Amino acid oxidation and alanine production in rat hemidiaphragm *in vitro*

Effects of dichloroacetate

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Dichloroacetate (an activator of pyruvate dehydrogenase) stimulates $^{14}\text{CO}_2$ production from $[\text{U}-^{14}\text{C}]$ glucose, but not from $[\text{U}-^{14}\text{C}]$ glutamate, $[\text{U}-^{14}\text{C}]$ aspartate, $[\text{U}-^{14}\text{C}]$ - and $[1-^{14}\text{C}]$ -valine and $[\text{U}-^{14}\text{C}]$ - and $[1-^{14}\text{C}]$ -leucine. It is concluded (1) that pyruvate dehydrogenase is not rate-limiting in the oxidation to CO_2 of amino acids that are metabolized to tricarboxylic acid-cycle intermediates, and (2) that carbohydrate (and not amino acids) is the main carbon precursor in alanine formation in muscle.

Alanine produced by skeletal muscle is a major precursor in hepatic gluconeogenesis. It was originally proposed (Felig, 1975) that the carbon skeleton of the alanine released was derived from glucose via glycolysis. More recently, it has been suggested that alanine is synthesized *de novo* from pyruvate derived from the carbon skeletons of those amino acids that are metabolized to tricarboxylic acid-cycle intermediates in muscle (see Snell, 1980), i.e. glutamate, valine, isoleucine, aspartate and asparagine. NADP^+ -dependent malate dehydrogenase (Garber *et al.*, 1976; but see Snell & Duff, 1982) or phosphoenolpyruvate carboxykinase plus pyruvate kinase (Goldstein & Newsholme, 1976; Snell & Duff, 1977; Palmer *et al.*, 1982) have been suggested to be involved in the synthesis of alanine *de novo* from amino acid carbon. These enzymes have also been implicated in gluconeogenesis (Oedra & Palmer, 1981) and amino acid oxidation (Goldstein & Newsholme, 1976; Palmer & Sugden, 1983).

There is still controversy regarding the origin of the carbon skeleton of the alanine released from muscle (Chang & Goldberg, 1978*a,b*; Lee & Davis, 1979; Snell, 1980). To elucidate this problem, the effects of dichloroacetate on amino acid oxidation to CO_2 and alanine production in rat hemidiaphragms were investigated. Dichloroacetate activates the pyruvate dehydrogenase complex via inhibition of the kinase responsible for phosphorylation and inactivation of the complex (see Crabb *et al.*, 1981). Such activation of the pyruvate dehydrogenase complex would be expected to

divert amino acid carbon into oxidation to CO_2 at the expense of alanine synthesis.

Materials and methods

Materials

L- $[\text{U}-^{14}\text{C}]$ Valine, L- $[1-^{14}\text{C}]$ valine, L- $[\text{U}-^{14}\text{C}]$ glutamate, L- $[\text{U}-^{14}\text{C}]$ aspartate, L- $[\text{U}-^{14}\text{C}]$ leucine, L- $[1-^{14}\text{C}]$ leucine and D- $[\text{U}-^{14}\text{C}]$ glucose were obtained from Amersham International, Amersham, Bucks., U.K. The final specific radioactivities of the ^{14}C -labelled amino acids was $0.083 \mu\text{Ci}/\mu\text{mol}$. Dichloroacetic acid was from BDH Chemicals, Poole, Dorset, U.K. Other biochemical reagents and amino acids were from Sigma or Boehringer. All reagent chemicals were of analytical grade.

Animals

Male Wistar rats (100–120 g) were maintained at approx. 17°C on a 12h-day/12h-night cycle (day started at 08:00 h). Animals were fed *ad libitum* or starved for 40 h before the experiments, which were commenced at 11:00–13:00 h.

Experimental

Hemidiaphragms from fed or 40h-starved rats were preincubated (30 min at 37°C) in substrate-free Krebs–Ringer bicarbonate buffer, pH 7.4, equilibrated with O_2/CO_2 (19:1), and then incubated as described elsewhere (Palmer *et al.*, 1982, 1983) for 2 h in 2 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, with the additions specified in the text. $^{14}\text{CO}_2$ (calculated as $\mu\text{mol}/2\text{h}$ per g wet wt.) was determined by entrapment in Hyamine 10-X

hydroxide solution (BDH Chemicals) after acidification of the incubation media with 0.2 ml of 60% (w/v) HClO_4 (Palmer *et al.*, 1983). The KOH-neutralized HClO_4 extracts of the media were assayed for lactate, pyruvate, alanine (Palmer *et al.*, 1982), glutamate (Bernt & Bergmeyer, 1974) and glutamine (Lund, 1974). Glycogen was determined in hemidiaphragms at the end of the incubation period on extraction (100°C, 5 min) in 0.3 ml of 30% (w/v) KOH (Palmer *et al.*, 1983).

