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Organic aciduria in fasted rats caused by 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate (etomoxir)

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Impairment of hepatic mitochondrial β-oxidation by disease or inhibitors is often associated with a massive organic aciduria [1–3]. Long-chain fatty acids that cannot be oxidized by the mitochondria undergo β-oxidation to form long-chain dicarboxylic acids in the endoplasmic reticulum [4]. These are then converted to their mono-acyl-CoA esters by an acyl-CoA synthase in the endoplasmic reticulum, which then undergo partial β-oxidation in the peroxisomes to medium-chain and short-chain decarboxylic acids [5]. 2-[6-(4-Chlorophenoxy)hexyl]oxirane-2-carboxylate (etomoxir) is hypoglycaemic in fasted animals and its CoA ester strongly inhibits mitochondrial β-oxidation at the stage of carnitine palmitoyltransferase I [6, 7]. It was therefore of interest to determine the effects of etomoxir on the excretion of organic acids in fasted rats.

Male Wistar rats from a local strain (200–250 g) were maintained on a 13 hr light/11 hr dark cycle. Food was withdrawn at 9.00 a.m. and they were put individually in metabolic cages and injected intraperitoneally with a solution of the sodium salt of *RS*-etomoxir (50 μmol/kg body wt) or 0.14 M NaCl at 3.00 p.m. Urine was collected for 18 hr. The rats were then killed by cervical dislocation at 9.00 a.m. the next day. Urinary organic acids were extracted and analysed by gas-liquid chromatography and their identities were confirmed by mass spectrometry [8]. Creatinine was determined by the standard alkaline picrate method. Rat livers were gently homogenized in 250 mM sucrose, 2 mM Hepes, 2 mM EGTA, pH 7.2, 5 mL of buffer for each gram of liver, and the oxidation of [1-^{14}C]-palmitate and [9,10-^{3}H]palmitate was determined in the homogenates. The homogenate (50 μL) containing 1–2 mg of protein was added to 950 μL of medium at pH 7 and 30° containing 110 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, 10 mM potassium phosphate, 5 mM ATP, 1 mM L-carnitine, 1 mM EGTA and 0.2 mg cytochrome *c*, in plastic vials in a shaking water bath (170 strokes/min). After a 5 min preincubation 0.12 mM palmitate bound to bovine serum albumin in a molar ratio of 5:1 containing 22 kBq [9,10-^{3}H]palmitate and 2 kBq [1-^{14}C]palmitate was added. At appropriate times the reaction was stopped with 200 μL of 5 M $HClO_4$, to precipitate unchanged and esterified palmitate, then centrifuged (100,000 g_{min}) and the supernatant was divided into three aliquots. One aliquot was counted to give the total acid-soluble radioactivity, the second passed down a 0.5 mL Dowex 1 column (Cl^- form, 200 mesh) and the column washed with 2 mL H_2O to give an eluate containing $^{3}H_2O$, [1-^{14}C]acetyl-carnitine and [2-^{3}H]-acetyl-carnitine, and the third adjusted to pH 12 with KOH and kept at 25° for 45 min to hydrolyse acetyl-carnitine and then passed down a Dowex 1 column and washed with 2 mL H_2O so that the eluate only contained $^{3}H_2O$ released from [9,10-^{3}H]palmitate. The amount of $^{14}CO_2$ formed from [1-^{14}C]palmitate by liver homogenates is small and so was not measured [9].

Administration of etomoxir caused a marked organic aciduria with the excretion of large amounts of the dicarboxylic acids hexanedioic acid, heptanedioic acid, octanedioic acid, decanedioic acid, undecanedioic acid and hexadecanedioic acid (Table 1). The excretion of other organic acids was not significantly different from the controls (Table 1).

