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The role of phosphoenolpyruvate carboxykinase in muscle alanine synthesis

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1. 3-Mercaptopicolinic acid (3-MPA) is reportedly a specific inhibitor of phosphoenolpyruvate (PEP) carboxykinase and has hitherto been used accordingly to elucidate the metabolic role of PEP carboxykinase *in vitro* and *in vivo*. 2. We show that 3-MPA has multiple effects on intermediary metabolism in hemidiaphragms from 40h-starved rats. It decreases the release of lactate+pyruvate and alanine in hemidiaphragms provided with no added substrate or with valine, leucine or isoleucine. Moreover, irrespective of the substrate provided (none, valine, leucine, isoleucine, glucose, acetate, oleate), 3-MPA decreases the [lactate]/[pyruvate] ratio. 3. 3-MPA is without effect on $^{14}CO_2$ production from [U-^{14}C]valine, [1-^{14}C]valine, [1-^{14}C]leucine, [U-^{14}C]isoleucine or [1-^{14}C]oleate, but stimulates $^{14}CO_2$ production from [U-^{14}C]glucose and [1-^{14}C]pyruvate and inhibits $^{14}CO_2$ production from [1-^{14}C]acetate. Glycolytic flux (measured as ^3H_2O formation from [5-^{3}H]glucose) is stimulated by 3-MPA. 4. It is concluded that 3-MPA has site(s) of actions other than PEP carboxykinase and that the putative role of PEP carboxykinase in alanine synthesis *de novo* in skeletal muscle from tricarboxylic acid-cycle intermediates and related amino acids requires reappraisal.

Amino acids released from skeletal muscle constitute an important source of carbon for gluconeogenesis in starvation (see Ruderman, 1975; Felig, 1975; Snell, 1980). Alanine and glutamine account for over 50% of the amino acids released by muscle in starvation, although they comprise less than 10% of the component amino acids present in skeletal-muscle proteins. The idea of a key role for alanine as a precursor in hepatic gluconeogenesis has been widely accepted (see Snell, 1980).

Alanine is formed in the alanine aminotransferase reaction by the transamination of glutamate and pyruvate. Leucine, isoleucine, valine and aspartate contribute amino groups to alanine synthesis via transamination with 2-oxoglutarate. However, controversy surrounds the origin of the pyruvate for the alanine aminotransferase reaction in muscle. One proposal has been that glucose taken up by the muscle from the bloodstream produces pyruvate via glycolysis for alanine synthesis (Felig, 1975). Although alanine synthesis by this mechanism serves to transport potentially toxic amino acid nitrogen from muscle to liver for ureogenesis, it clearly makes no addition to the total body glucose pool. An alternative proposal has been that a pathway exists in skeletal muscle for the conversion of the carbon skeletons of glutamate, valine, isoleucine, aspartate and asparagine into pyruvate for alanine synthesis *de novo* (Goldstein & Newsholme, 1976; Garber *et al.*, 1976; Snell & Duff, 1977; see Snell, 1980). The metabolism of these amino acids involves their conversion into tricarboxylic acid-cycle intermediates. It has been suggested that the oxaloacetate subsequently formed is converted into pyruvate via PEP carboxykinase (EC 4.1.1.32) and pyruvate kinase (EC 2.7.1.40) or $NADP^+$–malate dehydrogenase (malic enzyme; EC 1.1.1.40). Evidence in support of this pathway is reviewed by Snell (1980). By contrast, others, notably Chang & Goldberg (1978), conclude that tricarboxylic acid-cycle intermediates are not precursors in alanine synthesis, but contribute carbon to glutamine production.

Abbreviations used: 3-MPA, 3-mercaptopicolinate; PEP, phosphoenolpyruvate; n.s., not statistically significant.