Results are expressed as means $\pm$ S.E.M. with the numbers of observations in parentheses. For each diaphragm, one hemidiaphragm served as control, the second receiving additions as specified. Statistical analysis was by Student's *t* test for unpaired data.

Results

Effects of dichloroacetate on oxidation of glutamate and aspartate

In agreement with McAllister *et al.* (1973), dichloroacetate stimulates $[\text{U-}^{14}\text{C}]$ glucose oxidation to $^{14}\text{CO}_2$ in hemidiaphragms from 40h-starved rats (by approx. 3.7-fold; Table 1). There were accompanying decreases in release of lactate by (by 27%) and pyruvate (by 85%), but not of alanine (but see Snell & Duff, 1977; Goodman *et al.*, 1978). The [lactate]/[pyruvate] ratio in the incubation medium was increased by dichloroacetate from 9.99 ± 0.8 to 50.12 ± 5.4 ($P < 0.001$). This may result from the increase in glycolytic flux produced by dichloroacetate. Glycogen content of the hemidiaphragms at the end of the incubation period was decreased by approx. 50% by dichloroacetate.

Dichloroacetate was without effect on $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]$ glutamate or $[\text{U-}^{14}\text{C}]$ aspartate by hemidiaphragms from 40h-starved rats (Table 1), but decreased release of lactate,

pyruvate and alanine by hemidiaphragms provided with either $[\text{U-}^{14}\text{C}]$ glutamate or $[\text{U-}^{14}\text{C}]$ aspartate and increased the ratio of released [lactate]/[pyruvate] in the incubation media. With glutamate as substrate the ratio was increased by dichloroacetate from 13.32 ± 2.2 to 38.17 ± 5.1 ($P < 0.05$), whereas with aspartate as substrate the ratio increased from 8.78 ± 1.3 to 20.7 ± 3.5 ($P < 0.05$).

Dichloroacetate decreased glycogen (i.e. glycogen residual at the completion of the incubation period) in diaphragms provided with glucose and glutamate, but not aspartate. This lack of effect of dichloroacetate on glycogen content in the presence of aspartate may reflect the relatively low concentrations present under this condition.

Effects of dichloroacetate on the stoichiometry of oxidation of valine and leucine

The lack of stimulatory effect of dichloroacetate on $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]$ glutamate and $[\text{U-}^{14}\text{C}]$ aspartate indicates that, in hemidiaphragms from 40h-starved rats, flux through the pyruvate dehydrogenase complex does not limit $^{14}\text{CO}_2$ production from these substrates. This raises the question as to whether the pathway of oxidation to CO_2 of amino acids that are metabolized to tricarboxylic acid-cycle intermediates proceeds via this enzyme complex (see Palmer & Sugden, 1983). To examine this further, the effects of dichloroacetate on $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]$ valine and $[\text{U-}^{14}\text{C}]$ valine were assessed in hemidiaphragms from fed or 40h-starved rats. $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]$ valine estimates oxidative decarboxylation in the branched-chain 2-oxo acid dehydrogenase complex, whereas $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]$ valine measures total oxidation to CO_2 . (If oxidation were complete, this ratio would be 5.) The results are shown in Table 2.

Table 1. *Effects of 5 mM-dichloroacetate on metabolism of 5 mM- $[\text{U-}^{14}\text{C}]$ glucose, 3 mM- $[\text{U-}^{14}\text{C}]$ glutamate and 3 mM- $[\text{U-}^{14}\text{C}]$ aspartate in hemidiaphragms from 40h-starved rats*

Residual glycogen refers to the glycogen content of the hemidiaphragms at the end of the 2h incubation period.

