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COMPARISON OF THE EFFECTS OF 2-CHLOROPROPIONATE AND DICHLOROACETATE ON KETOGENESIS AND LIPOGENESIS IN ISOLATED RAT HEPATOCYTES

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Abstract—The respective effects of 2-chloropropionate and dichloroacetate on the pyruvate metabolic crossroads, lipogenesis and ketogenesis, were compared in hepatocytes isolated from fed rats. 2-Chloropropionate acts as an exclusive pyruvate dehydrogenase activator: it increases ketogenesis, lipogenesis, Krebs cycle intermediates and mitochondrial NADH/NAD^+ ratio. The effects of dichloroacetate depend on experimental conditions and the intensity of its catabolization into oxalate: the resultant action of dichloroacetate on tested parameters combines the effects of pyruvate dehydrogenase activation on the one hand, and pyruvate carboxylase inhibition by oxalate on the other. A mixture of 2-chloropropionate plus oxalate mimics the effects of dichloroacetate. In hepatocytes from fed rats, endogenous lipogenesis is correlated with the mitochondrial NADH/NAD^+ ratio, irrespective of the effector added.

Some halogenated carboxylic acids activate pyruvate dehydrogenase activity and have been used experimentally in the treatment of hyperlactacidemia. Among them, dichloroacetate (DCA) and 2-chloropropionate (2-CP) have been the most extensively studied on whole animals or isolated cells [1–9]. Beyond its pyruvate dehydrogenase activation effect, DCA exhibits some side effects essentially due to its catabolization into glyoxylate and then oxalate [7, 10], which is a strong inhibitor of pyruvate carboxylase [11] and pyruvate kinase [12] in liver cells. Gluconeogenesis from pyruvate has been shown to be influenced differently by 2-CP and DCA in liver cells [7]: it is stimulated by the former and inhibited by the latter, probably by its 'oxalate' effect. We have recently compared the modifications induced by DCA and oxalate on ketogenesis, lipogenesis and tricarboxylic acid substrate levels [13]. The aim of the present work was to test in parallel the effects of DCA and 2-CP on ketogenesis and lipogenesis in rat hepatocytes in order to determine whether these two compounds have different actions on these pathways, as they have on gluconeogenesis.

MATERIALS AND METHODS

Isolation and incubation of hepatocytes. Male Sprague–Dawley rats (body weight 200–250 g), fed *ad lib.*, were anaesthetized with pentobarbital (10 mg per animal, i.p.). Hepatocytes were isolated by the method of Berry and Friend [14] as modified by Krebs *et al.* [15]. Cells were washed in Krebs Henseleit saline buffer [16] containing dialyzed fatty acid-free albumin (2% w/v) and continuously gassed with O_2–CO_2 (19:1). Cell suspensions were made 5 (low cell concentration) or 10×10^6 (high cell concentration) cells per ml with Krebs Henseleit at 10 μM Ca^{2+}. This low Ca^{2+} concentration was chosen to avoid the precipitating insoluble oxalate. Moreover, in preliminary experiments [17], it has been shown that metabolic differences induced by either 2-CP or DCA were more apparent at low (10 μM) than usual (2.5 mM) Ca^{2+} concentration. Aliquots of cell suspensions (2 ml) were distributed into 25 ml Erlenmeyer flasks gassed with O_2–CO_2 (19:1), were stoppered and shaken (80 cycles/min). After 10 min pre-incubation time, effectors and ^3H_2O (200 μCi per vial) were added. After 60 min at 37°, the cell suspensions were deproteinized with $HClO_4$ (0.6 M final concentration). It was shown in some control experiments at low cell concentration that the rates of lipogenesis and ketogenesis were constant during 60 min. This was not verified at higher cell concentration, but it must be stressed that, referred to the same number of cells, the metabolic rates were quite similar in control incubations with either 5 or 10×10^6 cells per ml (compare Tables 1 and 2).

Analytical methods. Lipogenesis, conducted in separated vials, was estimated by measuring the incorporation of ^3H_2O into saponifiable lipids according to Harris [18]. Unlabelled perchloric acid extracts were neutralized with $KHCO_3$ for determination of metabolites: pyruvate, lactate, phosphorylated substrates and ATP [19]; acetoacetate and β-hydroxybutyrate [20]; malate [21] and citrate [22].

