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THE STARVATION INDUCED INCREASE IN MUSCLE PROTEIN DEGRADATION IS NON-LYSOSOMAL IN ORIGIN

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Evidence is presented that 20 mM NH_4^+ specifically inhibits lysosomal protein degradation in rat skeletal muscle *in vitro*. Using this inhibitor it is shown that the increased muscle proteolysis resulting from 48 hr starvation is non-lysosomal in origin. The implications of this result with respect to the mechanism and selectivity of intracellular protein degradation are discussed.

INTRODUCTION

Evidence has recently accumulated to support the existence of at least two separate pathways for mammalian intracellular protein degradation (1). The role of lysosomes in both normal (see refs in 2) and pathological conditions such as muscular dystrophy (3) is well established. However, *in vitro* studies using various inhibitors of lysosomal proteolysis have suggested that this accounts for at most 40% of intracellular protein degradation in rat yolk sac (4) and hepatoma cell preparations (5). Moreover, Dice *et al.* (6) have shown that the increased proteolysis in liver and muscle associated with short term starvation in rats shows a fundamentally different selectivity to normal degradation with respect to protein subunit size and charge. This result is consistent with a two pathway model for degradation with preferential stimulation of one pathway under these conditions. Whether a lysosomal or non-lysosomal system is stimulated has not been established.

20 mM NH_4^+ has been shown to inhibit 100% of lysosomal proteolysis, but to inhibit only 40% of total intracellular proteolysis in rat yolk sac

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preparations (4). Using rat extensor digitorum longus (E.D.L.) muscle preparations we have correlated *in vitro* NH_4^+ sensitive proteolysis with muscle acid protease activity. This inhibitor has then been used to assess the contributions of the two degradative pathways to the increased muscle proteolysis seen in short term starvation.

MATERIALS AND METHODS

Animals - Female Wistar rats (Body Wt 50-80 g) were obtained from the Animal Breeding Unit, Prince Henry Hospital, Sydney, Australia. The animals were allowed free access to water and a proprietary rat food (Allied Feeds Pty. Ltd. Sydney, Australia). Starved animals were deprived of food for 48 hrs (11.00 a.m. - 11.00 a.m.) prior to sacrifice by cervical dislocation.

Protein Degradation - Extensor digitorum longus muscles were dissected out and incubated essentially as described by Li and Golberg (7). Individual muscles were preincubated for 40 mins in 3 ml of Krebs-Ringer bicarbonate buffer containing 10 mM D-glucose, 5 mM HEPES and 0.5 mM cycloheximide in the presence or absence of 20 mM NH_4Cl. The buffer was saturated with 95% O_2/ 5% CO_2 and adjusted to pH 7.40 prior to use. The muscles were then transferred to fresh flasks and incubated for 2 hrs in 3 ml of the same buffer. All incubations were at 37°C in a metabolic shaker, shaking speed 80 cycles per min. Protein degradation was measured as tyrosine released into the medium (7) and was expressed as nmoles tyrosine released/2 hr/g wet wt. of tissue. Contralateral muscles incubated with and without NH_4Cl yielded NH_4^+ insensitive and total protein degradation respectively. NH_4^+ sensitive degradation was obtained by difference.

Acid Protease Activity - Total hind limb muscle minus soleus and E.D.L. was excised and homogenised according to Canonico and Bird (8). Total acid protease activity was measured by the release of U.V. absorbing (280 nm) acid soluble material from bovine haemoglobin at pH 3.0 (9) in the presence of 0.1% Triton X-100. Activity is expressed as μg tyrosine released/hr/mg Wet Wt. of tissue.

Statistical Treatments - Results are expressed as mean ± standard deviation. The significances of differences between means were determined by a two-tailed students t test.

RESULTS AND DISCUSSION

20 mM NH_4Cl inhibited protein degradation in normal E.D.L. by 38 ± 15% (n=38). This degree of inhibition is similar to those previously reported for a variety of other tissues, including hepatoma cells (5), heart atria (10) and yolk sac (4). NH_4^+ sensitive protein degradation was strongly correlated with acid protease activity ($r=0.41$ $p < .001$) (Fig. 1) whereas NH_4^+ insensitive protein degradation showed no such correlation ($r=0.12$ $p > .4$) (Fig. 2). Since it is probable that the acid protease

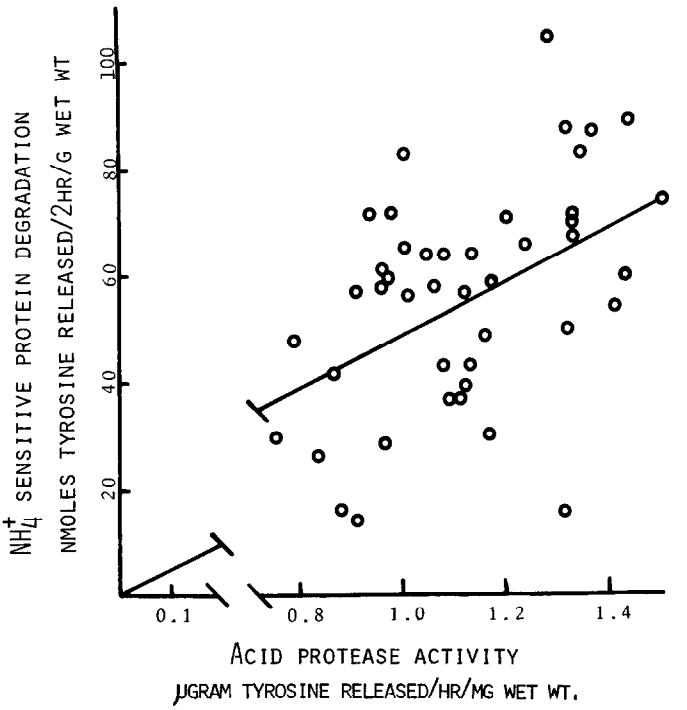

Fig. 1 Correlation of *in vitro* NH_4^+-sensitive protein degradation in E.D.L. with muscle acid protease activity. Pearson's Correlation Coefficient $r = 0.41$, $p < .001$. (n=46).