* $P < 0.05$, ** $P < 0.01$ for differences between substrate alone and substrate plus dichloroacetate

Additions to incubation medium	$^{14}\text{CO}_2$ ($\mu\text{mol}/2\text{h}$ per g wet wt.)	Metabolites released into medium ($\mu\text{mol}/2\text{h}$ per g wet wt.)			Residual glycogen ($\mu\text{mol}/\text{g}$ wet wt.)
		Lactate	Pyruvate	Alanine	
$[\text{U-}^{14}\text{C}]$ Glucose (6)	10.7 ± 0.8	25.1 ± 2.2	2.6 ± 0.2	3.2 ± 0.3	6.1 ± 0.6
$[\text{U-}^{14}\text{C}]$ Glucose + dichloroacetate (6)	$39.1 \pm 3.8^{**}$	$18.3 \pm 1.5^*$	$0.4 \pm 0.03^{**}$	3.2 ± 0.2	$3.3 \pm 0.7^*$
$[\text{U-}^{14}\text{C}]$ Glutamate (5)	9.2 ± 0.4	10.6 ± 0.4	0.9 ± 0.1	4.2 ± 0.5	3.0 ± 0.3
$[\text{U-}^{14}\text{C}]$ Glutamate + dichloroacetate (5)	8.9 ± 0.6	$8.8 \pm 0.1^*$	$0.3 \pm 0.1^{**}$	$1.4 \pm 0.1^{**}$	$1.7 \pm 0.4^*$
$[\text{U-}^{14}\text{C}]$ Aspartate (5)	6.6 ± 0.2	8.1 ± 0.3	1.0 ± 0.1	2.4 ± 0.1	1.1 ± 0.1
$[\text{U-}^{14}\text{C}]$ Aspartate + dichloroacetate (5)	6.7 ± 0.2	$7.5 \pm 0.2^*$	$0.4 \pm 0.04^{**}$	$1.4 \pm 0.2^{**}$	1.0 ± 0.3

Table 2. *Effects of 5 mM-dichloroacetate on metabolism of 3 mM-[U-¹⁴C]- and [1-¹⁴C]-valine and [U-¹⁴C]- and [1-¹⁴C]-leucine by hemidiaphragms from fed and starved rats: ¹⁴CO₂ production and residual glycogen*

Residual glycogen refers to the glycogen content of the hemidiaphragms at the end of the 2 h incubation period. *P < 0.05, **P < 0.01, ***P < 0.001 for differences between substrate alone and substrate plus dichloroacetate. The number of observations was 6 in each of the groups for ¹⁴CO₂ production and 12 otherwise. 'Ratio' is that of ¹⁴CO₂ production from [U-¹⁴C]valine or [U-¹⁴C]leucine to ¹⁴CO₂ production from [1-¹⁴C]valine or [1-¹⁴C]leucine. Release of metabolites in these experiments is shown in Table 3.

Additions to incubation medium	¹⁴ CO ₂ production (μmol/2h per g wet wt.)		Ratio	Residual glycogen (μmol/g wet wt.)
	[U- ¹⁴ C]amino acid	[1- ¹⁴ C]amino acid		
Fed				
Valine	5.77 ± 0.4	2.16 ± 0.2	2.72 ± 0.1	2.09 ± 0.2
Valine + dichloroacetate	3.01 ± 0.2***	1.68 ± 0.1**	1.88 ± 0.3***	1.20 ± 0.2*
40h-starved				
Valine	5.50 ± 0.6	2.45 ± 0.1	2.25 ± 0.2	1.61 ± 0.2
Valine + dichloroacetate	3.51 ± 0.2***	2.27 ± 0.2	1.57 ± 0.2***	0.74 ± 0.3*
Fed				
Leucine	22.59 ± 1.5	5.74 ± 0.4	3.96 ± 0.3	2.13 ± 0.3
Leucine + dichloroacetate	9.05 ± 0.4***	2.55 ± 0.3***	3.68 ± 0.3	2.34 ± 0.3

Table 3. *Effects of 5 mM-dichloroacetate on metabolism of 3 mM-[U-¹⁴C]- and [1-¹⁴C]-valine and [U-¹⁴C]- and [1-¹⁴C]-leucine by hemidiaphragms from fed and starved rats: metabolite release*

Residual glycogen refers to the glycogen content of the hemidiaphragms at the end of the 2 h incubation period. *P < 0.05, **P < 0.01, ***P < 0.001 for differences between substrate alone and substrate plus dichloroacetate. The number of observations was 6 in each of the groups for ¹⁴CO₂ production and 12 otherwise. Values for ¹⁴CO₂ production and residual glycogen in these experiments are shown in Table 2.