All results were expressed with reference to the number of hepatocytes. 10^9 hepatocytes correspond to 9.24 ± 0.09 g of liver wet weight [23]. Results of experiments run with effectors were always com-

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Table 1. Effects of DCA and 2-CP on hepatocytes incubated at 'low cell' concentration (5×10^6 cells per ml of incubation medium)

	n	Control	2 mM DCA	2 mM 2-CP
Ketone bodies	8	47.14 ± 2.93	65.25 ± 3.84†	59.20 ± 3.22†‡
β-Hydroxybutyrate/acetoacetate	8	0.38 ± 0.02	0.45 ± 0.05*	0.56 ± 0.04†§
Malate	6	4.23 ± 0.37	3.71 ± 0.25	5.41 ± 0.35†§
Citrate	6	4.81 ± 0.40	4.10 ± 0.55	5.32 ± 0.36‡
Phosphoenolpyruvate	4	1.30 ± 0.09	1.46 ± 0.05	1.13 ± 0.04‡
Lipogenesis	8	37.04 ± 2.87	48.49 ± 4.26†	51.73 ± 0.36†‡
Lactate + pyruvate	6	299.3 ± 35.6	72.8 ± 12.8†	75.3 ± 17.6†
Lactate/pyruvate	6	5.43 ± 0.41	11.23 ± 1.55†	12.44 ± 1.66†

Results are means ± S.E.M. of n experiments, and are expressed as μmole per 10^9 cells. Ketone bodies are the sum of β-hydroxybutyrate plus acetoacetate production. Lipogenesis is expressed as μmole ^3H_2O incorporated into lipids after 60 min. Results are compared to controls by a paired Student's t-test. *$P < 0.05$; †$P < 0.01$. Results from DCA or 2-CP-treated cells are compared to each other by a paired Student's t-test. ‡$P < 0.05$; §$P < 0.01$.

pared with controls performed on the same batch of cells.

Chemicals. Substrates, coenzymes and auxiliary enzymes for spectrophotometric determinations were purchased from Boehringer (Mannheim, F.R.G.) or Sigma (St Louis, MO); ^3H_2O was from the Radiochemical Centre (Amersham, U.K.); dichloroacetic acid (Merck Schuchardt, Darmstadt, F.R.G.) and 2-chloropionic acid (Eastman Kodak, Rochester, NY) were used as sodium salts (pH 7.4).

RESULTS

Maximal decrease (−85%) of pyruvate and lactate accumulation is reached at 1.8 mM DCA or 2-CP and remains constant up to 4 mM (data not shown). So all experiments were performed at 2 mM DCA or 2-CP.

The main data obtained from cells (5×10^6 cells per ml of incubation medium) incubated with or without pyruvate dehydrogenase activators are shown in Table 1. DCA and 2-CP had parallel action on pyruvate and lactate level, lipogenesis, ketogenesis, cytosolic and mitochondrial NADH/NAD^+ ratios (respectively calculated from lactate : pyruvate and β-hydroxybutyrate : acetoacetate ratios). However, as compared to 2-CP, DCA increased total ketone body formation more significantly and mitochondrial NADH/NAD^+ ratio less efficiently. Moreover, DCA did not modify citrate, malate and phosphoenolpyruvate levels as did 2-CP. Thus the effects of both pyruvate dehydrogenase activators are not quite similar. These differences might be due to a side effect of DCA-derived oxalate since oxalate lowers both NADH/NAD^+ ratios and Krebs cycle substrate concentration [13]. According to the data from Demaugre *et al.* [10] and Crabb and Harris [7], it can be extrapolated that after 60 min incubation the oxalate concentration is about 40 μM, when the cell suspension is made 5×10^6 cells per ml and incubated with 2 mM DCA. As far as differences between 2-CP and DCA effects are due to oxalate formation, they should be amplified by increasing cell concentration, which should enhance oxalate formation from DCA. Under these conditions (Table 2), modifications due to 2-CP were similar to those reported in Table 1, while in DCA-treated cells new variations with respect to control appeared: the mitochondrial NADH/NAD^+ ratio and tricarboxylic acid substrate level dropped, the phosphoenolpyruvate concentration increased whereas lipogenesis was no longer activated.

