Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle

Bradford B. LOWELL,† Neil B. RUDERMAN*† and Michael N. GOODMAN*†‡
Departments of *Medicine and †Physiology, Boston University School of Medicine, Boston, MA 02118, U.S.A.

To examine the role of lysosomes in the degradation of skeletal-muscle myofibrillar proteins, we measured the release of N^{τ}-methylhistidine from perfused muscle of starved and fed rats in the presence or absence of agents that inhibit lysosomal proteinase activity. After 1 day of starvation, the release of N^{τ}-methylhistidine by perfused muscle of 4-, 8- and 24-week-old rats increased by 322, 159 and 134% respectively. On the other hand, total protein breakdown, assessed by tyrosine release, increased by 62, 20 and 20% respectively. Inhibitors of lysosomal proteinases as well as high concentrations of insulin or amino acids failed to diminish the release of N^{τ}-methylhistidine by perfused muscle of starved and fed rats, despite a 25–35% inhibition of total protein breakdown. The data strongly suggest that the complete breakdown of myofibrillar proteins occurs via a non-lysosomal pathway. They also suggest that total proteolysis, which primarily reflects non-myofibrillar protein breakdown, occurs at least in part within lysosomes.

INTRODUCTION

The precise mechanism by which intracellular proteins are degraded is largely unknown, although it is accepted that proteolysis can occur both within and outside of lysosomes (Wibo & Poole, 1974; Dean, 1975; Hopgood *et al.*, 1977; Neff *et al.*, 1979; Seglen *et al.*, 1979; Wildenthal & Crie, 1980; Mortimore, 1982; Ballard & Gunn, 1982). The role of the lysosome in the regulation of protein breakdown in skeletal muscle is unclear, however. Although few in number, lysosomes have been identified morphologically in muscle (Bird *et al.*, 1980; Whitaker *et al.*, 1983). In addition, lysosomal proteinases have been consistently detected in skeletal muscle, and it has been shown that purified cathepsins B, D, H and L can degrade purified actin and myosin to a limited degree (Bird *et al.*, 1980). In addition, inhibitors of lysosomal proteinase activity such as NH_4Cl, chloroquine, leupeptin and EP 475 decrease proteolysis when added to muscles incubated *in vitro* (Libby & Goldberg, 1978; Jenkins *et al.*, 1979; Clark *et al.*, 1984). The latter studies, however, measured only total proteolysis and did not distinguish between the breakdown of myofibrillar and non-myofibrillar proteins. Approx. 60% of the protein in skeletal muscle is myofibrillar, and its rate of turnover has been reported to be several times slower than that of non-myofibrillar proteins (Bates & Millward, 1983). Because of this, measurements of total proteolysis may primarily reflect changes in non-myofibrillar protein breakdown. Thus the role of lysosomes in the degradation of myofibrillar proteins remains to be determined.

To evaluate this, we examined the effect of agents that inhibit lysosomal proteinase activity on the release of N^{τ}-methylhistidine (3-methylhistidine) and tyrosine by perfused rat muscle under basal and stimulated (i.e. starvation) conditions. As reviewed elsewhere (Young & Munro, 1978; Wassner & Li, 1982), N^{τ}-methylhistidine is a minor amino acid found only in actin and myosin and, on its release during proteolysis, it is neither degraded nor reutilized for protein synthesis by the muscle cell. Thus its release from muscle should reflect myofibrillar protein breakdown. On the other hand, tyrosine is found in all muscle proteins, and its release in the presence of an agent that inhibits protein synthesis provides an index of total proteolysis.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats were obtained from Charles River Breeding Laboratories (Willmington, MA, U.S.A.). They were kept singly in wire-bottom cages in animal quarters maintained at 22 °C with a light/dark cycle of 12 h (06:00–18:00 h). They were either fed on Purina Laboratory Chow and given water *ad lib.* or starved for 24 h.