Additions to incubation medium	Metabolites released into medium (μmol/2h per g wet wt.)		
	Lactate	Pyruvate	Alanine
Fed			
Valine	8.87 ± 0.7	0.62 ± 0.1	3.40 ± 0.1
Valine + dichloroacetate	5.93 ± 0.9**	0.17 ± 0.03***	2.26 ± 0.1***
40h-starved			
Valine	7.88 ± 0.6	9.68 ± 0.1	1.91 ± 0.1
Valine + dichloroacetate	2.47 ± 0.2***	0.18 ± 0.03***	1.26 ± 0.1***
Fed			
Leucine	11.66 ± 0.9	0.57 ± 0.02	3.73 ± 0.2
Leucine + dichloroacetate	6.48 ± 0.6***	0.32 ± 0.03***	2.17 ± 0.1***

In hemidiaphragms from fed rats, dichloroacetate decreased ¹⁴CO₂ production from [U-¹⁴C]-valine by 48% and from [1-¹⁴C]valine by 22%. Consequently, the ratio of ¹⁴CO₂ produced from [U-¹⁴C]valine to ¹⁴CO₂ produced from [1-¹⁴C]valine was decreased by dichloroacetate (by 31%). In hemidiaphragms from 40h-starved rats, dichloroacetate decreased ¹⁴CO₂ production from [U-¹⁴C]valine by 36%, but ¹⁴CO₂ production from [1-¹⁴C]valine was not significantly affected. The ratio of ¹⁴CO₂ production from [U-¹⁴C]valine to ¹⁴CO₂ production from [1-¹⁴C]valine was decreased by 30%. The ratio of ¹⁴CO₂ production from [U-¹⁴C]valine to ¹⁴CO₂ production from [1-¹⁴C]valine was <5 in both the absence and presence of

dichloroacetate. Thus activation of the pyruvate dehydrogenase complex does not result in the complete oxidation of valine to CO₂. In hemidiaphragms from both fed and starved rats provided with valine, dichloroacetate decreased release of lactate, pyruvate and alanine and increased the ratio of released [lactate]/[pyruvate] from 14.1 ± 1.1 to 41.4 ± 7.1 (*P* < 0.005) and from 11.8 ± 0.6 to 17.6 ± 2.3 (*P* < 0.05) in hemidiaphragms from fed and starved rats respectively (Table 3). These changes in metabolism are consistent with activation of the pyruvate dehydrogenase complex by dichloroacetate in the presence of valine. In addition to the effects on alanine output, dichloroacetate promoted glutamate re-

lease by hemidiaphragms provided with valine, but suppressed the release of glutamine (by $\geq 60\%$; results not shown).

Since dichloroacetate decreases *in toto* valine oxidation to CO_2 , despite its activation of the pyruvate dehydrogenase complex, it is implied that the oxidation of valine to CO_2 is not limited by the pyruvate dehydrogenase reaction (see Palmer & Sugden, 1983). The decreased $^{14}\text{CO}_2$ production from both $[1\text{-}^{14}\text{C}]\text{valine}$ and $[\text{U-}^{14}\text{C}]\text{valine}$ strongly suggested an inhibitory action of dichloroacetate at a locus before the pyruvate dehydrogenase reaction. For this reason, effects of dichloroacetate on the metabolism of $[1\text{-}^{14}\text{C}]\text{leucine}$ and $[\text{U-}^{14}\text{C}]\text{leucine}$ were studied (Tables 2 and 3). Leucine is metabolized via the branched-chain dehydrogenase complex to acetyl-CoA and acetoacetate, and thus pyruvate dehydrogenase is not implicated in its oxidation. Dichloroacetate decreased $^{14}\text{CO}_2$ production from both $[\text{U-}^{14}\text{C}]\text{leucine}$ (by 60%) and $[1\text{-}^{14}\text{C}]\text{leucine}$ (by 56%). The ratio of $^{14}\text{CO}_2$ produced from $[\text{U-}^{14}\text{C}]\text{leucine}$ to $^{14}\text{CO}_2$ produced from $[1\text{-}^{14}\text{C}]\text{leucine}$ was unchanged. As with diaphragms provided with valine, dichloroacetate decreased the release of lactate, pyruvate and alanine. In hemidiaphragms provided with leucine the $[\text{lactate}]/[\text{pyruvate}]$ ratio was unchanged (20.6 ± 1.3 without and 20.0 ± 1.7 with dichloroacetate). This may relate to the fact that, whereas dichloroacetate decreased residual glycogen in hemidiaphragms from fed or starved rats incubated with valine, it had no such effect in hemidiaphragms from fed rats incubated with leucine. The reason for this difference is not clear. It is noteworthy that changes in $[\text{lactate}]/[\text{pyruvate}]$ ratio in response to dichloroacetate are observed only in conjunction with increased glycogenolysis, suggesting that availability of glycolytic precursors is a precondition for this effect.

