Etomoxir, sodium 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate, inhibits triacylglycerol depletion in hepatocytes and lipolysis in adipocytes

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Abstract The effects of etomoxir, an inhibitor of mitochondrial long-chain fatty acid oxidation, on triacylglycerol metabolism in rat hepatocytes and adipocytes were investigated. Etomoxir inhibited the depletion of triacylglycerol stores in hepatocytes incubated without exogenous fatty acids and inhibited lipolysis in adipocytes. The effects on hepatocytes could be attributed to two mechanisms. At low concentrations (1–10 μM) *R*-etomoxir increased fatty acid esterification by inhibition of β-oxidation. This effect was specific for the *R*-enantiomer and was associated with increased triacylglycerol secretion. At higher concentrations (50–100 μM) *RS*-etomoxir inhibited lipolysis and triacylglycerol secretion, independently of inhibition of carnitine palmitoyltransferase I. These effects of *RS*-etomoxir on triacylglycerol metabolism and lipolysis may contribute to the chronic hypolipidaemic effects of etomoxir in vivo.

Key words: Etomoxir; Lipolysis; Triacylglycerol; Adipocyte; Hepatocyte (rat)

1. Introduction

The raised plasma fatty acid (FA) concentration in non-insulin-dependent diabetes mellitus (NIDDM) is a contributing factor to the impaired glucose utilization by muscle and the increased hepatic gluconeogenesis as a result of increased fatty acid oxidation in these tissues [1]. This led to the development of inhibitors of FA oxidation for the treatment of NIDDM. These include substituted 2-oxiranecarboxylates, e.g. ethyl-2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate (etomoxir) and 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA, clomoxir) and methyl tetradecyl glycidate (TDGA, palmoxirate). The CoA esters of these compounds inhibit mitochondrial carnitine palmitoyltransferase (CPT) I activity [2].

The 2-oxiranecarboxylates do not affect blood glucose concentrations in normal man in the fed state, but are hypoglycaemic in fasted and diabetic animals and man [3]. Their acute administration increases plasma [FA] [4,5], which results in a delayed increase in [triacylglycerol] in the blood in starved and diabetic rats [5,6] but not in normal rats [5]. This triglyceridemia has raised questions on use of these drugs for treatment of NIDDM. The acute effects of 2-oxiranecarboxylates on plasma FA and triacylglycerol (TAG) concentrations are probably due to inhibition of CPT I, which increases the long-chain fatty acyl-CoA/long-chain fatty acyl-carnitine ratio in the cytoplasm, enhances re-esterification of FA to TAG [7] and increases hepatic secretion of very low density lipoprotein (VLDL) [8].

By contrast, chronic administration of 2-oxiranecarboxylates to healthy or diabetic rats decreases plasma cholesterol and TAG while the [FA] remains unchanged or is slightly lowered [4,9–11]. The chronic lowering of plasma FA and cholesterol concentrations by etomoxir and other CPT I inhibitors was suggested to be due to the inhibition of de novo FA and cholesterol synthesis as 2-oxiranecarboxylates inhibit FA and cholesterol biosynthesis [12–14]. These effects, unlike the inhibition of CPT I, are not enantiomer-specific indicating that they are not secondary to inhibition of β-oxidation [14,15].

In this study we report that etomoxir inhibits lipolysis in adipocytes and depletion of cellular TAG stores in hepatocytes. The latter is due to both increased FA esterification and inhibition of lipolysis. These effects may contribute to the lowering of plasma [FA] and [TAG] during chronic treatment in vivo.

2. Materials and methods

2.1. Materials

[2-^{3}H]Glycerol was from Amersham International PLC (Amersham, Bucks, UK). Etomoxir (*R*- and *S*-enantiomers and *RS*(rac)-etomoxir) was a gift from Dr. H. Wolf, Byk Gulden Chemische Fabrik GmbH (Konstanz, Germany).

2.2. Hepatocyte culture

Hepatocytes were isolated from male Wistar rats (body wt 200–250 g) fed ad libitum, by collagenase perfusion of the liver [15] and cultured in MEM containing 5% calf serum in 24-well plates at a density of 6×10^4 cells/cm^2 [16].

