Biochemical Pharmacology, Vol. 36, No. 22, pp. 3917–3921, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

GLUCOSE KINETICS DURING ACUTE AND CHRONIC TREATMENT OF RATS WITH 2[6(4-CHLOROPHENOXY)HEXYL]OXIRANE-2-CARBOXYLATE, ETOMOXIR

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(*Received* 5 *February* 1987; *accepted* 18 *June* 1987)

Abstract—(1) The effects of 2[6(4-chlorophenoxy)hexyl]oxirane-2-carboxylate (etomoxir), a candidate antiketonaemic and antidiabetic drug, on glucose turnover and recycling of glucose carbon in rats were determined using [3-^{3}H,U-^{14}C]glucose. (2) Etomoxir (Na salt) was infused continuously at a rate of 2 mg/hr in fasted male Wistar ab Boots rats (250–280 g) that had been maintained on a standard diet, or on a diet containing 0.1% of etomoxir for 10 days. (3) In rats treated acutely with etomoxir, plasma glucose concentrations were decreased by about 1 mM, glucose turnover was decreased by 14%, and recycling of glucose carbon by 30% compared with the controls infused with 0.14 M NaCl. (4) Infusion of etomoxir in rats chronically pretreated with etomoxir had little effect on plasma glucose concentrations, but increased glucose turnover and recycling of glucose carbon by 40%. (5) Acute infusion of etomoxir caused dramatic lowering of blood 3-hydroxybutyrate concentrations from 1 mM to about 0.03 mM with little change in other intermediary metabolites. (6) In rats chronically fed etomoxir, the proportion of pyruvate dehydrogenase in quadriceps muscle in the active form was 31% compared with 15% in the controls. (7) It was concluded that etomoxir in the acute dose given had only moderate effects on glucose turnover and that chronic administration of etomoxir caused increased glucose turnover and glucose recycling in the rat.

Etomoxir* has been proposed as a candidate antiketonaemic and antidiabetic drug, which closely resembles POCA and TDGA. The CoA-esters of these drugs are powerful inhibitors of mitochondrial β-oxidation of long-chain fatty acids at the stage of CPT I on the outer face of the inner mitochondrial membrane [1–4]. POCA also inhibits gluconeogenesis from lactate and pyruvate in isolated rat hepatocytes [5, 6] and perfused livers [4]. The antiketonaemic and hypoglycaemic effects of etomoxir in fasted rats are similar to those of POCA [7].

Blood glucose concentrations during fasting are determined by the rates of hepatic glucose production and of glucose utilisation by extrahepatic tissues. Fatty acids are the preferred fuel for most tissues during fasting and gluconeogenesis is usually coupled to fatty acid oxidation. Inhibitors of fatty acid oxidation which may increase utilisation of glucose or decrease gluconeogenesis, or both, are of potential interest for the management of diabetes (see ref. 8). We therefore studied the effects of etomoxir on glucose turnover in fasted rats.

* Abbreviations used: etomoxir, 2[6(4-chlorophenoxy)hexyl]oxirane-2-carboxylate; POCA, 2[5(4-chlorophenyl)pentyl]oxirane-2-carboxylate; TDGA, 2-tetradecyloxirane-2-carboxylate; CPT I, carnitine palmitoyltransferase I (EC 2.3.1.21); PDH, pyruvate dehydrogenase (EC 1.2.4.1); GT, glucose turnover; MCR, metabolic clearance rate.

MATERIALS AND METHODS

Materials. Enzymes, cofactors and substrates were from Boehringer Corp., except 4(4-aminophenyl)azobenzenesulphonic acid which was a gift from Dr R. M. Denton. Etomoxir, both the ethyl ester and the sodium salt, were gifts from Dr Gerhard Ludwig, Byk Gulden Lomberg Chemische Fabrik GmbH, Konstanz, G.F.R. Radiochemicals were from Amersham International. Arylamine acetyltransferase was prepared from pigeon liver [9]. Bovine kidney PDH phosphatase was generously donated by Dr S. J. Yeaman.

