Is regulation of proteolysis associated with redox-state changes in rat skeletal muscle?

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In isolated rat diaphragms, only those substrates that increased the tissue NADH/NAD⁺ ratio lowered the rate of proteolysis. However, direct inhibition of proteinase activity by leupeptin promoted oxidation of the NAD couple of the muscles. These results suggest that changes in muscle reduction–oxidation state may be important in the regulation of proteolysis.

In both skeletal muscle (Fulks *et al.*, 1975; Buse & Weigand, 1977) and heart (Chua *et al.*, 1979a) leucine, but no other amino acids, can decrease the rate of protein degradation. It now appears that leucine must at least be transaminated to 4-methyl-2-oxopentanoic acid to produce this inhibitory effect (Tischler & Goldberg, 1980). Indeed, this transamination product of leucine also inhibits proteolysis in skeletal and cardiac muscle (Chua *et al.*, 1979a; Tischler & Goldberg, 1980). Besides these two metabolites, only glucose at high concentrations (10–20 mM) has been shown to lower the rate of protein degradation in skeletal muscle (Fulks *et al.*, 1975). On the other hand, in heart, a variety of substrates (*e.g.* lactate, acetate, acetoacetate and propionate) can also inhibit protein breakdown (Chua *et al.*, 1979a,b).

The mechanism by which these metabolites decrease proteolysis remains unknown. In investigating possible mechanisms several questions must be considered: first, why is the inhibitory effect specific for certain metabolites; secondly, why are some metabolites effective in heart but not in skeletal muscle; and thirdly, is catabolism of the effective metabolites required? It seems likely that catabolism of the substrates is a requirement for this regulation. If so, then some common intermediate or cofactor may account for the action of these compounds. Acetyl-CoA, a common end-product of these various substrates, is an unlikely candidate, since its formation in the mitochondria would not readily alter acetyl-CoA concentrations in the cytoplasm, where most proteolysis takes place. On the other hand, the ability of these metabolites to alter the overall tissue reduction–oxidation state could provide a pathway for regulating proteolysis. To test this possibility, lactate/pyruvate ratios in the medium, tissue NADH and NAD⁺ concentrations

and rates of proteolysis were measured in rat diaphragms incubated under various conditions. Aoki *et al.* (1975) have reported that ratios of lactate/pyruvate in the plasma reflect changes in the NAD couple of skeletal muscle, and more recently Buse *et al.* (1980) have used this ratio to demonstrate alterations of the NAD couple in incubated diaphragms.

Materials and methods

Chemicals and animals

Leupeptin and enzymes were from Boehringer. Substrates, coenzymes and enzymes and buffers were from Sigma. All reagents were of analytical grade. Male Sprague–Dawley rats (60–90 g), bred by Animal Resources at the University of Arizona, were provided with Purina Laboratory chow and water *ad libitum*. Where indicated, food was removed 48 h before killing.

Experimental procedures

Rats were killed by cervical dislocation. Diaphragms were dissected out and preincubated for 30 min at 37°C in Krebs–Henseleit (1932) buffer equilibrated with O₂/CO₂ (19:1, v/v) containing the additions indicated, as described previously (Odessey & Goldberg, 1972). They were then transferred to flasks containing 3 ml of fresh medium and incubated for 90 or 120 min. Incubations were terminated by removing the tissues and heating the medium in boiling water for 3 min. For measurement of NAD⁺ or NADH concentrations, tissues were homogenized in 1.5 ml of ice-cold 0.2 M-HClO₄ or 0.25 M-KOH in 50% (w/v) ethanol, respectively. After centrifugation, 1 ml of the supernatant solution was adjusted either with 2.5 M-KOH/0.1 M-Pipes (1,4-piperazinediethanesulphonic acid) to pH 6.0–

6.5 (acid extract) or with 1.5 M-HClO₄ and 0.25 M-Tris to pH 8.1–8.5 (basic extract). The KClO₄ precipitates were removed by centrifugation. NADH and NAD⁺ in the extracts were measured fluorimetrically by a procedure adapted from that of Williamson & Corkey (1969). For NADH, 0.2 ml of tissue extract was added to 1.8 ml of 20 mM-KH₂PO₄/6 mM-EDTA/0.5 mM-pyruvate, pH 7.4. For NAD⁺, 0.1 ml of extract was added to 1.9 ml of 0.1 M-Tris/0.4 M-hydrazine hydrate/5 mM-EDTA. Lactate (Hohorst, 1965) and pyruvate (Bücher *et al.*, 1965) in the medium was assayed spectrophotometrically. Tyrosine released into the medium was determined fluorimetrically (Waalkes & Udenfriend, 1957). Pyruvate, NAD⁺ and NADH were assayed on the day of the experiment to decrease the chance of chemical breakdown.

