Branched-chain amino acid metabolism and alanine formation in rat diaphragm muscle *in vitro*

Effects of dichloroacetate

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(Received 25 June 1984/Accepted 28 August 1984)

Dichloroacetate (which activates pyruvate dehydrogenase) decreases the release of alanine, pyruvate and lactate in hemidiaphragm incubations with valine. Dichloroacetate interferes with alanine formation by diverting pyruvate into oxidative pathways, which not only limits pyruvate availability for direct transamination to form alanine but also indirectly affects branched-chain amino acid transamination by limiting 2-oxoglutarate regeneration from glutamate.

During starvation amino acids are a major source of carbon for hepatic gluconeogenesis. Much experimental evidence in animals and man has indicated alanine as predominant in the gluconeogenic flow of amino acid precursors from protein in skeletal muscle to the liver (for review see Snell, 1980). The formation of muscle alanine in this circumstance represents synthesis *de novo* from other amino acids produced during protein degradation. Attention has been focused on the branched-chain amino acids as major precursors: muscle appears to be the principal site of their metabolism; they are released in a smaller proportion than their abundance in muscle protein; and they are the only amino acids at physiological concentrations that are capable of increasing alanine production (see Snell, 1980). It has been argued that for muscle alanine (via hepatic gluconeogenesis) to contribute to the net body glucose requirement in gluconeogenic situations, such as starvation, the carbon as well as the nitrogen for its synthesis must originate from other amino acids (Goldstein & Newsholme, 1976; Snell & Duff, 1977). Evidence for the conversion of the carbon skeletons of valine and isoleucine (which can contribute C₄ fragments to the citric acid cycle at the level of succinyl-CoA) into pyruvate for alanine formation has been presented (Goldstein & Newsholme, 1976; Snell & Duff, 1977, 1981, 1982; Palmer *et al.*, 1982). This pathway involves the withdrawal of oxaloacetate from the citric acid cycle and its conversion into pyruvate by the consecutive actions of phosphoenolpyruvate carboxykinase and pyruvate kinase. In theory the availability of pyruvate, generated in this way, for

transamination to form alanine could be influenced by the activity of pyruvate dehydrogenase, which serves to divert pyruvate into oxidative metabolism. For example, during starvation increased alanine production by skeletal muscle (Snell & Duff, 1979) is accompanied by increased branched-chain amino acid oxidation (Goldberg & Odessey, 1972; Snell & Duff, 1982), elevated phosphoenolpyruvate carboxykinase activity (Newsholme & Williams, 1978; Snell & Duff, 1979) and inactivation of the pyruvate dehydrogenase complex (Hennig *et al.*, 1975; Caterson *et al.*, 1982).

Dichloroacetate is an agent that activates the pyruvate dehydrogenase complex through inhibition of the kinase subunit, which phosphorylates and inactivates the complex (Whitehouse *et al.*, 1974; Crabb *et al.*, 1981). Activation of the pyruvate dehydrogenase complex would be expected to diminish alanine formation by promoting the oxidation of pyruvate. In the present study, the effects of dichloroacetate on alanine formation and branched-chain amino acid metabolism in rat diaphragm muscle *in vivo* have been investigated.

Materials and methods

Materials

Animals. Male rats (70–100 g) of an inbred Wistar albino strain (University of Surrey Animal Unit) were maintained under conditions of 12 h light–12 h darkness at 12°C and were allowed water *ad libitum* during starvation for 48 h before being killed at 10:00 h.

Chemicals. L-[U- ^{14}C]Leucine and L-[1- ^{14}C]leucine were obtained from Amersham International, Amersham, Bucks., U.K. Dichloroacetic acid, D-glucose, L-valine and L-leucine were from BDH Chemicals, Poole, Dorset, U.K. Enzymes, co-enzymes and other biochemicals were from Boehringer Co., Lewes, Sussex, U.K.

