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STUDIES ON THE HYPOGLYCAEMIC COMPOUND CYCLOPROPANECARBOXYLIC ACID

EFFECTS ON GLUCONEOGENESIS *IN VITRO*

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Abstract—The effects of the species specific hypoglycaemic agent, cyclopropanecarboxylic acid, on gluconeogenesis in rat and guinea-pig kidney slices were investigated. Cyclopropanecarboxylate (0·1 mM) inhibited glucose production from pyruvate by 86 per cent in guinea-pig but by only 22 per cent in rat. ATP levels in guinea-pig kidney slices were decreased by cyclopropanecarboxylate, but by an amount insufficient to account totally for the inhibition of gluconeogenesis. The site of inhibition of gluconeogenesis in guinea-pig kidney cortex slices was found to be located at the level of fructose-1,6-diphosphatase. Cyclopropanecarboxylate had no effect on partially purified FDPase and no detectable effect on gluconeogenesis in kidney homogenates. The mechanism of the cyclopropanecarboxylate inhibition of gluconeogenesis is discussed, together with its possible hypoglycaemic action.

THE EFFECTS of cyclopropanecarboxylic acid, an orally active hypoglycaemic agent in some species, on fatty acid oxidation and ketogenesis were reported in the preceding paper.[1] We found that the oxidation of long chain fatty acids is inhibited by cyclopropanecarboxylate in both rat and guinea-pig liver mitochondria. This finding could partially explain the hypoglycaemic effect in sensitive species (e.g. guinea-pig) but not the lack of effect in other species (e.g. rat). Holt, Holt and Böhm[2] and Patrick[3] have suggested that the action of the hypoglycaemic compound hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid) is partly due to the inhibition of gluconeogenesis. Senior and Sherratt[4] reported that cyclopropanecarboxylate (1 mM) had only a minimal inhibitory effect on glucose formation from pyruvate in kidney slices of the insensitive species, rat. They did not examine the effect in guinea-pigs.

We now report the effects and possible mechanisms of action of cyclopropanecarboxylate on gluconeogenesis in rat and guinea-pig kidney and liver slices. In the light of these results and those previously reported,[1] the mechanism of the hypoglycaemic action of cyclopropanecarboxylate is discussed.

MATERIALS AND METHODS

Reagents. Dithiothreitol and glycerol were obtained from British Drug Houses Ltd., Poole, Dorset; gluconeogenic substrates and glucose oxidase kits were purchased from Boehringer, Mannheim. AMP, ATP and desiccated whole fireflies for ATP determinations were obtained from Sigma Chemical Co., London. The preparation of sodium cyclopropanecarboxylate and its carnitine ester was as described in the preceding paper.[1]

Measurement of gluconeogenesis. The preparation of tissue slices from animals fasted for 24 hr to deplete tissue glycogen levels and the determinations of glucose were carried out by the methods of Krebs, Bennett, de Gasquet, Gascoyne and Yoshida.[5] All gluconeogenic substrates were at a concentration of 10 mM and incubations at 37° were for a period of 2 hr. When gluconeogenesis was to be determined in kidney homogenates, cortex slices were homogenized in Krebs–Ringer bicarbonate medium[6] but containing half the usual bicarbonate concentration, followed by centrifugation at 600 *g* for 10 min to give a nuclei-free supernatant. Of this fraction, 0·5 ml (approx. 50 mg wet wt. tissue) was added to 4 ml of the gluconeogenic incubation medium and incubations were carried out in the normal way.

Statistical analysis. The P values for the significance of the difference between means were calculated, where applicable, by the Student's *t*-test using a computer program.

Determinations of tissue ATP levels. The pre-weighed slices were removed rapidly from the incubation medium, placed in liquid nitrogen and ground in a precooled pestle and mortar. ATP was determined by the luciferin–luciferase method as modified by Stanley and Williams.[7]

Determination of fructose-1,6-diphosphatase. (a) *With the partially purified enzyme.* Crude fructose-1,6-diphosphatase (EC 3.1.3.11) was prepared from guinea-pig liver and kidney by the method of Gomori.[8] Of this enzyme fraction 0·2 ml was incubated for 1 hr at 37° in 2·0 ml of the standard incubation medium. This contained: tris–HCl buffer, 0·05 M, pH 9·1; FDP,* 4 mM; $MgSO_4$, 10 mM; dithiothreitol, 1·0 mM. The reaction was stopped by the addition of 2·0 ml of 0·35 N $HClO_4$ and the inorganic phosphate released was determined on 1 ml of the supernatant (after centrifugation) by the method of Fiske and Subbarow.[9]

(b) *In tissue homogenates.* Tissue homogenate (1 ml approx. 100 mg wet weight of tissue) was incubated for 1 hr at 30° with 2·0 ml of medium containing 1·0 mM FDP; 0·05 M Tris buffer, pH 9·0, 5 mM $MnCl_2$, 1·0 mM mercapto-ethanol. The reaction was stopped and the inorganic phosphate was determined as above.