Results and discussion

Diaphragms from starved rats were incubated with glucose at a low concentration (2 mM) in the absence or presence of substrates that can be metabolized in skeletal muscle. Cycloheximide was also included in the medium to inhibit protein synthesis and thereby permit direct measurement of muscle proteolysis by the release of tyrosine from the tissue into the medium (Fulks *et al.*, 1975). Although cycloheximide inhibits muscle proteolysis (Goldberg & Dice, 1974), in diaphragms under these experimental conditions it decreases protein degradation by less than 6% (M. E. Tischler & A. L. Goldberg, unpublished results). Furthermore, in incubations of diaphragms with 5 mM-glucose or 0.5 mM-leucine and 5 mM-glucose, 0.5 mM-cycloheximide did not alter the ratio of lactate/pyruvate in the medium or the muscle NADH concentration ($P > 0.1$ by using the unpaired Student's *t* test).

As reported previously (Fulks *et al.*, 1975; Buse & Weigand, 1977), I found that, in diaphragm,

amino acids other than leucine (*i.e.* isoleucine, valine, aspartate, asparagine and glutamine) did not alter the rate of proteolysis. In addition neither lactate, 3-hydroxybutanoate, octanoate or acetate had any influence on this process. The combined observations made with these substrates are given in Table 1. Addition of these metabolites to the medium also did not affect the ratio of lactate/pyruvate in the medium (Table 1) or in the tissue (results not shown). Previous studies have reported that the ratio of lactate/pyruvate reflects the oxidation state of the NAD couple in muscle (Aoki *et al.*, 1975; Buse *et al.*, 1980) as it does in liver (Williamson *et al.*, 1967). The muscle concentrations of NADH and NAD⁺ were also similar in the presence or absence of this group of substrates (Table 1).

In contrast with these results, those metabolites (*i.e.* leucine, 4-methyl-2-oxopentanoate and glucose) that can decrease proteolysis in skeletal muscle (Table 1; Fulks *et al.*, 1975; Buse & Weigand, 1977), altered significantly the reduction–oxidation state of diaphragm. The total NADH content in the muscle increased on the average by 30 nmol/g of muscle and this change was paralleled by a fall in the NAD⁺ content by a similar amount (27 nmol/g of muscle) (Table 1). The opposite changes of NADH and NAD⁺ resulted in a fall of the measured (*i.e.* total) ratio of NAD⁺/NADH from 2.9 to 2.1. The cytoplasmic free ratio of NAD⁺/NADH, which would be inversely related to the lactate/pyruvate ratio, also fell markedly. Therefore the more oxidized state of the muscle NAD couple in control incubations and in those with non-inhibitory substrates was associated with a more rapid rate of proteolysis.

That proteolysis, like substrate catabolism, may be linked to the cellular redox potential is an attractive idea, since the tissue reduction–oxidation state is altered during degradation of substrates (as shown herein), in response to hormone action on

Table 1. *Effect on reduction–oxidation state in diaphragms of substrates that inhibit or have no effect on proteolysis* Quarter-diaphragms from 48 h-starved rats were incubated with 2 mM-glucose and 0.5 mM-cycloheximide (control) as described in the Materials and methods section. The non-inhibitory substrates added separately included: isoleucine, valine or sodium octanoate, 2 mM; sodium aspartate, asparagine, glutamine or sodium 3-hydroxybutanoate, 5 mM; sodium lactate or sodium acetate, 10 mM. The inhibitory substrates were: leucine, 2 mM; sodium 4-methyl-2-oxopentanoate, 0.5 mM or glucose, 18 mM. Values are means $\pm$ S.E.M. for all measurements within each group. The total number of observations is given in parentheses. Statistical analysis (paired Student's *t* test) comparing incubations with and without substrates is expressed by * $P < 0.001$.