Experimental

Cut hemidiaphragm preparations from 48 h-starved rats were incubated for 2 h at 37°C in 2 ml of albumin-free bicarbonate buffer, pH 7.4, equilibrated with O_2/CO_2 (19:1), following a 30 min pre-incubation period under the same conditions, as previously described (Snell & Duff, 1977). Additions to the incubation media were made as indicated in the Tables. $^{14}\text{CO}_2$ production was determined after collection in Hyamine 10-X hydroxide solution (BDH Chemicals) contained in suspended plastic wells (Kontes Scientific Glassware, Vineland, NJ, U.S.A.), after acidification of the incubation media with 0.5 ml of 0.5 M- H_2SO_4 . Rates of leucine metabolism were calculated on the basis of equal molar specific radioactivities (67 $\mu\text{Ci}/\text{mmol}$) of [U- ^{14}C]- and [1- ^{14}C]-leucine in the incubation medium. $^{14}\text{CO}_2$ produced by decarboxylation of [1- ^{14}C]leucine or oxidation of [U- ^{14}C]leucine, and 4-methyl-2-oxo[1- ^{14}C]pentanoate released into the incubation medium after transamination of [1- ^{14}C]leucine, are expressed in $\mu\text{mol}/2\text{h}$ per g wet wt. 4-Methyl-2-oxo[1- ^{14}C]pentanoate released into the incubation medium during incubations was determined as $^{14}\text{CO}_2$ after chemical decarboxylation with H_2O_2 as described by Cappucino *et al.* (1978). Rates of leucine transamination were calculated as the sum of the rates of $^{14}\text{CO}_2$ and 4-methyl-2-oxo[1- ^{14}C]pentanoate produced from [1- ^{14}C]leucine. Branched-chain 2-oxo acid dehydrogenase activity was

calculated from the rate of $^{14}\text{CO}_2$ production from [1- ^{14}C]leucine. In other experiments, HClO_4 extracts of the incubation media, after removal of the hemidiaphragms, were neutralized with KOH and assayed enzymically for alanine, lactate and pyruvate (Snell & Duff, 1977). Leucine aminotransferase (EC 2.6.1.42) activity was assayed in homogenates of rat diaphragm by the colorimetric assay described by Cappucino *et al.* (1978).

Results are expressed as mean values $\pm$ S.E.M. for the numbers of observations indicated in the Tables. For each diaphragm, one hemidiaphragm served as control, with the other receiving appropriate additions as indicated in the Tables. Statistical analysis was by Student's *t* test for paired observations.

Results and discussion

Effect of dichloroacetate on metabolite release by hemidiaphragms from 48 h-starved rats

Dichloroacetate decreased the release of alanine by hemidiaphragms incubated in bicarbonate medium alone or in the presence of 3 mM-valine or 5 mM-glucose by 50, 68 and 55% respectively (Table 1). In all cases the diminished alanine release was accompanied by decreases in the release of pyruvate (by 86, 91 and 95%) and lactate (by 78, 59 and 73%). In addition, the [lactate]/[pyruvate] ratio in the incubation medium at 2 h was increased by dichloroacetate from 4.5 to 21.0 with valine as substrate, and from 4.8 to 26.2 with glucose as substrate. These findings are similar to those reported in part by ourselves (Snell & Duff, 1977), and by others in the perfused hind-limb preparation (Goodman *et al.*, 1978) and the hepatectomized rat *in vivo* (Blackshear *et al.*, 1975). The decrease in pyruvate and lactate release is due to the stimulation of pyruvate oxidation by the

Table 1. *Effect of 10 mM-dichloroacetate on metabolite release in vitro by hemidiaphragms from 48 h-starved rats*

Hemidiaphragms prepared from 48 h-starved rats were pre-incubated for 30 min in medium without any additions, and then incubated for 2 h in fresh medium containing the additions indicated, as described in the Materials and methods section. Paired incubations were carried out in the presence and in the absence of 10 mM-sodium dichloroacetate. Alanine, pyruvate and lactate released into the medium were determined as described in the Materials and methods section and are expressed as μmol of metabolite released/2h per g wet wt. of tissue. Values are the means $\pm$ S.E.M. for five experiments, and statistical analysis (paired Student's *t* test) showed significant differences for all paired measurements at $P < 0.01$ or better.

