Specificity of the effects of leucine and its metabolites on protein degradation in skeletal muscle

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The effects of leucine, its metabolites, and the 2-oxo acids of valine and isoleucine on protein synthesis and degradation in incubated limb muscles of immature and adult rats were tested. Leucine stimulated protein synthesis but did not reduce proteolysis when leucine transamination was inhibited. 4-Methyl-2-oxopentanoate at concentrations as low as 0.25 mM inhibited protein degradation but did not change protein synthesis. The 2-oxo acids of valine and isoleucine did not change protein synthesis or degradation even at concentrations as high as 5 mM. 3-Methylvalerate, the irreversibly decarboxylated product of 4-methyl-2-oxopentanoate, decreased protein degradation at concentrations ≥ 1 mM. This was not due to inhibition of 4-methyl-2-oxopentanoate catabolism, because 0.5 mM-3-methylvalerate did not suppress proteolysis, even though it inhibited leucine decarboxylation by 30%; higher concentrations of 3-methylvalerate decreased proteolysis progressively without inhibiting leucine decarboxylation further. During incubation with [1- 14 C]- and [U- 14 C]-leucine, it was found that products of leucine catabolism formed subsequent to the decarboxylation of 4-methyl-2-oxopentanoate accumulated intracellularly. This pattern was not seen during incubation with radiolabelled valine. Thus, the effect of leucine on muscle proteolysis requires transamination to 4-methyl-2-oxopentanoate. The inhibition of muscle protein degradation by leucine is most sensitive to, but not specific for, its 2-oxo acid, 4-methyl-2-oxopentanoate.

Branched-chain amino acids have unique anabolic effects on muscle protein metabolism. For example, in perfused hearts, the stimulation of protein synthesis attained with a complete mixture of amino acids was achieved with branched-chain amino acids alone (Morgan *et al.*, 1971; Rannels *et al.*, 1974). In incubated and perfused skeletal muscle, protein synthesis could be stimulated by leucine but not by valine or isoleucine (Buse & Reid, 1975; Buse & Weigand, 1977; Fulks *et al.*, 1975; Li & Jefferson, 1978; Tischler *et al.*, 1982). Leucine also inhibited protein degradation in incubated skeletal muscle of immature rats (Buse & Reid, 1975; Fulks *et al.*, 1975) and in perfused rat hearts (Chua *et al.*, 1979). However, in perfused hindquarters of adult rats, leucine did not reduce protein degradation (Li & Jefferson, 1978).

In studying the mechanism of these anabolic effects of leucine, Tischler *et al.* (1982) found that the degree of inhibition of protein degradation in rat hemidiaphragms increased with increasing

concentrations of leucine in the incubation media and in the intracellular space. Chua *et al.* (1979) reported that high levels of the decarboxylated products of leucine, valine, and isoleucine decreased protein degradation in perfused rat hearts, but only the decarboxylated products of leucine and valine stimulated protein synthesis. Thus, the degradation of branched-chain amino acids and/or the intracellular accumulation of one or more of their catabolic products appear to be necessary for suppression of proteolysis. Indeed, Tischler *et al.* (1982) found that after inhibiting branched-chain amino acid transamination, leucine no longer decreased protein degradation in incubated rat hemidiaphragms, though protein synthesis was still stimulated by leucine. They also reported that an increased rate of leucine decarboxylation was associated with progressive suppression of muscle proteolysis. Since branched-chain amino acids share common transamination and decarboxylation pathways (Odessey & Goldberg, 1979;

Smith *et al.*, 1983) and products of branched-chain 2-oxo acid catabolism reduce protein degradation in perfused hearts (Chua *et al.*, 1980), it is unclear why proteolysis is suppressed in hemidiaphragms incubated with leucine but not with valine or isoleucine (Buse & Reid, 1975; Fulks *et al.*, 1975; Tischler *et al.*, 1982). Differences in the metabolism of branched-chain amino acids in cardiac muscle and in diaphragm and other types of skeletal muscle (Veerkamp & Wagenmakers, 1981) might account for this. Therefore, we studied the effect of 4-methyl-2-oxopentanoate on protein degradation in incubated limb muscles of different fibre types from both immature and adult rats. To determine the specificity of an antiproteolytic effect of this compound, we also measured rates of protein synthesis and degradation in limb muscles incubated with 3-methylvalerate, the irreversibly decarboxylated product of 4-methyl-2-oxopentanoate, and with the 2-oxo acids of valine and isoleucine. Finally, the metabolism of branched-chain amino acids, 4-methyl-2-oxopentanoate and 3-methylvalerate was examined to determine whether any effects of these compounds on muscle proteolysis could be related to differences in their metabolism.

