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STEREOSPECIFICITY OF THE INHIBITION BY ETOMOXIR OF FATTY ACID AND CHOLESTEROL SYNTHESIS IN ISOLATED RAT HEPATOCYTES

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Abstract—The racemates of substituted 2-oxiranecarboxylates are potent inhibitors of fatty acid oxidation and fatty acid and cholesterol synthesis. We show in the accompanying paper [Agius L, Peak M and Sherratt HSA, *Biochem Pharmacol* **42**: 1711–1715, 1991] that only the *R*-enantiomer of etomoxir, a potent hypoglycaemic compound, inhibits fatty acid oxidation in hepatocytes. We demonstrate in this paper that although the *R*-enantiomer of etomoxir is esterified to its CoA-ester more readily than the *S*-enantiomer, both the *R*- and *S*-enantiomers are equally potent inhibitors of fatty acid and cholesterol synthesis from acetate in rat hepatocytes. The inhibition of fatty acid synthesis is not due to direct inhibition of fatty acid synthetase and the inhibition of cholesterol synthesis occurs at a site proximal to formation of mevalonate. Since the *S*-enantiomer inhibits fatty acid and cholesterol synthesis but not fatty acid oxidation the inhibition of the biosynthetic pathways is not coupled to inhibition of fatty acid oxidation.

In recent years the ethyl-esters or sodium salts of a number of substituted 2-oxiranecarboxylic acids have been shown to be potent hypoglycaemic compounds in fasted animals and man [1]. Their CoA esters, formed in the cytosol from the free acids, inhibit long-chain fatty acid oxidation at the stage of carnitine palmitoyltransferase I (CPT I) in liver and extrahepatic tissues and secondarily increase glucose oxidation and decrease hepatic gluconeogenesis [1]. These compounds include palmoxirate (tetradecyl-2-oxiranecarboxylate, 2-TDGA‡), 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (clomoxir, POCA) and etomoxir (2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylate [1].

NEFA concentrations increase after acute doses of palmoxirate or clomoxir [2, 3]. However, both plasma NEFA and cholesterol concentrations decrease by about 50% with chronic administration of clomoxir to rats [3]. *RS*-Palmoxirate and *RS*-clomoxir inhibit the biosynthesis of fatty acids in isolated rat hepatocytes [4–6] and *RS*-clomoxir inhibits cholesterol biosynthesis [6]. The mechanisms by which these compounds inhibit these biosynthetic pathways have not been elucidated.

Carbon 2 of the 2-oxiranecarboxylates is asymmetric and most work with these compounds has been done with the racemates. The CoA ester of *R*-palmoxirate, but not of *S*-palmoxirate, inhibits CPT 1 [7] and only the *R*-enantiomer of etomoxir is hypoglycaemic (H.P.O. Wolf, personal communication) and inhibits palmitate oxidation [8]. It was therefore of interest to determine the effects of the *R*- and *S*-etomoxir on the biosynthesis of long-chain fatty acids and cholesterol by isolated rat hepatocytes. We show that both the *R*- and *S*-enantiomers inhibit fatty acid and cholesterol biosynthesis, clearly indicating that the inhibition of these pathways is independent of inhibition of β-oxidation.

MATERIALS AND METHODS

[2-^{14}C]Acetate, [2-^{14}C]mevalonate, [4-^{14}C]cholesterol and [1-^{14}C]palmitate were obtained from Amersham International (Amersham, U.K.). Biochemicals were obtained from the Sigma Chemical Co. (Poole, U.K.). OptiScint 'HiSafe' scintillation fluid was obtained from LKB. The *R*- and *S*-etomoxir and *R*- and *S*-etomoxir-CoA were provided by Dr H. P. O. Wolf, Byk Gulden Chemische Fabrik GmbH (Konstanz, Germany); these were more than 95% enantiomerically pure.

Hepatocytes were prepared from fed male Wistar rats (body wt: 250–300 g) bred in the Medical School, University of Newcastle upon Tyne, and incubated in 25 mL plastic conical flasks in 1.5 mL of Earle's balanced salts containing 5 mM glucose with shaking at 37° [5] in an atmosphere of 95% O_2/5% CO_2. Protein was determined by the Lowry method [9].

Long-chain acyl-CoA synthetase (EC 6.2.1.2) activity was assayed at pH 7.0 and 37° by the uptake of CoASH [10] in rat liver, mitochondrial outer membrane fractions [11]. Fatty acid synthetase was measured spectrophotometrically at pH 7.0 and 30° in the particle-free supernatant fraction from the

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‡ Abbreviations: etomoxir, 2-[6-(4-chlorophenoxy)-hexyl]oxirane-2-carboxylate; etomoxir-CoA, 2-[6-(chlorophenoxy)hexyl]oxirane-2-carbonyl-CoA; clomoxir, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate; clomoxir-CoA, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carbonyl-CoA; palmoxirate, tetra-decyl-2-oxirane-carboxylate; CPT I, carnitine palmitoyltransferase I (EC 2.3.1.21); NEFA, non-esterified fatty acids; NSL, non-saponifiable lipid fraction (includes cholesterol and squalene).

livers of rats that been fasted for 48 hr and then refed on a fat free diet for 48 hr [12].