The rates of palmitate oxidation measured using [9,10-^{3}H]palmitate or [1-^{14}C]palmitate agreed within 10%. About 70% of the ^{3}H released from [9,10-^{3}H]palmitate was as

Table 1. Organic aciduria in fasted rats induced by etomoxir

	Metabolite (μg/μmol creatinine 18 hr)	
	Control rats (N = 3)	Etomoxir-treated rats (N = 3)
Malonic acid	1.0 ± 0.5	0.9 ± 0.3
2-Methylmalonic acid	0.3 ± 0.2	0.8 ± 0.3
2-Ethylpropionic acid	1.2 ± 0.5	1.2 ± 0.1
Succinic acid	2.0 ± 0.2	1.1 ± 0.3
Isocitric acid	1.1 ± 0.5	1.9 ± 0.3
Hippuric acid	5.0 ± 0.5	3.2 ± 0.4
Hexanedioic acid	0.2 ± 0.1	1.3 ± 0.3
Heptanedioic acid	ND	1.8 ± 0.3
Octanedioic acid	0.4 ± 0.2	23.7 ± 0.1
Nonanedioic acid	ND	4.1 ± 0.5
Decanedioic acid	0.8 ± 0.4	57.4 ± 12.3
Undecanedioic acid	ND	2.4 ± 0.5
Dodecanedioic acid	ND	15.0 ± 2.4
Hexadecanedioic acid	ND	2.2 ± 0.5

Values are means ± SD.
Urine was collected and analysed as described in the text after administration of etomoxir (50 μmol/kg body wt).
ND, not detected.

Table 2. Inhibition of palmitate oxidation in liver homogenates by etomoxir

Substrate	Control animals	Etomoxir-treated animals
[1-^{14}C]Palmitate		
Acid-soluble metabolites	11.9 ± 1.8	2.5 ± 1.5†
[9,10-^{3}H]Palmitate		
Acid-soluble metabolites	11.1 ± 1.5	3.2 ± 2.0*
$^{3}H_2O$ released	7.8 ± 0.7	1.7 ± 0.5†

Palmitate oxidation was measured as described in the text. The rates are expressed as nmol of acetyl units formed/min per mg protein (means ± SEM, N = 3) assuming that each molecule of palmitate is oxidized completely and that 75% of the ^{3}H of [9,10-^{3}H]palmitate is released as $^{3}H_2O$.
Significances of differences from the controls.
* $P < 0.005$, † $P < 0.0005$.

$^{3}H_2O$ and 30% as ^{3}H-labelled negatively charged species in the controls (Table 2) compared with the theoretical values of 75 and 25%, respectively, for complete oxidation to acetyl-units [10]. The oxidation of palmitate was inhibited by about 80% in liver homogenates from etomoxir-treated rats compared with those from the controls (Table 2).

Substituted 2-oxiranecarboxylic acids, including etomoxir, are a class of compounds which are candidate antidiabetic drugs [6, 11]. These have several metabolic effects as a consequence of inhibition of long-chain fatty acid oxidation in mitochondria. We have now shown marked organic aciduria following an acute dose of etomoxir in fasted rats which is consistent with the strong inhibition of mitochondrial long-chain fatty acid oxidation in liver, but not of peroxisomal β-oxidation [12]. This dicarboxylic aciduria resembles that caused by administration of methylenecyclopropylglycine to fasted rats, a hypoglycaemic amino acid from seeds of *Litchi chinensis*, whose metabolites inhibit mitochondrial β-oxidation and the stage of the 3-oxoacyl-CoA thiolases [13]. Longer-term administration of substituted 2-oxiranecarboxylic acids to rodents causes peroxisomal proliferation, presumably as a result of impaired mitochondrial β-oxidation of long-chain fatty acids [12]. These findings are similar to those in inherited defects of mitochondrial β-oxidation [1], for which these drugs provide a good model.

In summary, administration of etomoxir to fasted rats induces a massive organic aciduria as a consequence of the inhibition of hepatic mitochondrial oxidation of long-chain fatty acids.

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*Division of Clinical Neuroscience and †Department of Pharmacological Sciences
University of Newcastle upon Tyne
Newcastle upon Tyne
NE2 4HH, U.K.
‡University of Konstanz
PO Box 5560
D-7750 Konstanz, Germany

RAJINDER SINGH KLER*
H. STANLEY A. SHERRATT†
DOUGLASS M. TURNBULL*
KLAUS MELDE‡

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