Experimental

Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratory Inc. and maintained on Purina RMH 1000 (Agway Country Foods) chow and water *ad libitum*.

Rates of protein synthesis and degradation were measured in incubated soleus and extensor digitorum longus muscles obtained from fed, immature rats weighing less than 75 g and in epitrochlearis muscles from fed adult rats weighing 200–220 g by using procedures described previously (Clark & Mitch, 1983a,b). After induction of anaesthesia with pentobarbital (5 mg/100 g body wt.), the muscles were removed and incubated at 37°C in 3 ml of Krebs-Henseleit (1932) bicarbonate buffer containing 10 mM-glucose, 0.5 mM-phenylalanine and other additions as indicated. The medium was gassed with O₂/CO₂ (19:1) and before incubation the pH was checked and adjusted to 7.4 when necessary. After a 30-min equilibration period, the muscles were transferred to flasks containing fresh medium, regassed, and incubated for 2 h. Muscles were then removed and 2 ml of medium was added to 0.5 ml of cold 50% (w/v) trichloroacetic acid so that tyrosine and phenylalanine concentrations could be measured fluorometrically (Andrews *et al.*, 1973; Faulkner, 1965; Waalkes & Udenfriend, 1957). In incubations containing L-cycloserine, HNO₃ and the nitroso-2-naphthol reagents were mixed before adding the sample of medium since

L-cycloserine prevents the reaction of tyrosine with these reagents (Tischler *et al.*, 1982).

Protein synthesis was measured as the rate of incorporation of [U-¹⁴C]phenylalanine (0.05 µCi/ml) into muscle protein divided by the extracellular specific radioactivity of phenylalanine. In the presence of a 5-fold-physiological concentration of phenylalanine, we and others (Clark & Mitch, 1983a,b; Fulks *et al.*, 1975) have found that there is rapid equilibration between the intra- and extra-cellular specific radioactivity of [U-¹⁴C]phenylalanine.

Muscle protein degradation was calculated as the rate of release of tyrosine into the medium during incubation with 0.5 mM-cycloheximide (Fulks *et al.*, 1975). Tyrosine release was measured because this amino acid is neither synthesized nor degraded by muscle. Moreover, it does not accumulate intracellularly during the experimental period (Buse & Weigand, 1977; Clark & Mitch, 1983a); this was confirmed in the muscles used in these studies (results not shown).

Rates of uptake and catabolism of 3-methylvalerate, 4-methyl-2-oxopentanoate, leucine, valine and isoleucine were calculated by measuring the radioactivity recovered in protein, in the intracellular space of muscle and as ¹⁴CO₂ evolved during incubation with the respective radio-labelled compounds. To calculate the intracellular concentration of radiolabelled compounds (Morgan *et al.*, 1971), the extracellular spaces of soleus, extensor digitorum longus, and epitrochlearis muscles were determined with [¹⁴C]inulin. These values were 28 ± 2% for soleus (*n* = 5), 27 ± 3% for extensor digitorum longus (*n* = 5) and 24 ± 3% for epitrochlearis muscles (*n* = 5). Incorporation of radiolabelled compounds into muscle protein was measured by scintillation counting after washing trichloroacetic-acid-insoluble material successively with 10% (w/v) trichloroacetic acid and ethanol/diethyl ether (1:1, v/v). The protein was dissolved in Soluene (Packard Instruments Co) and the radioactivity accumulated was divided by the extracellular specific radioactivity to calculate a rate of muscle protein synthesis (Odessey & Goldberg, 1972).

To measure ¹⁴CO₂ evolved, the flask was cooled on ice, and 2 ml of the medium was transferred by syringe to another stoppered flask containing 0.5 ml of 50% (w/v) trichloroacetic acid and a suspended centre well containing 0.2 ml of phenethylamine. This flask was incubated for 1 h and the ¹⁴CO₂ trapped in the well was measured by scintillation counting.

To determine the extent of inhibition of leucine transamination during incubation with 5 mM-cycloserine, [1-¹⁴C]leucine (0.5 mM; 0.1 mCi/mmol) was added to the medium and, after the experimental

period, the flask was cooled on ice, the muscle removed and a stopper with a suspended well containing phenethylamine was placed in the flask. Trichloroacetic acid was added and the $^{14}\text{CO}_2$ evolved during leucine decarboxylation was measured. The centre well was then exchanged for one containing fresh phenethylamine and 0.5 ml of H_2O_2 was injected to convert 4-methyl-2-oxo[1- ^{14}C]pentanoate to $^{14}\text{CO}_2$ plus 3-methylvalerate during an additional 1 h incubation (Odessey & Goldberg, 1979). The sum of $^{14}\text{CO}_2$ measured in these two collections was used to calculate the rate of leucine transamination (Odessey & Goldberg, 1979; Tischler *et al.*, 1982).

