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CHARACTERIZATION OF OXALATE AS A CATABOLITE OF DICHLOROACETATE RESPONSIBLE FOR THE INHIBITION OF GLUCONEOGENESIS AND PYRUVATE CARBOXYLATION IN RAT LIVER CELLS

France DEMAUGRE, Claude CEPANEC and Jean-Paul LEROUX

Laboratoire de Biochimie Médicale (INSERM U 75) - Faculté de Médecine Necker-Enfants Malades, 156. rue de Vaugirard 75730 PARIS CEDEX 15, France

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SUMMARY

In isolated hepatocytes, dichloroacetate decreased glucose synthesis from lactate, pyruvate and alanine, but not from substrates which bypass pyruvate carboxylase (propionate, glycerol). It was also found to inhibit pyruvate carboxylation in isolated mitochondria, but only after a preincubation period, and had no effect on partially purified pyruvate carboxylase. Hepatocytes and liver mitochondria metabolized [^{14}C] dichloroacetate to oxalate which inhibits pyruvate carboxylase and mimics, without preincubation, the effects of dichloroacetate in mitochondrial pyruvate carboxylation. Thus, oxalate appears to be responsible for the inhibition of gluconeogenesis by dichloroacetate at the level of pyruvate carboxylation.

INTRODUCTION

Dichloroacetate decreases the blood glucose of diabetic or fasted rats (1). This action has been ascribed to an activation of muscle pyruvate dehydrogenase (E.C. 1.2.4.1)(2) which results in a local overconsumption, then a decreased peripheral release of gluconeogenic precursors (1). However, direct effects of dichloroacetate on liver metabolism have been described, especially an inhibition of gluconeogenesis (3,4,5,6) also demonstrated in rat kidney (7). Indirect evidence suggested that inhibition of gluconeogenesis by dichloroacetate was localized at the pyruvate carboxylase (E.C. 6.4.1.1) step in rat liver (6,8) as well as kidney (7). As also postulated by Whitehouse et al (9), Harris et al (10) and Crabb (11), dichloroacetate might be hydrolyzed to glyoxylate, then oxidized to oxalate which is a powerful inhibitor of pyruvate carboxylase (12). The aim of this work was to give experimental evidence for the transformation of dichloroacetate to oxalate in liver cells and show that oxalate may be responsible for the inhibitory effects of dichloroacetate on gluconeogenesis and pyruvate carboxylation.

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MATERIAL AND METHODS

Glucose synthesis was measured with or without dichloroacetate in hepatocytes isolated from 24 h starved rats and incubated as described previously (6). The effects of dichloroacetate on pyruvate carboxylation and decarboxylation were studied in mitochondria isolated from fed or fasted rats and incubated as described by Adam and Haynes (13). Mitochondria showed a respiratory control rate of about 4.5 with 3 mM succinate as substrate. Mitochondrial protein was assayed by the biuret method (14). Pyruvate carboxylase was partially purified from freeze-dried rat liver mitochondria by ammonium sulphate precipitation (30 to 40 percent of saturation), and its activity measured by the method of Ballard and Hanson (15) with slight modifications (16). For measurements of oxalate formation, hepatocytes isolated from fed rats were incubated at 37°C as described in (6) with 1 mM [U-^{14}C] dichloroacetate (New England Nuclear, Specific activity : 5700 dpm/nmol). No exogenous substrate was added. At the end of the incubation, the cell suspension was heated at 100°C for 3 min and centrifuged, and [^{14}C] oxalate was measured on aliquots of the supernatant. Mitochondria were incubated at 37°C as described in (13), but without substrate, in the presence of 1.5 mM [U-^{14}C] dichloroacetate. Reaction was stopped as described above. [^{14}C] oxalate was assayed by measurement of [$^{14}CO_2$] evolved owing to oxalate decarboxylase (E.C. 4.1.1.2)(17,18). 0.5 ml of boiled supernatant and 0.05 U of oxalate decarboxylase from Collybia velutipes (Sigma, Grade II) were added to 0,5 ml of 0.52 M citrate buffer, pH 3.0, in the main cavity of Warburg flasks. [$^{14}CO_2$] was trapped by a filter paper moistened with 0.15 ml of 2 M NaOH placed in the centerwell. The reaction was allowed to proceed for 8 hours at room temperature. Then the paper was removed, dried, and counted for radioactivity in 10 ml of toluene - PPO (4 g/l) POPOP (0.2 g/l). A blank containing all reactants except oxalate decarboxylase was simultaneously processed. 10 nmoles of [U-^{14}C] oxalate (Commissariat à l'Energie Atomique, Specific activity : 22 000 dpm/nmol) were added as internal standard to one ml of the hepatocyte or mitochondrial suspension processed as previously described : recovery was usually better than 90 percent.

