmitoyl-CoA, it is suggested that POCA-CoA may inhibit fatty acid synthase directly.

Inhibitors of fatty acid oxidation are of potential interest as hypoketonaemic and hypoglycaemic drugs [1]. Following the introduction of 2-tetradecylglycidate, a long-chain fatty acid containing a 2-oxirane ring by Tutwiler *et al.* [2], 2[5(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA)‡, a powerful hypoketonaemic and hypoglycaemic agent in fasted animals and a candidate antidiabetic and cardioprotective drug, was described by Wolf *et al.* [3, 4]. The CoA ester of POCA (POCA-CoA) is a potent inhibitor of beta-oxidation of long-chain fatty acids at the stage of carnitine palmitoyltransferase I (CPT I, EC 2.3.1.21) located on the outer face of the inner mitochondrial membrane [5–7, and is more effective in liver mitochondria from fed ($I_{0.5} < 1$ μm) than from 48 hr fasted rats ($I_{0.5} < 4$ μM) [7]. In this respect POCA-CoA resembles malonyl-CoA [8] which appears important in the reciprocal regulation of fatty acid synthesis and the mitochondrial oxidation of long-chain fatty acids [9]. It is likely that POCA-CoA is the active metabolite of POCA formed in intact cells or *in vivo* [7].

A key question is whether POCA-CoA directly affects other metabolic pathways in addition to fatty acid oxidation? Since both POCA-CoA and malonyl-CoA inhibit CPT I, it is possible that POCA-CoA also competes with malonyl-CoA in the pathway for fatty acid biosynthesis at sites where malonyl-CoA is involved. Further, 2-tetradecylglycidate is a weak inhibitor of fatty acid biosynthesis in isolated rat hepatocytes [10]. In this study the effects of POCA on fatty acid synthesis and on ketogenesis from palmitate in isolated hepatocytes were compared.

‡ Abbreviations: POCA, 2[5(4-chlorophenyl)pentyl]oxirane-2-carboxylate; POCA-CoA, 2[5(4-chlorophenyl)-pentyl]oxirane-2-carbonyl-CoA; CPT I, carnitine palmitoyltransferase I; PDH, pyruvate dehydrogenase.

MATERIALS AND METHODS

POCA (sodium salt) was a gift from Dr Gerhard Ludwig, Byk Gulden Lomberg Chemische Fabrik GmbH (Konstanz, F.R.G.). Collagenase (Type 1), bovine serum albumin (Fraction V), substrates and cofactors were from Sigma Chemical Co., and 3-hydroxybutyrate dehydrogenase was from Boehringer Corp. POCA-CoA was prepared as previously described [7]. Radiochemicals were from Amersham International. Male albino rats (180–220 g) were used. They were fed standard laboratory chow *ad lib.*, or, where indicated, deprived of food for 24 hr before use.

Parenchymal hepatocytes were isolated after perfusion of livers with collagenase [11]. The hepatocytes were preincubated for 20 min and then washed by centrifugation [12] and suspended in Krebs–Henseleit buffer [13] containing 10 mM glucose and 2% (w/v) defatted serum albumin [14]. Cell viability estimated by Trypan Blue exclusion was > 90%.

Fatty acid synthesis was measured by incorporation of ^3H from ^3H_2O into saponifiable fatty acids [12]. The oxidation of 0.5 mM [$U^{14}C$] palmitate was measured as in [15] and acetoacetate and 3-hydroxybutyrate were determined enzymically [16] in the neutralized perchlorate extracts. The rates of fatty acid synthesis and ketone body formation were linear for 60 min and all incubations were in duplicate for 60 min. Rates were determined from the 60 min values corrected for zero-time values and are expressed as μmoles of substrate formed or

transformed/hour per gram wet-wt of hepatocytes (1 g wet-wt of hepatocytes corresponds to approximately 118×10^6 cells). Results are expressed as means ± S.E.M. for N hepatocyte preparations. Statistical analysis was performed by the paired *t*-test.

