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Adrenergic inhibition of branched-chain 2-oxo acid dehydrogenase in rat diaphragm muscle *in vitro*

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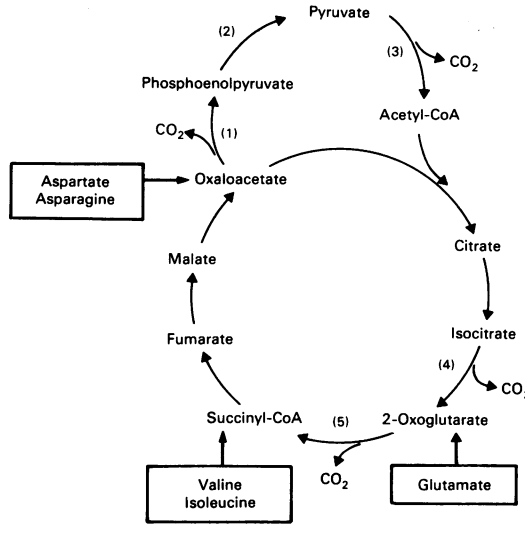
1. In theory, the complete oxidation to CO_2 of amino acids that are metabolized by conversion into tricarboxylic acid-cycle intermediates may proceed via their conversion into acetyl-CoA. The possible adrenergic modulation of this oxidative pathway was investigated in isolated hemidiaphragms from 40h-starved rats. 2. Adrenaline (5.5 μM), phenylephrine (0.49 mM) and dibutyryl cyclic AMP (10 μM) inhibited $^{14}CO_2$ production from 3 mM-[U-^{14}C]valine by 35%, 28% and 19% respectively. At the same time, these agents stimulated glycogen mobilization (measured as a decrease in glycogen content) and glycolysis (measured as lactate release). 3. Adrenaline, phenylephrine and dibutyryl cyclic AMP did not inhibit $^{14}CO_2$ production from 3 mM-[U-^{14}C]aspartate or 3 mM-[U-^{14}C]glutamate, although, as in the presence of valine, the agents stimulated glycogen mobilization and glycolysis. The rate of proteolysis (measured as tyrosine release in the presence of cycloheximide) was not changed by adrenaline. 4. The data indicate that the adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]valine was not a consequence of radiolabel dilution. Inhibition was apparently specific for branched-chain amino acid metabolism in that the adrenergic agonists also inhibited $^{14}CO_2$ production from [1-^{14}C]valine, [1-^{14}C]leucine and [U-^{14}C]isoleucine. Since $^{14}CO_2$ production from the 1-^{14}C-labelled substrates is a specific measure of decarboxylation in the reaction catalysed by the branched-chain 2-oxo acid dehydrogenase complex, it is at this site that the adrenergic agents are concluded to act.

Certain amino acids are metabolized by conversion into tricarboxylic acid-cycle intermediates. Skeletal muscle has a restricted capacity to utilize amino acids and is only able to convert glutamate (cycle entry at 2-oxoglutarate), valine and isoleucine (cycle entry at succinyl-CoA), and aspartate and asparagine (cycle entry at oxaloacetate) into tricarboxylic acid-cycle intermediates (Chang & Goldberg, 1978*b*). Complete oxidation to CO_2 of such intermediates cannot be achieved simply by turnover of the cycle (with the concomitant input of acetyl-CoA), but requires conversion of the intermediates into acetyl-CoA (Goldstein & Newsholme, 1976; Palmer & Sugden, 1983; Palmer *et al.*, 1983). In skeletal muscle, oxaloacetate may be converted into acetyl-CoA by the enzymes phosphoenolpyruvate carboxykinase (EC 4.1.1.32), pyruvate kinase (EC 2.7.1.40) and the pyruvate dehydrogenase complex (Goldstein & Newsholme, 1976). This pathway is shown in Scheme 1. Alternatively, malic enzyme [EC 1.1.1.40; malate dehydrogenase (decarboxylating), $NADP^+$-dependent] may act to decarboxylate malate to pyruvate (Garber *et al.*, 1976*a*). Tricarboxylic acid-cycle intermediates diverted into pyruvate (and phosphoenolpyruvate) formation not only may be oxidized (via acetyl-CoA) but may also in theory act as precursors in the synthesis *de novo* of glycogen (Connett, 1979; Odedra & Palmer, 1981), alanine and lactate.