Measurement of glucose turnover. Male Wistar ab Boots rats (250–280 g) were used. Jugular and femoral cannulae were implanted under ether anaesthesia 24 hr before use and the animals were then fasted for 20 hr. Three groups of six rats were used. Groups 1 and 2 had been maintained on standard laboratory chow, while group 3 was fed chow containing etomoxir (0.1% w/w, ethyl ester, added as a 10% w/w solution in acetone followed by air drying) for 10 days to provide a model for chronic use of etomoxir. The animals were not anaesthetised and were unrestrained during the experiments and care was taken to maintain normal body temperature. The metabolic clearance rate of etomoxir was estimated from the fractional clearance and volume of distribution of the drug in the rat [10]. On the basis of plasma concentrations of etomoxir after bolus

injections producing a 25% decrease in blood glucose concentrations [10], the dose of etomoxir was calculated by multiplying the metabolic clearance rate by the desired steady state plasma concentration of etomoxir. Group 1 was infused with 0.14 M NaCl as control. Infusion of etomoxir (Na salt) solution (100 mg in 15.5 ml of water) via the femoral vein to group 2 was started at 09.00 hr; 5.2 mg of etomoxir was given over the first 30 min, and then at a rate of 2 mg/hr for the next 5.5 hr, representing a total dose of 58–65 mg/kg body wt. Group 3 was infused with etomoxir at a rate of 2 mg/hr from the time of implantation of the cannulae, but without the priming dose, representing a total dose of 143–160 mg/kg body wt. At 11.00 hr all rats received a bolus dose of [3-^{3}H]glucose (10 μCi) and [U-^{14}C]glucose (6 μCi), and blood samples (200 μl) were taken from the jugular venous cannula every 3 min until 15 min, and then at 30, 45 and 60 min, and subsequently every 30 min until 240 min. Plasma glucose concentrations at each time point were measured by a glucose oxidase method (Yellow Springs Glucose Analyser, Clandon Scientific, London, U.K.). Plasma was separated immediately and 100 μl was deproteinised with $Ba(OH)_2$ and $ZnSO_4$ and the neural supernatant passed down a column of Ag2-X8 resin. The column was washed with ion-free water to elute glucose and the specific radioactivity of ^{14}C and ^{3}H determined by dual-counting in the residue after freeze-drying the eluate. It was assumed that glucose was the only significant source of radioactivity present. Recovery of added glucose was greater than 94%. Glucose turnover (GT) was defined

$$GT = \frac{\text{Injected dose of radioactivity (dpm/kg)}}{\int_0^\infty \text{Specific activity of plasma glucose} \cdot dt}$$

and the percentage recycling of glucose carbon

$$= \frac{GT^3 - GT^{14}}{GT^3} \times 100$$

where GT^3H is the turnover of glucose measured with [3-^{3}H]glucose and $CT^{14}C$ measured with [U^{14}C]glucose [11]. This method assumes that the blood glucose is in a steady state during sampling and this appeared to be justified by the small coefficient of variation (7%) for all 16 samples collected for each rat (Table 1). The percentage recycling of glucose carbon is taken as an estimate of gluconeogenesis from pyruvate.

Concentrations of metabolic intermediates in blood. To assess the effects of etomoxir on the concentrations of some intermediary metabolites in blood, a further group of six rats was infused with etomoxir as in group 2 (above) and a control group was infused with 0.14 M NaCl. Blood was taken at 0, 2 and 6 hr after the start of infusion and metabolites were determined by standard fluorimetric techniques [12].