The conclusion that PEP carboxykinase is implicated in the pathway of alanine synthesis *de novo* rests largely on the finding that 3-MPA (an inhibitor of PEP carboxykinase; Di Tullio *et al.*, 1974) inhibits valine-stimulated alanine synthesis in diaphragms from starved rats (Snell & Duff, 1977). This conclusion obviously presupposes that 3-MPA is a specific inhibitor of PEP carboxykinase. Decreased alanine release could equally be a consequence of decreased glycolytic flux, preferential conversion of available pyruvate into lactate and/or increased activity of the pyruvate dehydrogenase complex. The present work has therefore examined the effects of 3-MPA on the metabolism of the branched-chain amino acids, acetate, glucose or pyruvate, and on cytoplasmic redox state in hemidiaphragms from 40h-starved rats.

Materials and methods

Materials

L-[U-^{14}C]Valine, L-[1-^{14}C]valine, L-[1-^{14}C]-leucine, L-[U-^{14}C]isoleucine, D-[U-^{14}C]glucose, D-[5-^{3}H]glucose, [1-^{14}C]acetate, [1-^{14}C]oleate and [1-^{14}C]pyruvate were obtained from Amersham International, Amersham, Bucks., U.K. 3-Mercaptopicolinic acid was a gift from Dr. N.W. Di Tullio, SKF Laboratories, Philadelphia, PA, U.S.A. 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-dark/12h-light cycle (day started at 08:00h). Animals were fed *ad libitum* or starved for 40h before the experiments, which were commenced at 11:00–13:00h.

Experimental

Hemidiaphragms from fed or 40h-starved rats were preincubated (30min at 37°C) in substrate-free Krebs–Ringer bicarbonate buffer, pH7.4, equilibrated with O_2/CO_2 (19:1). Thereafter, hemidiaphragms were incubated as described elsewhere (Palmer *et al.*, 1982, 1983) for 2h in 2ml of Krebs–Ringer bicarbonate buffer, pH7.4, with the additions specified in the text. [1-^{14}C]Oleate (1mM) was prepared in 1.5% (w/v) bovine serum albumin in Krebs–Ringer bicarbonate. $^{14}CO_2$ (calculated as μmol/2h per g wet wt.) was determined by entrapment in Hyamine 10-X hydroxide solution (BDH Chemicals) after acidification of the incubation media with 0.2ml 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 by extraction (100°C, 5min) in 0.3ml of 30% (w/v) KOH (Palmer *et al.*, 1983).

Results are expressed as means ± S.E.M. for 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.

Results

Effects of 3-MPA on metabolite release by hemidiaphragms from 40h-starved rats

Hemidiaphragms from 40h-starved rats were incubated for 2h in Krebs–Ringer bicarbonate containing no added substrate, or valine, iso-

Table 1. *Effects of 1mM-3-MPA on release of lactate and pyruvate by hemidiaphragms from 40h-starved rats provided with different substrates*

The release of other metabolites in these experiments is shown in Table 2. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (by Student's paired *t* test) for differences between control and plus 3-MPA; †$P<0.05$, ††$P<0.01$, †††$P<0.001$ (by Student's unpaired *t* test) for differences between no added substrate and plus substrate.

		Metabolites released into medium (μmol/2h per g wet wt.)			
		Lactate + pyruvate		Ratio [lactate]/[pyruvate]	
Substrate added	No. of observations	Control	+3-MPA	Control	+3-MPA
None	12	9.8 ± 1.0	7.7 ± 0.8**	17.6 ± 2.3	7.6 ± 0.9***
Valine (3mM)	16	9.1 ± 0.6	6.7 ± 0.5**	6.7 ± 0.5†††	3.7 ± 0.13***
Isoleucine (3mM)	8	8.3 ± 0.7	7.0 ± 0.5	6.8 ± 0.7††	4.9 ± 0.6*
Leucine (3mM)	8	7.8 ± 0.8	6.5 ± 0.7**	7.6 ± 0.5††	5.0 ± 0.5*
Glucose (5mM)	18	25.9 ± 1.1†††	25.8 ± 1.0	9.3 ± 0.6††	7.4 ± 0.4*
Acetate (5mM)	6	6.5 ± 0.4†	8.4 ± 0.8*	19.7 ± 3.8	9.0 ± 0.9*
Oleate (1mM)	6	10.0 ± 0.9	10.5 ± 0.2	10.7 ± 1.4†	7.8 ± 1.6*