RESULTS AND DISCUSSION

Dichloroacetate significantly decreases gluconeogenesis of fasted rat hepatocytes from lactate, pyruvate and alanine, but not from glycerol (5,6). Moreover, it has no effect on glucose synthesis from 10 mM propionate (140 $\pm$ 15 nmol/min/10^7 cells without dichloroacetate, 138 $\pm$ 17 nmol/min/10^7 cells after 10 min of preincubation with 2 mM dichloroacetate ; mean of 3 different experiments $\pm$ standard deviation). Thus, an inhibition of pyruvate carboxylation by dichloroacetate may be postulated. Therefore, the effects of dichloroacetate on pyruvate carboxylation were tested in isolated mitochondria (fig. 1). No significant inhibition was observed when the effector was added simultaneously with pyruvate. However, preincubation of mitochondria with dichloroacetate resulted in an important inhibition of pyruvate carboxylation ; inhibition increased with dichloroacetate concentration and preincubation time, and was observed in mitochondria prepared from fed as well as fasted animals. The rate of pyruvate carboxylation in controls (preincubated without dichloroacetate) decreased with preincubation time (fig. 1), which

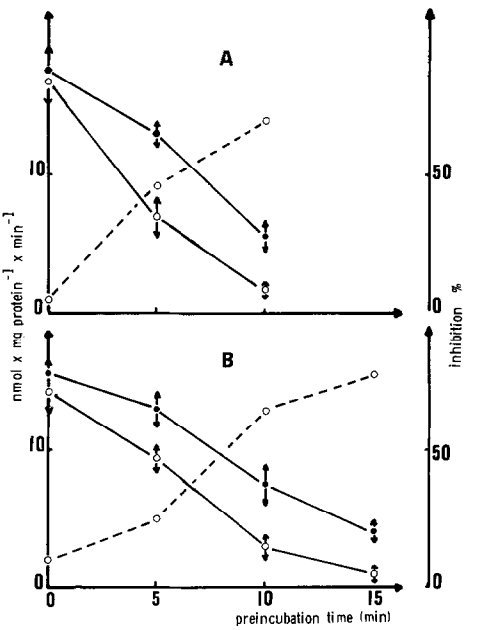

Fig. 1 : Effect of preincubation with dichloroacetate on pyruvate carboxylation by mitochondria isolated from fed (A) or fasted (B) rats

Mitochondria were either immediately incubated with substrates or preincubated for the indicated time without ●—● or with ○—○ 0.5 mM dichloroacetate. Then 5 mM pyruvate and 12 mM[^{14}C]potassium bicarbonate were added and 5 min incubations at 37°C were performed as described by Adam and Haynes (8). Results are the means of three experiments ± standard deviation. Dashed lines refer to the inhibition by dichloroacetate, expressed as a percentage of controls similarly processed.

may be related to the spontaneous decrease of the ATP content of mitochondria (about 40 percent after 5 min of preincubation). However, as previously demonstrated in intact hepatocytes (5,6), dichloroacetate did not significantly modify mitochondrial ATP. Dilution of labelled [$^{14}CO_2$] by cold CO_2 arising from higher rate of pyruvate decarboxylation in experiments with dichloroacetate can be excluded. We observed no activation of pyruvate decarboxylation : 12.3 ± 1.4 without and 10.3 ± 1.3 nmol/mg protein/min with dichloroacetate (means of three determinations ± standard deviation). This lack of effect of dichloroacetate may be the result of the high concentration of pyruvate used (5 mM) which maximally activates pyruvate dehydrogenase. However Lacey and Randle (7) also observed no activation of pyruvate dehydrogenase by dichloroacetate in kidney slices preincubated with lactate. For the same reasons, a decreased pyruvate concentration due to pyruvate dehydrogenase activation cannot explain inhibition of pyruvate gluconeogenesis or pyruvate carboxylation by dichloroacetate. The non-competitive character towards pyruvate of pyruvate

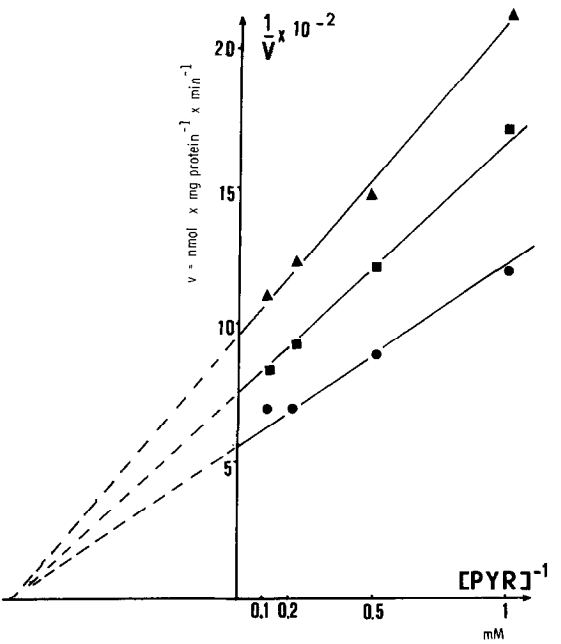

Fig. 2 : The effect of dichloroacetate on the kinetics of pyruvate carboxylation in mitochondria.

