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Metabolic consequences of methylenecyclopropylglycine poisoning in rats

Klaus MELDE,* Sandra JACKSON,† Kim BARTLETT,‡ H. Stanley A. SHERRATT†§ and Sandro GHISLA*
*University of Konstanz, P.O. Box 5560, D-7750 Konstanz, Federal Republic of Germany and
Departments of †Pharmacological Science and ‡Child Health, The Medical School, Human Metabolism Research Centre, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, U.K.

We describe the effects of methylenecyclopropylglycine in fasted rats. A 75% decrease in the blood glucose concentration and an increase of lactate and pyruvate were observed 6 h after administration of 100 mg of this amino acid/kg. By contrast with the effects reported for hypoglycin [Williamson & Wilson (1965) Biochem. J. **94**, 19c–21c], the plasma concentrations of ketone bodies decreased after administration of methylenecyclopropylglycine and the concentrations of branched-chain amino acids in the plasma were increased 6-fold. The oxidation of decanoylcarnitine or of palmitate was nearly completely inhibited in rat liver mitochondria from methylenecyclopropylglycine-poisoned rats. The activities of acetoacetyl-CoA and of 3-oxoacyl-CoA thiolase were decreased to 25% and < 10% of the controls. There was a pronounced aciduria, due to the excretion of dicarboxylic acids and of oxidation products of branched-chain amino acids. The accumulation of the toxic metabolite methylenecyclopropylformyl-CoA in the mitochondrial matrix was detected after administration of methylenecyclopropylglycine. Similarly we confirmed experimentally that methylenecyclopropylacetyl-CoA accumulates in mitochondria incubated with methylenecyclopropylpyruvate.

INTRODUCTION

Methylenecyclopropylglycine (MCPG), the lower homologue of the better-known hypoglycaemic amino acid hypoglycin (methylenecyclopropylalanine), was isolated in 1962 by Gray & Fowden and shown to be hypoglycaemic in mice [1]. It is also hypoglycaemic in fasted rats [2]. Hypoglycin is converted into its active metabolite methylenecyclopropylacetyl-CoA (MCPA-CoA) by the combined action of branched-chain-amino-acid aminotransferase and branched-chain-oxoacid dehydrogenase (Scheme 1). MCPA-CoA inactivates medium-chain-acyl-CoA and short-chain-acyl-CoA dehydrogenases involved in the β-oxidation of fatty acids or in the degradation of branched-chain amino acids [3–11] by covalent modification of the flavin prosthetic groups of the acyl-CoA dehydrogenases by a 'suicide' reaction [12–15]. The pharmacological effects of hypoglycin, namely hypoglycaemia [3], hyperketonaemia [11] and a pronounced organic aciduria [16,17], are the results of these inhibitions [8,18]. By analogy with hypoglycin, it has been suggested that MCPG is converted into a toxic metabolite, methylenecyclopropylformyl-CoA (MCPF-CoA) (Scheme 1) [2,19]. Metabolites of MCPG inhibit β-oxidation, but at a different stage from those of hypoglycin [2]. In this work we describe the similarities and, more importantly, the differences between the effects of MCPG and of hypoglycin in fasted rats. We found that administration of MCPG to rats causes a marked hypoketonaemia and strong inhibition of the mitochondrial 3-oxoacyl-CoA and acetoacetyl-CoA thiolases. We also provide direct evidence for the formation of MCPF-CoA from MCPG *in vivo* and for the formation of MCPF-CoA from methylenecyclopropylglyoxalate and MCPA-CoA from methylenecyclopropylpyruvate (MCPP) *in vitro*. In addition we describe a marked organic aciduria after administration of MCPG, which in part is comparable with that after the administration of hypoglycin [16,17].

MATERIALS AND METHODS

Materials

Hypoglycin and MCPG were isolated from dried kernels of litchi (*Litchi chinensis*) fruits as described by Boschert [20]. The kernels were provided by Dr. A. Munzhuber (Taipei, Taiwan), and were obtained from litchi-fruit canneries. MCPG used for animal feeding was 80% pure and free of hypoglycin as determined by amino acid analysis. Alanine, glycine and valine were the only amino acids present as contaminants. It was assumed that they would not affect the results in the concentrations used.