leucine, leucine, glucose, acetate or oleate, and measurements were made of lactate, pyruvate, alanine, glutamate and glutamine released into the medium. The results are shown in Tables 1 and 2. The total release of lactate and pyruvate was not increased by the addition of valine, isoleucine or leucine. These additions, however, decreased the [lactate]/[pyruvate] ratio by approx. 60% and increased the release of (1) alanine (by 139%, 154% and 119% by valine, isoleucine and leucine respectively), (2) glutamate (by 71%, 92% and 56% by valine, isoleucine and leucine respectively) and (3) glutamine (by 106%, 112% and 121% by valine, isoleucine and leucine respectively). Thus, despite the diversity of their metabolic fates, valine, isoleucine and leucine promote alanine, glutamate and glutamine production to similar extents. Addition of glucose (5mM) not only increased lactate + pyruvate release and decreased (by 47%) the [lactate]/[pyruvate] ratio, but also stimulated alanine production by 109%. There was no equivalent stimulation of glutamate and glutamine release. Addition of acetate decreased lactate + pyruvate release (by 33%), whereas addition of oleate decreased the [lactate]/[pyruvate] ratio by 34%. Otherwise the provision of these fat fuels had little effect on metabolite release.

In the absence of added substrate and on addition of valine, isoleucine and leucine, 3-MPA decreased the release of lactate + pyruvate by 16–26% and decreased the [lactate]/[pyruvate] ratio by 28–57%. Whereas basal alanine release was unaffected by 3-MPA, rates of valine-stimulated, isoleucine-stimulated and leucine-stimulated alanine release were decreased by 3-MPA by 13%, 45% and 19% respectively (such that stimulation was decreased to 106%, 38% and 75% respectively above basal). The decreased rates of release of lactate, pyruvate and alanine were not a consequence of decreased glycogenolysis: residual glycogen (i.e. that residual at the end of the incubation period) was not significantly affected by 3-MPA (results not shown). Glutamate and glutamine release (basal and on addition of branched-chain amino acids) was little affected by 3-MPA, except that valine-stimulated glutamine release was decreased by 15%.

With glucose as substrate, 3-MPA decreased alanine release (by 23%) and decreased the [lactate]/[pyruvate] ratio (by 21%), but release of lactate + pyruvate, glutamate and glutamine was unaffected. On provision of acetate as substrate, output of lactate + pyruvate was increased by 21% by 3-MPA. Notwithstanding, 3-MPA decreased the [lactate]/[pyruvate] ratio (by 54%). Residual glycogen was slightly decreased by 3-MPA in the presence of acetate [control (6), $1.8 \pm 0.2 \mu$mol/g wet wt.; +3-MPA (6), 1.2 ± 0.2; $P < 0.05$], and this

Table 2. *Effects of 1mM-3-MPA on release of alanine, glutamate and glutamine by hemidiaphragms from 40h-starved rats provided with different substrates*

The release of other metabolites in these experiments is shown in Table 1. $*P < 0.05$, $**P < 0.01$ (by Student's paired *t* test) for differences between control and plus 3-MPA. $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ (by Student's unpaired *t* test) for differences between no added substrate and plus substrate.