Substrates	Content (nmol/g of muscle) of:		Lactate/ pyruvate	Rate of proteolysis (nmol/2 h per mg)
	NADH	NAD		
None	101 $\pm$ 2 (84)	299 $\pm$ 8 (12)	12.5 $\pm$ 0.4 (84)	0.66 $\pm$ 0.02 (108)
Non-inhibitory	101 $\pm$ 2 (96)	297 $\pm$ 5 (66)	13.0 $\pm$ 0.3 (78)	0.64 $\pm$ 0.01 (120)
Inhibitory	131 $\pm$ 2 (114)*	273 $\pm$ 5 (42)*	21.6 $\pm$ 0.6 (78)*	0.48 $\pm$ 0.01 (108)*

muscle and after changes of cellular pH (see below). Aoki *et al.* (1977) showed that administration of insulin to fasted humans seemed to cause reduction of the NAD couple in their forearm muscles, and the ability of insulin to inhibit proteolysis in skeletal muscle is well known (Fulks *et al.*, 1975). On the other hand, infusion of cortisol or tri-iodothyronine promoted oxidation of the NAD couple of forearm muscles, and, in contrast with insulin, these hormones accelerate the degradation of muscle protein (Tomas *et al.*, 1979; Goldberg *et al.*, 1980). In the perfused hemicorpus, isoproterenol reduced the rate of proteolysis, but increased the perfusate ratio of lactate/pyruvate from 33 to 51 (Li & Jefferson, 1977). Finally, lowering the external pH of perfused hearts from 7.3 to 6.8 causes the perfusate lactate-pyruvate ratio to increase (Williamson *et al.*, 1978), whereas a drop of pH from 7.4 to 7.0 is accompanied by a fall in the rate of proteolysis (Chua *et al.*, 1979b).

The concurrent effects of substrates, and possibly hormones and pH, on proteolysis and the oxidation state of the NAD couple in muscle support the idea that the cellular redox potential may, in some way, regulate protein degradation. Leupeptin, which slows proteolysis in isolated muscles (Libby & Goldberg, 1978) by inhibiting directly certain proteinases (e.g. cathepsin B and Ca^{2+} -activated proteinase) would not be expected to decrease protein degradation through a reduction of the NAD couple. Leupeptin, like leucine and glucose (Table 1), lowered significantly the rate of proteolysis in the isolated diaphragms (Table 2). However unlike the inhibitory substrates, leupeptin caused an oxidation of the NAD couple, as shown by significant decreases of both the NADH concentration in the muscles and the lactate/pyruvate ratio in the medium (Table 2).

This effect of leupeptin on the NAD couple may provide further evidence for an important link

between protein degradation and the redox potential in muscle. As shown herein, degradation of leucine by muscle causes a reduction of the NAD couple (Table 1) and the inhibition of its catabolism by L-cycloserine (Tischler & Goldberg, 1980) leads to an oxidation of the NAD couple (M. E. Tischler, unpublished results). By analogy, the direct inhibition of proteinases by leupeptin could result in an oxidation of the NAD couple if changes in the rate of proteolysis, like other degradative pathways, cause significant alterations of the cellular redox potential and thereby of the oxidation state of the NAD couple.

If proteolysis proceeds more rapidly when the muscle redox potential is more positive (*i.e.* more oxidized), then variation of the muscle reduction-oxidation state by substrates and hormones could provide a means of regulating protein degradation. This theory could also explain why only some substrates are capable of decreasing proteolysis. For instance, the specificity of leucine may reflect the inability of the other amino acids to produce a more reduced state in muscle. Lactate may inhibit protein breakdown in heart, but not in skeletal, muscle because in cardiac muscle it is a more effective substrate and can alter thereby the redox state. Similar analogies can probably be found for other substrates. In any case, the data suggest that an important relationship seems to exist between proteolysis and the redox potential in muscle, but it is unknown at this time how they may be linked.

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Table 2. *Effect of leupeptin on reduction-oxidation state and rate of proteolysis in diaphragm*

Hemidiaphragms from 48-h starved rats were incubated with 5 mM-glucose and 0.5 mM-cycloheximide in the absence or presence of 5 μM -leupeptin, as described in the Materials and methods section. Values are means $\pm$ S.E.M. for diaphragms from six rats. Statistical analysis of the paired observations (Student's *t* test) is given as: * $P < 0.005$, † $P < 0.01$.

Incubation	Content of		Rate of proteolysis (nmol/90 min per mg)
	NADH (nmol/g of muscle)	Lactate/ pyruvate	
No leupeptin	104 $\pm$ 3	14.6 $\pm$ 0.9	0.71 $\pm$ 0.03
Leupeptin	66 $\pm$ 11	10.5 $\pm$ 0.6	0.56 $\pm$ 0.03
Difference	−38 $\pm$ 9†	−4.1 $\pm$ 0.8*	−0.15 $\pm$ 0.03*

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