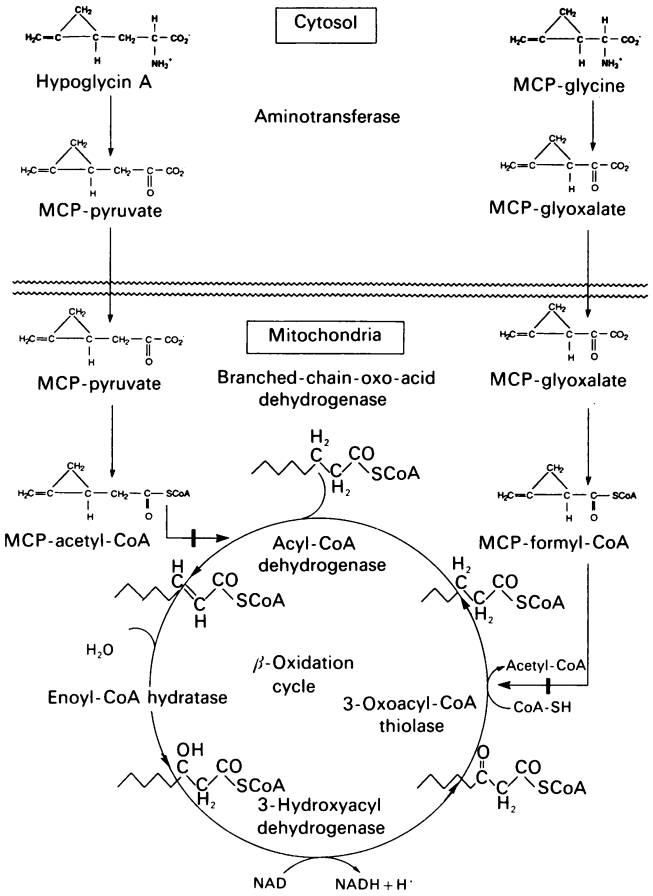
MCPA-CoA was synthesized according to Wenz *et al.* [12] and MCPF-CoA by the method of Boschert [20]. 3-Oxohexadecanoyl-CoA was synthesized by the method of El-Fakhri & Middleton [21] and hexadec-2,3-enoyl-CoA as described in [22]. MCPP was prepared from hypoglycin, and methylenecyclopropylglyoxalate from MCPG using L-amino-acid oxidase and catalase as described by Rüdiger *et al.* [23]. Decanoyl-L-carnitine and palmitoyl-L-carnitine were synthesized and characterized as described by Holland & Sherratt [24]. L-Carnitine was a gift from Sigma Tau, Rome, Italy. All commercially available acyl-CoA thioesters were from Sigma. L-Amino acid oxidase and catalase were from Boehringer, Mannheim, Germany.

Animals

Male rats of the local Sprague–Dawley strain (The Medical School, University of Newcastle upon Tyne, U.K.) weighing 200–250 g at the start of the experiment were used. The animals were fed on a standard diet with water ad libitum. They were fasted for 24 hours, and then given a single dose of MCPG (100 mg/kg body weight) intraperitoneally. For the collection of urine samples, the animals were held individually in metabolic cages for 6 h. The animals were killed by cervical dislocation and

Abbreviations used: MCPA-CoA, methylenecyclopropylacetyl-CoA; MCPF-CoA, methylenecyclopropylformyl-CoA; MCPG, methylenecyclopropylglycine; MCPP, methylenecyclopropylpyruvate; LC-, long-chain-specific; MC-, medium-chain-specific; SC-, short-chain-specific.

§ To whom correspondence should be sent.



Scheme 1. Conversion of MCPG and hypoglycin into active metabolites and their sites of inhibition of β-oxidation

Abbreviation: MCP-, methylenecyclopropyl.

blood was obtained by cardiac puncture. The livers were removed and mitochondrial fractions prepared as described below.

Standard Methods

Plasma glucose, lactate, pyruvate, acetoacetate and 3-hydroxybutyrate concentrations were determined as described by Lloyd *et al.* [25]. The plasma concentrations of free amino acids were determined as described by Goodman & Vale [26], using a Biotronix amino acid analyser. Protein concentrations were determined as described by Lowry *et al.* [27], with BSA as standard. Statistical significances of the differences between means were calculated by Student's *t* test ($*P < 0.05$; $**P < 0.01$; $***P < 0.005$). Owing to the wide range of the amounts of the organic acid extracts and of free amino acids in the plasma, no tests for significance were performed for these values.

Measurement of mitochondrial β-oxidation

Mitochondrial fractions were prepared in 0.3 M-sucrose/10 mM-Hepes/1 mM-EGTA, pH 7.2, and their O_2 uptake, as determined polarographically at 30 °C, as described by Van Hoof *et al.* [18]. 20 μM-Decanoylcarnitine, 10 mM-glutamate plus 1 mM-malate or 20 mM-succinate were used as substrates. Coupling conditions were obtained by the addition of 0.5 mM-ADP and uncoupling conditions with 20 μM-2,4-dinitrophenol.

Measurement of enzyme activity

Enzyme activities were measured in mitochondria solubilized with Triton X-100 (1 mg/mg of mitochondrial protein) in 0.25 mM-sucrose/10 mM-Hepes/1 mM-EGTA, pH 7.2, for 30 min at 0 °C, followed by centrifugation (115000 $g_{av.}$ · min). 3-Oxoacyl-CoA thiolase and acetoacetyl-CoA thiolase were measured spectrophotometrically at 303 nm by monitoring the disappearance of the Mg^{2+}–enolate complex of 3-oxohexadecanoyl-CoA or acetoacetyl-CoA [28]. The activities were calculated by using a molar absorption coefficient of 9900 $M^{-1} \cdot cm^{-1}$ for 3-oxohexdecanoyl-CoA and of 21400 $M^{-1} \cdot cm^{-1}$ for acetoacetyl-CoA [29]. The activities of long-chain-specific (LC-) and short-chain-specific (SC-)3-hydroxyacyl-CoA dehydrogenases were measured and calculated as described by El-Fakhri & Middleton [21]. The activities of LC- and SC-enoyl-CoA hydratases were determined by monitoring the decrease of A_{263} due to the reduction of the double bond of hexadec-2,3-enoyl-CoA and crotonyl-CoA. A molar absorption coefficient of 15400 $M^{-1} \cdot cm^{-1}$ was used [22]. Acyl-CoA dehydrogenases were measured by using dichlorophenol-indophenol and phenazine methosulphate as artificial electron acceptors as described by Veitch *et al.* [17]. Branched-chain-amino-acid aminotransferase activities were measured [30] with L-leucine as substrate.