Synthesis of long-chain fatty acids. The reactions were started by the addition of 0.5 mM [2-^{14}C]-sodium acetate (30 μCi/μmol) to 1.5 mL of a suspension of hepatocytes (2–4 mg protein). After 90 min the incubations were quenched with 3.0 mL of 5% (w/v) KOH and the flask contents washed into a stoppered glass tube with three batches of 3 mL of ethanol. Unlabelled palmitic acid (1 mg) was added as carrier. The cell preparations were saponified for 90 min at 70° and then acidified by the addition of 3.0 mL of 5 N H_2SO_4. Fatty acids were extracted three times with 10 mL of light petroleum (b.p. 40–60°), and the extracts washed three times with 1 mM acetic acid. The combined extracts were evaporated to 1–2 mL at 70° and transferred to scintillation vials and, after the addition of 2 mL acetone containing 1% (w/v) acetic acid, were evaporated to dryness at 20°. Radioactivity was then determined after addition of 5 mL scintillation fluid.

Synthesis of cholesterol. Hepatocytes were incubated with [2-^{14}C]mevalonate (54 μCi/μmol) for 60 min and saponified as described above with the addition of unlabelled cholesterol (1 mg) and squalene (1 mg) as carriers, and the alkaline solution extracted three times with light petroleum (b.p. 40–60°) containing butylated hydroxytoluene (0.01%, w/v) as antioxidant [13], washed three times with 1.0 mM KOH and evaporated to dryness at room temperature to obtain the non-saponifiable lipid (NSL) fraction. The residue was dissolved in 100 μL of light petroleum (b.p. 95–105°) and 10 μL was taken for determination of radioactivity. Samples (10 μL) were used for TLC on silica gel and were developed with acetone/light petroleum (b.p. 40–60°): 20/80 (v/v) [13]. Radioactive spots were detected by autoradiography for 3 days at −85°. Unlabelled squalene and cholesterol markers were detected with iodine vapour, [4-^{14}C]cholesterol was also used as a marker. The R_f values were: cholesterol, 0.5; squalene, 0.9; in this system squalene and squalene-2,3-epoxide are not well resolved [13]. Areas corresponding to cholesterol and squalene were scraped off the chromatographic plates and transferred to scintillation vials and 5 mL of scintillant added and their radioactivity determined. Cholesterol synthesis from [2-^{14}C]-acetate was also measured in a few experiments. After saponification, the alkaline solution was extracted with light petroleum to obtain the NSL fraction as described above. The digest was then acidified and the long-chain fatty acid fraction isolated. Recoveries were checked using [1-^{14}C]-palmitate and [4-^{14}C]cholesterol as standards and were greater than 95%.

RESULTS

Inhibition of long-chain fatty acid synthesis

Fatty acid synthesis was determined from the incorporation of [2-^{14}C]acetate into saponifiable fatty acid. This accounted for 85–90% of the incorporation of radioactivity into the total lipid fraction and was linear with time during 90 min (not shown). There was a concentration-dependent inhibition of fatty acid synthesis by both *R*- and *S*-etomoxir with no significant differences between the enantiomers (Table 1). There was some variation in the absolute rates of fatty acid synthesis with different preparations of hepatocytes. In other studies measuring the synthesis of fatty acids *in vivo* by the incorporation of ^3H from ^3H_2O, the incorporation into cholesterol was less than 5% of that into fatty acid [14].

Table 1. Effects of *R*- and *S*-etomoxir on the synthesis of free fatty acids from [2-^{14}C]acetate in isolated hepatocytes

Etomoxir concentration (μM)	Inhibition of incorporation of [2-^{14}C]acetate	
	R-Etomoxir	*S*-Etomoxir
0	100.0 ± 4.2	100.0 ± 4.2
2	62.8 ± 10.8*	67.7 ± 10.7*
10	28.0 ± 13.3†	21.9 ± 13.4†
50	7.2 ± 0.6†	5.8 ± 0.6†

Incorporation of [2-^{14}C]acetate into the total lipid fraction was measured in triplicate with three hepatocyte preparations during a 90 min incubation as described in Materials and Methods and expressed as percentages of the control; this includes an estimated 10–15% incorporation of radioactivity into the NSL fraction. The contribution of acetyl-CoA formed from glucose to the cytosolic pool of acetyl-CoA was assumed to be small in the presence of added 0.5 mM [2-^{14}C]acetate. The control rate was 4.79 ± 2.04 nmol [2-^{14}C]acetate incorporated/hr/mg protein (mean ± SD, N = 9).