Substrate added	No. of observations	Metabolites released into medium (μmol/2h per g wet wt.)					
		Alanine		Glutamate		Glutamine	
		Control	+3-MPA	Control	+3-MPA	Control	+3-MPA
None	12	1.6±0.3	1.6±0.1	2.6±0.2	2.4±0.1	2.0±0.3	1.5±0.2
Valine (3mM)	16	3.7±0.3†††	3.3±0.4**	4.4±0.2†††	4.3±0.1	4.0±0.2†††	3.4±0.3**
Isoleucine (3mM)	8	4.0±0.5†††	2.2±0.1*	5.0±0.3†††	4.8±0.1	4.2±0.3††	4.3±0.2
Leucine (3mM)	8	3.4±0.3†††	2.8±0.3*	4.0±0.2†††	4.0±0.1	4.3±0.4††	4.6±0.2
Glucose (5mM)	18	3.3±0.3††	2.5±0.1*	3.1±0.2	2.8±0.2	2.0±0.3	2.0±0.2
Acetate (5mM)	6	1.6±0.4	1.4±0.7	1.8±0.2	2.2±0.2	2.2±0.3	2.1±0.2
Oleate (1mM)	6	2.2±0.1	2.0±0.2	—	—	—	—

may account in part for the increased release of lactate+pyruvate. In common with other substrates, with added oleate 3-MPA decreased the [lactate]/[pyruvate] ratio (by 31%); total lactate, pyruvate and alanine release was unaffected. With glucose and lactate as substrates, rates of glutamate and glutamine release were unaffected by 3-MPA.

Effects of 3-MPA on $^{14}CO_2$ production from ^{14}C-labelled substrates in hemidiaphragms from 40h-starved rats

3-MPA did not affect $^{14}CO_2$ production from [1-^{14}C]valine or [1-^{14}C]leucine (Table 3). $^{14}CO_2$ production from [1-^{14}C]valine or [1-^{14}C]leucine is a measure of decarboxylation in the branched-chain 2-oxo acid dehydrogenase (EC 1.2.4.4) reaction. The implication, therefore, is that the decreased rates of lactate+pyruvate and alanine release observed with 3-MPA with valine or leucine as substrates are not a consequence of inhibited flux through the branched-chain 2-oxo acid dehydrogenase complex. $^{14}CO_2$ production from [U-^{14}C]valine and [U-^{14}C]isoleucine was also unaffected by 3-MPA.

3-MPA increased $^{14}CO_2$ production from [U-^{14}C]glucose by 38% (Table 3). ^3H_2O formation from [5-^{3}H]glucose, a measure of glycolytic flux (Ashcroft *et al.*, 1972), was also increased by 3-MPA [control 12.3±0.7μmol/2h per g wet wt. (12); +3-MPA 16.8±0.9 (12), 37% increase, $P<0.001$]. Residual glycogen in hemidiaphragms provided with glucose was not significantly affected by 3-MPA [control (12), 3.6±0.3μmol/g wet wt.; +3-MPA (12), 4.4±0.6μmol/g wet wt.; n.s.]. It is therefore suggested that a major effect of 3-MPA is to increase utilization of extracellular glucose. Since output of lactate+pyruvate and alanine in the presence of glucose is not increased by 3-MPA (Tables 1 and 2), the 'extra' glucose taken up is presumably metabolized via the pyruvate dehydrogenase complex. Consistent with this idea, 3-MPA increased $^{14}CO_2$ production from [1-^{14}C]pyruvate by 20% (Table 3). There was a concomitant 28% decrease in the [lactate]/[pyruvate] ratio [control (8), 3.40±0.4μmol/2h per g wet wt.; +3-MPA (8), 2.5±0.4; $P<0.05$]. Thus 3-MPA may increase flux via the pyruvate dehydrogenase complex.