H.p.l.c. analysis of MCPF-CoA and MCPA-CoA generated in mitochondrial incubations

Liver mitochondrial fractions from rats treated with MCPG were used to detect MCPF-CoA formed *in vivo*. Mitochondria (20 mg of protein) were suspended in 2 ml of water and 200 μl of 5 mM-$HClO_4$ and 10 nmol of hexanoyl-CoA as internal standard were added, and the precipitated protein was removed by centrifugation. The supernatant was adjusted to pH 7 with K_2CO_3 and freeze-dried after removal of $KClO_4$. The residue was dissolved in 500 μl of h.p.l.c.-grade water and a 200 μl sample was analysed by h.p.l.c. with photodiode-array detection of u.v.-absorbing compounds as described by Watmough *et al.* [31]. Acyl-CoA esters were eluted from the column (Lichrosorb 10-RP18; 250 mm × 4.6 mm) by a gradient of methanol in water containing 50 mM-KH_2PO_4, pH 5.3, using the conditions described by Causey *et al.* [32]. MCPF-CoA had a retention time of 48.23 min and was co-eluted with an unidentified peak with a retention time of 47.82 min. MCPF-CoA was therefore purified further [31] and identified. MCPF-CoA was absent from extracts of mitochondria from control rats. In addition, the formation of MCPF-CoA was demonstrated in mitochondria from control rats incubated with methylenecyclopropylglyoxalate. Mitochondria (20 mg of protein) were incubated at 30 °C in a shaking water bath (120 strokes/min) in 2 ml of medium containing 100 mM-KCl, 2.5 mM-KH_2PO_4, 5 mM-$MgCl_2$, 1 mM-EDTA, 5 mM-ATP, 0.1 mM-CoA, 0.5 mM-carnitine, 40 μM-2,4-dinitrophenol, pH 7.2 [31]. After 3 min preincubation, 20 μM-methylenecyclopropylglyoxalate was added and incubated for 5 min, the reaction stopped by addition of 200 μl of 5 M-$HClO_4$ and then treated as described above and analysed by h.p.l.c. The formation of MCPA-CoA was also demonstrated in mitochondria incubated with MCPP (Fig. 3, below).

Analysis of urinary organic acids

The urine samples, each containing 10 μmol of creatinine, were saturated with NaCl and transferred to liquid/liquid extractors. The samples were acidified by addition of 200 μl of 6 M-HCl and the internal standard (20 μg of phenylbutyrate/μmol of creatinine) was added. A saturated NaCl solution was added to give a final volume of 5 ml. Organic acids were extracted with 50 ml of redistilled diethyl ether for 8 h. The diethyl ether was removed under a gentle stream of N_2 at room temperature. The residue was treated with an excess of ethereal diazomethane

and left at room temperature for at least 30 min. The methylated extracts were analysed by g.l.c.–m.s. using the system and conditions described by Veitch *et al.* [17].

RESULTS AND DISCUSSION

Effects of MCPG on blood metabolites

Administration of MCPG (100 mg/kg body wt.) to 24 h-fasted rats caused a 72% decrease in plasma glucose concentrations after 4 h (Table 1). There were small increases in lactate and pyruvate, with a change in the lactate/pyruvate ratio from 20 to 9. Acetoacetate and 3-hydroxybutyrate concentrations were lowered to less than 0.01 mM (Table 1). The plasma concentrations of most amino acids were increased (Table 1); the largest changes were increases in the branched-chain amino acids (500–550%) and the basic amino acids (200–260%) (Table 1).

Effects of methylenecyclopropylglyoxalate on mitochondrial oxidations

There was no inhibition of the oxidation of 10 mM-succinate or of 10 mM-glutamate plus 1 mM-malate, and there was no effect on the stimulation of State 3 respiration by 10 μM-ADP, in mitochondria preincubated for 3 min with 10 μM-methylenecylcopropylglyoxalate, or in mitochondria from MCPG-treated rats (results not shown). This indicates that metabolites of MCPG have little effect on the citrate cycle, the respiratory chain or oxidative phosphorylation.

No oxidation of 10 μM-decanoylcarnitine was detected in coupled or uncoupled liver mitochondria from MCPG-treated rats, although 10 μM-decanoylcarnitine was rapidly and completely oxidized to acetoacetate by the controls (Fig. 1). The oxidation of decanoylcarnitine was also completely suppressed in uncoupled mitochondria from control rats after 3 min preincubation with 10 μM-methylenecyclopropylglyoxalate. Similar results were obtained when 10 μM-palmitoylcarnitine was used as substrate (results not shown). There was no inhibition of the oxidation of 10 μM-decanoylcarnitine in normal mitochondria that had been incubated for 3 min with 20 μM-MCPG, or with 20 μM-MCPF-CoA plus 0.5 mM-carnitine, indicating that MCPF-CoA cannot cross the mitochondrial inner membrane directly and that it is not a substrate for the carnitine palmitoyltransferases.