In starvation, alanine released by skeletal muscle is an important precursor in hepatic gluconeogenesis, and together with glutamine this amino acid accounts for over 50% of the amino acids released by skeletal muscle (even though alanine and glutamine comprise only approx. 10% of muscle protein). There is some controversy as to whether alanine is synthesized *de novo* from amino acids via phosphoenolpyruvate carboxykinase (Snell & Duff, 1977, 1981, 1983; Duff & Snell, 1982; reviewed by Snell, 1980) and pyruvate kinase (Palmer *et al.*,



Scheme 1. *Putative pathway whereby amino acids that enter intermediary metabolism via conversion into tricarboxylic acid cycle-intermediates undergo complete oxidation to CO_2*

Glutamate, valine, isoleucine, aspartate and asparagine are presumed to enter the cycle and be converted into oxaloacetate. The phosphoenolpyruvate carboxykinase, pyruvate kinase and pyruvate dehydrogenase reactions convert oxaloacetate into acetyl-CoA via the reaction sequence:

$$\text{Oxaloacetate} \longrightarrow \text{phosphoenolpyruvate} \dashrightarrow \text{pyruvate} \longrightarrow \text{acetyl-CoA}$$

The acetyl-CoA so formed enters the cycle in the citrate synthase reaction. The overall stoichiometry is:

$$1\ \text{Oxaloacetate} \longrightarrow 4CO_2$$

Key to enzymes: (1) phosphoenolpyruvate carboxykinase; (2) pyruvate kinase; (3) pyruvate dehydrogenase; (4) isocitrate dehydrogenase; (5) 2-oxoglutarate dehydrogenase.

1982), or whether the carbon skeleton of alanine is predominantly derived from glycolytically produced pyruvate (Chang & Goldberg, 1978*a*,*b*). If the latter is correct, then flux via phosphoenolpyruvate carboxykinase and/or malic enzyme must be presumed to be minimal and the question is posed whether skeletal muscle is able to oxidize tricarboxylic acid-cycle intermediates completely to CO_2.

Given the importance of alanine as a gluconeogenic precursor, it is hardly surprising that alanine production in skeletal muscle is under complex multisite control. The rate of proteolysis is subject to dietary and endocrine regulation (Karl *et al.*, 1976; Garber *et al.*, 1976*b*). The activities of key enzymes change in starvation: phosphoenolpyruvate carboxykinase activity is increased (Snell & Duff, 1979), whereas the concentration of active (dephosphorylated) pyruvate dehydrogenase complex (but not total complex) is decreased (Caterson *et al.*, 1982). The present study investigates the acute adrenergic control of amino acid oxidation in isolated rat hemidiaphragm.

Materials and methods

Materials

L-[U-^{14}C]Valine, L-[1-^{14}C]valine, L-[U-^{14}C]-glutamate, L-[U-^{14}C]aspartate, L-[1-^{14}C]leucine and L-[U-^{14}C]isoleucine were obtained from Amersham International, Amersham, Bucks., U.K. Adrenaline was obtained from Phoenix Pharmaceuticals (Gloucester, U.K.), phenylephrine hydrochloride from Sigma (London) Chemical Co. (Poole, Dorset, U.K.), and N^6,$O^{2'}$-dibutyryladenosine 3′:5′-monophosphate (dibutyryl cyclic AMP) was from Boehringer Corp. (London) Ltd. (Lewes, East Sussex, U.K.). Other biochemical reagents and amino acids were from Sigma or Boehringer. All reagent chemicals were of analytical grade.

Animals

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

Experimental

Hemidiaphragms from 40h-starved rats, isolated as described elsewhere (Odedra & Palmer, 1981), were preincubated for 30min at 37°C in 2ml of substrate-free Krebs–Ringer bicarbonate buffer, pH 7.4 [equilibrated with O_2/CO_2 (19:1)] to deplete glycogen stores. Hemidiaphragms were then blotted dry and transferred to 25ml flasks (containing central glass wells) containing 2ml of Krebs–Ringer bicarbonate buffer with the additions specified in the text. These flasks were incubated and treated as described elsewhere (Palmer *et al.*, 1982), and $^{14}CO_2$ production from labelled substrates (calculated as μmol/2h per g wet wt.) was determined by $^{14}CO_2$ entrapment in Hyamine 10-X hydroxide solution (scintillation grade, 1M in methanol; BDH Chemicals, Poole, Dorset, U.K.) after acidification [with $HClO_4$ (60%, w/v)] of the incubation media. The KOH-neutralized $HClO_4$ extracts were assayed for lactate, pyruvate and alanine (calculated as μmol/g wet wt.) as described previously (Palmer *et al.*, 1982). Glycogen was determined in hemidiaphragms extracted (100°C, 5min) in 0.3ml of 30% (w/v) KOH. Extracts were cooled and treated overnight at 0.4°C with 0.8ml of ethanol containing one drop of saturated Na_2SO_4. The resultant precipitate was isolated by centrifugation (approx. 10000rev./min for 6min: Eppendorf centrifuge), washed with 1ml of ethanol and taken up in 1ml of water. Glycogen content (referred to as residual glycogen) was determined at the end of the 2h incubation period, and after a further 2h period during which the flasks (treated with $HClO_4$) were shaken to allow equilibration of $^{14}CO_2$ with Hyamine (diaphragm glycogen was stable to the latter treatment). This method of glycogen determination yielded fractionally higher values for diaphragm glycogen content than those reported previously from this laboratory (Palmer *et al.*, 1982). Tyrosine determinations were kindly undertaken by Dr. P. H. Sugden, by the method of Rubin & Goldstein (1970).