activity is predominantly lysosomal, these results suggest that NH_4^+ sensitive proteolysis may be equated with lysosomal proteolysis. The contribution of any non lysosomal proteolytic system to NH_4 sensitive degradation is likely to be minimal since the regression line relating NH_4 sensitive degradation and acid protease activity passes through the origin (zero intercept = 0 ± 18 nmoles/2 hr/g) (Fig. 1). This conclusion regarding the specifity of NH_4^+ inhibition for lysosomal proteolysis is similar to that drawn by Ballard *et al.* for yolk sac preparations (4).

Starvation for 48 hrs resulted in an increase in total E.D.L. proteolysis of 84 nmoles/2 hr/g Wet Wt. (Table 1). This increase was predominantly due to an 81% increase (72 nmoles/2 hr/g) in NH_4^+-insensitive proteolysis. In contrast, NH_4^+-sensitive proteolysis showed insignificant

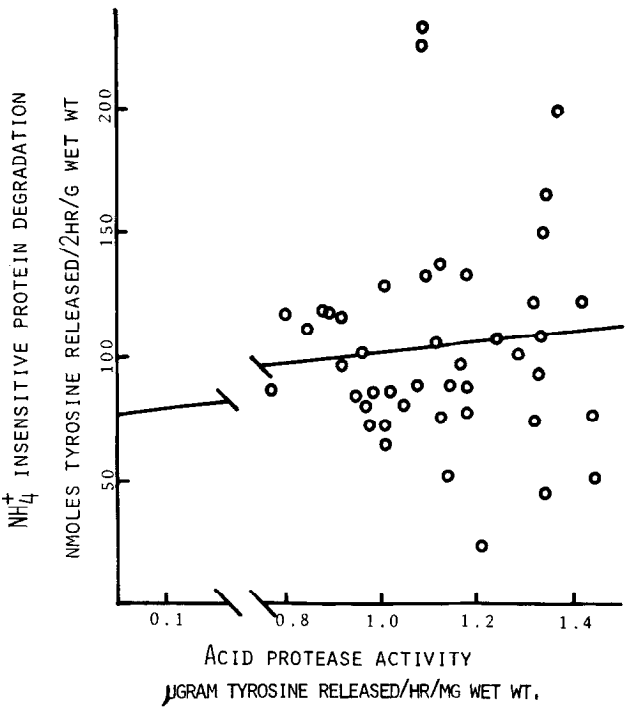

Fig. 2 Correlation of *in vitro* NH_4^+ insensitive protein degradation with muscle acid protease activity. Pearson's Correlation Coefficient r = 0.12, p > .4. (n=46).

TABLE 1

The effect of 48 hr starvation on *in vitro* protein degradation in E.D.L. and muscle acid protease activity.

	nmoles tyrosine released/2 hr/g wet wt		
	Normal (n=18)	Starved (n=10)	Starved/Normal
Total degradation	144 ± 18	228 ± 50 (a)	1.58
NH_4^+ insensitive degradation	91 ± 22	163 ± 47 (a)	1.81
NH_4^+ sensitive degradation	53 ± 22	65 ± 15	1.23
	µg tyrosine released/hr/mg wet wt.		
Acid protease activity	1.03 ± .16	1.31 ± .20 (a)	1.27

(a) Significantly different from normal controls p < .001

increase under these conditions. Simultaneously, acid protease activity showed a small but significant increase. This change is similar in magnitude to that observed for Cathepsin D activity in gastrocnemius muscle after 48 hrs starvation (11).

It therefore appears that the major proteolytic response in the starved state involves the NH_4^+-insensitive system and hence, by inference, the non-lysosomal system. Although the precise nature of the NH_4^+-insensitive proteolytic system is unknown, a considerable number of neutral or alkaline proteases have been found in a variety of tissues including skeletal muscle (see refs in 2). It may be relevant that one such enzyme, an alkaline myofibrillar protease showed a 7 fold increase in activity during 6 days starvation (12).

It is well established that normal intracellular proteolysis is selective with respect to protein sub-unit size and charge (2). However, in starvation this selectivity is extensively modified (6). This has been interpreted by Dice *et al.* (6) as the preferential stimulation of a non selective proteolytic system which obscures the selectivity of the basal system. Accepting this interpretation and assuming that the increase in NH_4^+-insensitive proteolysis represents stimulation of a prexisting proteolytic system, our results implicate the NH_4^+-insensitive or non-lysosomal system as the non selective system. Conversely they imply that the lysosome is responsible for the size and charge selectivity of protein degradation under normal conditions.

Acknowledgement

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