Activities of enzymes of β-oxidation and some enzymes involved in the degradation of branched-chain amino acids

3-Oxoacyl-CoA and acetoacetyl-CoA thiolases are the major targets in the β-oxidation pathway for the toxic metabolites of MCPG (Table 2). Their activities were depressed to less than 10% and about 25% respectively of those in the controls. That the inhibition of the thiolases persisted in the final assay medium when the matrix contents had been diluted approx. 100000-fold indicates that the inhibition was irreversible or that MCPF-CoA has a very high affinity for these enzymes. In addition, the activities of the two chain-length-specific 3-hydroxyacyl-CoA dehydrogenases were decreased to 73 and 60% of control values. The two chain-length-specific enoyl-CoA hydratases and, as reported previously [2], the three acyl-CoA dehydrogenases of the β-oxidation pathway, are not inhibited significantly in liver mitochondria from MCPG-treated rats. By contrast, the two acyl-CoA dehydrogenases involved in the degradation of branched-chain amino acids are both inhibited: 2-(methyl)-branched-chain acyl-CoA dehydrogenase to 70% of the control values and isovaleryl-CoA dehydrogenase to 40% respectively, confirming our earlier observations [2]. Branched-chain-amino-acid aminotransferase was also inhibited by 50% (Table 2). Any reversible enzyme inhibitions by high concentrations of metabolites in the mitochondrial matrix would not have been detected.

The strong inhibition of mitochondrial β-oxidation after administration of MCPG can be explained by inactivation of

Table 1. Effects of administration of MCPG to fasted rats on the concentrations of some blood metabolites

Blood was obtained 6 h after the intraperitoneal administration of MCPG (100 mg/kg body wt.) to 24 h-fasted rats and analysed as described in the Materials and methods section. The results are means ± S.E.M. for five rats. Significances of differences from the controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

Metabolite	Units of concn.	Concn.	
		Control rats	MCPG-treated rats
Glucose	mM	6.92±0.93	1.90±0.42***
Lactate	mM	2.01±0.33	3.44±0.18**
Pyruvate	mM	0.10±0.02	0.38±0.06**
3-Hydroxybutyrate	mM	0.35±0.06	< 0.01***
Acetoacetate	mM	0.22±0.04	< 0.01***
Glutamate	μM	838±211	422±165
Glycine	μM	667±108	427±73
Alanine	μM	350±93	530±106
Valine	μM	133±25	694±119***
Leucine	μM	141±31	717±146***
Isoleucine	μM	76±18	414±146*
Lysine	μM	300±126	607±110
Cysteine	μM	18±12	65±26
Ornithine	μM	53±18	209±69
Histidine	μM	70±33	220±74

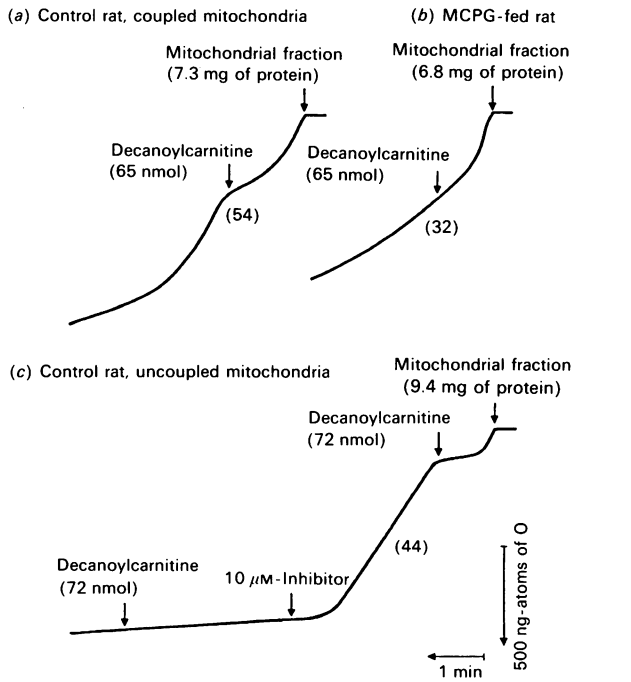


Fig. 1. Oxidation of decanoylcarnitine by (*a*) coupled mitochondria from a control rat, (*b*) coupled mitochondria from a MCPG-fed rat and (*c*) uncoupled mitochondria from a control rat incubated with 10 μM-methylenecyclopropylglyoxalate

Conditions were as described in the Materials and methods section.

Table 2. Effects of administration of MCPG to fasted rats on the activities of some enzymes in liver mitochondria

MCPG (100 mg/kg body wt.) or 0.14 M-NaCl was administered to 24 h-fasted rats and enzyme activities were measured at 30 °C in extracts of liver mitochondria as described in the Materials and methods section. The results are expressed as μmol of substrate consumed/min per mg of protein and are means ± S.E.M. for three animals in each group. Significances of differences from the control $^{**}P < 0.01$; $^{***}P < 0.005$.