Materials

Bovine serum albumin (Cohn fraction V) was obtained from Miles Laboratory, Kankakee, IL, U.S.A. It was dissolved in Krebs–Ringer bicarbonate solution (Goodman *et al.*, 1981) and dialysed for 24 h against the latter before use. All chemicals were of reagent grade (Fisher). Cycloheximide, N^{τ}-methylhistidine (standard), and fluorescamine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H.p.l.c. reagents were from Fisher.

Perfused-hindquarter experiments

Details of the perfusion apparatus and the operative preparation for the hindquarter were as described previously (Goodman *et al.*, 1981). To eliminate the skin as a potential source of N^{τ}-methylhistidine (Wassner & Li, 1982), it was removed from all hindquarters before perfusion. The perfusion medium consisted of Krebs–Ringer bicarbonate solution, 4% (w/v) bovine serum

‡ To whom reprint requests should be addressed.

albumin, 6 mM-glucose, 1 mM-lactate, 0.1 mM-pyruvate, and washed rejuvenated outdated erythrocytes (haematocrit 22%). Unless stated otherwise, hormones and amino acids were not added to the initial perfusate. The initial non-esterified fatty acid concentration in the perfusate was 100–200 μM, and if necessary its pH was adjusted to 7.4. During the experiment the perfusion medium was continuously gassed with O_2/CO_2 (19:1), and its temperature was maintained at 37 °C. The initial volume of perfusate was 150 ml; the first 25 ml that passed through the tissue was discarded, and thereafter the medium was recycled. Perfusate flow rate was maintained at 12 ml/min.

Evaluation of protein degradation

Total protein degradation by the perfused hindquarter was estimated by measuring the release of tyrosine (Goodman *et al.*, 1981), and myofibrillar degradation by the release of N^{τ}-methylhistidine, both in the presence of 200 μM-cycloheximide. At this concentration of cycloheximide, protein synthesis is inhibited by at least 90% with 30 min. After pre-perfusion for 30 min, a sample of the left tibialis anterior muscle was removed and frozen in liquid N_2, after which a sample of perfusion medium was taken. After an additional 60 min of perfusion, a second sample of perfusate was taken and the tibialis anterior of the right leg was removed and frozen. The perfusate was deproteinized with $HClO_4$ (final concn. 3%, v/v), and the muscle was homogenized in ice-cold 3% $HClO_4$. Perfusage and muscle samples were centrifuged at 4 °C for 30 min (2500 *g*), and the supernatants were frozen at −20 °C until analysed. Release of tyrosine and N^{τ}-methylhistidine was calculated by summing the changes in perfusate and tissue as described by Li & Wassner (1984). We assume that changes in the tibialis anterior muscle reflect the total musculature perfused, since it is a fast-twitch muscle containing both oxidative and glycolytic fibres. Tyrosine in the $HClO_4$ extracts was measured as described previously (Goodman *et al.*, 1981), and N^{τ}-methylhistidine by a slight modification of the h.p.l.c. method of Wassner *et al.* (1980). In some perfusions, the release of N^{τ}-methylhistidine was measured in the absence of cycloheximide (results not shown). These results were similar to those in the presence of cycloheximide, indicating that, at the concentration used and during the duration of the perfusion, cycloheximide did not inhibit myofibrillar protein degradation. Fed and starved rats, as well as control and experimental groups, were always perfused on the same day.

Statistics

Experimental groups were compared with controls by Student's *t* test.

RESULTS

Myofibrillar and total protein breakdown were studied under both basal (fed) and stimulated (starved) conditions. Starvation has been reported to increase total muscle protein breakdown (Li & Goldberg, 1976; Li *et al.*, 1979; Goodman *et al.*, 1981) and more recently to increase myofibrillar protein breakdown (Li & Wassner, 1984). Since these responses have been shown to depend on the age of the rat (Goodman *et al.*, 1981; Li *et al.*, 1979), rats of various ages were used. As shown in Table 1, and in agreement with previous reports (Goodman *et al.*, 1981; Li *et al.*, 1979), after 1 day of starvation total protein breakdown (i.e. tyrosine release) increased by 62% in 4-week-old rats, whereas it was only modestly increased in 8- and 24-week-old rats. In contrast, 1 day of starvation greatly augmented the release of N^{τ}-methylhistidine by the perfused hindquarter in all three groups.