If DCA effects result from both direct pyruvate dehydrogenase activation on the one hand, and pyruvate carboxylase and pyruvate kinase inhibition on the other, a mixture of 2-CP and oxalate is expected to mimic them. Indeed, as shown in Fig. 1, the trend and intensity of variations induced by 2 mM 2-CP plus 40 μM oxalate mixture were quite similar to the DCA-provoked modifications collected in Table 1. As already reported [13] oxalate lowered NADH/NAD^+ ratio, malate and citrate levels and enhanced phosphoenolpyruvate concentration. 2-CP, by pyruvate dehydrogenase activation, greatly

Table 2. Effects of DCA and 2-CP on hepatocytes incubated at 'high cell' concentration (10×10^6 cells per ml of incubation medium)

	n	Control	2 mM DCA	2 mM 2-CP
Ketone bodies	5	50.94 ± 4.91	77.08 ± 3.93†	71.49 ± 4.82†‡
β-Hydroxybutyrate/acetoacetate	5	0.44 ± 0.03	0.34 ± 0.04†	0.58 ± 0.05†§
Malate	5	7.49 ± 0.79	5.95 ± 0.85*	7.59 ± 0.81‡
Citrate	5	3.31 ± 0.33	2.13 ± 0.36†	3.26 ± 0.36‡
Phosphoenolpyruvate	4	1.60 ± 0.17	2.14 ± 0.11*	1.32 ± 0.18*§
Lipogenesis	6	38.28 ± 2.93	42.04 ± 3.36	49.61 ± 3.74†

Data and significance are expressed as explained in the footnote of Table 1.

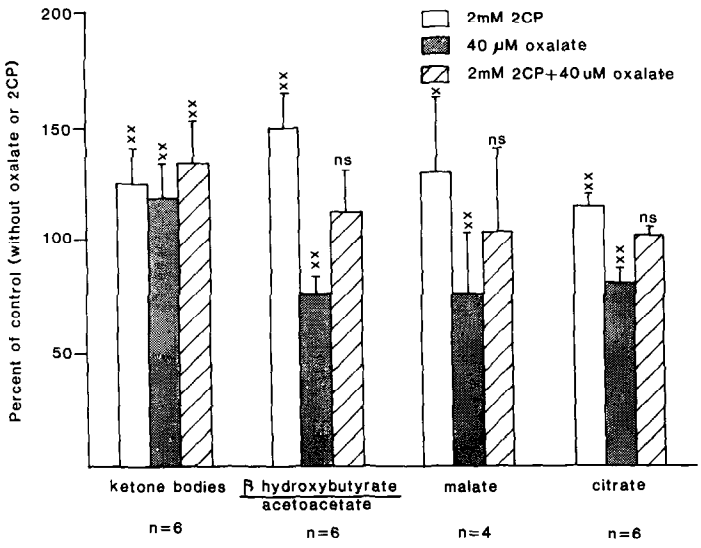

Fig. 1. Effects of 2-CP, oxalate, and 2-CP plus oxalate on Krebs cycle intermediates and ketogenesis in hepatocytes. Cell suspensions were made 5×10^6 cells per ml. Results, means ± S.E.M. of n separate determinations, are expressed as the percentage of control values: β-hydroxybutyrate/acetoacetate ratio was 0.38 ± 0.02 and, expressed as μmole/10^9 hepatocytes ketone bodies, malate and citrate were, respectively, 49.32 ± 6.92, 4.31 ± 1.14 and 4.21 ± 0.38. Results are compared to controls by a paired Student's t-test: $^{x}P < 0.05$; $^{xx}P < 0.01$.

lowered pyruvate and lactate accumulation as did DCA (Table 1) but raised the level of tricarboxylic acid cycle substrates and NADH/NAD^+ ratios. Finally, the opposite effects of 2-CP and oxalate on NADH/NAD^+ ratio and tricarboxylic cycle intermediates neutralized one another.