Statistics: * $P < 0.01$, † $P > 0.005$, relative to the respective controls.

Formation of the CoA esters of etomoxir

The formation of the CoA esters of etomoxir was determined in mitochondrial outer membrane fractions. The rates (nmol/min/mg protein ± SD, N = 4) at 37° were *R*-etomoxir (62 ± 7), *S*-etomoxir (33 ± 14) and palmitate (253 ± 29). Little formation of CoA esters was detected in liver homogenates or microsome fractions, apparently due to acyl-CoA hydrolase (EC 3.1.2.2) activity. The CoA ester of *R*-palmoxirate was reported to be formed nine times more rapidly than that of *S*-palmoxirate [15].

Fatty acid synthase activity

After fasting and refeeding the activity of fatty acid synthetase in liver cytosolic fractions increased 4-fold to 10.5 + 1.2 nmol NADPH oxidized/min/mg protein at 30° (N = 4), (1.75 moles of NADPH are required for each mole of acetate incorporated into palmitate). Fatty acid synthase activity was not affected by *R*- and *S*-etomoxir and *R*- and *S*-etomoxir-CoA (100 μM), or by palmitate and palmitoyl-CoA (50 μM).

Inhibition of cholesterol synthesis

R- and *S*-etomoxir (10 μM) inhibited the conversion of [2-^{14}C]acetate into the NSL fraction by about 70% (Table 2), and 50 μM *R*-etomoxir caused a similar inhibition (not shown). The highest

Table 2. Effects of *R*- and *S*- etomoxir on the synthesis of non-saponifiable lipids from [2-^{14}C]acetate in isolated hepatocytes

Time (min)	Incorporation of [2-^{14}C]acetate* (nmol/mg protein) (mean ± SD, N = 3)		
	Control	*R*-Etomoxir (10 μM)	*S*-Etomoxir (10 μM)
30	1.55 ± 0.12	0.37 ± 0.00†	0.46 ± 0.01†
60	2.32 ± 0.69	0.71 ± 0.06*	0.83 ± 0.13*
90	2.98 ± 0.19	0.94 ± 0.07†	0.87 ± 0.07†

Incorporation of [2-^{14}C]acetate into the NSL fraction was measured as described in Materials and Methods using a different hepatocyte preparation from Table 1. $^{14}CO_2$ is not lost during the formation of isopentenyl pyrophosphate from [2-^{14}C]acetate.
Statistics: *$P < 0.05$, † $P < 0.005$, relative to the respective controls.

Table 3. Effects of *R*- and *S*-etomoxir on the incorporation of [2-^{14}C]mevalonate into non-saponifiable lipid in isolated hepatocytes

Etomoxir concentration (μM)	*R*-Etomoxir	*S*-Etomoxir
0	100.0 ± 6.1	100.0 ± 6.1
2	95.9 ± 10.0	86.8 ± 7.7
10	79.1 ± 14.2	85.1 ± 18.0
50	72.6 ± 18.1	77.3 ± 21.6

Incorporation of [2-^{14}C]mevalonate in the NSL fraction was measured during a 90 min incubation as described in Materials and Methods. The results for triplicate incubations with three preparations of hepatocytes are expressed as percentages of the controls, mean ± SD (N = 9) as the absolute rates cannot be calculated as the pool sizes of mevalonate were not known.

Table 4. Effects of *R*- and *S*-etomoxir on the ratio of cholesterol to squalene in the non-saponifiable lipid fraction formed from [2-^{14}C]mevalonate

	Ratio of radioactivities cholesterol/ squalene	Percentage of radioactivity of cholesterol plus squalene in NSL fraction
Control	10.7 ± 1.2	77.5 ± 6.4
R-Etomoxir 2 μM	6.1 ± 1.9	74.0 ± 12.2
10 μM	5.6 ± 0.4*	72.0 ± 4.4
50 μM	5.2 ± 0.5*	74.7 ± 3.5
S-Etomoxir 2 μM	6.3 ± 0.6*	85.7 ± 8.5
10 μM	5.0 ± 0.3*	80.3 ± 6.7
50 μM	5.2 ± 0.5*	74.3 ± 2.5

Equal volumes of the NSL fraction in light petroleum were applied to TLC plates or were counted. Radioactivities of areas corresponding to cholesterol and squalene after TLC were determined as described in Materials and Methods. Results are means ± SD (N = 3).
Statistics: * $P < 0.005$, relative to the respective controls.

concentrations of *R*- and *S*-etomoxir used (50 μM) only slightly inhibited (by about 25%) incorporation of [2-^{14}C]mevalonate into the NSL lipid fraction with no significant differences between the enantiomers (Table 3).