Enzyme	Activity		Inhibition (%)
	Control	MCPG-treated	
SC-acyl-CoA dehydrogenase	14.8 ± 0.9	10.9 ± 0.7**	26
MC-acyl-CoA dehydrogenase	12.4 ± 2.4	11.6 ± 2.7	7
LC-acyl-CoA dehydrogenase	8.6 ± 0.3	7.9 ± 0.7	9
SC-enoyl-CoA hydratase	15570 ± 1800	14000 ± 1500	11
LC-enoyl-CoA hydratase	1560 ± 280	1255 ± 190	10
SC-3-hydroxyacyl-CoA dehydrogenase	1219 ± 215	730 ± 45**	40
LC-3-hydroxyacyl-CoA dehydrogenase	520 ± 20	380 ± 79***	27
Acetoacetyl-CoA thiolase	390 ± 55	100 ± 18***	75
3-Oxoacyl-CoA thiolase	265 ± 34	20 ± 6***	93
Isovaleryl-CoA dehydrogenase	1.4 ± 0.24	0.82 ± 0.12**	41
2-Methylbutyryl-CoA dehydrogenase	0.64 ± 0.10	0.19 ± 0.06***	80
Branched-chain aminotransferase	0.59 ± 0.08	0.33 ± 0.11***	45

Table 3. Effects of administration of MCPG to fasted rats on the excretion of organic acids and glycine conjugates

Urine was collected and analysed after the administration of MCPG (100 mg/kg body wt.) or 0.14 M-NaCl to 24 h-fasted rats as described in the Materials and methods section. The results are expressed as g/mol of creatinine, determined by a standard alkaline picrate method, and are means ± S.E.M. for three animals. *, Metabolite not detected.

Metabolite	Concn. (g/mol of creatinine)	
	Control	MCPG-treated
3-Hydroxyisobutyrate	0.2 ± 0.1	4.3 ± 3.2
3-Hydroxyisovalerate	0.3 ± 0.2	7.5 ± 2.3
2-Methyl-3-hydroxybutyrate	0*	8.4 ± 1.7
Malonate	1.0 ± 0.5	1.2 ± 0.2
Methylmalonate	1.3 ± 0.1	1.4 ± 0.3
Orthophosphate	2.6 ± 1.1	4.3 ± 0.8
Hexanoate	0*	2.0 ± 0.3
Hexanedioate	0*	4.0 ± 1.3
2-Methylbutyrylglycine	0*	5.0 ± 2.2
Isovalerylglycine	0*	5.0 ± 1.4
Heptanedioate	0*	4.0 ± 1.6
Octanedioate	0.42 ± 0.26	6.8 ± 3.2
Citrate	2.5 ± 1.4	3.0 ± 0.7
Nonanedioate	0*	2.8 ± 1.1
Decanedioate	0.8 ± 0.5	100 ± 26.4
Undecanedioate	0*	5.6 ± 2.4
Dodecanedioate	0*	23.1 ± 6.9
Hexadecanedioate	0*	8.6 ± 3.4

mitochondrial 3-oxoacyl-CoA and acetoacetyl-CoA thiolases. No inactivation of rat liver enoyl-CoA hydratases was found (Table 3). This result contrasts with the strong inhibition of purified pig kidney crotonase *in vitro* by MCPF-CoA [2,33]. The effects of MCPG metabolites on the activities of the different enoyl-CoA hydratases is puzzling. This may be due to the large differences in the kinetic properties of pig kidney crotonase, which are different from those of all other known mammalian crotonases [34]. Metabolites of several inhibitors of mitochondrial β-oxidation are known to inhibit mitochondrial thiolases: pent-4-enoate [35,36], pent-4-ynoate (H. S. A. Sherratt, unpublished work), mecaptoacetate [37], 2-bromo-octanoate [38] and 4-bromocrotonate [39]. Of these, pent-4-enoate, pent-4-ynoate and mercaptoacetate have been shown to be hypoglycaemic [35,36,40].

Formation of MCPF-CoA from methylenecyclopropylglyoxalate, and of MCPA-CoA from MCPP, in the mitochondrial matrix

Fig. 2 shows chromatograms of acid-soluble mitochondrial intermediates from a control animal (*a*), from an animal treated with MCPG (100 mg/kg body wt.) (*b*) and from control mitochondria incubated with 20 μM-methylenecyclopropylglyoxalate (*c*). None of the peaks had the typical absorption spectrum of a CoA ester, except peak 1, which had a relative retention time of 0.77 (*b*) or 0.78 (*c*) (MCPF-CoA), and of the internal standard hexanoyl-CoA (peak 2; relative retention time 1.00). The relative retention times of the standards under the conditions were: butyryl-CoA, 0.70; MCPF-CoA, 0.78; MCPA-CoA, 0.87; and hexanoyl-CoA, 1.00. The absence of other short-chain acyl-CoA esters is consistent with the inhibition of long-chain-fatty-acid oxidation at the stage of 3-oxoacyl-CoA thiolase. Treatment of extracts with 2 M-KOH for 60 min at 55 °C caused complete loss of all peaks identified as CoA esters (results not shown). This, taken together with the spectral and chromatographic data, strongly suggests that these peaks were CoA-esters.

Fig. 3 shows typical chromatograms for mitochondria from control and MCPP-treated animals. In extracts of mitochondria incubated with MCPP, an additional peak with a retention time of 58.80 min (relative retention time 0.87) with the typical spectrum of a CoA ester was detected, and it was concluded that this was MCPA-CoA. In addition, there was a high concentration of butyryl-CoA (retention time 42.53 min; 0.70), consistent with the known inhibition of butyryl-CoA dehydrogenase by MCPA-CoA [7,18].