The influence of 2-CP on ketogenesis and lipogenesis at increasing oxalate concentrations is shown in Fig. 2A. 2-CP alone, as well as oxalate, enhanced ketogenesis. When both compounds were added together, their effects were cumulative but not strictly additive. As already reported [13, 23] lipogenesis was inhibited by oxalate which, even at low concentration (60 μM), also suppressed the 2-CP-induced increase of lipogenesis (Fig. 2). Once more this observation is consistent with oxalate-induced side effects of DCA (Table 2). From the latter data it appears that under our experimental conditions

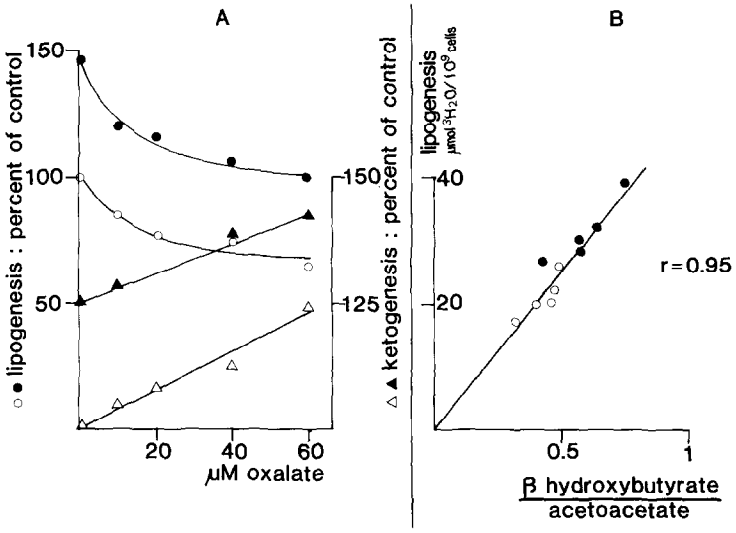

Fig. 2. (A) Effect of oxalate concentration on lipogenesis (○ ●) and ketogenesis (△ ▲) of hepatocytes (5×10^6 cells per ml of incubation medium) incubated with (closed symbols) and without (open symbols) 2 mM 2-CP. Results are means for duplicate incubations on the same batch of cells. They were reproduced at 40 μM oxalate in five different experiments. (B) Relation between β-hydroxybutyrate/acetoacetate ratio and lipogenesis. Data are drawn from experiments reported in (A). ○, Lipogenesis without 2-CP with or without oxalate; ●, lipogenesis with 2-CP with or without oxalate.

the lipogenesis of hepatocytes is related to the mitochondrial NADH/NAD^+ ratio. Indeed, in Fig. 2B such a correlation is obvious.

DISCUSSION

It had previously been shown that the modifications of ketogenesis induced by DCA were mainly mediated by its effect on mitochondrial oxaloacetate metabolism [6, 24]. However, Buc *et al.* [13] had deduced from a comparison of oxalate to DCA effects that the latter did not appear to derive from catabolization to oxalate. In order to define more accurately the mechanisms of the metabolic actions of DCA, the effects of this compound were compared to those of 2-CP, a halogenated acid which cannot be transformed to oxalate. Though not precisely identified, the main 2-CP-catabolites should be D-lactate, L-lactate and propionate; however, in hepatocytes incubated with 2-CP, the concentration of the postulated metabolites remains too low to modify the tested metabolic pathways [7]. Moreover, at concentrations up to 5 mM, D-lactate has no measurable metabolic effects (results not shown). Thus 2-CP acts as an exclusive pyruvate dehydrogenase activator. It always produces similar modifications of ketogenesis, lipogenesis, Krebs cycle intermediates and mitochondrial NADH/NAD^+ ratios, irrespective of the experimental conditions (Tables 1 and 2). On the contrary, DCA effects depend on the cell concentration in the suspension: at low cell concentration (Table 1) they are not very different from those of 2-CP whereas at high cell concentration, the postulated increased oxalate formation adds its effects to those of 2-CP. Under those conditions the resultant action of DCA on lipogenesis, ketogenesis and mitochondrial redox potential appears to combine the effects of pyruvate dehydrogenase activation and pyruvate carboxylase inhibition. As a confirmation, a mixture of 2-CP and oxalate mimics the metabolic action of DCA alone (Tables 1 and 2, Fig. 1).