Results are shown as means ± S.E.M., with the numbers of observations in parentheses. For each rat diaphragm, one hemidiaphragm was treated with specified additions, whereas the second served as a control. Accordingly, statistical analysis, unless otherwise specified, was by Student's *t* test for paired data. To simplify the construction of the Tables, the controls for each treatment group are not shown. Instead the means of these controls are shown.

Results

Adrenergic inhibition of [U-^{14}C]valine oxidation

Hemidiaphragms from 40h-starved rats were incubated for 2h in Krebs–Ringer bicarbonate with 3mM-[U-^{14}C]valine. Adrenaline, phenylephrine and dibutyryl cyclic AMP inhibited $^{14}CO_2$ production from [U-^{14}C]valine by 35%, 28% and 19% respectively (Table 1). $^{14}CO_2$ production from 3mM-[U-^{14}C]valine was not strictly linear with time, but increased somewhat disproportionately with time, i.e. in one experiment $^{14}CO_2$ production (calculated as μmol/g wet wt.) at 30, 60 and 120min incubation was 1.1 ± 0.04 (4), 2.6 ± 0.2 (4) and 6.7 ± 0.3 (7) respectively. Nonetheless, in the same experiment, inhibition by adrenaline of $^{14}CO_2$ production from [U-^{14}C]valine was constant with time, i.e. inhibition (%) at 30, 60 and 120min was 34.5 ± 4.8 (4), 39.1 ± 4.6 (4) and 37.0 ± 4.2 (7).

The procedure adopted of preincubating the hemidiaphragms in substrate-free Krebs–Ringer bicarbonate did not result in the total depletion of glycogen stores. After preincubation, glycogen content was 6.8 ± 0.4 μmol of glucose equivalents/g wet wt. Incubation of hemidiaphragms with 3mM-[U-^{14}C]valine led to glycogen mobilization [4.1 ± 0.8 μmol of glucose equivalents mobilized/2h per g wet wt. ($n = 5$)]. The adrenergic agents decreased residual glycogen (i.e. diaphragm glycogen content at the completion of the 2h incubation period) by 50–80% (Table 1). Lactate production was increased by 44%, 49% and 21% by adrenaline, phenylephrine and dibutyryl cyclic AMP

Table 1. *Metabolism of 3mM-[U-^{14}C]valine by hemidiaphragms from 40h-starved rats*

The concentrations used of adrenaline, phenylephrine and dibutyryl cyclic AMP were 5.5μM, 0.49mM and 10μM respectively. For details see the Experimental section. 'Residual glycogen' refers to the glycogen content of the hemidiaphragms at the end of the 2h incubation period. *$P < 0.05$; **$P < 0.01$.

Additions to incubation media	$^{14}CO_2$ (μmol/2h per g wet wt.)	Metabolites released into medium (μmol/2h per g wet wt.)		Residual glycogen (μmol/g wet wt.)
		Lactate	Alanine	
None	6.9 ± 0.3 (23)	7.5 ± 0.7 (28)	4.1 ± 0.4 (28)	1.6 ± 0.2 (21)
Adrenaline	4.5 ± 0.3 (8)**	10.8 ± 0.8 (14)**	4.4 ± 0.5 (12)	0.3 ± 0.1 (6)**
Phenylephrine	5.0 ± 0.3 (8)**	11.2 ± 1.0 (8)*	3.9 ± 0.4 (7)	0.5 ± 0.04 (8)**
Dibutyryl cyclic AMP	5.6 ± 0.4 (7)**	9.1 ± 0.6 (6)*	4.3 ± 0.5 (7)	0.8 ± 0.2 (7)

respectively (Table 1). Adrenaline and phenylephrine (but not dibutyryl cyclic AMP) markedly increased the [lactate]/[pyruvate] ratio [control ratio 8.3 ± 1.1 (28); + adrenaline (13), 13.5 ± 1.6 ($P < 0.05$); + phenylephrine (8), 12.4 ± 1.7 ($P < 0.05$)]. Despite the apparent inhibition of valine oxidation and the accompanying stimulation of glycolytic flux, production of pyruvate and alanine was not significantly altered by adrenaline, phenylephrine or dibutyryl cyclic AMP. The mean rate of release (μmol/2h per g wet wt.) of pyruvate was 0.9 ± 0.1 ($n = 56$) (results not shown).