Pyruvate dehydrogenase activity in muscle. A group of four rats was fed etomoxir (0.1% in the diet) for 40 days. They were killed by cervical dislocation and the quadriceps muscle immediately frozen in liquid nitrogen. PDH was measured spectrophotometrically [13] by the increase in absorbance at 460 nm due to acetylation of 4(4-aminophenyl)azobenzenesulphonate by acetyl-CoA formed, catalysed by arylamine acetyltransferase. Skeletal muscle (0.3 g) was homogenised at 0° (Polytron Kinematica, setting No. 4 for 30 sec) in 2.5 ml of medium containing 100 mM Tris–HCl, pH 7.3, 1.2 mM dithiothreitol. PDH activity was measured in a medium containing 100 mM Tris–HCl, pH 7.8, 0.7 mM EDTA, 1.2 mM dithiothreitol, 1 mM thiaminepyrophosphate, 1 mM pyruvate, 0.2 mM CoASH, 0.05 mM 4(4-aminophenyl)azobenzenesulphonate and 0.25 units of arylamine acetyltransferase at 30°. For determination of total activity, PDH was converted to its active form by preincubating muscle extracts for 25 min at 30° in 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM dichloroacetate and PDH phosphatase.

RESULTS

Infusion of etomoxir in untreated rats (group 2) caused small but significant falls in plasma glucose concentrations (about 1 mM, $P < 0.001$). There were small decreases in glucose turnover (14%, $P < 0.05$), and moderate decreases in recycling of glucose carbon (32%, $P < 0.05$), compared with the controls (Table 1). In animals pretreated with etomoxir for 10 days (group 3) the turnover of [3-^{3}H]glucose was increased by 14% ($P < 0.05$) while that of [U-^{14}C]glucose was the same as in the controls so that recycling of glucose carbon was increased by 41% ($P < 0.05$). In this strain of rats liver glycogen is depleted after a 20 hr fast so that blood glucose

Table 1. Effects of intravenous infusion of etoximir on glucose turnover and recycling in 20 hr fasted normal rats, and in rats pretreated with etoximir

	Plasma glucose concentrations (mM)	Turnover of [3-^{3}H]glucose (μmol/min/kg)	Turnover of [U-^{14}C]glucose (μmol/min/kg)	Percentage recycling of glucose carbon
Normal controls	6.5 ± 0.1	36.4 ± 1.2	28.2 ± 1.9	23 ± 3
Short-term infusion of etoximir	5.4 ± 0.1***	31.5 ± 1.0*	26.7 ± 1.2	16 ± 1*
Infusion of etoximir in rats pretreated with etoximir for 10 days	6.3 ± 0.3	41.1 ± 1.4*	27.5 ± 0.7	32 ± 3*

The results were calculated assuming that plasma glucose concentrations were in a steady state during the turnover studies (coefficient of variation 7%). The specific radioactivities of glucose at each time point were calculated using the mean of the 16 glucose determinations over 4 hr. Values are means ± SEM (N = 6). Significance of differences from controls; *$P < 0.05$; ***$P < 0.001$.