Reagent grade chemicals were purchased from Sigma. [U- ^{14}C]Phenylalanine and radiolabelled amino acids were purchased from Schwartz/Mann; 3-methyl[1- ^{14}C]valerate and 4-methyl-2-oxo[1- ^{14}C]pentanoate were purchased from New England Nuclear. L-Cycloserine was generously supplied by Dr. W. E. Scott of Hoffmann-La Roche Inc. (Nutley, NJ, U.S.A.).

Values are presented as means $\pm$ S.E.M. Paired comparisons were used and $P < 0.05$ was judged to be significant statistically.

Results

Rates of protein synthesis and degradation in incubated soleus and extensor digitorum longus muscles varied from day to day, but as noted by others (Goldberg *et al.*, 1975; Libby & Goldberg, 1980; Lundholm *et al.*, 1981), the variability was smaller and more consistent within each experiment. One factor that could have contributed to

this variability was differences in the dimensions of the muscle, causing variation in diffusion of oxygen and substrates (Goldberg *et al.*, 1975). Preliminary studies revealed that soleus and extensor digitorum longus muscles obtained from rats weighing more than 75 g had more variable rates of protein synthesis and degradation than did muscles from smaller rats. For this reason, immature rats were not studied if their weight exceeded 75 g. We found that the day-to-day coefficient of variation in the rate of protein degradation in epitrochlearis muscles (28–34 mg) of rats weighing between 200 and 220 g was 5.7%; for soleus and extensor digitorum longus, it was 14.4% and 11.3%, respectively.

The effects of leucine on protein synthesis and degradation in incubated soleus and extensor digitorum longus muscles are shown in Table 1 (Expt. 1). Leucine increased the rate of protein synthesis by 21% in soleus and 16% in extensor digitorum longus; it decreased the rate of protein degradation by 20% in soleus and 14% in extensor digitorum longus. To determine whether this effect on limb muscle protein degradation required conversion of leucine to 4-methyl-2-oxopentanoate, as it does in rat hemidiaphragms (Tischler *et al.*, 1982), transamination of leucine was inhibited by using 5 mM-L-cycloserine (Table 1, Expt. 2). L-Cycloserine was used instead of amino-oxyacetate because we found previously (Mitch, 1980) that the latter compound reduced protein synthesis in incubated muscle. In preliminary experiments, we confirmed that 5 mM-L-cycloserine inhibited transamination of [1- ^{14}C]leucine by more than 50% (Tischler *et al.*, 1982); it did not

Table 1. Effects of leucine on protein turnover in limb muscles from immature rats incubated in the presence or absence of L-cycloserine

In Expt. 1, muscles from fed rats were incubated in Krebs–Henseleit bicarbonate buffer containing 10 mM-glucose, 0.5 mM-phenylalanine with and without leucine as indicated. Protein synthesis was calculated from the rate of incorporation of [U- ^{14}C]phenylalanine into protein and the specific radioactivity of phenylalanine (0.1 mCi/mmol) in the medium (Clark & Mitch, 1983a). Protein degradation was measured as the rate of release of tyrosine during incubation with 0.5 mM-cycloheximide. In Expt. 2, the effects of 0.5 mM-leucine on protein synthesis and degradation were examined with and without 5 mM-L-cycloserine. This concentration of L-cycloserine inhibited leucine transamination by more than 50% (see the text). Values are means $\pm$ S.E.M. for 10–12 rats in Expt. 1 and 16 rats in Expt. 2; * $P < 0.05$, † $P < 0.02$ by paired analysis compared with control incubations.

	Leucine (0.5 mM)	Protein synthesis (nmol of phenylalanine/h per g)		Protein degradation (nmol of tyrosine/h per g)	
		Soleus	Extensor digitorum longus	Soleus	Extensor digitorum – longus
Expt. 1					
Control	–	83.3 $\pm$ 2.9	71.6 $\pm$ 3.1	233.6 $\pm$ 14.3	187.9 $\pm$ 7.2
Experimental	+	100.7 $\pm$ 3.4†	83.4 $\pm$ 2.9†	186.8 $\pm$ 13.9*	161.6 $\pm$ 5.8*
Expt. 2					
Control	+	104.5 $\pm$ 5.1	83.7 $\pm$ 5.5	179.3 $\pm$ 6.4	164.4 $\pm$ 3.4
Cycloserine (5 mM)	+	107.4 $\pm$ 5.5	84.5 $\pm$ 6.1	227.2 $\pm$ 5.7*	192.5 $\pm$ 5.0†

suppress leucine-stimulated protein synthesis (Table 1, Expt. 2). Since leucine did not inhibit protein degradation in the presence of 5 mM-L-cycloserine, we concluded that the antiproteolytic effect of leucine requires its transamination to 4-methyl-2-oxopentanoate.