The finding that MCPG has no effect on β-oxidation in isolated mitochondria, but that methylenecyclopropylglyoxalate is strongly inhibitory, also indicates that MCPG must first be converted into an active metabolite *in vivo*. Therefore it can be assumed that MCPG is metabolized in the cytosol to methylenecyclopropylglyoxalate, which is then oxidatively decarboxylated in the mitochondrial matrix to MCPF-CoA, by analogy with hypoglycin [8] (Scheme 1).

MCPF-CoA only differs chemically from MCPA-CoA by the absence of a methylene group. This may weaken binding to the active sites of medium-chain acyl-CoA and butyryl-CoA dehydrogenases compared with MCPA-CoA, so that α-proton abstraction leading to suicide inhibition is impaired [12]. The proximity of the carbonyl group of MCPF-CoA to the methylenecyclopropyl ring may activate the methylene group so that it can undergo nucleophilic attack by thiol groups of the thiolases required for catalytic activity with the formation of an inactive adduct.

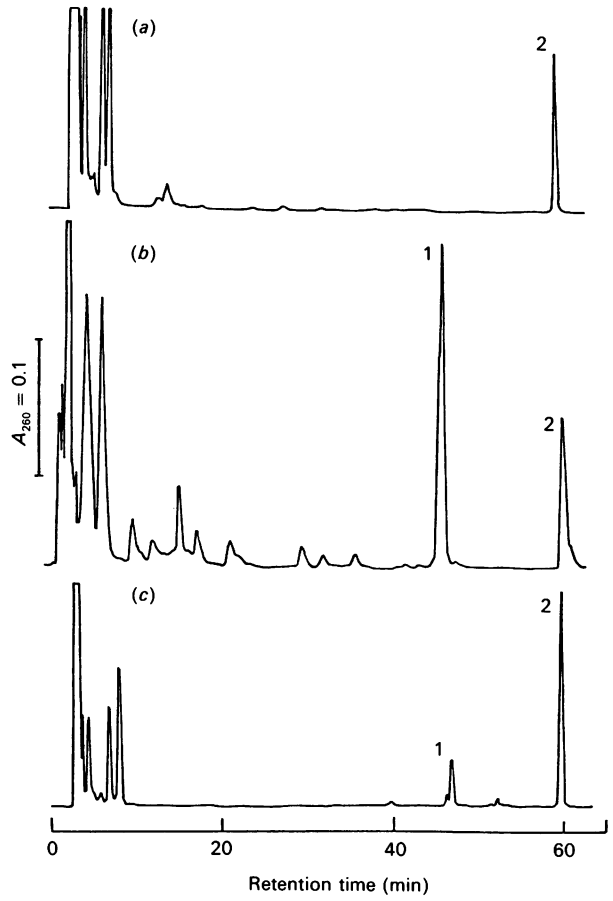


Fig. 2. H.p.l.c. analysis of acyl-CoA esters from extracts of (*a*) mitochondria from a control rat, (*b*) mitochondria from a MCPG-fed rat (100 mg/kg) and (*c*) mitochondria treated with 20 μM-methylenecyclopropylglyoxalate

Conditions of incubation and analysis were as described in the Materials and methods section. The relative retention times were as follows: (1) methylenecyclopropylformyl-CoA, 0.77; (2) hexanoyl-CoA (internal standard), 1.00.

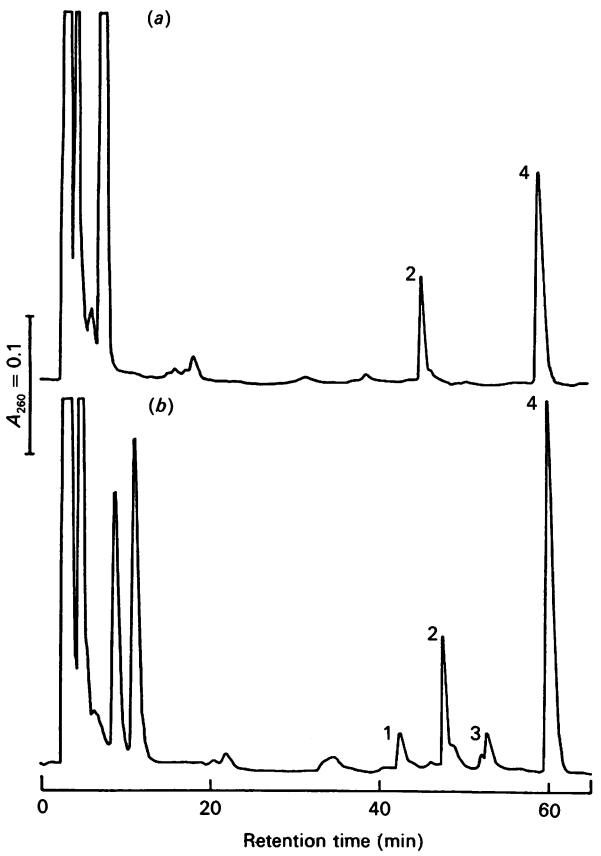


Fig. 3. H.p.l.c. analysis of acyl-CoA esters from extracts of (*a*) mitochondria from a control rat and (*b*) mitochondria from a control rat incubated with 20 μM-methylenecyclopropylpyruvate.

Conditions of incubation and analysis were as described in the Materials and methods section. The relative retention times were as follows: (1) butyryl-CoA, 0.70; (2) unidentified; (3) methylenecyclopropylacetyl-CoA; (4) hexanoyl-CoA (internal standard), 1.00.