2.3. Determination of cellular and secreted triacylglycerol in hepatocytes

After cell attachment (7 h) hepatocytes were cultured in serum-free MEM containing the concentrations of etomoxir indicated for 24 h. On termination of the incubation, cellular and medium TAG were determined as in [16] and are expressed as nmol/mg of cell protein. Medium was deproteinized with perchloric acid for determination of acetoacetate and D-3-hydroxybutyrate [17]. Protein was determined as in [18].

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Abbreviations: CPT I, carnitine palmitoyltransferase I; FA, fatty acid; TAG, triacylglycerol; etomoxir, 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate; POCA, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (clomoxir); TDGA, methyl tetradecyl glycidate (palmoxirate); MEM, minimal essential medium.

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Table 1
Effects of *RS*-etomoxir on cellular and secreted TAG in hepatocytes

[Etomoxir] (μM)	Ketones formed (nmol/mg protein)	D-3-Hydroxybutyrate/ acetoacetate	Triacylglycerol (nmol/mg protein)		
			Secreted	Cellular	Total
0	545 ± 61	0.55 ± 0.06	40 ± 5	48 ± 4	88 ± 9
1	333 ± 51[b]	0.44 ± 0.02	59 ± 9[a]	54 ± 4	113 ± 13[a]
5	323 ± 53[b]	0.41 ± 0.05[b]	55 ± 9[a]	57 ± 6[a]	113 ± 14[a]
10	318 ± 48[b]	0.39 ± 0.06[b]	52 ± 9[a]	59 ± 5[a]	111 ± 14[a]
50	272 ± 37[b]	0.36 ± 0.06[a]	29 ± 5[a]	59 ± 3[a]	87 ± 8
100	252 ± 29[a]	0.35 ± 0.05[a]	16 ± 5[a]	64 ± 6	80 ± 9

Hepatocytes were incubated for 24 h with the concentrations of *RS*-etomoxir indicated. Ketone body (acetoacetate+3-hydroxybutyrate) formation is expressed as nmol/mg protein. Cellular and secreted TAG is expressed as nmol/mg protein. Cellular TAG content at start of 24 h culture was 80 ± 4 nmol/mg protein. Values are means ± S.E.M. ($n = 4$). Statistics: [a] $P < 0.05$, [b] $P < 0.005$ relative to respective control.

2.4. Incorporation of [2-^{3}H]glycerol into cellular and secreted triacylglycerol

After culture in serum-free MEM for 14–18 h the hepatocytes were incubated for 3 h in medium containing 3 mM [2-^{3}H]glycerol (0.5 Ci/mol) and the additions indicated and incorporation of radiolabel into cell and medium TAG was determined as in [19]. Incorporation was linear during 3 h.

2.5. Adipocyte isolation and determination of lipolysis

Adipocytes were isolated from epididymal fat pads from 24 h fasted male Wistar rats (body wt 150–180 g) by collagenase digestion as in [20]. They were suspended in Krebs Ringer buffer containing 4% (w/v) defatted BSA to a cell volume of 6% (v/v). Adipocytes were preincubated without or with etomoxir for 30 min, washed and resuspended to 6% (v/v). Adipocyte suspension (200 μl) was added to 200 μl of Krebs Ringer buffer containing 4% (w/v) BSA and the additions indicated. After 10 min incubation in a shaking water bath at 37°C, 200 μl of medium was deproteinized with 40 μl of ice-cold 10% (w/v) perchloric acid for determination of glycerol [20]. Results are expressed as nmol glycerol formed/min per unit volume of packed cells.

3. Results and discussion

When hepatocytes were incubated without exogenous FA for 24 h, the cellular TAG content declined from 80 nmol/mg to 48 nmol/mg in incubations without inhibitor and to 57–59 nmol/mg in incubations with 5–50 μM etomoxir (Table 1). This inhibition of depletion of endogenous TAG stores by *RS*-etomoxir was associated with increased TAG secretion at low [etomoxir] (1–10 μM) but with inhibition of secretion at 50 μM etomoxir. Ketogenesis was progressively inhibited with increasing [etomoxir] up to a maximum of 50% indicating that in the absence of exogenous FA, ketone bodies are formed in part from endogenous FA presumably derived from TAG stores. The D-3-hydroxybutyrate/acetoacetate ratio was decreased by etomoxir indicating a more oxidised mitochondrial NADH/NAD redox state.