To investigate whether the inhibition of protein degradation was unique for this branched-chain 2-oxo acid, the effects of 0.5 mM concentrations of 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate and 3-methyl-2-oxopentanoate on protein synthesis and degradation in three types of skeletal muscle were studied (Table 2). In soleus, extensor digitorum longus, and epitrochlearis muscles, 4-methyl-2-oxopentanoate consistently decreased protein degradation by 22, 15 and 16% respectively, but did not affect protein synthesis. During incubation of each muscle with 0.5 mM of the 2-oxo acids of valine or isoleucine, neither protein synthesis nor degradation was altered. Further studies revealed that incubation of epitrochlearis muscles with higher concentrations (1 and 5 mM) of 3-methyl-2-oxovalerate or 3-methyl-2-oxopentanoate did not change the rate of protein degradation. In contrast, incubation with a lower concentration (0.25 mM) of 4-methyl-2-oxopentanoate reduced protein degradation from 137.4 ± 6.4 to 120.5 ± 6.9 nmol of tyrosine/h per g ($P < 0.05$ by paired analysis).

To determine whether inhibition of muscle protein degradation could be reproduced by 3-methylvalerate, the irreversibly decarboxylated product of 4-methyl-2-oxopentanoate, we measured the dose-response relationship between this compound and protein degradation in incubated limb muscles of mature and immature rats. At 0.5 mM-3-

methylvalerate, we measured CO_2 production from this compound by using 3-methyl[1- ^{14}C]-valerate and found that it was $0.51 \pm 0.04 \mu\text{mol/h}$ per g in soleus, $0.19 \pm 0.02 \mu\text{mol/h}$ per g in extensor digitorum longus and $0.29 \pm 0.02 \mu\text{mol/h}$ per g in epitrochlearis muscles, indicating that the compound entered muscle and was degraded. However, 0.5 mM-3-methylvalerate did not affect muscle protein degradation (Table 3). When the concentration of 3-methylvalerate was 1 or 5 mM, protein degradation decreased by 16–32% in soleus, extensor digitorum longus and epitrochlearis muscles. At 10 mM-3-methylvalerate, protein degradation in epitrochlearis muscles decreased even further ($P < 0.05$ by unpaired *t*-test) but in limb muscles of immature rats, protein degradation increased.

To compare the metabolism of 3-methylvalerate and 4-methyl-2-oxopentanoate, limb muscles were incubated with different concentrations of 3-methyl[1- ^{14}C]-valerate or 4-methyl-2-oxo[1- ^{14}C]-pentanoate. As shown in Fig. 1, the rate of CO_2 released from 4-methyl-2-oxopentanoate was substantially greater than that evolved from 3-methylvalerate. The difference between the rates of CO_2 evolved from 4-methyl-2-oxopentanoate and from 3-methylvalerate was remarkably constant when the extracellular concentration of these compounds was varied from 0.5 to 10 mM.

Besides being converted to $^{14}\text{CO}_2$, 4-methyl-2-oxo[1- ^{14}C]-pentanoate can be transaminated to form [1- ^{14}C]leucine in muscle. At 0.5 mM-4-methyl-2-oxopentanoate, we calculated that the intracellular concentration of leucine plus its 2-oxo acid was $0.40 \pm 0.02 \mu\text{mol/ml}$ (Table 4) while the rate of incorporation of 4-methyl-2-oxopentanoate into

Table 2. Effect of the 2-oxo acids of branched-chain amino acids on protein turnover in incubated muscles. Protein synthesis and degradation were measured as in Table 1. Values are means $\pm$ S.E.M. from 6–12 rats; * $P < 0.05$ by paired analysis compared with incubations without 4-methyl-2-oxopentanoate.