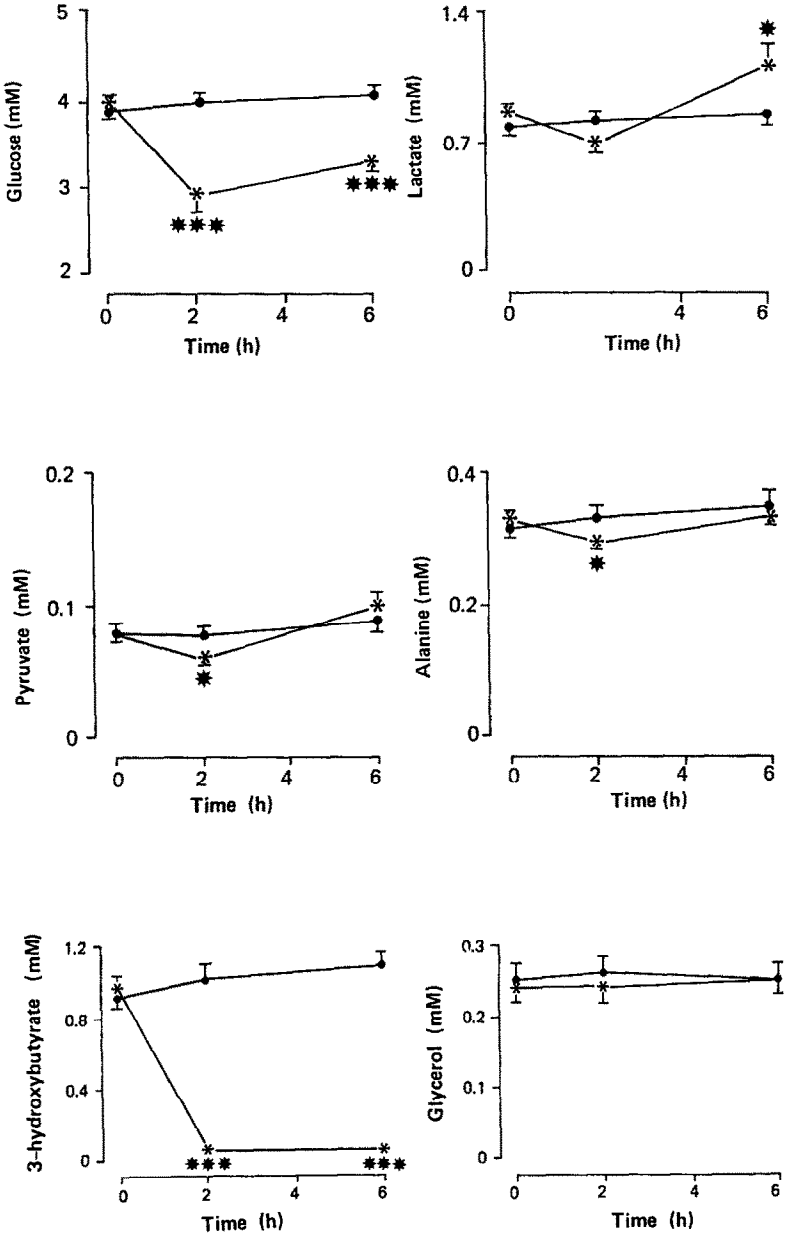

Fig. 1. Effects of etomoxir on the concentrations of glucose, lactate, pyruvate, alanine, 3-hydroxybutyrate and glycerol in blood. Controls (●), etomoxir-treated (*). Values are expressed as means ± SEM (N = 6). Significances of differences from controls; *P < 0.05, ***P < 0.001.

Table 2. Pyruvate dehydrogenase activities in quadriceps muscle from etoximir-fed rats

	Etoximir-fed	Control
Initial activity	0.32 ± 0.02*	0.13 ± 0.01
Total activity	1.01 ± 0.07**	0.78 ± 0.04
Percentage PDH in active form	31 ± 3***	16 ± 1

PDH activities at 30° are expressed as μmol/min/g wet wt. Values are means ± SEM for 4 animals. Total activities are after fully activating PDH by dephosphorylation. Significance of differences from controls, *P < 0.01, **P < 0.05, ***P < 0.005.

concentrations then become dependent on gluconeogenesis [14]. Rats maintained on the etomoxir-containing diet behaved normally, similarly to rats fed on a diet containing 0.2% POCA for 12 weeks [15]. During and after infusion of etomoxir with our experimental conditions both groups showed marked muscle weakness. However, muscle weakness was not observed in other experiments in which etomoxir was also infused intravenously daily in doses of 20 mg/kg body wt for 14 days [10].

Infusion of etomoxir caused dramatic lowering of blood 3-hydroxybutyrate concentrations from 1 mM to almost undetectable values (0.03 mM), without change in blood glycerol (Fig. 1) indicating profound inhibition of heptatic ketogenesis as was previously noted withy POCA [7]. Etomoxir decreased blood glucose concentrations, from 4.0 ± 0.1 mM to 2.9 ± 0.2 mM after 2 hr ($P < 0.001$), and to 3.3 ± 0.1 mM after 6 hr ($P < 0.001$) while there was little change in pyruvate, lactate and alanine (Fig. 1).