Mitochondria isolated from fasted rats were preincubated for 5 min, before pyruvate addition, with or without dichloroacetate : control ●–● , 0.5 mM dichloroacetate ■–■ , 1 mM dichloroacetate ▲–▲ . (incubation conditions as under fig. 1).

carboxylation inhibition by dichloroacetate (fig. 2), as well as the effects of preincubation (fig. 1), preclude a competitive inhibition of pyruvate entry into mitochondria as responsible for the experimental results (19).

The important increase of inhibition with preincubation time (fig. 1) suggests a prior metabolization of dichloroacetate : as a confirmation it must be stressed that the catalytic activity of pyruvate carboxylase partially purified by $(NH_4)_2\ SO_4$ precipitation was not modified by dichloroacetate even after preincubation at 37°C with the effector (results not shown). Oxalate is a known inhibitor of pyruvate carboxylase (12) ; as shown in tables I and II, oxalate was formed when intact hepatocytes or liver mitochondria were incubated with 1 to 1.5 mM [^{14}C] dichloroacetate. Oxalate formation and effects of dichloroacetate on gluconeogenesis (6) were measured after similar incubation periods ; after 40 min incubations of hepatocytes with 1 mM dichloroacetate, a final concentration of 15 to 20 μM oxalate was observed (table I) : this concentration is in the same order of magnitude as the Ki of oxalate towards pyruvate carboxylase (12) ;

Table I : [^{14}C] Oxalate formation from [^{14}C] dichloroacetate in isolated rat hepatocytes

	Incubation time				
	0	20 min		40 min	
Expt No	Total cpm recovered	Total cpm recovered	Oxalate formed nmol/10^7 cells	Total cpm recovered	Oxalate formed nmol/10^7 cells
I	3056	18 936	19.3	34 356	37.9
II	3296	12 340	11.0	26 971	28.7

11 x 10^6 hepatocytes were incubated in a total volume of 2 ml, as described in the text. 1 mM[U-^{14}C] dichloroacetate (3.35 x 10^6 cpm) was added at zero time. Total cpm refer to radioactivity recovered after incubation of deproteinized supernatants with oxalate decarboxylase, corrected from radioactivity evolved during similar incubations without oxalate decarboxylase. Oxalate production was calculated after subtraction of cpm recovered at zero time. No spontaneous decomposition of [^{14}C] dichloroacetate was observed when incubated for 40 min without hepatocytes (total cpm recovered : exp I, 4228 ; exp II, 3825). Each value is the mean of duplicate determinations.

Table II : [^{14}C] Oxalate formation from [^{14}C] dichloroacetate in isolated liver mitochondria

	Incubation time				
	0	5 min		10 min	
Expt No	Total cpm recovered	Total cpm recovered	Oxalate formed nmol/mg protein	Total cpm recovered	Oxalate formed nmol/mg protein
I	2370	6223	0.22	14 129	0.66
II	2175	5419	0.28	9 095	0.60

Mitochondria (23 mg of mitochrondrial protein in exp I and 15.5 in exp II) were incubated in a total volume of 1.5 ml as described in the text. 1.5 mM [U-^{14}C] dichloroacetate (3.84 x 10^6 cpm) was added at zero time. Oxalate formation was calculated as indicated in the legend of table I. No spontaneous decomposition of [U-^{14}C] dichloroacetate was observed when incubated for ten minutes without mitochondria (total cpm recovered : exp I, 2440 ; exp II, 2235). Each value is the mean of duplicate determinations.

moreover, oxalate inhibited partially purified pyruvate carboxylase by 32 percent at a 10 μM concentration, and 75 percent at a 50 μM concentration (mean of 2 experiments ; concentrations of substrates and effectors, mM : acetylCoA, 0.09 ; ATP, 2.5 ; bicarbonate, 50 ; pyruvate, 10 ; $MgCl_2$, 5). In intact mitochondria, oxalate inhibited pyruvate carboxylation, without preincubation, by 73 percent at a 0.25 mM concentration and 84 percent at a 1 mM concentration (mean of 2 experiments ; concentration of substrates and effectors, mM : bicarbonate, 12 ; pyruvate, 5 ; $MgCl_2$, 12 ; EDTA, 1.2). Taking into account the concentration ratio $MgCl_2$: oxalate, inhibition of pyruvate carboxylation by oxalate cannot be ascribed to a decreased concentration of free Mg^{++} due to complexation by oxalate. In the same range of concentration glyoxylate and glycolate had no measurable effect on partially purified pyruvate carboxylase.

It may be concluded that, in isolated hepatocytes and mitochondria, dichloroacetate is metabolized to oxalate which inhibits gluconeogenesis and pyruvate carboxylation. The mechanism of this biotransformation is under investigation.

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