	Protein synthesis (nmol of phenylalanine/h per g)		Protein degradation (nmol of tyrosine/h per g)		
	Soleus	Extensor digitorum longus	Soleus	Extensor digitorum longus	Epitrochlearis
Control	137.3 ± 5.1	97.4 ± 3.2	267.3 ± 23.9	212.5 ± 17.5	144.6 ± 4.0
+0.5 mM-4-Methyl-2-oxopentanoate	132.2 ± 5.6	91.1 ± 5.2	$208.5 \pm 17.8^*$	$181.7 \pm 9.9^*$	$118.4 \pm 4.6^*$
Change (%)	-4.0	-6.0	-21.9	-14.5	-16.3
Control	88.9 ± 3.7	71.1 ± 2.9	184.0 ± 12.2	220.4 ± 12.8	134.5 ± 5.1
+0.5 mM-3-Methyl-2-oxovalerate	81.6 ± 3.0	67.0 ± 2.8	202.4 ± 10.2	212.2 ± 8.0	136.5 ± 8.9
Change (%)	-8.2	-5.7	+10.0	-3.7	+1.4
Control	72.7 ± 4.8	60.6 ± 3.8	227.2 ± 11.4	195.0 ± 21.2	143.6 ± 7.2
+0.5 mM-3-Methyl-2-oxopentanoate	78.0 ± 4.1	63.9 ± 2.7	211.9 ± 16.8	221.2 ± 10.8	136.8 ± 6.8
Change (%)	+7.2	+5.4	-6.7	+13.0	-4.7

Table 3. Effect of 3-methylvalerate on muscle protein degradation

Protein degradation was measured as the release of tyrosine during incubations with 0.5 mM-cycloheximide. Values are means $\pm$ S.E.M. from experiments on 6–10 rats in each group; * $P < 0.05$, † $P < 0.01$ by paired analysis compared with incubation without 3-methylvalerate.

	Protein degradation (nmol of tyrosine/h per g)		
	Soleus	Extensor digitorum longus	Epitrochlearis
Control	257.3 $\pm$ 21.4	209.5 $\pm$ 26.1	133.5 $\pm$ 4.6
+ 3-Methylvalerate (0.5 mM)	265.3 $\pm$ 24.2	221.9 $\pm$ 18.2	137.8 $\pm$ 5.3
Change (%)	+ 3.1 $\pm$ 5.6	+ 6.2 $\pm$ 6.3	+ 3.3 $\pm$ 3.1
Control	200.9 $\pm$ 16.1	150.2 $\pm$ 7.8	141.5 $\pm$ 6.6
+ 3-Methylvalerate (1.0 mM)	134.8 $\pm$ 13.9†	119.2 $\pm$ 9.2*	117.8 $\pm$ 4.8†
Change (%)	− 32.0 $\pm$ 5.8	− 20.3 $\pm$ 5.7	− 16.4 $\pm$ 3.2
Control	172.0 $\pm$ 9.5	174.9 $\pm$ 7.5	146.8 $\pm$ 7.1
+ 3-Methylvalerate (5.0 mM)	140.6 $\pm$ 7.7*	143.9 $\pm$ 4.9*	114.1 $\pm$ 4.3†
Change (%)	− 16.2 $\pm$ 8.0	− 16.8 $\pm$ 5.3	− 21.2 $\pm$ 6.3
Control	158.9 $\pm$ 4.3	163.2 $\pm$ 5.3	138.5 $\pm$ 6.5
+ 3-Methylvalerate (10.0 mM)	187.7 $\pm$ 5.6*	182.1 $\pm$ 7.3†	99.5 $\pm$ 5.6*
Change (%)	+ 18.6 $\pm$ 5.3	+ 11.8 $\pm$ 5.0	− 28.1 $\pm$ 3.0

Fig. 1. Comparison of the rates of oxidation of 4-methyl-2-oxo[1-14C]pentanoate (●) and 3-methyl[1-14C]valerate (○) by epitrochlearis muscle

Muscles from fed rats were incubated in Krebs–Henseleit buffer with 10 mM-glucose, 0.5 mM-phenylalanine and different concentrations of radiolabelled substrate (as indicated on the abscissa). The specific radioactivity was the same (0.1 mCi/mmol) at each concentration studied. CO₂ was collected as described in the Experimental section and expressed as the molar quantity of substrate oxidized as calculated from the specific radioactivity in the medium. Values are means $\pm$ S.E.M. for six paired muscles.

protein was 129 $\pm$ 8 nmol/h per g. In contrast, with 0.5 mM-3-methylvalerate, the intracellular concentration of this compound and its degradation products was lower (0.24 $\pm$ 0.04 μ mol/ml) and its

incorporation into trichloroacetic acid-insoluble material (presumably glycogen) was only 24 $\pm$ 3 nmol/h per g. However, when muscle was incubated with 1 mM-3-methylvalerate, the intracellular concentration of this compound and its catabolic products increased to 0.95 μ mol/ml and protein degradation was inhibited. These results suggest that accumulation of a catabolic product of 4-methyl-2-oxopentanoate inhibits protein degradation in muscle.