Acetyl-CoA carboxylase (EC 6.4.1.2) was measured in a high speed (100,000 *g*) supernatant fraction from rat liver homogenized in 1.5 vol. of 0.3 M mannitol/5 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid/0.1 mM EGTA, pH 7.2 after filtration on sephadex G25 to remove pyruvate [17]. The supernatant was preincubated for 30 min at 30° with 20 mM citrate to activate acetyl-CoA carboxylase. The assay contained 100 mM/Tris/HCl, 20 mM acetate, 5 mM ATP, 1 mM dithiothreitol, 0.5 mM EDTA, 200 μM acetyl-CoA, 20 mM citrate, 20 mM $NaH^{14}CO_3$ (0.5 Ci/mole), 10 mg bovine serum albumin, 2–4 mg liver protein, pH 7.4 in a final volume of 1.0 ml at 30°. Activities were determined by stopping the reaction with 0.5 M HCl after 15, 30, 60 and 90 sec and determining the ^{14}C fixed into acid-stable metabolites. Fixation of $H^{14}CO_3$ in the controls was linear for at least 60 sec. Protein was determined by the method of Lowry [18] using bovine serum albumin as standard.

RESULTS

Fatty acid synthesis

POCA had a biphasic effect on fatty acid synthesis in hepatocytes from fed rats. In the absence of exogenous palmitate, 2 μM POCA caused a small but significant increase in the rate of fatty synthesis, 5 μM POCA had little effect and higher concentrations inhibited (Table 1). Fatty acid synthesis was also inhibited (30–54%) by added palmitate (0.25–0.5 mM, Table 2), presumably by inhibition of acetyl-CoA carboxylase by palmitoyl-CoA (Table 5) [19]. The inhibition of fatty acid synthesis by 100 μM-POCA (70%) in the absence of exogenous palmitate (Tables 1 and 2) was greater than the inhibition of fatty acid synthesis by 0.25–0.5 mM palmitate. In the presence of 0.5 mM palmitate, the additional inhibition of fatty acid synthesis by 10 μM POCA (58%) was greater ($P < 0.05$) than the inhibition by

Table 1. Effects of POCA on fatty acid synthesis in hepatocytes from fed rats

POCA (μM)	Fatty acid synthesis (μmoles ^3H_2O/hr per g wet-wt)	Percentage of control value
0	7.8 ± 1.1	
2	9.8 ± 0.7*	124
5	7.9 ± 0.6	101
10	5.0 ± 0.5*	64
20	4.6 ± 0.6*	59
50	4.2 ± 0.2†	54
100	2.5 ± 0.2†	32

Hepatocyte suspensions (2×10^6 cells/ml) were incubated for 60 min in Krebs–bicarbonate buffer containing 10 mM glucose and ^3H_2O (0.2 mCi/ml) and the concentrations of POCA indicated. Fatty acid synthesis was determined as described in the Methods Section. Rates of fatty acid synthesis are expressed as μmole of ^3H_2O incorporated/hr per g wet-wt of hepatocytes. Values are means ± S.E.M. for 5–6 hepatocyte preparations. Values that are significantly different (paired *t*-test) from the control with no added POCA are shown: * $P < 0.05$; † $P < 0.005$.

Table 2. Inhibition of fatty acid synthesis by POCA in hepatocytes from fed rats incubated with different palmitate concentrations

	Fatty acid synthesis (% of control)		Ketogenesis (% of control)	
Palmitate (mM)	10 μM POCA	100 μm POCA	10 μM POCA	100 μm POCA
0	61.8 ± 7.2	28.5 ± 0.3	35.5 ± 3.5	32.5 ± 10.4
0.25	44.8 ± 6.6	29.5 ± 4.3	38.5 ± 3.4	24.0 ± 3.2
0.50	42.0 ± 2.5	24.0 ± 4.1	35.0 ± 3.6	21.5 ± 2.0

Hepatocyte suspensions (10^6 cells/ml) were incubated with the concentrations of palmitate indicated without or with (10 μM and 100 μM) POCA. Fatty acid synthesis and ketone body formation (acetoacetate plus 3-hydroxybutyrate) in incubations containing POCA are expressed as the percentage of the corresponding rate without POCA. The basal rate of fatty acid synthesis (7.5 + 1.5 μmoles of ^3H_2O incorporated/hr per g wet-wt) was decreased by 30% and 54% with 0.25 and 0.5 mM palmitate respectively. The basal rate of ketone body formation (4.7 ± 1.7 μmoles of ketone bodies/hr per g wet-wt of hepatocytes) was increased by 350 and 620% with 0.25 and 0.5 mM palmitate respectively. Values are means ± S.E.M. for 3 hepatocyte preparations.