Additions to incubation medium	Metabolite release ($\mu\text{mol}/2\text{h}$ per g of tissue)		
	Alanine	Pyruvate	Lactate
None	1.24 $\pm$ 0.05	1.40 $\pm$ 0.10	5.06 $\pm$ 0.37
10 mM-Dichloroacetate	0.62 $\pm$ 0.09	0.19 $\pm$ 0.08	1.11 $\pm$ 0.18
3 mM-Valine	2.10 $\pm$ 0.12	1.03 $\pm$ 0.13	4.63 $\pm$ 0.58
3 mM-Valine + 10 mM-dichloroacetate	0.67 $\pm$ 0.11	0.09 $\pm$ 0.03	1.89 $\pm$ 0.15
5 mM-Glucose	1.61 $\pm$ 0.18	5.26 $\pm$ 0.28	25.3 $\pm$ 2.3
5 mM-Glucose + 10 mM-dichloroacetate	0.73 $\pm$ 0.05	0.26 $\pm$ 0.07	6.82 $\pm$ 0.04

dichloroacetate-mediated activation of the pyruvate dehydrogenase complex (McAllister *et al.*, 1973; Whitehouse *et al.*, 1974; Goodman *et al.*, 1978). The present results show that alanine release is also influenced by the availability of pyruvate. In particular, the incremental increase in alanine release in the presence of 3 mM-valine (compared with unsupplemented incubation medium) is almost completely abolished by the addition of dichloroacetate (see Table 1). This suggests that the extra alanine released by diaphragm muscle in the presence of valine is dependent on pyruvate availability and transamination (i.e. involves synthesis *de novo*) and not on the generation of pre-formed alanine via muscle proteolysis. It is also possible that valine itself may provide the carbon for alanine formation, via net intracellular pyruvate generation, as well as the amino nitrogen by transamination (see Snell & Duff, 1977, 1981, 1982).

Effect of dichloroacetate on branched-chain amino acid metabolism by hemidiaphragm from 48 h-starved rats

An increase in branched-chain amino acid release in the presence of dichloroacetate by the perfused hind-limb preparation (Goodman *et al.*, 1978) and by the intact rat *in vivo* (Blackshear *et al.*, 1975) led to the suggestion that this agent may inhibit muscle branched-chain amino acid metabolism. In contrast, dichloroacetate stimulates branched-chain amino acid metabolism in liver (Harris *et al.*, 1978; Crabb *et al.*, 1981) and in heart preparations (Sans *et al.*, 1980), although by different mechanisms. In cardiac muscle, dichloroacetate stimulates leucine oxidation by activation of the branched-chain oxo acid dehydrogenase (possibly by affecting the phosphorylation state of the enzyme), whereas in liver the stimulation is

secondary to enhanced transamination of leucine, with no effect on the branched-chain oxo acid dehydrogenase (Crabb *et al.*, 1981).

In contrast with the effects observed in other tissues, in diaphragm muscle from 48 h-starved rats 10 mM-dichloroacetate inhibited the oxidation of 3 mM-[1-¹⁴C]leucine by 49 and 64% respectively in the absence and in the presence of 5 mM-glucose in the incubation medium (Table 2). An inhibitory effect of dichloroacetate on ¹⁴CO₂ production from [1-¹⁴C]leucine has also been reported by Pardridge *et al.* (1983) in cultured myoblasts. In the present study, the locus of action of dichloroacetate on leucine oxidation was investigated by measuring 4-methyl-2-oxo[1-¹⁴C]pentanoate release in order to calculate the rate of total [1-¹⁴C]leucine transamination (see the Materials and methods section). In the present experiments the addition of glucose to the medium stimulated the total transamination of [1-¹⁴C]leucine by only 6% (statistically insignificant) in these unpaired experiments (Table 2). However, other work with the use of a paired experimental design (unsupplemented medium versus glucose-containing medium) has shown a significant (*P* < 0.01) stimulation of [1-¹⁴C]leucine transamination by an average of 19% in hemidiaphragms from starved rats (Duff, 1983). Thus glucose, by increasing the availability of pyruvate, can indirectly (see below) promote leucine transamination; this effect is abolished by dichloroacetate (Table 2). The percentage of 4-methyl-2-oxo[1-¹⁴C]pentanoate formed by leucine transamination that was further decarboxylated to ¹⁴CO₂ was not significantly affected by the presence of dichloroacetate: 82.0 and 80.7% in the presence and in the absence of dichloroacetate respectively in glucose-free medium, and 86.4 and 88.3% respectively in glucose-supplemented medium (Table 2). These findings suggest that the