To evaluate whether products of 4-methyl-2-oxopentanoate catabolism accumulate intracellularly, we compared the metabolism of [1-14C]- and [U-14C]-branched-chain amino acids. In muscle incubated with a branched-chain amino acid labelled at C-1, radioactivity in the intracellular space would reflect the concentrations of the amino acid plus its 2-oxo acid. Subtraction of this value from the intracellular concentration of radiolabelled compounds arising during incubation with the U-14C-labelled branched-chain amino acid (at the same concentration and specific radioactivity) would yield an estimate of the intracellular concentration of intermediates arising after decarboxylation of the 2-oxo acid. During incubation of epitrochlearis muscles with 1 mM-leucine, the intracellular concentration of metabolites formed after decarboxylation of 4-methyl-2-oxopentanoate was calculated to be 0.91 μ mol/ml (Table 4). Accumulation of products of 4-methyl-2-oxopentanoate catabolism was confirmed by the higher rate of ¹⁴CO₂ production from [1-14C]-leucine compared with [U-14C]leucine. In contrast, during incubations with 1 mM-valine, we calculated that the intracellular concentration of valine metabolites subsequent to the decarboxylation reaction was 0.078 μ mol/ml. Likewise, ¹⁴CO₂

Table 4. *Metabolism of branched-chain amino acids, 4-methyl-2-oxopentanoate and 3-methylvalerate by epitrochlearis muscle*
Epitrochlearis muscles of fed rats were incubated in Krebs–Henseleit buffer containing 10 mM-glucose, 0.5 mM-phenylalanine and the respective compound as indicated. The specific radioactivity was the same in all experiments. The amount of radioactivity recovered as CO₂, in the acid-soluble and in the acid-precipitable fractions is expressed as the molar quantity of the substrate calculated from the specific radioactivity in the medium. Values are means $\pm$ S.E.M. from at least six muscles in each group.

Substrate	Concentration (mM)	Recovery of ¹⁴ C		
		CO ₂ (μ mol/h per g)	Trichloroacetic acid-soluble (μ mol/ml)	Trichloroacetic acid-insoluble (nmol/h per g)
[U- ¹⁴ C]Leucine	0.5	0.168 $\pm$ 0.012	0.77 $\pm$ 0.05	345 $\pm$ 16
[1- ¹⁴ C]Leucine	0.5	0.470 $\pm$ 0.032	0.48 $\pm$ 0.03	289 $\pm$ 25
4-Methyl-2-oxo[1- ¹⁴ C]pentanoate	0.5	0.773 $\pm$ 0.043	0.40 $\pm$ 0.02	129 $\pm$ 8
3-Methyl[1- ¹⁴ C]valerate	0.5	0.292 $\pm$ 0.016	0.24 $\pm$ 0.04	24 $\pm$ 3
[U- ¹⁴ C]Valine	0.5	0.024 $\pm$ 0.004	0.46 $\pm$ 0.01	134 $\pm$ 10
[U- ¹⁴ C]Isoleucine	0.5	0.069 $\pm$ 0.008	0.47 $\pm$ 0.02	162 $\pm$ 14
[U- ¹⁴ C]Leucine	1.0	0.484 $\pm$ 0.025	1.33 $\pm$ 0.06	331 $\pm$ 12
[1- ¹⁴ C]Leucine	1.0	1.122 $\pm$ 0.034	0.42 $\pm$ 0.04	267 $\pm$ 8
[U- ¹⁴ C]Valine	1.0	0.046 $\pm$ 0.006	0.78 $\pm$ 0.02	142 $\pm$ 4
[1- ¹⁴ C]Valine	1.0	0.043 $\pm$ 0.007	0.70 $\pm$ 0.02	150 $\pm$ 6
[U- ¹⁴ C]Isoleucine	1.0	0.164 $\pm$ 0.009	0.76 $\pm$ 0.03	133 $\pm$ 6

production from [1-¹⁴C]valine was comparable with that from [U-¹⁴C]valine, suggesting that in muscle valine is more completely catabolized following initial decarboxylation than is leucine. During incubation of epitrochlearis muscles with 0.5 or 1 mM concentrations of isoleucine and valine, production of ¹⁴CO₂ from [U-¹⁴C]isoleucine exceeded that from [U-¹⁴C]valine but was only 30–40% of that arising from [U-¹⁴C]leucine. Thus, in skeletal muscle, the metabolism of individual branched-chain amino acids differs (Table 4). Leucine is taken up and decarboxylated more rapidly and products arising from its degradation accumulate intracellularly to a greater extent compared with valine or isoleucine.