Table 3. Effects of POCA on the oxidation of [U-^{14}C] palmitate by hepatocytes from fasted rats

[POCA] (μM)	[U-^{14}C]palmitate converted/hr per g wet-wt		Ketone body formation (μmoles/hr/g)	Hydroxybutyrate / acetoacetate
	$^{14}CO_2$	^{14}C-acid soluble metabolites		
0	1.35 ± 0.20	9.88 ± 41	32.9 ± 1.8	0.76
5	1.05 ± 0.07(78)	5.01 ± 0.43(51)	14.4 ± 1.2(44)	0.51
10	1.00 ± 0.10(74)	4.58 ± 0.39(46)	13.6 ± 1.2(41)	0.45
20	0.66 ± 0.22(49)	3.12 ± 0.19(32)	8.3 ± 0.1(25)	0.45
100	0.43 ± 0.07(32)	1.64 ± 0.06(17)	5.3 ± 0.2(16)	0.24

Hepatocyte suspensions (10^6 cells/ml) were incubated with 0.5 mM[U-^{14}C] palmitate and the concentrations of POCA indicated. Rates of [U-^{14}C] palmitate conversion to $^{14}CO_2$ and ^{14}C-acid-soluble metabolites are expressed as μmoles of palmitate converted/hr per g wet-wt of hepatocytes. Values are means ± S.E.M. for 3–4 hepatocyte preparations. The numbers in parentheses represent the rates in the presence of POCA expressed as a percentage of the controls without added POCA.

Table 4. Effects of POCA on ketogenesis in hepatocytes from fed and fasted rats

Additions	Ketone body formation			
	Fed rats		Fasted rats	
	—	100 μM POCA	—	100 μM POCA
2 mM Lactate	4.95 ± 1.05	4.44 ± 1.09	10.0 ± 3.9	1.10 ± 0.03*
2 mM Pyruvate	7.09 ± 1.40	6.00 ± 1.10	10.4 ± 3.0	2.60 ± 1.50*
1 mM Palmitate	18.1 ± 3.50	7.60 ± 1.80*	50.3 ± 11.4	19.7 ± 6.10*

Hepatocyte suspension (10^6 cells/ml) were incubated with the additions indicated, without or with 100 μM POCA. Ketone body formation represents acetoacetate plus 3-hydroxybutyrate expressed as μmol/hr per g wet-wt of hepatocytes, means ± S.E.M. (N = 4). Values that are significantly different (paired t test) from the corresponding controls without POCA are shown; *$P < 0.01$.

10 μM POCA in the absence of added palmitate (38%) (Table 2).

Fatty acid oxidation and ketogenesis

POCA (5–100 μM) inhibited the oxidation of 0.5 mM [U-^{14}C] palmitate as measured by the formation of $^{14}CO_2$ and ^{14}C-labelled acid-soluble metabolites in hepatocytes from 24 hr-fasted rats. Ketone body formation was also inhibited and the 3-hydroxybutyrate/acetoacetate ratio was decreased (Table 3), indicating a more oxidised state of the NADH/NAD couple in the mitochondrial matrix. The percentage inhibition of formation of ^{14}C-labelled products and ketone body formation was greater than the inhibition of $^{14}CO_2$ formation (Table 3). Similar results were obtained with hepatocytes from fed rats (results not shown). This agrees with the study by Schudt and Simon [20] who found inhibition of ketogenesis from oleate by POCA ($I_{0.5}$ 1.5 μM).

POCA also inhibited ketogenesis during incubations with 2 mM lactate or 2 mM pyruvate (without added palmitate) in hepatocytes from fasted rats, but not in hepatocytes from fed rats (Table 4). This suggests that lactate and pyruvate are precursors of ketone bodies in the fed state but not in the fasted state.