Discussion

Dichloroacetate decreases $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{valine}$ and $[1\text{-}^{14}\text{C}]\text{leucine}$ in hemidiaphragms from fed rats. Since $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{valine}$ or $[1\text{-}^{14}\text{C}]\text{leucine}$ is a specific measure of flux through the branched-chain 2-oxo acid dehydrogenase reaction, it is inferred that dichloroacetate inhibits flux through this reaction in hemidiaphragms from fed rats via interaction with branched-chain 2-oxo acid dehydrogenase or inhibition of transamination or effects on uptake. The reason for the lack of effect of dichloroacetate on $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{valine}$ in hemidiaphragms from starved rats is not clear. $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{valine}$ and $[1\text{-}^{14}\text{C}]\text{leucine}$ depends on the specific radioactivities of 3-methyl-2-oxovalerate and 4-methyl-2-oxovalerate, and

thus it cannot be precluded that $^{14}\text{CO}_2$ production is decreased because of radiolabel dilution produced by dichloroacetate. Dichloroacetate stimulates glycolytic flux, and therefore the apparent failure of dichloroacetate to stimulate $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{valine}$, $[\text{U-}^{14}\text{C}]\text{glutamate}$ and $[\text{U-}^{14}\text{C}]\text{aspartate}$ might theoretically be a consequence of radiolabel dilution in the cytosolic pyruvate pool. This is, however, unlikely, as the addition of 20 mM-glucose has no effect on $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{-aspartate}$ or -glutamate (Palmer *et al.*, 1983).

Dichloroacetate decreased the ratio of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{valine}$ to $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{valine}$ by approx. 30% in hemidiaphragms from both fed and starved rats. This may indicate that dichloroacetate inhibits decarboxylation steps before pyruvate dehydrogenase in addition to the branched-chain 2-oxo acid dehydrogenase reaction. Since the ratio of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{leucine}$ to that from $[1\text{-}^{14}\text{C}]\text{leucine}$ is not similarly decreased, it seems likely that this site of action of dichloroacetate is before entry of valine carbon into the tricarboxylic acid cycle, possibly methylmalonate semialdehyde dehydrogenase. The inhibition of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{leucine}$ is presumably secondary to inhibition of the branched-chain 2-oxo acid dehydrogenase complex. Other work has indicated that dichloroacetate inhibits branched-chain amino acid oxidation in skeletal muscle (Goodman *et al.*, 1978; Partridge *et al.*, 1983), but not in heart (Sans *et al.*, 1980).

The pyruvate dehydrogenase complex in theory is implicated in the pathway of oxidation to CO_2 of valine, glutamate and aspartate (Goldstein & Newsholme, 1976). Dichloroacetate (via activation of the pyruvate dehydrogenase complex) may therefore divert amino acid carbon from alanine synthesis into CO_2 production. Dichloroacetate increased flux through the pyruvate dehydrogenase reaction irrespective of the substrate provided (i.e. glucose, glutamate, aspartate, valine, leucine), as indicated by decreased release of lactate, pyruvate and alanine (see also Snell & Duff, 1977; Goodman *et al.*, 1978). However, whereas dichloroacetate stimulated $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glucose}$, it did not stimulate $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamate}$, $[\text{U-}^{14}\text{C}]\text{aspartate}$ or $[\text{U-}^{14}\text{C}]\text{valine}$. This may imply that these amino acids do not act as carbon precursors in alanine synthesis *de novo*. Dichloroacetate promoted glycogen mobilization in hemidiaphragms from starved rats provided with glutamate or valine. It therefore seems reasonable to conclude that glycogen (rather than the carbon skeletons of amino acids) is the source of the carbon skeletons of pyruvate, lactate and alanine. The present results therefore support

the conclusion of Chang & Goldberg (1978*a,b*). Glycogen breakdown was also increased in starved rat hemidiaphragms incubated with glucose. It is noteworthy that amino nitrogen diverted from alanine formation by dichloroacetate activation of pyruvate dehydrogenase in the presence of valine or leucine does not appear in glutamine (results not shown).

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