Isovaleryl-CoA has been shown to inhibit branched-chain oxo acid dehydrogenase activity in kidney and liver (Randle *et al.*, 1981). To determine whether 3-methylvalerate inhibited decarboxylation of 4-methyl-2-oxopentanoate in skeletal muscle, we incubated epitrochlearis muscles with 4-methyl-2-oxo[1-¹⁴C]pentanoate (0.5 mM) and different concentrations of 3-methylvalerate. Production of CO₂ was significantly inhibited by the addition of 0.5 mM-3-methylvalerate (0.77 $\pm$ 0.06 to 0.54 $\pm$ 0.04 μ mol/h per g; $P < 0.05$); the degree of inhibition of CO₂ production did not increase when the concentration of 3-methylvalerate was raised either to 1 mM (0.56 $\pm$ 0.05 μ mol/h per g) or 5 mM (0.52 $\pm$ 0.03 μ mol/h per g). In these same experiments, protein degradation was not depressed further by the addition of 3-methylvalerate to 4-methyl-2-oxopentanoate in the medium. Thus, we found that 0.5 mM-3-methylvalerate inhibited

decarboxylation, but, as shown in Table 3, 0.5 mM-3-methylvalerate did not inhibit proteolysis.

Discussion

These results indicate that the inhibitory effect of leucine on protein degradation in limb muscles of the rat, as in the hemidiaphragm (Tischler *et al.*, 1982), requires its transamination to the 2-oxo acid. Incubation with 0.5 mM-4-methyl-2-oxopentanoate decreased protein degradation by 15–22% in muscles of both adult and immature rats, but concentrations of the 2-oxo acids of valine and isoleucine as high as 5 mM did not change muscle protein degradation. 3-Methylvalerate, the irreversibly decarboxylated product of 4-methyl-2-oxopentanoate, also decreased proteolysis, but only when the concentration was at least 1 mM (Table 3). In contrast, protein degradation was inhibited by the 2-oxo acid of leucine at 0.25 mM concentration.

The mechanism by which 4-methyl-2-oxopentanoate reduces muscle proteolysis is unclear. The observation that 3-methylvalerate inhibited the decarboxylation of the 2-oxo acid of leucine and also decreased muscle protein degradation (Table 3) could mean that the antiproteolytic effect of 3-methylvalerate was a consequence of increased intracellular levels of 4-methyl-2-oxopentanoate. This appears unlikely for two reasons. Firstly, 0.5 mM-3-methylvalerate inhibited 4-methyl-2-oxopentanoate decarboxylation in epitrochlearis muscles by 30% but did not affect the rate of protein degradation. As the concentration

of 3-methylvalerate was raised to 1 and 5 mM, inhibition of decarboxylation did not increase further, even though muscle protein degradation was progressively inhibited (Table 3). Therefore, these two effects of 3-methylvalerate on muscle metabolism appear to be independent. Secondly, the 2-oxo acids of valine and isoleucine also inhibit 4-methyl-2-oxopentanoate decarboxylation in muscle (Odessey & Goldberg, 1979; Smith *et al.*, 1983) yet in our experiments, 0.5–5 mM concentrations of these compounds did not inhibit proteolysis. These observations provide indirect evidence that the antiproteolytic effect of the 2-oxo acid of leucine in muscle is not related to increasing intracellular levels of the compound. Because 4-methyl-2-oxopentanoate is rapidly transaminated to leucine (Rhead *et al.*, 1981) or decarboxylated (Fig. 1) to isovaleryl-CoA in muscle, proof that 4-methyl-2-oxopentanoate has a unique inhibitory effect on muscle protein degradation would require blocking both enzymatic reactions. Unfortunately, we and others (Smith *et al.*, 1977) have found that amino-oxycetate and cycloserine do not inhibit branched-chain amino acid transamination effectively in the presence of 4-methyl-2-oxopentanoate, and non-toxic inhibitors of branched-chain amino acid dehydrogenase are not available. Thus, we cannot exclude the possibility that muscle protein degradation can be specifically inhibited by changes in the intracellular concentration of 4-methyl-2-oxopentanoate.

The antiproteolytic effect of the 2-oxo acid of leucine is most likely caused by the intracellular accumulation of a product of its catabolism. This would be consistent with the observation that concentrations of 3-methylvalerate ≥ 1 mM led to accumulation of 3-methylvalerate and its metabolites in intracellular fluid ($0.95 \mu\text{mol/ml}$; Table 4) and depressed protein degradation. During incubation with 0.5 mM-3-methylvalerate, we found that the intracellular concentration of this compound and its degradation products was only $0.24 \mu\text{mol/ml}$ and that protein degradation was unchanged. To examine whether products arising after decarboxylation of 4-methyl-2-oxopentanoate accumulate during incubation with leucine, we compared the $^{14}\text{CO}_2$ production from [U- ^{14}C]- and [1- ^{14}C]-leucine and found that the rate of leucine decarboxylation was more than twice the rate of complete oxidation. Furthermore, we subtracted the intracellular concentration of leucine and its 2-oxo acid measured during incubation with [1- ^{14}C]leucine from that of leucine and its metabolites measured during incubation with [U- ^{14}C]leucine. Using this method, we found that the intracellular concentration of 3-methylvalerate and its catabolic products was $0.30 \mu\text{mol/ml}$ with 0.5 mM-leucine and increased to

