Metabolic consequences of methylenecyclopropylglycine poisoning in rats

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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 (MCP) *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]. MCPG 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; MCP, methylenecyclopropylpyruvate; LC-, long-chain-specific; MC-, medium-chain-specific; SC-, short-chain-specific.
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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 μ M-ADP, in mitochondria preincubated for 3 min with 10 μ M-methylenecyclopropylglyoxalate, 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 μ M-decanoylcarnitine was detected in coupled or uncoupled liver mitochondria from MCPG-treated rats, although 10 μ M-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 μ M-methylenecyclopropylglyoxalate. Similar results were obtained when 10 μ M-palmitoylcarnitine was used as substrate (results not shown). There was no inhibition of the oxidation of 10 μ M-decanoylcarnitine in normal mitochondria that had been incubated for 3 min with 20 μ M-MCPG, or with 20 μ M-MCPG-CoA plus 0.5 mM-carnitine, indicating that MCPG-CoA cannot cross the mitochondrial inner membrane directly and that it is not a substrate for the carnitine palmitoyl-transferases.

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. 100 000-fold indicates that the inhibition was irreversible or that MCPG-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 $\pm$ 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 $\pm$ 0.93	1.90 $\pm$ 0.42***
Lactate	mM	2.01 $\pm$ 0.33	3.44 $\pm$ 0.18**
Pyruvate	mM	0.10 $\pm$ 0.02	0.38 $\pm$ 0.06**
3-Hydroxybutyrate	mM	0.35 $\pm$ 0.06	< 0.01***
Acetoacetate	mM	0.22 $\pm$ 0.04	< 0.01***
Glutamate	μ M	838 $\pm$ 211	422 $\pm$ 165
Glycine	μ M	667 $\pm$ 108	427 $\pm$ 73
Alanine	μ M	350 $\pm$ 93	530 $\pm$ 106
Valine	μ M	133 $\pm$ 25	694 $\pm$ 119***
Leucine	μ M	141 $\pm$ 31	717 $\pm$ 146***
Isoleucine	μ M	76 $\pm$ 18	414 $\pm$ 146*
Lysine	μ M	300 $\pm$ 126	607 $\pm$ 110
Cysteine	μ M	18 $\pm$ 12	65 $\pm$ 26
Ornithine	μ M	53 $\pm$ 18	209 $\pm$ 69
Histidine	μ M	70 $\pm$ 33	220 $\pm$ 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 μ M-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 $\pm$ 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 $\pm$ 0.9	10.9 $\pm$ 0.7**	26
MC-acyl-CoA dehydrogenase	12.4 $\pm$ 2.4	11.6 $\pm$ 2.7	7
LC-acyl-CoA dehydrogenase	8.6 $\pm$ 0.3	7.9 $\pm$ 0.7	9
SC-enoyl-CoA hydratase	15 570 $\pm$ 1800	14 000 $\pm$ 1500	11
LC-enoyl-CoA hydratase	1560 $\pm$ 280	1255 $\pm$ 190	10
SC-3-hydroxyacyl-CoA dehydrogenase	1219 $\pm$ 215	730 $\pm$ 45**	40
LC-3-hydroxyacyl-CoA dehydrogenase	520 $\pm$ 20	380 $\pm$ 79***	27
Acetoacetyl-CoA thiolase	390 $\pm$ 55	100 $\pm$ 18***	75
3-Oxoacyl-CoA thiolase	265 $\pm$ 34	20 $\pm$ 6***	93
Isovaleryl-CoA dehydrogenase	1.4 $\pm$ 0.24	0.82 $\pm$ 0.12**	41
2-Methylbutyryl-CoA dehydrogenase	0.64 $\pm$ 0.10	0.19 $\pm$ 0.06***	80
Branched-chain aminotransferase	0.59 $\pm$ 0.08	0.33 $\pm$ 0.11***	45

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 MCPG-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), mercaptoacetate [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 MCPG-CoA from methylenecyclopropylglyoxalate, and of MCPA-CoA from MCPG, 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) (MCPG-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; MCPG-CoA, 0.78; MCPA-CoA, 0.87; and

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 $\pm$ S.E.M. for three animals. *, Metabolite not detected.

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

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 MCPG-treated animals. In extracts of mitochondria incubated with MCPG, 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 MCPG-CoA, by analogy with hypoglycin [8] (Scheme 1).

MCPG-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 MCPG-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 μ M-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.

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

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 μ M-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.

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. Isovaleryl-glycine, 2-methylbutyryl-glycine and benzoyl-glycine 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 hypoglycemic 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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