Theoretically $^{14}CO_2$ may be produced from [U-^{14}C]valine in the decarboxylation of α-oxoisovalerate (3-methyl-2-oxobutanoate), in the decarboxylation of methylmalonate semialdehyde, and in the reactions catalysed by phosphoenolpyruvate carboxykinase, the pyruvate dehydrogenase complex, NAD^+:isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex (see Scheme 1). If the metabolism of valine in diaphragm muscle proceeds via pyruvate and acetyl-CoA, then the apparent adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]valine could result from ^{14}C-label dilution in the cytosolic pyruvate pool (secondary to adrenergic stimulation of glycogenolysis and pyruvate production via glycolysis). Were the specific radioactivity of cytosolic pyruvate (derived from [U-^{14}C]valine) to be decreased in this manner, then $^{14}CO_2$ production in the pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase reactions would be decreased. To investigate this possibility hemidiaphragms from 40h-starved rats were incubated with 3mM-[U-^{14}C]-valine in the presence of 20mM-glucose (Table 2). Under these conditions there was net glycogen synthesis and a 5-fold increase in the rates of lactate and pyruvate release (cf. Tables 2 and 1). Nonetheless, lactate production was further increased by adrenaline (by 34%) and the glycogen content was decreased. Adrenaline was without significant effect on the rate of pyruvate release: mean 4.2 ± 0.6 μmol/2h per g wet wt. ($n = 20$). The [lactate]/[pyruvate] ratio increased with adrenaline from 9.2 ± 0.6 to 12.9 ± 1.1 ($P < 0.01$). Alanine release was inhibited by adrenaline (by 15%). Therefore, although the provision of glucose increased glycolytic flux, adrenaline still inhibited (by 32%) $^{14}CO_2$ production from [U-^{14}C]valine. Moreover, the rates of $^{14}CO_2$ production from [U-^{14}C]valine were similar, irrespective of whether glucose was present, implying that the mechanism of adrenergic inhibition of $^{14}CO_2$ production is not dependent on the specific radioactivity of pyruvate (cf. Tables 1 and 2).

Lack of adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]glutamate and [U-^{14}C]aspartate

To attempt to identify the site(s) of adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]valine, the effect of the agents on $^{14}CO_2$ production from [U-^{14}C]glutamate and [U-^{14}C]aspartate was examined. The metabolism of glutamate and aspartate, like that of valine, proceeds by their conversion into tricarboxylic acid-cycle intermediates. If the putative pathway for the complete oxidation of these intermediates is correct (shown in Scheme 1), four decarboxylation reactions are involved, catalysed by phosphoenolpyruvate carboxykinase, the pyruvate dehydrogenase complex, NAD^+:isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex.

$^{14}CO_2$ production from 3mM-[U-^{14}C]glutamate was not inhibited by adrenaline, phenylephrine or dibutyryl cyclic AMP (Table 3). Nonetheless, these agents increased lactate production (by 40%, 21% and 62% with adrenaline, phenylephrine and dibutyryl cyclic AMP respectively). Alanine production was approx. 70% greater with glutamate than with valine as substrate (cf. Tables 3 and 1). Pyruvate production was 0.4 ± 0.03 μmol/2h per g wet wt. ($n = 29$) and was not altered significantly by adrenaline, phenylephrine or dibutyryl cyclic AMP. Analogous results were obtained from [U-^{14}C]-aspartate as substrate: $^{14}CO_2$ production from [U-^{14}C]aspartate was insensitive to inhibition by adrenaline, phenylephrine and dibutyryl cyclic AMP (Table 4). This insensitivity of $^{14}CO_2$ production to adrenergic agonists was evident despite the fact that glycolytic flux was increased. Lactate release was increased by 84%, 64% and 46% with adrenaline, phenylephrine and dibutyryl cyclic AMP respectively.