Mechanism of the metabolic disturbances caused by MCPG

The profound hypoglycaemia caused by MCPG in fasted rats whose hepatic glycogen stores have been depleted (Table 1) can be attributed to inhibition of fatty acid oxidation. In the liver, inhibition of β-oxidation decreases gluconeogenesis by decreasing the supply of NADH, which is required for glucose synthesis from many precursors, and of acetyl-CoA, which allosterically activates pyruvate carboxylase necessary for glucose synthesis from pyruvate and other precursors which are first converted into pyruvate [41]. Any inhibition of β-oxidation in extrahepatic tissues might be expected to increase glucose utilization and contribute to hypoglycaemia [42]. However, in hypoglycin-treated rats, glucose disposal is actually decreased, but to a lesser extent than gluconeogenesis [43].

Inactivation of acetoacetyl-CoA thiolase in liver can explain the suppression of ketogenesis by MCPG (Table 1), since this enzyme is involved in the synthesis of acetoacetate from acetyl-CoA. Acetyl-CoA is formed by β-oxidation, and to a lesser extent by the oxidation of pyruvate and some amino acids. By contrast there is marked hyperketonaemia in hypoglycin-treated rats [11]. This has been attributed to an even greater inhibition of the extrahepatic oxidation of ketone bodies than of their formation by the liver [7,11]. Inhibition of MC-acyl-CoA and SC-acyl-CoA dehydrogenases, but not LC-acyl-CoA dehydrogenase, by hypoglycin metabolites inhibits the maximum rate of mitochondrial β-oxidation of long-chain fatty acids by about 50% and which then only proceeds as far as butyrate, with continuous recycling of CoA required for this limited oxidation [6–8,35,36].

The large increases in the concentrations of the branched-chain amino acids in plasma caused by MCPG can be attributed to inhibition of their degradation, probably at the stage of branched-chain-amino-acid transferase (Tables 1 and 2). There may also be some inhibition at the stage of branched-chain-acyl-CoA dehydrogenase (Table 2). This contrasts with the lack of such changes after administration of hypoglycin [26], which inhibits the degradation of branched-chain amino acids mainly at the stage of dehydrogenation of isovaleryl-CoA, 2-methylbutyryl-CoA or isobutyryl-CoA [16] (Scheme 1), but not at the level of the aminotransferases [44]. However, MCPG metabolites also inhibit isovaleryl-CoA dehydrogenase by 40% and 2-methylbutyryl-CoA dehydrogenase by 70% (Table 2).

Effects of MCPG on urinary organic acids

Few organic acids except benzoylglycine (hippuric acid) were detected in urine from the control rats. By contrast, there was a marked organic aciduria in MCPG-treated rats (Table 3). Some monocarboxylic acids were identified were 3-hydroxyisovaleric acid, 2-methyl-3-hydroxybutanoic acid and 3-hydroxybutanoic

acid. Dicarboxylic acids detected were: hexane-1,6-dioic acid, heptane-1,7-dioic acid, octane-1,8-dioic acid, nonane-1,9-dioic acid, undecane-1,11-dioic acid, dodecane-1,12-dioic acid and hexadecane-1,16-dioic acid. Isovalerylglycine, 2-methylbutyrylglycine and benzoylglycine were also identified.

Administration of MCPG to fasted rats causes a marked organic aciduria (Table 3). Long-chain fatty acids accumulate because of inhibition of β-oxidation (Scheme 1). These are oxidized to ω-dicarboxylic acids in the endoplasmic reticulum, where they are converted into their mono-CoA esters, which then undergo β-oxidation to shorter-chain dicarboxylic acids in the peroxisomes (presumably either the specific peroxisomal thiolase [45] is not inhibited or metabolites of MCPG are not formed in peroxisomes) [46,47]. Some free acids and glycine conjugates derived from the degradation of the branched-chain amino acids were also created (Table 3), corresponding to inhibition at the stage of the branched-chain-acyl-CoA dehydrogenases. Hypoglycin also causes a massive organic aciduria, but the pattern of metabolites differs from that caused by MCPG with large amounts of some unsaturated dicarboxylic acids and relatively more metabolites of branched-chain amino acids [16].

Conclusions

There has been little interest in the metabolic effects of MCPG since its discovery nearly 30 years ago [1], because it was assumed to have a similar mechanism of action to that of hypoglycin. However, the present investigation has revealed that, although there are some similarities, there are also marked differences. Both are powerfully hypoglycamic and appear to be converted into active metabolites by very similar pathways (Scheme 1). MCPG is strongly hypoketonaemic but, by contrast, hypoglycin is hyperketonaemic in fasted rats.