Table 2. *Effect of 10 mM-dichloroacetate on 3 mM-[1-¹⁴C]leucine metabolism by hemidiaphragms from 48 h-starved rats*

Hemidiaphragms prepared from 48 h-starved rats were incubated (see Table 1 and the Materials and methods section) with 3 mM-[1-¹⁴C]leucine and other additions as shown below. Paired incubations were carried out in the presence and in the absence of 10 mM-sodium dichloroacetate. The production of ¹⁴CO₂ and the release of 4-methyl-2-oxo[1-¹⁴C]pentanoate were measured as described in the Materials and methods section. The total rate of transamination of [1-¹⁴C]leucine was calculated as the sum of ¹⁴CO₂ production and 4-methyl-2-oxo[1-¹⁴C]pentanoate release. Values are the means ± S.E.M. for four experiments, and statistical analysis (paired Student's *t* test) showed significant differences for all paired measurements at *P* < 0.001 or better.

Additions to incubation medium	Metabolic activity (μmol/2h per g of tissue)		
	¹⁴ CO ₂ production	4-Methyl-2-oxo-[1- ¹⁴ C]pentanoate release	Total transamination of [1- ¹⁴ C]leucine
None	5.44 ± 0.28	1.30 ± 0.15	6.74 ± 0.22
10 mM-Dichloroacetate	2.77 ± 0.03	0.61 ± 0.07	3.38 ± 0.08
5 mM-Glucose	6.33 ± 0.31	0.84 ± 0.14	7.17 ± 0.39
5 mM-Glucose + 10 mM-dichloroacetate	2.28 ± 0.15	0.36 ± 0.09	2.64 ± 0.23

inhibitory action of dichloroacetate on leucine oxidation may be due to decreased transamination rather than an inhibition at the branched-chain oxo acid dehydrogenase step. However, no direct effect of dichloroacetate on leucine aminotransferase activity in diaphragm homogenates was found [0.517 ± 0.029 (4) and 0.539 ± 0.031 (4) $\mu\text{mol/min}$ per g of tissue for incubations in the absence and in the presence of 10mM-dichloroacetate respectively].

To investigate further the action of dichloroacetate on branched-chain amino acid metabolism, a comparison was made of the effects on $[1-^{14}\text{C}]$ - and $[\text{U}-^{14}\text{C}]$ -leucine oxidation. Leucine was studied because, unlike the other branched-chain amino acids, it is metabolized ultimately to acetyl-CoA and acetoacetate without the participation of the pyruvate dehydrogenase complex, which is known to be affected by dichloroacetate. As in the previous experiments (Table 2), dichloroacetate inhibited $[1-^{14}\text{C}]$ leucine metabolism by decreasing transamination and by decreasing the release of 4-methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate without any appreciable effect on the percentage of total 4-methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate that is further decarboxylated (Table 3). Dichloroacetate decreased $^{14}\text{CO}_2$ production from both $[1-^{14}\text{C}]$ leucine (by 62%) and $[\text{U}-^{14}\text{C}]$ leucine (by 61%) by similar extents. The ratio of $^{14}\text{CO}_2$ produced from $[\text{U}-^{14}\text{C}]$ leucine to that produced from $[1-^{14}\text{C}]$ leucine was not affected by dichloroacetate (Table 3). The ratio of $^{14}\text{CO}_2$ produced from $[\text{U}-^{14}\text{C}]$ leucine to that produced from $[1-^{14}\text{C}]$ leucine provides a measure of the extent to which the amino acid

subsequent to decarboxylation at the C-1 position are completely oxidized to CO_2 , the expected ratio would be 6.0. If decarboxylation at C-1 is the only source of $^{14}\text{CO}_2$, the ratio would be 1.0. The intermediate values obtained in the present work show that oxidation (of $[\text{U}-^{14}\text{C}]$ leucine) involves additional decarboxylation reactions to that involving branched-chain oxo acid dehydrogenase, but also that the carbon skeleton was not completely oxidized. Dichloroacetate therefore has no effect on the subsequent oxidation of leucine beyond the branched-chain oxo acid dehydrogenase step.