(c) *In kidney cortex slices.* Kidney cortex slices (approx. 100 mg wet weight of tissue) were incubated for 2 hr at 37° in 4 ml of the usual gluconeogenic incubation medium[5] containing 10 mM FDP. Two ml were deproteinized with 0·2 ml of 3 N perchloric acid and the inorganic phosphate released was determined as above.

RESULTS

The effect of cyclopropanecarboxylate on gluconeogenesis. The results shown in Table 1 demonstrate that cyclopropanecarboxylate at a concentration of 0·1 mM inhibited renal gluconeogenesis from pyruvate by 86 per cent in guinea-pigs and by only 22 per cent in rats. In other experiments there was still only a 40 per cent inhibition in rats when the concentration was raised to 5·0 mM, compared with total inhibition at this concentration in guinea-pigs, thus demonstrating a considerable quantitative species difference. Results using various gluconeogenic substrates suggested that the site of action of cyclopropanecarboxylate in guinea-pig kidney slices was located at the level of fructose-1,6-diphosphatase (FDPase) as there was no

*Abbreviations used: FDP, fructose-1,6-diphosphate; FDPase, fructose-1,6-diphosphatase; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; DNP, dinitrophenol; PFK, phosphofructokinase; P_i, inorganic phosphate.

TABLE 1. THE EFFECT OF CYCLOPROPANECARBOXYLATE ON GLUCONEOGENESIS IN KIDNEY CORTEX SLICES

Species	Substrates	Glucose production	
		Control (μmoles/g dry wt./2 hr)	Cyclopropanecarboxylate (% of control)
Rat	Pyruvate	379 ± 21	78 ± 9*
	Oxaloacetate	98 ± 6	76 ± 6
	FDP	142 ± 13	118 ± 27†
	F-6-P	415 ± 83	87 ± 8†
Guinea-pig	Pyruvate	109 ± 18	14 ± 6‡
	Oxaloacetate	97 ± 15	18 ± 3‡
	Glycerol	23 ± 3	52 ± 4
	FDP	69 ± 16	11 ± 8‡
	F-6-P	457 ± 81	96 ± 8†
	G-6-P	518 ± 71	79 ± 7*

* $P = 0{\cdot}05$. † P is not significant. ‡ $0{\cdot}01 > P > 0{\cdot}001$.

Experimental details were as given in the Methods section. All gluconeogenic substrates were 10 mM and cyclopropanecarboxylate was 0·1 mM. The results are the means ± S.E.M. of two to six experiments. The endogenous rate of glucose production was always less than 6 per cent of the pyruvate controls.

inhibition when the product of this enzyme, F-6-P, was used as the glucose precursor. It is unlikely that the inhibition when using FDP was due to an effect by cyclopropanecarboxylate on the permeability of the tissue, since in two experiments using [^{14}C]FDP there was no significant difference from the control on the incorporation of radioactivity into the kidney slices. It is also unlikely that the apparent decrease in glucose synthesis was in fact due to an increase in glucose utilization in the presence of the hypoglycaemic agent. In two other experiments 0·2 μmole of [U-^{14}C]glucose were added either to samples containing FDP (10 mM), incubation time 1 hr, or samples previously incubated for 1 hr with FDP, additional incubation time 1 hr. At the end of the incubation period, both the $^{14}CO_2$ evolution and the incorporation of radioactivity into the kidney slices were determined. These experiments indicated that the maximum amount of glucose utilization was 1·5 and 1·6 μmoles/hr/g dry weight of kidney for control and cyclopropanecarboxylate samples respectively.

There was also a similar degree of inhibition of glucose synthesis from FDP (70 per cent) when using guinea-pig liver slices.

The addition of DL-carnitine (0·1 mM) did not overcome the cyclopropanecarboxylate inhibition of gluconeogenesis but when cyclopropanecarboxyl carnitine was used there was no effect on glucose production (Table 2).