The percentage of PDH in the active form in muscle from fed rats maintained on the diet containing etomoxir was 32% compared with 16% in the controls, while the total activity of PDH (expressed after dephosphorylation) was 1.01 μmol/min g wet wt compared with 0.78 μmol/min/g wet wt in the controls (Table 2). The expressed activity of PDH in muscle from treated rats was therefore about 2.5 times greater than in the controls. PDH activity in normal rats would not be expected to change after 20 hr of fasting.

DISCUSSION

Hypoglycaemia following acute doses of POCA or TDGA in fasted animals is thought to be due to impaired gluconeogenesis secondary to inhibition of long-chain fatty acid oxidation, causing lack of acetyl-CoA necessary to activation pyruvate carboxylase and a decreased supply of mitochondrial reducing equivalents, but with no direct effects on the pathway of gluconeogenesis [16]. It is also supposed that decreased generation of acetyl-CoA and of NADH by β-oxidation allows activation of PDH thus increasing pyruvate oxidation and hence that of glucose. As expected, glucose turnover was decreased in rats treated acutely with etomoxir and there was some decrease in recycling of glucose carbon. However, in etomoxir-pretreated rats infused with etomoxir there were moderate increases in glucose turnover. Despite activation of muscle PDH (Table 2) glucose carbon recycling was increased by 41% ($P < 0.05$) in rats (Table 1). Similar results were obtained in pigs indicating that increased utilization of glucose rather than decreased gluconeogenesis is the cause of the hypoglycaemic effects of etomoxir observed in this species [17].

Small doses of TDGA did not prevent the hyperglycaemic effects of adrenaline in fed or fasted rats, not the stimulation of glucose synthesis from lactate plus pyruvate in isolated hepatocytes [18]. By contrast, the hypoglycaemic effects of direct inhibitors of gluconeogenesis were not overcome by adrenaline [18]. Gluconeogenesis may sometimes depend on the oxidation of alternative substrates such as pyruvate and the carbon skeletons of some amino acids to provide energy, and such substrates may be partitioned between oxidation and gluconeogenesis, thus permitting some glucose production when fatty acid oxidation is impaired. In rats, but not necessarily in other species, chronic pretreatment with etomoxir, POCA and TDGA may cause adaptive changes allowing gluconeogenesis to become less dependent on mitochondrial fatty acid oxidation in liver, for example chronic administration of POCA in the food causes a limited increase in the capacity for hepatic peroxisomal β-oxidation in rats [19]. In hypoglycin-poisoning there is virtually complete supression of carbon recycling in fasted animals associated with inhibition of fatty acid oxidation, although the metabolic disturbances are not confined to β-oxidation and glucose turnover is decreased by 70% [20, 21]. The oxidation of palmitoyl-CoA plus carnitine by skeletal muscle mitochondrial fractions from muscle of rats treated chronically with POCA is decreased by 50% (and some inhibition may have been reversed during their preparation) [2], and palmitate oxidation is largely suppressed in perfused hearts taken from rats given POCA (30 mg/kg body wt) 3 hr previously, together with a 2–2.5-fold increase in the rate of glucose utilisation [22]. This study confirms that gluconeogenesis is not obligatorily coupled to fatty acid oxidation in rats. It is important to determine the effects of a range of doses of etomoxir on glucose turnover in diabetic states where excessive rates of gluconeogenesis may be much more dependent on hepatic mitochondrial fatty acid oxidation.

Acknowledgements—We thank Drs. G. Ludwig and H. P. O. Wolf for valuable discussions and Mr. G. George for skilled technical assistance. Y.T.K. was supported by the Medical Research Council and H.S.A.S. by the Wellcome Trust.

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