Autoradiographs of chromatograms of the NSL fractions showed that a spot which co-chromatographed with [4-^{14}C]cholesterol was the major product, with some radioactivity in the region corresponding to squalene. There were only traces of other radioactive products and about 70–80% of the applied radioactivity was recovered from the cholesterol and squalene regions. About 90% of tracer amounts of [4-^{14}C]cholesterol incubated with hepatocytes for 60 min was recovered, and no radioactive metabolites with different R_f values were detected. The ratios of radioactivity in 'cholesterol' and 'squalene' derived from [2-^{14}C]mevalonate were less in the presence of *R*- and *S*-etomoxir (Table 4). However, there were no significant differences in the ratios of radioactivity in 'cholesterol' and 'squalene' derived from [2-^{14}C]acetate (not shown).

DISCUSSION

Inhibition of fatty acid and cholesterol synthesis

Both fatty acids and cholesterol are derived from acetyl-CoA in the cytosol; but by different pathways. Most cytosolic acetyl-CoA is derived from glucose which is first converted to pyruvate which is then oxidized in the mitochondrial matrix to acetyl-CoA. This condenses with oxaloacetate to form citrate which leaves the matrix on a specific carrier. In the cytosol acetyl-CoA is reformed from citrate by citrate lyase (EC 4.1.3.7). Acetyl-CoA is also formed from acetate by acetyl-CoA synthase (EC 6.2.1.1). We have now shown that both *R*- and *S*-etomoxir (10–50 μM) strongly inhibit the biosynthesis of fatty acids and of cholesterol from acetate to an equal extent in rat hepatocytes (Tables 1 and 2), presumably by the same mechanism. This finding was surprising in view of the lack of effect of the *S*-etomoxir on β-oxidation [8]. It was known that palmoxirate and clomoxir (which were only available as the racemates) strongly inhibit fatty acid synthesis from glucose assessed by incorporation of ^3H_2O [4–6]. *RS*-Clomoxir and *RS*-clomoxir-CoA only weakly inhibit acetyl-CoA carboxylase, similar to palmitoyl-CoA [5]. It was therefore suggested that a likely site of inhibition of fatty acid synthesis was a direct effect

on fatty acid synthetase by clomoxir or its CoA-ester [5]. However, 100 μM concentrations of *R*- and *S*-etomoxir, and of *R*- and *S*-etomoxir-CoA, had no effect on the activity of fatty acid synthetase in liver supernatant fractions.

One explanation for inhibition of fatty acid synthesis from [2-^{14}C]acetate might be depletion of cytosolic CoASH required for the formation of acetyl-CoA and malonyl-CoA by sequestration as etomoxir-CoA. This is unlikely for three reasons: it would be opposed by long-chain acyl-CoA hydrolase and the CoASH concentrations in livers of rats after chronic administration of clomoxir are higher than in controls [3]. Further, the *R*- and *S*-enantiomers inhibited equally yet *R*-etomoxir-CoA was formed more readily than *S*-etomoxir-CoA by acyl-CoA synthetase. The supply of NADPH required for fatty acid and cholesterol synthesis may not be impaired since there is evidence that clomoxir stimulates the pentose phosphate pathway in cultured human fibroblasts [16]. Incorporation of [2-^{14}C]acetate into the NSL fraction was strongly inhibited (by about 70%) by both *R*- and *S*-etomoxir (Table 2). Both *R*- and *S*-etomoxir weakly inhibited the synthesis of cholesterol from [2-^{14}C]mevalonate by 20% at 50 μM). *RS*-Palmoxirate was reported to inhibit cholesterol synthesis in isolated rat hepatocytes by (about 50% at 400 μM) measured by incorporation of ^{3}H from $^{3}H_2O$ [6]. The strong inhibition of incorporation of radioactivity into the NSL fraction by *R*- and *S*-etomoxir from [2-^{14}C]acetate contrasts with the weak inhibition from mevalonate (Tables 2 and 3). The increased cholesterol/squalene ratio caused by *R*- and *S*-etomoxir (Table 4) suggests that there is also weak inhibition of the conversion of squalene to cholesterol.

Conclusions

The similar inhibition of fatty acid and cholesterol synthesis by both *R*- and *S*-etomoxir contrasts with the powerful inhibition of CPT I by the CoA-esters of *R*-etomoxir but not by *S*-etomoxir. However, the mechanisms of inhibition of fatty acid and cholesterol synthesis are still unknown. Inhibition of fatty acid synthesis may partly explain the chronic lowering of plasma NEFA concentrations in rats after chronic, but not acute, administration of clomoxir or etomoxir [1]. Inhibition of cholesterol synthesis may explain the lowering of plasma cholesterol by this group of compounds [1–3].

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