When guinea-pig kidney cortex slices were pre-incubated for 30 min before transferring to medium containing FDP, there was again the same degree of inhibition, no matter at which stage the cyclopropanecarboxylate was added (Table 3). In one experiment, using [1-^{14}C]cyclopropanecarboxylate in the pre-incubation medium, it was found that about 10 per cent of the radioactivity was taken up by the tissue, 50 per cent of which remained during the subsequent 2 hr incubation period. It would therefore appear that less than 0·4 μmole of intracellular cyclopropanecarboxylate are required to inhibit the production of at least 30 μmoles of glucose from FDP.

TABLE 2. THE EFFECT OF CYCLOPROPANECARBOXYLATE AND CARNITINE ON GLUCONEOGENESIS FROM PYRUVATE IN GUINEA-PIG KIDNEY CORTEX SLICES

Addition	% of control	P
Cyclopropanecarboxylate (0·002 mM)	112 ± 10	—
Cyclopropanecarboxylate (0·02 mM)	22 ± 6	—
Cyclopropanecarboxylate (0·1 mM)	14 ± 6	0·001
Cyclopropanecarboxylate (0·5 mM)	9 ± 2	0·001
Cyclopropanecarboxylate (5·0 mM)	0	0·001
Cyclopropanecarboxyl carnitine (0·1 mM)	92 ± 3	N.S.*
Carnitine (0·1 mM)	115 ± 10	—
Cyclopropanecarboxylate (0·1 mM) + carnitine (0·1 mM)	30 ± 2	—
DNP (0·12 mM)	47 ± 3	0·01

* N.S. = not significant.

Experimental details were as given in the Methods section. Results are expressed as the percentage glucose production of control slices ± S.E.M. for two to six experiments.

As found by other workers,[10] the gluconeogenic capacity of kidney homogenates was much lower than that of slices. Surprisingly, cyclopropanecarboxylate had no effect on glucose production from FDP in this system, the values, expressed as μmoles/g wet weight, being 2·5 ± 0·1 (control) and 2·4 ± 0·1 (treated).

The effect of cyclopropanecarboxylate on FDPase activity. As can be seen from Table 4, cyclopropanecarboxylate at the normal gluconeogenic inhibitory concentration had no effect on the activity of isolated guinea-pig liver or kidney FDPase. That the crude enzyme was active and specific for FDP was evident since the addition of either AMP (a non-competitive inhibitor of FDPase[11,12]) or KCN or the omission of Mg^{2+} (a known activator of FDPase[13]) from the incubation medium decreased the enzyme activity and the amount of inorganic phosphate released was low when either G-6-P or F-6-P replaced FDP. There was again no inhibition when FDPase activity was measured in kidney cortex homogenates (two experiments—data not shown).

TABLE 3. THE EFFECT OF CYCLOPROPANECARBOXYLATE ON GLUCONEOGENESIS FROM FDP IN PRE-INCUBATED GUINEA-PIG KIDNEY CORTEX SLICES

Pre-incubation medium	Addition	Glucose μmoles/g dry wt./2 hr ± S.E.M.
A	None (control)	30 ± 2
A	Cyclopropanecarboxylate (0·1 mM)	6 ± 1
B	None	7 ± 1
A	None (control)	51 ± 3
B*	None	15 ± 1

* Tissue pre-incubated with [1-^{14}C]cyclopropanecarboxylate.

The tissue was incubated for 30 min in medium gassed with O_2/CO_2 (95:5) prior to transference to medium containing FDP (10 mM). The pre-incubation medium contained A, water; or B, cyclopropanecarboxylate (0·1 mM). Determinations were carried out in triplicate.

TABLE 4. THE EFFECT OF CYCLOPROPANECARBOXYLATE ON THE ACTIVITY OF PARTIALLY PURIFIED GUINEA-PIG FRUCTOSE-1,6-DIPHOSPHATASE

Tissue	Addition	P_i released
Liver	None (control)	1·22 ± 0·02
	KCN (0·01 M)	0·90 ± 0·00
	Cyclopropanecarboxylate (0·2 mM)	1·21 ± 0·02
Kidney	None (control)	1·12 ± 0·01
	KCN (0·01 M)	0·95 ± 0·01
	Cyclopropanecarboxylate (0·2 mM)	1·15 ± 0·00
Kidney	None (control)	0·54 ± 0·01
	Cyclopropanecarboxylate (0·1 mM)	0·54 ± 0·00
	AMP (2·0 mM)	0·34 ± 0·00
	AMP (2·0 mM) + Cyclopropanecarboxylate (0·1 mM)	0·33 ± 0·00
Liver	None (control)	1·23 ± 0·00
	Mg^{2+} omitted	0·70 ± 0·05
	Mg^{2+} omitted + cyclopropanecarboxylate (0·1 mM)	0·65 ± 0·03
	G-6-P (4 mM)—No FDP	0·15 ± 0·03
	F-6-P (4 mM)—No FDP	0·12 ± 0·00

Experimental details were as given in the Methods section. Enzyme activity is expressed as μmoles of inorganic phosphate released per ml of deproteinized supernatant ± S.E.M. Determinations were carried out in triplicate.