3-MPA produced marked (36%) inhibition of $^{14}CO_2$ production from [1-^{14}C]acetate (Table 3). $^{14}CO_2$ is lost from [1-^{14}C]acetate in the reactions of the tricarboxylic acid cycle (and also, at least in theory, via randomization of radiolabel in the cycle in the PEP carboxykinase and pyruvate dehydrogenase reactions; see Palmer & Sugden, 1983). The inhibition of $^{14}CO_2$ production from [1-^{14}C]-acetate may therefore be the result of inhibition of tricarboxylic acid-cycle flux or, alternatively, may arise secondarily to the activation of pyruvate dehydrogenase flux via radiolabel dilution in the [^{14}C]acetyl-CoA pool. $^{14}CO_2$ production from [1-^{14}C]oleate is refractory to inhibition by 3-MPA, presumably because transport is rate-limiting to oxidation.

In hemidiaphragms from fed rats, 3-MPA had no significant effect on $^{14}CO_2$ production from 1mM-[1-^{14}C]pyruvate [control (6), 12.14±0.9μmol/2h per g wet wt.; +3-MPA (6), 11.25±0.7; n.s.]. However, 3-MPA decreased the release of alanine [control (12), 3.40±0.1μmol/2h per g wet wt.; +3-MPA (12), 3.08±0.1; 9% decrease, $P<0.05$] and glutamine [control (12), 4.63±0.3μmol/2h per g wet wt.; +3-MPA (12), 2.44±0.2; 47% decrease, $P<0.001$] by hemidiaphragms from fed rats provided with 3mM-valine, the release of other metabolites being unaffected.

Discussion

Branched-chain amino acids stimulate alanine, glutamate and glutamine production by hemi-

Table 3. *Effect of 1mM-3-MPA on $^{14}CO_2$ production from ^{14}C-labelled substrates by hemidiaphragms from 40h-starved rats*
**$P<0.01$ for differences between control and plus 3-MPA.

Substrate added	No. of observations	$^{14}CO_2$ production (μmol/2h per g wet wt.) Control	+3-MPA
[U-^{14}C]Valine (3mM)	8	5.5±0.4	5.5±0.3
[1-^{14}C]Valine (3mM)	8	2.4±0.1	2.3±0.2
[1-^{14}C]Leucine (3mM)	8	4.4±0.2	4.7±0.3
[U-^{14}C]Isoleucine (3mM)	8	12.4±0.7	12.3±1.1
[U-^{14}C]Glucose (5mM)	6	11.3±0.5	15.5±1.2**
[1-^{14}C]Acetate (5mM)	6	14.7±0.9	9.4±0.9**
[1-^{14}C]Oleate (1mM)	6	1.0±0.2	0.9±0.1
[1.^{14}C]Pyruvate (1mM)	8	4.8±0.3	5.7±0.2**

diaphragms from starved rats (see also Snell & Duff, 1977; Chang & Goldberg, 1978). It warrants mention that, whereas studies *in vivo* indicate significant release by muscle of glutamine, with concomitant net uptake of glutamate (Felig, 1975), muscle preparations *in vitro* invariably release both glutamate and glutamine (Garber *et al.*, 1976; Chang & Goldberg, 1978). Leucine is predominantly (Poston, 1984), if not exclusively, ketogenic and cannot be converted to any significant extent into tricarboxylic acid-cycle intermediates or pyruvate. Nevertheless leucine has a similar capacity to valine and isoleucine (glucogenic amino acids) in promoting alanine, glutamate and glutamine output. This suggests that branched-chain amino acids stimulate alanine, glutamate and glutamine production primarily by acting as amino-group donors (see also Chang & Goldberg, 1978).