Table 2. *Metabolism of 3mM-[U-^{14}C]valine by hemidiaphragms from 40h-starved rats in the presence of 20mM-glucose*
For details see the Experimental section. 'Residual glycogen' refers to the glycogen content of the hemidiaphragms at the end of the 2h incubation period. $^*P < 0.05$; $^{**}P < 0.01$.

Additions to incubation media	$^{14}CO_2$ (μmol/2h per g wet wt.)	Metabolites released into medium (μmol/2h per g wet wt.)		Residual glycogen (μmol/g wet wt.)
		Lactate	Alanine	
None	7.2 ± 0.4 (10)	39.6 ± 2.9 (10)	7.8 ± 0.3 (10)	9.6 ± 0.3 (10)
Adrenaline (5.5 μM)	4.9 ± 0.4 (10)**	59.9 ± 3.8 (10)**	6.6 ± 0.3 (10)*	1.9 ± 0.9 (10)**

Table 3. *Metabolism of 3 mM-[U-^{14}C]glutamate by hemidiaphragms from 40 h-starved rats*
The concentrations used of adrenaline, phenylephrine and dibutyryl cyclic AMP are as specified in Table 1. For other details see the Experimental section. 'Residual glycogen' refers to the glycogen content of the hemidiaphragms at the end of the 2 h incubation period. $*P < 0.05$; $**P < 0.01$.

Additions to incubation media	$^{14}CO_2$ (μmol/2 h per g wet wt.)	Metabolites released into medium (μmol/2 h per g wet wt.)		Residual glycogen (μmol/g wet wt.)
		Lactate	Alanine	
None	10.2 ± 0.6 (18)	7.7 ± 1.1 (21)	7.1 ± 0.4 (11)	1.8 ± 0.2 (12)
Adrenaline	9.8 ± 0.6 (4)	10.8 ± 1.1 (4)*	6.8 ± 0.3 (3)	0.3 ± 0.1 (3)**
Phenylephrine	11.6 ± 0.6 (4)	9.3 ± 0.9 (3)	7.3 ± 0.5 (4)	0.6 ± 0.2 (3)*
Dibutyryl cyclic AMP	9.6 ± 0.7 (4)	12.5 ± 0.6 (3)	7.1 ± 0.3 (4)	0.6 ± 0.1 (4)*

Table 4. *Metabolism of 3 mM-[U-^{14}C]aspartate by hemidiaphragms from 40 h-starved rats*
The concentrations used of adrenaline, phenylephrine and dibutyryl cyclic AMP are as specified in Table 1. For other details see the Experimental section. 'Residual glycogen' refers to the glycogen content of the hemidiaphragms at the end of the 2 h incubation period. $*P < 0.05$; $**P < 0.01$.

Additions to incubation media	$^{14}CO_2$ (μmol/2 h per g wet wt.)	Lactate released into medium (μmol/2 h per g wet wt.)	Residual glycogen (μmol/g wet wt.)
None	7.9 ± 0.2 (17)	9.1 ± 0.9 (18)	1.5 ± 0.4 (17)
Adrenaline	8.0 ± 0.4 (4)	16.7 ± 0.9 (3)*	0.2 ± 0.02 (3)*
Phenylephrine	8.2 ± 0.1 (4)	14.9 ± 0.5 (4)**	0.3 ± 0.03 (3)*
Dibutyryl cyclic AMP	8.0 ± 0.3 (3)	13.3 ± 1.8 (3)	0.9 ± 0.3 (4)

Adrenergic inhibition of the branched-chain 2-oxo acid dehydrogenase complex

Since $^{14}CO_2$ production from [U-^{14}C]valine, but not from [U-^{14}C]glutamate or [U-^{14}C]aspartate, is inhibited by adrenaline, phenylephrine and dibutyryl cyclic AMP, it is suggested that inhibition occurs at a site(s) specific to the pathway of valine conversion into succinyl-CoA. It cannot be precluded, however, that the cellular and/or mitochondrial uptake of glutamate and aspartate (but not of valine) is rate-limiting to their oxidation to CO_2. $^{14}CO_2$ production from [1-^{14}C]valine (as opposed to [U-^{14}C]valine) is a specific measure of flux through the branched-chain 2-oxo acid dehydrogenase (EC 1.2.4.4) reaction. Adrenaline and phenylephrine (but not dibutyryl cyclic AMP) inhibited $^{14}CO_2$ production from 3 mM-[1-^{14}C]valine by 29% and 23% respectively ($P < 0.05$). The rates of $^{14}CO_2$ production (μmol/2 h per g wet wt.) were: control, 3.1 ± 0.3 (12); + adrenaline (5.5 μM), 2.2 ± 0.1 (5); + phenylephrine (0.49 mM), 2.4 ± 0.1 (4); + dibutyryl cyclic AMP (10 μM), 3.1 ± 0.1 (3). The corresponding inhibition by adrenaline and phenylephrine of $^{14}CO_2$ production from [U-^{14}C]valine was 35% and 28% respectively (see Table 1). Values determined for lactate, pyruvate, alanine and glycogen with hemidiaphragms provided with [1-^{14}C]valine were confirmed to be similar (results not shown) to those obtained with [U-^{14}C]valine (shown in Table 1).