It has been suggested in previous papers [13, 24] that the mitochondrial oxaloacetate level regulated the metabolic crossroads of mitochondrial acetyl-CoA. Thus it was concluded that ketogenesis should be increased by an imbalance between acetyl CoA and oxaloacetate relative synthesis, resulting from either pyruvate dehydrogenase activation or pyruvate carboxylase inhibition. Present results with 2-CP confirm this assumption. Moreover, when a pyruvate dehydrogenase activator (2-CP) and a pyruvate carboxylase inhibitor (oxalate) are added simultaneously their effects on ketogenesis appear to be cumulative (Figs. 1 and 2A). It is also consistent that DCA behaves as a more powerful activator of ketogenesis than 2-CP.

As shown by Table 1 and 2, 2-CP activates lipogenesis under all the tested experimental conditions: 2-CP increases the production of mitochondrial acetyl CoA which in its turn activates pyruvate carboxylase and oxaloacetate formation, thus citrate production (as shown by the increased concentration of measured Krebs cycle intermediates); as a result the cytosolic production of acetyl CoA increases. Oxalate (60 μM), which by itself inhibits lipogenesis [13], also suppresses the 2-CP-induced activation of fatty acid synthesis as a probable result of oxaloacetate shortage; oxalate alone decreases the concentration of malate and citrate whereas the 2-CP plus oxalate mixture does not significantly modify Krebs cycle intermediates. It may be postulated that the excess acetyl CoA formed under the influence of 2-CP sufficiently activates pyruvate carboxylase to overcome the inhibition of the enzyme by oxalate so that the two effectors neutralize one another.

The effects of DCA on lipogenesis are less straightforward than those of 2-CP, depending on the relative action of DCA itself on pyruvate dehydrogenase and of DCA-derived oxalate on pyruvate carboxylase. At low cell concentration (Table 1) only the activating effect of DCA on pyruvate dehydrogenase is observed and lipogenesis is activated, whereas at high cell concentration (Table 2) more oxalate is formed and there is no modification of fatty acid synthesis. In a previous series of experiments [13], no significant effects of DCA were observed on lipogenesis (and ketogenesis) at cell concentrations of $5–6 \times 10^6$ per ml. Such differences with the present data may be due to (i) the smaller number of experiments, (ii) a somewhat higher concentration of cells, up to 6×10^6 per ml, (iii) different statistical treatment (unpaired *t*-test), and (iv) the absence of albumin in the incubation medium. The effects of DCA concentration might also be explained by differences in oxalate formation: Crabb *et al.* [3] have shown that 1 mM DCA activates lipogenesis whereas 4 mM does not.

The stimulation of lipogenesis by DCA at low cell concentration is not accompanied by increased citrate concentration (Table 1): this apparent discrepancy may be due to the fact that citrate concentration, but not flux, was measured; moreover, citrate content was determined at the end of the incubation period, when oxalate concentration may have risen to inhibitory levels towards pyruvate carboxylase.

Irrespective of the effector used, lipogenesis is quite tightly correlated to the mitochondrial NADH/NAD^+ ratio deduced from β-hydroxybutyrate/acetoacetate ratio (Fig. 2B). As a whole, citrate production determines both the intensity of lipogenesis and the production of reducing equivalents by tricarboxylic acid cycle. Moreover, the increase of the NADH/NAD^+ ratio may, by itself, activate lipogenesis as shown by experiments with ethanol [25].

Recent work from Assimacopoulos-Jeannet *et al.* [26] dealing with the activation of fatty acid synthesis by insulin mentions that no modification of the mitochondrial redox potential was noticeable. However, their experimental conditions are different, and in that report insulin action on lipogenesis seems to be related to acetyl CoA carboxylase more than pyruvate dehydrogenase activation.

To conclude, 2-CP appears to be a pure pyruvate dehydrogenase activator without side effects: as such it is an excellent tool in the study of the metabolic roles of pyruvate dehydrogenase.

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