To investigate the mechanism involved in the decreased depletion of TAG stores we determined the effects of *RS*-etomoxir on the incorporation of [^{3}H]glycerol in cellular and secreted TAG (Table 2). Low concentrations of *RS*-etomoxir (1–5 μM) increased glycerol incorporation into cellular but not in secreted TAG with a net increase in glycerol incorporation into total TAG suggesting increased esterification. However, 100 μM etomoxir caused a net decrease in glycerol incorporation into total TAG, suggesting either inhibition of esterification or inhibition of lipolysis of endogenous TAG with consequent decreased availability of FA for esterification. The demonstration of both stimulation and inhibition of [^{3}H]glycerol incorporation into TAG is consistent with previous studies using other β-oxidation inhibitors (POCA and TDGA) which reported either inhibition of esterification [21] or increased esterification (and increased secretion) because of inhibition of β-oxidation [8,12].

To investigate the mechanisms of the opposite effects of low and high concentrations of etomoxir we used the *R*- and *S*-enantiomers of etomoxir, because the inhibition of β-oxidation [15] but not the inhibition of FA and cholesterol biosynthesis is specific for the *R*-enantiomer [14]. We confirmed that only *R*-etomoxir inhibited ketogenesis (Table 3). Surprisingly however, both enantiomers lowered the D-3-hydroxybutyrate/acetoacetate ratio indicating that this is not simply due to inhibition of β-oxidation. Only the *R*-enantiomer increased secretion and cellular TAG at low concentrations (10 μM). However, both enantiomers inhibited TAG secretion at 100 μM (Table 3). Incorporation of [^{3}H]glycerol into total TAG was increased ($P < 0.05$) by 1 μM and 10 μM *R*-etomoxir (control, 3261 ± 203; 1 μM, 4361 ± 452; 10 μM, 4291 ± 447 dpm/well, means ± S.E.M., $n = 4$) but not by *S*-etomoxir (1 μM, 3144 ± 452; 10 μM, 3329 ± 757).

These results suggest that the effects that are specific for low concentrations of the *R*-enantiomer (increased TAG secretion, increased incorporation of [2-^{3}H]glycerol into total TAG and decreased depletion of endogenous TAG) are secondary to inhibition of β-oxidation. By contrast, the inhibition of TAG secretion (Table 1) and net esterification (Table 2) by 100 μM *RS*-etomoxir are at least in part due to a mechanism that is independent of inhibition of β-oxidation since they are also elicited by the *S*-enantiomer. Since the inhibition of esterification by high [etomoxir] is not associated with increased depletion of cellular TAG, etomoxir may have an additional inhibitory effect on lipolysis and indirectly decrease esterifica-

Table 2
Effect of *RS*-etomoxir on incorporation of [2-^{3}H]glycerol into TAG

[Etomoxir] (μM)	[^{3}H]Glycerol incorporated into triacylglycerol (dpm/well)		
	Secreted	Cellular	Total
0	358 ± 76	2878 ± 399	3216 ± 441
1	296 ± 81	4169 ± 399[b]	4465 ± 447[b]
5	295 ± 105	4025 ± 345[b]	4319 ± 400[b]
10	296 ± 109	3631 ± 549	3926 ± 609
50	251 ± 116	3077 ± 323	3327 ± 344
100	187 ± 77	2603 ± 307	2789 ± 324[a]

Hepatocytes were incubated for 3 h with the concentrations of *RS*-etomoxir indicated plus 3 mM [2-^{3}H]glycerol (0.5 Ci/mol) as described in Section 2. Incorporation of glycerol into cellular and secreted TAG is expressed as dpm/well. Values are means ± S.E.M. ($n = 4$). Statistics: [a] $P < 0.05$, [b] $P < 0.005$, relative to respective control.