The branched-chain 2-oxo acid dehydrogenase complex is also implicated in the metabolism of isoleucine and leucine. $^{14}CO_2$ production from [U-^{14}C]isoleucine by hemidiaphragms was inhibited by adrenaline (21%). Although phenylephrine and dibutyryl cyclic AMP inhibited $^{14}CO_2$ production (by 10% and 8% respectively), inhibition was not statistically significant (Table 5). Glycogenolysis and lactate production were stimulated by the adrenergic agents, and the [lactate]/[pyruvate] ratio was increased from 7.3 ± 0.6 (control, $n = 20$) to 9.5 ± 0.5 (+ adrenaline; $P < 0.05$) or 10.8 ± 0.7 (+ phenylephrine; $P < 0.02$) or 8.7 ± 0.9 (+ dibutyryl cyclic AMP; $P < 0.05$). Production of pyruvate and alanine was not significantly altered by the adrenergic agonists in the presence of 3 mM-[U-^{14}C]isoleucine.

$^{14}CO_2$ production from [1-^{14}C]leucine, unlike that from [U-^{14}C]leucine, depends specifically on flux through the branched-chain 2-oxo acid dehydrogenase reaction. $^{14}CO_2$ production from 3 mM-[1-^{14}C]leucine (like that from [1-^{14}C]valine) was inhibited by adrenaline (14%), phenylephrine (7%) and dibutyryl cyclic AMP (12%) (Table 5). Lactate production in the presence of 3 mM-[1-^{14}C]leucine was significantly less ($P < 0.02$, unpaired t test; see also Chang & Goldberg, 1978*c*) than in the presence of valine, isoleucine, aspartate or glutamate (see Tables 1 and 5). Nonetheless, lactate production in the presence of [1-^{14}C]leucine was stimulated by adrenaline (142%), phenylephrine (113%) and

Table 5. *Metabolism of 3 mM-[U-^{14}C]isoleucine or 3 mM-[1-^{14}C]leucine by hemidiaphragms from 40 h-starved rats*
The concentrations used of adrenaline, phenylephrine and dibutyryl cyclic AMP are as specified in Table 1. For other details see the Experimental section. 'Residual glycogen' refers to the glycogen content of the hemidiaphragms at the end of the 2 h incubation period. $*P < 0.05$; $**P < 0.01$.

Substrate	Additions to incubation media	CO_2 (μmol/2 h per g wet wt.)	Metabolite released into medium (μmol/2 h per g wet wt.) Lactate	Alanine	Residual glycogen (μmol/g wet wt.)
[U-^{14}C]Isoleucine	None	23.9 ± 1.7 (18)	7.0 ± 0.5 (21)	3.3 ± 0.2 (23)	2.2 ± 0.3 (14)
	Adrenaline	18.8 ± 1.4 (6)**	13.7 ± 1.0 (7)**	3.4 ± 0.3 (7)	0.04 ± 0.02 (4)**
	Phenylephrine	21.6 ± 2.3 (5)	9.8 ± 1.0 (7)*	3.5 ± 0.2 (7)	0.1 ± 0.1 (5)**
	Dibutyryl cyclic AMP	21.9 ± 1.5 (7)	9.1 ± 0.7 (7)*	2.8 ± 0.1 (9)	0.8 ± 0.1 (5)**
[1-^{14}C]Leucine	None	4.2 ± 0.4 (24)	5.5 ± 0.4 (20)	2.4 ± 0.3 (21)	2.0 ± 0.3 (15)
	Adrenaline	3.6 ± 0.5 (8)*	13.3 ± 2.1 (7)**	2.6 ± 0.1 (6)	0.1 ± 0.1 (5)*
	Phenylephrine	3.9 ± 0.5 (8)*	11.7 ± 1.9 (6)**	2.8 ± 0.2 (7)	0.3 ± 0.03 (5)**
	Dibutyryl cyclic AMP	3.7 ± 0.1 (8)*	7.1 ± 0.7 (7)**	2.6 ± 0.1 (8)	0.3 ± 0.04 (5)**