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REFERENCES

1. Grey, D. O. & Fowden, L. (1962) Biochem. J. **82**, 385–389
2. Melde, K., Buttner, H., Boschert, W., Wolf, H. P. O. & Ghisla, S. (1989) Biochem. J. **259**, 921–924
3. von Holt, C. & Leppla, W. (1956) Bull. Soc. Chim. Belg. **65**, 113–123
4. von Holt, C. (1966) Biochim. Biophys. Acta **125**, 1–10
5. von Holt, C., von Holt, M. & Bohm, H. (1966) Biochim. Biophys. Acta **125**, 11–21
6. Sherratt, H. S. A. & Osmundsen, H. (1976) Biochem. Pharmacol. **25**, 743–750
7. Osmundsen, H. & Sherratt, H. S. A. (1975) FEBS Lett. **55**, 38–41
8. Sherratt, H. S. A. (1986) Trends Pharmacol. Sci. **7**, 186–191
9. Tanaka, K. & Ikeda, Y. (1990) in Progress in Clinical and Biological Science (Tanaka, K. & Cotes, P. M., eds.), pp. 176–184, Alan R. Liss, New York
10. Ikeda, Y. & Tanaka, K. (1990) Biochim. Biophys. Acta **1058**, 167–184
11. Williamson, D. H. & Wilson, M. B. (1965) Biochem. J. **94**, 19c–21c
12. Wenz, A., Thorpe, C. & Ghisla, S. (1981) J. Biol. Chem. **256**, 9809–9812
13. Ghisla, S., Melde, K., Zeller, H.-D. & Boschert, W. (1990) Prog. Clin. Biol. Sci. **321**, 185–192
14. Zeller, H. D. & Ghisla, S. (1984) in Flavins and Flavoproteins (Bray, R. C., Engel, P. C. & Mayhew, S. G., eds.), pp. 447–450, Walter de Gruyer, Berlin and New York
15. Zeller, H. D. & Ghisla, S. (1987) in Flavins and Flavoproteins (Edmondson, D. E. & McCormick, D. B., eds.), pp. 161–164, Walter de Gruyer, Berlin and New York
16. Hine, D. G. & Tanaka, K. (1984) Biomed. Mass Spectrom. **11**, 332–339
17. Veitch, R. K., Sherratt, H. S. A. & Bartlett, K. (1987) Biochem. J. **246**, 775–778
18. Van Hoof, F., Hue, L., Vamecq, J. & Sherratt, H. S. A. (1985) Biochem. J. **229**, 387–397
19. Yardley, H. & Godfrey, G. (1967) Arch. Dermatol. **96**, 98–93
20. Boschert, W. (1989) Ph.D. Thesis, University of Konstanz
21. El-Fakhri, M. & Middleton, B. (1982) Biochim. Biophys. Acta **713**, 270–279
22. Waterson, T. M. & Conway, R. S. (1981) Methods Enzymol. **71**, 421–430
23. Rüdiger, H. W., Langenbeck, U. & Goedde, H. W. (1972) Biochem. J. **126**, 445–446
24. Holland, P. C. & Sherratt, H. S. A. (1973) Biochem. J. **136**, 157–171
25. Lloyd, B., Burrin, J., Smyth, P. & Alberti, K. G. M. M. (1978) Clin. Chim. Acta **24**, 1724–1729
26. Goodman, S. J. & Vale, D. (1984) Biochem. Med. **31**, 97–103
27. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
28. Lynen, F. & Ochoa, S. (1953) Biochim. Biophys. Acta **12**, 299–311
29. Schulz, H. & Staack, H. (1981) Methods Enzymol. **71**, 398–403
30. Kido, R. (1988) Methods Enzymol. **166**, 274–281
31. Watmough, N. J., Turnbull, D. M., Sherratt, H. S. A. & Bartlett, K. (1989) Biochem. J. **262**, 261–269
32. Causey, A. G., Middleton, B. & Bartlett, K. (1986) Biochem. J. **235**, 343–350
33. Schulz, H. & Fong, J. M. (1981) Methods Enzymol. **72**, 604–610
34. Büttner, H. (1989) Ph.D. Thesis, University of Konstanz
35. Billington, D., Osmundsen, H. & Sherratt, H. S. A. (1978) Biochem. Pharmacol **27**, 2879–2890
36. Billington, D., Osmundsen, H. & Sherratt, H. S. A. (1978) Biochem. Pharmacol. **27**, 2891–2900
37. Veitch, R. K., Sherratt, H. S. A., Causey, A. G., Bartlett, K. & Middleton, B. (1986) Biochem. Soc. Trans. **14**, 703–704
38. Raaka, B. M. & Lowenstein, J. M. (1981) Methods Enzymol. **72**, 559–577
39. Olowe, Y. & Schultz, H. (1982) J. Biol. Chem. **257**, 5408–5413
40. Brendel, K. (1969) Proc. Int. Congr. Pharmacol. 4th, Basle, Switzerland, 508
41. Sherratt, H. S. A. (1981) in Short-term Regulation of Liver Metabolism (Hue, L. & van der Werve, G., eds.), pp. 199–227, Elsevier, Amsterdam
42. Randle, P. J., Hales, C. N., Garland, P. B. & Newsholme, E. A. (1963) Lancet **i**, 785–789
43. Osmundsen, H., Billington, D., Taylor, J. R. & Sherratt, H. S. A. (1978) Biochem. J. **170**, 337–342
44. Patrick, S. J. (1963) Can. J. Biochem. Physiol. **41**, 1163–1167
45. Mizazwa, S., Osmi, T. & Hasimoto, T. (1980) Eur. J. Biochem. **103**, 589–596
46. Vamecq, J. & Draye, J.-P. (1989) Essays Biochem. **24**, 115–225
47. Pourfarzam, M. & Bartlett, K. (1990) Biochem. J. **273**, 205–210