Table 3
Effect of *R*- and *S*-etomoxir on TAG metabolism in hepatocytes

Additions	Ketones formed (nmol/mg protein)	D-3-Hydroxybutyrate/ acetoacetate	Triacylglycerol (nmol/mg protein)		
			Secreted	Cellular	Total
Control	498 ± 55	0.57 ± 0.03	38 ± 6	48 ± 2	86 ± 5
R-etomoxir					
1 μM	311 ± 44[b]	0.48 ± 0.05[a]	52 ± 7	55 ± 4	107 ± 9[a]
10 μM	274 ± 35[b]	0.41 ± 0.02[a]	50 ± 8[a]	57 ± 4[a]	107 ± 11[a]
100 μM	255 ± 32[b]	0.42 ± 0.06[a]	13 ± 3[a]	61 ± 5[a]	74 ± 3[a]
S-etomoxir					
1 μM	490 ± 56	0.53 ± 0.04[a]	38 ± 5	48 ± 3	86 ± 7
10 μM	497 ± 62	0.52 ± 0.04[a]	34 ± 8	52 ± 2	84 ± 9
100 μM	518 ± 65	0.44 ± 0.05[a]	24 ± 4[a]	47 ± 3	70 ± 5[b]

Hepatocytes were incubated for 24 h with the concentrations of *R*- and *S*-etomoxir indicated. Ketone body (acetoacetate+3-hydroxybutyrate) formation is expressed as nmol/mg protein. Cellular and secreted TAG is expressed as nmol/mg protein. Cellular TAG content at the start of experiment was 80 ± 4 nmol/mg protein. Values are means ± S.E.M. ($n = 4$). Statistics: [a]$P < 0.05$, [b]$P < 0.005$ relative to respective control.

tion of glycerol to TAG because of decreased availability of FA.

The effect of etomoxir on lipolysis was determined in isolated rat adipocytes from glycerol release as glycerol formed during hydrolysis of TAG is not further metabolised (Table 4). *RS*-etomoxir did not affect lipolysis in the absence of adenosine deaminase. However, it inhibited the stimulated rate by 20% at 100 μM.

It is noteworthy that the effects of etomoxir on adipocytes were determined in the presence of 4% BSA, whereas the experiments on hepatocytes were without albumin, and binding of etomoxir to albumin has been reported [23]. In additional experiments on hepatocytes where the effects of etomoxir on β-oxidation were determined in both the absence and presence of 4% BSA, the sensitivity to etomoxir was greater in the absence of albumin (70% inhibition at 0.2 μM etomoxir [22]) than in 4% BSA (70% inhibition at 10 μM etomoxir, results not shown), suggesting that the free [etomoxir] in the adipocyte experiments was much lower (about 50-fold) than in the hepatocyte experiments. This may account for the apparently lower sensitivity in adipocytes compared with hepatocytes.

4. Conclusions

This study shows that low and high etomoxir concentrations have opposite effects on TAG metabolism in hepatocytes. Low concentrations increase FA esterification and TAG secretion and inhibit TAG depletion by inhibition of β-oxidation and diversion of FA to esterification. An additional inhibitory effect on hepatocyte lipolysis at low [etomoxir] cannot be excluded. High concentrations of etomoxir inhibit lipolysis, esterification and TAG secretion. These effects are not enantiomer-specific but were not associated with cytotoxic effects (as determined from release of lactate dehydrogenase, results not shown). The metabolic effects of β-oxidation inhibitors have been investigated mainly in hepatocytes and muscle. In the perfused rat heart etomoxir was reported to inhibit lipolysis as a result of inhibition of β-oxidation [24]. However the effects of β-oxidation inhibitors on adipocytes have remained undocumented. The present study shows that etomoxir inhibits lipolysis in adipocytes and has biphasic effects on TAG secretion by hepatocytes. It is suggested that these effects of etomoxir on adipocytes and hepatocytes may explain the observations in vivo where chronic treatment with etomoxir was not associated with elevated plasma FA or TAG [4,9–11].

Table 4
Effect of *RS*-etomoxir on the rate of lipolysis in adipocytes

Addition	Rate of lipolysis	
	Basal rate	Stimulated rate (+adenosine deaminase)
RS-etomoxir		
0 μM	158 ± 44	925 ± 61
1 μM	151 ± 54	892 ± 60
10 μM	150 ± 41	763 ± 84
100 μM	141 ± 30	734 ± 41[a]

Adipocytes were preincubated with 0–100 μM *RS*-etomoxir in the presence of 4% (w/v) defatted BSA for 30 min. Lipolysis was measured over a 10 min incubation in the absence or presence of adenosine deaminase with the additions indicated. The rate of lipolysis is expressed as nmol of glycerol released/min per ml packed cell volume. Results are means ± S.E.M. for 4 experiments (8 animals). Statistics: [a]$P < 0.05$.

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