Acetyl CoA carboxylase activity

POCA-CoA (100 μM) inhibited acetyl-CoA carboxylase activity in liver cell extracts by 22% during a 60 sec incubation compared with 33% inhibition by 100 μM palmitoyl-CoA and 28% by 100 μM POCA (Table 5).

Table 5. Effects of POCA-CoA and palmitoyl-CoA on acetyl-CoA carboxylase activity

Incubation time (sec)	Acetyl-CoA carboxylase activity		
	100 μM POCA-CoA	100 μM POCA	100 μM palmitoyl-CoA
30	89 ± 6	93 ± 7	77 ± 4
60	78 ± 8	72 ± 7	67 ± 4
90	86 ± 6	72, 82	58 ± 4

The effects of POCA-CoA and of palmitoyl-CoA on acetyl-CoA carboxylase activity in liver extracts were measured as described in the Methods Section. The activities are expressed as percentages of the control rates at the respective time points. Values are means ± S.E.M. for 4 experiments. Control rates were 3.1 ± 0.3 nmoles/min per mg protein and were linear for at least 60 sec.

DISCUSSION

POCA inhibited fatty acid synthesis in the absence and the presence of palmitate (Tables 1 and 2). It was considered that POCA-CoA may inhibit fatty acid synthesis by competing for a site which binds malonyl-CoA since inhibition of CPT I by POCA-CoA is analogous to that by malonyl-CoA [7–9]. However, inhibition of acetyl-CoA carboxylase, which converts acetyl-CoA to malonyl-CoA the immediate precursor of fatty acids, by 100 μM POCA-CoA was similar to that by 100 μM palmitoyl-CoA. By contrast, inhibition of fatty acid synthesis by 100 μM POCA (71%) was greater than by 250 μM palmitate (30%) (Table 2). Inhibition of fatty acid synthesis is presumably therefore due to a direct effect of POCA-CoA and/or free POCA on fatty acid synthetase.

The small stimulation of fatty acid synthesis by 2 μM POCA may be related to an increased flux through PDH (EC 1.2.4.1) which provides acetyl-CoA, and to a more oxidized state of the mitochondrial matrix favouring the availability of oxaloacetate which condenses with acetyl-CoA to form citrate. Citrate serves as a vehicle to transport acetyl-units to the site of fatty acid synthesis in the cytosol and is also an activator of acetyl-CoA carboxylase [19]. An activation of PDH by 2 μM POCA is indicated by an increase (37%) in the rate of formation of $^{14}CO_2$ from 0.5 mM [1-^{14}C] pyruvate (control, 17.1 ± 3.5; with 2 μM POCA, 24.2 ± 4.7; μmoles $^{14}CO_2$ formed/hr per g wet-wt means ± S.E.M., N = 4). An increased rate of decarboxylation of [1-^{14}C] pyruvate was also observed in human fibroblasts in the presence of POCA [6]. Inhibition of beta-oxidation in rat hearts perfused with 0.5 mM palmitate and 100 μM tetradecylglycidate or 0.5 mM POCA is associated with activation of PDH [21, 22]. Activation of PDH is presumably secondary to the more oxidized state of the matrix resulting from inhibition of the oxidation of endogenous fatty acids [23] by concentrations of POCA insufficient to impair fatty acid synthesis.

Inhibition of CPT I by POCA-CoA in the direction of palmitoylcarnitine formation is partly competitive with palmitoyl-CoA, and is greater in mitochondria from fed than from fasted animals [7]. Both POCA and palmitate would be expected to compete as substrates for palmitoyl-CoA synthase (EC 6.2.1.3), and POCA-CoA and palmitoyl-CoA for palmitoyl-CoA hydrolase (EC 3.1.2.2). The intracellular concentrations of POCA-CoA and palmitoyl-CoA and their variation with different concentrations of POCA and of palmitate are not known. Since POCA has a biphasic effect on fatty acid synthesis in hepatocytes, the balance between fatty acid synthesis and oxidation in animals given POCA presumably depends on both the dose and on the nutritional state of the animal. This may explain the 20% lower total body fat content of rats fed 0.2% POCA in their diet for 12 weeks, while that of rats fed 0.05% POCA was similar to the controls [23].

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