TABLE 5. THE EFFECT OF CYCLOPROPANECARBOXYLATE ON FRUCTOSE-1,6-DIPHOSPHATASE ACTIVITY IN GUINEA-PIG KIDNEY CORTEX SLICES

Addition	P_i released
None (control)	59·5 ± 5·8
Mg^{2+} omitted	47·4 ± 12·3
Cyclopropanecarboxylate (0·1 mM)	29·9 ± 4·2
None (control)	101·0 ± 19·4
Cyclopropanecarboxylate (0·1 mM)	94·3 ± 0·8
None (control)	55·2 ± 3·1
Cyclopropanecarboxylate (0·1 mM)	45·2 ± 1·2
Phenformin (0·4 mM)	48·0 ± 5·1

Experimental details were as given in the Methods section. Enzyme activity is expressed as μmoles of inorganic phosphate released per gram wet weight of tissue above endogenous levels ± S.E.M., and determinations were carried out in triplicate.

When FDPase activity was measured in guinea-pig kidney slices (Table 5), cyclopropanecarboxylate slightly inhibited the release of inorganic phosphate from FDP. However, neither the omission from the incubation medium of Mg^{2+}, nor the addition of phenformin (phenethyldiguanide), a hypoglycaemic agent shown by Patrick[3] to inhibit gluconeogenesis from FDP in rat kidney slices, had any significant effect on the release of inorganic phosphate. Since neither the contribution of other phosphatases nor the direction in which FDP is metabolized was determined, the results from this type of experiment cannot be of great significance. However, from the determinations of FDPase activity in all three systems, it can be concluded that it is unlikely that the inhibition of gluconeogenesis is due to a direct effect of cyclopropanecarboxylate on an active or allosteric site of fructose-1,6-diphosphatase.

The effect of cyclopropanecarboxylate on ATP levels in guinea-pig kidney cortex slices. Cyclopropanecarboxylate had a small but significant effect on ATP levels in guinea-pig cortex slices (Table 6). It is unlikely that these decreased levels would totally account for the inhibition of gluconeogenesis since DNP (0·12 mM) greatly reduced the ATP concentration but only inhibited glucose synthesis by about 50 per cent (Table 2).

TABLE 6. THE EFFECT OF CYCLOPROPANECARBOXYLATE ON ATP LEVELS IN GUINEA-PIG KIDNEY CORTEX SLICES

Control	Cyclopropanecarboxylate (0·1 mM)	DNP (0·12 mM)
102 ± 9	67 ± 5	15 ± 6
63 ± 7	45 ± 8	8 ± 2
84 ± 1	68 ± 1	—

Experimental details were as given in the Methods section. ATP levels are expressed as nmoles/g wet wt. ± S.E.M. Determinations were carried out in triplicate with tissue from three animals.

DISCUSSION

The present results show that renal and hepatic gluconeogenesis in guinea-pig tissues is strongly inhibited by cyclopropanecarboxylate *in vitro*, and that this inhibition is apparently at the level of FDPase but not due to a direct effect on the enzyme. In rat tissues the inhibition of gluconeogenesis was very much smaller.

Some other hypoglycaemic compounds (e.g. pent-4-enoic acid, hypoglycin, phenformin, 5-methoxyindole-2-carboxylic acid) inhibit gluconeogenesis in rats.[4,14,15] It has been suggested that this is due to a lack of ATP, and this could explain the small inhibition that we find in rat tissues, since cyclopropanecarboxylate inhibits the oxidation of tricarboxylic acid cycle intermediates and strongly inhibits the oxidation of palmitic acid,[1] thus leading to the accumulation of long chain fatty acids and the uncoupling of oxidative phosphorylation. The inhibition of fatty acid oxidation would also lead to a decrease in the level of acetyl CoA, which is necessary for the activation of pyruvate carboxylase (EC 6.4.1.1).[16]

Although cyclopropanecarboxylate significantly lowers ATP levels in guinea-pig kidney slices it is unlikely that this factor alone would explain the inhibition of glucose

production for several reasons. First, dinitrophenol decreases ATP levels to a greater extent than does cyclopropanecarboxylate, but it only inhibits gluconeogenesis by about 50 per cent; secondly, there is a lack of inhibition at the ATP-requiring reactions of the gluconeogenic pathway and thirdly, the inhibition by AMP of the partially purified FDPase does not seem to be sufficient to explain the decreased rate of glucose synthesis caused by cyclopropanecarboxylate.