dibutyryl cyclic AMP (40%) to rates equivalent to those observed with valine, isoleucine, aspartate or glutamate in the presence of adrenergic agonists. Production of pyruvate and alanine was not altered markedly by adrenaline, phenylephrine and dibutyryl cyclic AMP, and the [lactate]/[pyruvate] ratio was increased from 6.1 ± 0.6 (control) to 11.1 ± 0.7 (+adrenaline; $P < 0.02$), 10.6 ± 0.8 (+phenylephrine; $P < 0.02$) and 8.9 ± 0.6 (+dibutyryl cyclic AMP; $P < 0.05$).

Effects of adrenergic agonists on proteolysis

Diaphragms from 40 h-starved rats were incubated with 3 mM-valine + 0.5 mM-cycloheximide, and tyrosine release was determined. Adrenaline did not alter tyrosine release: mean tyrosine release was 870 ± 38 nmol/h per g wet wt. ($n = 8$). Tyrosine release under conditions when protein synthesis is blocked is a specific measure of the rate of proteolysis (Fulks *et al.*, 1975).

Discussion

Adrenaline, phenylephrine and dibutyryl cyclic AMP clearly inhibit $^{14}CO_2$ production from [U-^{14}C]valine by rat hemidiaphragms from 40 h-starved rats *in vitro*. The complete oxidation of valine to CO_2 is presumed (Scheme 1) to involve six possible decarboxylation reactions, namely those catalysed by branched-chain 2-oxo acid dehydrogenase, methylmalonate semialdehyde dehydrogenase, phosphoenolpyruvate carboxykinase, the pyruvate dehydrogenase complex, NAD^+:isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex. It is concluded that the adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]valine results from the inhibition of the branched-chain 2-oxo acid dehydrogenase complex. The evidence in support of this conclusion is as follows.

(1) Adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]valine is not simply a function of ^{14}C-label dilution in the cytosolic [^{14}C]pyruvate pool secondary to the adrenergic stimulation of glycogenolysis and glycolysis. $^{14}CO_2$ is produced exclusively from [1-^{14}C]valine in the branched-chain 2-oxo acid dehydrogenase reaction. Therefore the ratio of $^{14}CO_2$ produced from [U-^{14}C]valine to $^{14}CO_2$ produced from [1-^{14}C]valine is a specific measure of the number of decarboxylation reactions in valine catabolism. In theory, complete oxidation of valine to CO_2 would result in a ratio of 5. In the present study the ratio was calculated to be 2.1. This agrees with a ratio of 2.2 observed with rat hemidiaphragms from 48 h-starved rats provided with 3 mM-[U-^{14}C]- or -[1-^{14}C]-valine and 5 mM-glucose (Snell & Duff, 1982). A ratio of 2.1–2.2 indicates that valine oxidation to CO_2 is incomplete and may be consistent with the sequential decarboxylation of oxoisovalerate and methylmalonate semialdehyde coupled with partial decarboxylation in the phosphoenolpyruvate carboxykinase reaction. In starvation, the pyruvate dehydrogenase complex in rat diaphragm is predominantly (>98%) in the inactive phosphorylated form (Caterson *et al.*, 1982), and this may partly account for the incomplete oxidation of valine to CO_2. It is presumably because the pyruvate dehydrogenase, NAD^+:isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase reactions make such a minor contribution to net $^{14}CO_2$ production from ^{14}C-labelled tricarboxylic acid-cycle intermediates that dilution of the specific radioactivity of cytosolic [^{14}C]pyruvate does not appear to inhibit $^{14}CO_2$ production from [U-^{14}C]valine, [U-^{14}C]aspartate and [U-^{14}C]glutamate. It is impractical to quantify flux through the phosphoenolpyruvate carboxykinase reaction, because $^{14}CO_2$ production from U-^{14}C-labelled valine, aspartate and glutamate need not involve flux through the phosphoenolpyruvate

carboxykinase reaction. Entry of acetyl-CoA (produced by endogenous metabolism) into the tricarboxylic acid cycle coupled with label randomization in the fumarase reaction may allow $^{14}CO_2$ release without the net decarboxylation of cycle intermediates (Palmer *et al.*, 1983).