The attenuation of leucine oxidation by dichloroacetate could be due to inhibition of branched-chain oxo acid dehydrogenase. However, the proportion of 4-methyl-2-oxopentanoate that is oxidized through the branched-chain oxo acid dehydrogenase step is not affected by dichloroacetate (Table 2). Furthermore, if dichloroacetate blocked branched-chain oxo acid dehydrogenase, leading to 4-methyl-2-oxopentanoate accumulation, it is unlikely that release of the oxo acid would be decreased (Table 2). A simpler explanation for the effect of dichloroacetate is that the enhanced oxidation of pyruvate limits its availability for the regeneration of 2-oxoglutarate required for branched-chain amino acid transamination. [An increase in intracellular glutamate content in muscle from starved rats treated with dichloroacetate *in vivo* (Goodman *et al.*, 1978) is consistent with this explanation.] Since branched-chain amino acids do not transaminate directly with pyruvate but are metabolized by coupled transamination reactions:

carbon skeleton is totally oxidized (Zapalowski *et al.*, 1981; Snell & Duff, 1982). If all carbon atoms

the increased oxidation of pyruvate, through activation of pyruvate dehydrogenase by dichloro-

Table 3. Effect of 10mM-dichloroacetate on the decarboxylation of 3mM- $[1-^{14}\text{C}]$ - and $[\text{U}-^{14}\text{C}]$ -leucine by hemidiaphragms from 48h-starved rats

Incubations were as indicated in Table 2. Rates of leucine decarboxylation are expressed on the basis of equal specific radioactivities of $[1-^{14}\text{C}]$ - or $[\text{U}-^{14}\text{C}]$ -leucine in the incubation medium. The ratio of leucine decarboxylation refers to the ratio of $^{14}\text{CO}_2$ production from $[\text{U}-^{14}\text{C}]$ leucine to $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ leucine expressed on the basis of equal radioactivities of radiolabelled carbon atoms in the two radiolabelled derivatives. Other details are as indicated in Table 2. Values are the means $\pm$ S.E.M. for four experiments, and statistical analysis (paired Student's *t* test) showed significant differences for all paired measurements at $P < 0.01$ or better, except for the leucine decarboxylation ratios.

Incubation additions . . .	Metabolic activity ($\mu\text{mol/2h}$ per g of tissue)	
	5mM-Glucose	5mM-Glucose + 10mM-dichloroacetate
$[1-^{14}\text{C}]$ Leucine decarboxylation	6.50 ± 0.62	2.45 ± 0.07
4-Methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate release	1.05 ± 0.03	0.53 ± 0.09
$[1-^{14}\text{C}]$ Leucine transamination	7.55 ± 0.63	2.98 ± 0.02
$[\text{U}-^{14}\text{C}]$ Leucine decarboxylation	19.20 ± 2.40	7.44 ± 0.48
$[\text{U}-^{14}\text{C}]/[1-^{14}\text{C}]$ -Leucine decarboxylation	2.95 ± 0.42	3.04 ± 0.24

acetate, leads to diminished alanine formation by a dual mechanism. It limits the availability of pyruvate carbon for transamination to form alanine, and indirectly it diminishes branched-chain amino acid transamination. The impaired transamination of branched-chain amino acids would account for the decrease in oxo acid production and release and the decrease in amino acid decarboxylation observed in the present study. To the extent that oxidation of valine and isoleucine can provide carbon for alanine formation (see the introduction), dichloroacetate would also act in part to diminish the flow of amino acid carbon for alanine formation. The interference by dichloroacetate in the gluconeogenic flow of amino acid precursors to muscle alanine formation will diminish the availability of alanine for hepatic gluconeogenesis and contribute to the hypoglycaemic action of this agent *in vivo*.

We are grateful to the Wellcome Trust for financial support.

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