The hypoglycaemic agent has no direct effect on isolated FDPase activity, on inorganic phosphate release from FDPase in kidney slices or homogenates, or on gluconeogenesis in tissue homogenates. However the isolation of FDPase and the preparation of homogenates involve disruption of the tissue: the compartmentation essential for gluconeogenesis is then largely lost[5] and the loss of cyclopropanecarboxylate inhibition may be due to the loss of structural requirements for FDPase activity. (As mentioned in the Results section, the lack of effect on FDPase activity in kidney slices is of doubtful significance.) The association of rat FDPase with a network of extremely fine filamentous material in the cytoplasmic matrix has recently been demonstrated.[17] We found that only a small concentration of cyclopropanecarboxylate is required for inhibition and once the compound has entered the cell it is apparently tightly bound. It is thus possible that in intact cells an interaction between the enzyme, the inhibitor and the filamentous material leads to inhibition of gluconeogenesis by blocking the normal substrate binding site. In this connection it is of interest that cyclopropanecarboxylcarnitine does not inhibit glucose production, possibly because of its inability to bind at the same site. If the inhibitory effect is due to such blocking then the species difference would require some difference in the filamentous material or in the FDPase structure of guinea-pigs and rats. The inhibition of glucose production from FDP could also be due to the effect of cyclopropanecarboxylate on other enzymes, resulting in the metabolism of FDP via a non-glucogenic pathway, or by preventing the release of F-6-P from the active site of FDPase.

The catalytic properties of FDPase are very complex[13] and it has recently been reported[18] that the enzyme isolated from rabbit liver and kidney is activated by CoA. It would be of interest to find whether an interaction between CoA and cyclopropanecarboxylate, of importance in the inhibition of long-chain fatty acid oxidation,[1] also has an effect on the metabolism of FDP. Unfortunately this type of experiment would involve the disruption of tissue and the loss of the inhibitory effect.

It appears then that cyclopropanecarboxylate may inhibit gluconeogenesis in guinea-pigs by alteration of adenine nucleotide levels and by interaction with the normal substrate–enzyme complex at the site of FDPase. Both these effects would tend to increase FDP levels, which at concentrations greater than 0·1 mM markedly inhibit FDPase activity.[13]

Our results on the inhibition of oxidation of long-chain fatty acids in liver mitochondria[1] could not alone explain the difference in hypoglycaemic activity of cyclopropanecarboxylate in rats and guinea-pigs.[19] This difference is, however, well correlated with the difference in the effect on gluconeogenesis reported in this paper. It is now well established that the direction of carbohydrate metabolism, glycolysis or gluconeogenesis is partially regulated at the level of phosphofructokinase (EC 2.7.1.11) and FDPase. During starvation and acute alloxan diabetes the concentration of F-6-P falls by about 50 per cent,[20] thereby reducing PFK activity, and the recycling which exists between FDP and F-6-P[21] may act to stimulate gluconeogenesis. Since

cyclopropanecarboxylate almost totally inhibits renal and hepatic glucose synthesis in guinea-pigs and partially inhibits long chain fatty acid oxidation, then once glycogen levels have been depleted, extracellular glucose is the only major fuel remaining and its cellular uptake is enhanced. In adipose tissue cyclopropanecarboxylate increased the oxidation of [^{14}C]glucose[19,22] but in this tissue the situation must be different as gluconeogenesis does not occur.[23] It is of interest that during starvation and alloxan diabetes the levels of the major glycolytic and gluconeogenic intermediates vary to similar degrees in liver and adipose tissue, except for citrate, which is decreased in the liver and increased in adipose tissue.[24] It is therefore possible that it is the combined influence on fatty acid metabolism of both cyclopropanecarboxylate[25] and citrate that is partly responsible for the increased glucose oxidation in adipose tissue. Also, since it has been reported that cyclopropanecarboxylate produces a marked synergistic action with injected insulin,[19] it is possible that this is due to an effect by the former hypoglycaemic agent on renal and hepatic metabolism and an effect by insulin and cyclopropanecarboxylate on adipose tissue and possibly other peripheral tissues.

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