(2) Glutamate, aspartate and valine are presumed to be converted into tricarboxylic acid-cycle intermediates and thereafter share a common pathway of oxidative decarboxylation (Scheme 1). The finding therefore that $^{14}CO_2$ production from [U-^{14}C]-glutamate and [U-^{14}C]aspartate is insensitive to adrenergic inhibition suggests that the adrenergic agonists act on valine metabolism at a site not common to the metabolism of glutamate and aspartate. It cannot be precluded, however, that the uptake (cellular/mitochondrial) of glutamate and aspartate is rate-limiting to their oxidation to CO_2. Were this to apply, in theory it is feasible that adrenergic inhibition at a site common to the metabolism of valine, glutamate and aspartate would not necessarily decrease $^{14}CO_2$ production from [U-^{14}C]glutamate and [U-^{14}C]aspartate.

(3) Catecholamines inhibit alanine and glutamine formation by rat epitrochlearis muscle *in vitro* (Garber *et al.*, 1976*b*). The effect is mediated by a β-adrenoceptor and the adenylate cyclase system, and is thought to be the result of inhibition of muscle proteolysis. There is no analogous catecholamine-mediated inhibition of proteolysis (measured as tyrosine release) in isolated rat hemidiaphragm. Therefore the adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]valine cannot reasonably be attributed to alterations in the valine pool(s) size.

(4) In support of the branched-chain 2-oxo acid dehydrogenase complex being the specific site of action of adrenergic agonists is the fact that adrenaline, phenylephrine and dibutyryl cyclic AMP were non-specific in their inhibition of branched-chain amino acid oxidation: they inhibited $^{14}CO_2$ production from [U-^{14}C]valine, [1-^{14}C]valine, [U-^{14}C]isoleucine and [1-^{14}C]leucine. $^{14}CO_2$ production from [1-^{14}C]valine and [1-^{14}C]leucine is a specific measure of decarboxylation in the branched-chain 2-oxo acid dehydrogenase reaction. Since the metabolism of the three branched-chain amino acids distal to the branched-chain 2-oxo acid dehydrogenase proceeds via essentially distinct pathways, it is unlikely that there is adrenergic inhibition of a rate-limiting enzyme(s) within the distal portions of these pathways. However, the present results are not totally compatible with single-site adrenergic inhibition of branched-chain 2-oxo acid dehydrogenase: (i) whereas adrenaline and phenylephrine inhibited to an equivalent extent (23–35%) $^{14}CO_2$ production from [U-^{14}C]- and [1-^{14}C]-valine, dibutyryl AMP inhibited $^{14}CO_2$ production from [U-^{14}C]valine (by 19%) but not from [1-^{14}C]valine, and (ii) in general, adrenergic inhibition of $^{14}CO_2$ production from [U-^{14}C]isoleucine and [1-^{14}C]-leucine was less marked with [U-^{14}C]- or [1-^{14}C]-valine.

The activity of the branched-chain 2-oxo acid dehydrogenase complex is regulated by a specific phosphorylation/dephosphorylation mechanism in a manner analogous to that of the pyruvate dehydrogenase complex (Odessey, 1980): phosphorylation of the complex produces inactivation. The complex is regulated by the relative concentrations of products and substrates (inhibited by increased mitochondrial [NADH]/[NAD$^+$] and [branched-chain acyl-CoA]/[CoA] ratios. Presumably adrenaline, phenylephrine and dibutyryl cyclic AMP inhibit branched-chain 2-oxo acid dehydrogenase in diaphragm muscle via perturbation of the activation/inactivation cycle. In perfused rat liver branched-chain 2-oxo acid dehydrogenase is inhibited by α-adrenergic agonists (Buxton *et al.*, 1982) via a mechanism which involves redistribution of cell Ca^{2+}, but which is not strictly coupled to increases in the mitochondrial [NADH]/[NAD$^+$] ratio. Our results indicate that the adrenergic inhibition of the enzyme in diaphragm is not specific for α-adrenoceptors (since dibutyryl cyclic AMP, a β-adrenoceptor agonist, is also inhibitory) and therefore may not be strictly Ca^{2+}-dependent. It may be related to increases in the mitochondrial [NADH]/[NAD$^+$] ratio. This assumes that adrenergic agents promote increases in both cytosolic and mitochondrial [NADH]/[NAD$^+$] ratios. The availability to cells of oxidizable substrates may be important in regulating dehydrogenase activity. Incubation of heart mitochondria in the absence of substrate caused a 5–15-fold activation of the enzyme (Parker & Randle, 1978). Perfusion of isolated rat hearts with medium containing glucose (10mM) or pyruvate (10mM) is reported to phosphorylate and inactivate the 2-oxo acid dehydrogenase complex (Buxton & Olson, 1982), and subsequent substrate-free perfusion resulted in dephosphorylation and partial re-activation of the complex.

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