That 3-MPA inhibits valine-stimulated alanine synthesis in rat diaphragm (Snell & Duff, 1977) and extensor digitorum longus (Snell & Duff, 1981) *in vitro* is cited (see Snell, 1980) as evidence of the involvement of PEP carboxykinase in the putative pathway of alanine synthesis *de novo* from tricarboxylic acid-cycle intermediates and related amino acids. Consistent with this presumed role for PEP carboxykinase, Snell & Duff (1977) showed that 3-MPA inhibited valine-stimulated but not leucine-stimulated alanine synthesis. The presumption is that 3-MPA acts as a specific inhibitor of the cytosolic and mitochondrial forms of PEP carboxykinase (Robinson & Oei, 1975; Kostos *et al.*, 1975). A major finding of the present work is that 3-MPA has multiple metabolic effects. It stimulates glucose metabolism in isolated rat hemidiaphragm. Thus the hypoglycaemic effect of 3-MPA *in vivo* (Blackshear *et al.*, 1975; Goodman, 1975) may be attributed in part to increased peripheral glucose utilization, in addition to inhibition of gluconeogenesis. 3-MPA also increases glucose uptake in livers of starved rats (Nordlie *et al.*, 1982); this action may result from decreased hepatic concentrations of glucose 6-phosphate.

It was also observed that 3-MPA decreased efflux of lactate+pyruvate and alanine irrespective of whether the hemidiaphragms were provided with valine, isoleucine or leucine. This contrasts with the finding (Snell & Duff, 1977) that 3-MPA inhibits valine-stimulated, but not leucine-stimulated, alanine synthesis. The reason for this apparent discrepancy is not clear. In addition, whereas rates of $^{14}CO_2$ production from these amino acids were unchanged (cf. acetate, pyruvate or glucose), 3-MPA increased pyruvate formation and decreased the [lactate]/[pyruvate] ratio. Indeed, irrespective of the substrate provided (none, branched-chain amino acids, glucose, acetate, oleate), 3-MPA decreased the latter ratio. It is suggested firstly that 3-MPA may have site(s) of action other than PEP carboxykinase (a conclusion supported by its effects of glycolytic flux and glucose oxidation), and secondly that flux through PEP carboxykinase is quantitatively unimportant for $^{14}CO_2$ production from [U-^{14}C]valine and [U-^{14}C]isoleucine. These findings cast doubt on the participation of PEP carboxykinase in the putative pathway of alanine synthesis *de novo* from valine and isoleucine (and presumably from the other amino acids metabolized to tricarboxylic acid-cycle intermediates). This confirms the findings of Chang & Goldberg (1978). Whatever the mechanism of action of 3-MPA, it is clear that its effects on intermediary metabolism *in vitro* cannot be used as evidence in favour of a neogenic role for PEP carboxykinase in alanine synthesis. That 3-MPA stimulates glycolytic flux and $^{14}CO_2$ production from [U-^{14}C]glucose, decreases the [lactate]/[pyruvate] ratio, promotes $^{14}CO_2$ production from [1-^{14}C]pyruvate and inhibits $^{14}CO_2$ production from [1-^{14}C]acetate may imply multiple sites of action on the pyruvate dehydrogenase complex, PEP carboxykinase, tricarboxylic acid-cycle flux, cytosolic (and mitochondrial) redox states and glucose utilization. However, it cannot be precluded that the multiplicity of effects of 3-MPA results from its action at a single site, i.e. via inhibition of PEP carboxykinase.

The above discussion does not discount totally the existence of a pathway of alanine synthesis *de novo* in skeletal muscle. It does, however, question the strength of the evidence cited in support of the neogenic pathway involving PEP carboxykinase (see Snell, 1980; Snell & Duff, 1981, 1982) and the extent of the contribution of this presumptive pathway to total alanine formation relative to that of glycolysis. If, for the sake of argument, neogenic flux were to contribute 5% of the carbon skeleton of alanine+pyruvate+lactate (and glycolysis 95%), the synthesis *de novo* (1) would be difficult to identify and quantify by the techniques used hitherto (Garber *et al.*, 1976; Goldstein & Newsholme, 1976; Snell & Duff, 1977, 1981, 1982; Duff & Snell, 1982; see Snell, 1980) and yet (2) may still be a quantitatively important source of carbon precursor for hepatic gluconeogenesis. These considerations notwithstanding, the role of alanine released from skeletal muscle as the primary source of carbon precursor *de novo* in gluconeogenesis needs to be re-evaluated.

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