$0.91 \mu\text{mol/ml}$ with 1 mM-leucine. These results contrast sharply with those obtained with 1 mM-valine, which did not affect proteolysis; the intracellular concentration of products arising after decarboxylation of 3-methyl-2-oxovalerate was only $0.08 \mu\text{mol/ml}$ (Table 4). Thus, the antiproteolytic effect of the 2-oxo acid of leucine, which is not shared by valine or isoleucine (Buse & Reid, 1975; Fulks *et al.*, 1975; Tischler *et al.*, 1982) or their 2-oxo acids (Table 2), could be linked to the intracellular accumulation of a metabolite arising after decarboxylation of 4-methyl-2-oxopentanoate.

The greater sensitivity of muscle proteolysis to 4-methyl-2-oxopentanoate (Table 2) could be explained as follows. Firstly, the rate of 4-methyl-2-oxopentanoate uptake into muscle was found to be greater than that of 3-methylvalerate. As shown in Fig. 1, the rate of CO_2 production from 4-methyl-2-oxopentanoate was always greater than the rate from 3-methylvalerate, as was its utilization calculated as the sum of CO_2 production, intracellular accumulation, and incorporation into protein (Table 4). Secondly, the catabolism of 3-methylvalerate could be limited because it may not be readily charged with CoA after it enters muscle. The more rapid catabolism of 4-methyl-2-oxopentanoate (Fig. 1, Table 4) would lead to a greater accumulation of its catabolic products.

Tischler (1980) reported that protein degradation is inhibited and the NADH/NAD^+ ratio is increased when rat hemidiaphragms are incubated with leucine or its 2-oxo acid. This led him to suggest that the antiproteolytic effect of 4-methyl-2-oxopentanoate is linked to changes in the redox state of muscle. Our observations suggest that the cellular redox state and the rate of muscle protein degradation may not be closely linked. We found that 1 mM-3-methylvalerate decreased proteolysis in incubated epitrochlearis muscles while it inhibited 4-methyl-2-oxopentanoate decarboxylation. A lower rate of decarboxylation of 4-methyl-2-oxopentanoate would decrease NADH formation and the NADH/NAD^+ ratio. Moreover, if 3-methylvalerate were converted to isovaleryl-CoA and then catabolized, this would consume reducing equivalents and decrease the NADH/NAD^+ ratio further. In addition, we found that incubation of muscle with concentrations of 3-methyl-2-oxovalerate as high as 5 mM did not change protein degradation, even though catabolism of this 2-oxo acid of valine (Table 4) would result in a net increase in cellular NADH (Smith *et al.*, 1983). Finally, incubation of epitrochlearis muscles with 2 mM-pyruvate, which increased lactate production ($+7.3 \pm 2.2 \mu\text{mol/h per g}$) and therefore would consume NADH and decrease the NADH/NAD^+ ratio, did not change the rate of proteolysis ($-8.3 \pm 5.6 \text{ nmol of tyrosine/h per g}$; $n = 11$).

The potential therapeutic benefit of the 2-oxo acid of leucine has been suggested by clinical studies of catabolic conditions. Infusion of 4-methyl-2-oxopentanoate decreased urea production and improved nitrogen balance without producing adverse effects in starving, obese humans (Mitch *et al.*, 1981) and in post-operative patients (Sapir *et al.*, 1983). In these same studies, infusion of leucine did not improve nitrogen balance. In addition, supplements of branched-chain 2-oxo acids containing large amounts of 4-methyl-2-oxopentanoate have been reported to decrease the rate of 3-methylhistidine excretion by patients with Duchenne's muscular dystrophy (Stewart *et al.*, 1982). Based on these reports and the finding that muscle protein degradation *in vitro* is more sensitive to 4-methyl-2-oxopentanoate than to 3-methylvalerate and is unaffected by other branched-chain 2-oxo acids, it would appear that the 2-oxo acid of leucine, rather than leucine, 3-methylvalerate, 3-methyl-2-oxovalerate or 3-methyl-2-oxopentanoate, would be the most likely compound to reduce protein hypercatabolism *in vivo*.

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