Using 8-week-old rats starved for 24 h, we next determined whether the breakdown of myofibrillar protein was associated with a lysosomal or non-lysosomal pathway. For this purpose agents that inhibit lysosomal proteinase activity, such as NH_4Cl, chloroquine or leupeptin, were added to the perfusion medium. As shown in Table 2, whereas all of these agents decreased tyrosine release by 25–35%, none affected the release of N^{τ}-methylhistidine from the perfused hindquarter. These findings suggest that the complete degradation of myofibrillar proteins (i.e. actin and myosin) to free amino acids is regulated via a non-lysosomal pathway. In addition, since leupeptin also inhibits a non-lysosomal Ca^{2+}-activated proteinase in muscle (Brooks *et al.*, 1983), it would appear that this enzyme may not be involved in myofibrillar proteolysis, at least during brief starvation. NH_4Cl added to the perfusate of fed rats similarly diminished the release of tyrosine but not of N^{τ}-methylhistidine. We also tested the effects of insulin and

Table 1. Influence of starvation for 24 h on tyrosine and N^{τ}-methylhistidine release by perfused hindquarters of 4-, 8- and 24-week-old rats

Values are means ± S.E.M. for four to six rats. Within each group all values at day 1 of starvation are significantly different from those of rats fed *ad lib.* (day 0) ($P < 0.05$). Values in parentheses are percentage increases compared with day 0.

Age of rat (weeks)	Body wt. (g)	Period of starvation (days)	Release (nmol/h per g of muscle perfused)	
			Tyrosine	N^{τ}-Methylhistidine
4	103 ± 7	0	154 ± 10	0.81 ± 0.04
	75 ± 4	1	250 ± 25 (62)	3.42 ± 0.17 (322)
8	240 ± 5	0	100 ± 5	0.51 ± 0.03
	215 ± 4	1	120 ± 6 (20)	1.32 ± 0.07 (159)
24	555 ± 20	0	70 ± 4	0.58 ± 0.03
	475 ± 10	1	84 ± 4 (20)	1.36 ± 0.07 (134)

Table 2. Influence of NH_4Cl, chloroquine, leupeptin, insulin and amino acids on N^{τ}-methylhistidine and tyrosine release by perfused hindquarters of fed and 1-day-starved 8-week-old rats

Values are means ± S.E.M. for four to six rats per group. Additions were made to the initial perfusion medium; '5 × amino acids' refers to 5 times physiological plasma concentrations of all amino acids except tyrosine, which was omitted (Fulks *et al.*, 1975). *$P < 0.05$ compared with controls.

Group	Release (nmol/h per g of muscle perfused)	
	Tyrosine	N^{τ}-Methylhistidine
1. Starved 24 h		
Control	133 ± 5	1.30 ± 0.08
NH_4Cl (10 mM)	86 ± 5*	1.40 ± 0.06
Chloroquine (250 μM)	95 ± 5*	1.35 ± 0.25
Leupeptin (100 μg/ml)	98 ± 4*	1.20 ± 0.10
Insulin (20 munits/ml)	104 ± 4*	1.38 ± 0.15
5 × amino acids	140 ± 5	1.35 ± 0.20
2. Fed *ad lib.*		
Control	109 ± 5	0.48 ± 0.21
NH_4Cl (10 mM)	75 ± 7*	0.61 ± 0.16

high concentrations of amino acids on N^{τ}-methylhistidine release, since previous studies have shown that these agents can modulate the lysosomal pathway in heart (Long *et al.*, 1984) and liver (Mortimore, 1982) respectively. As shown in Table 2, insulin decreased tyrosine release by 22%, but neither it nor amino acids at 5 times normal plasma concentrations affected N^{τ}-methylhistidine release.

DISCUSSION

The results in this paper suggest that two pathways for protein degradation exist in skeletal muscle: (1) a non-lysosomal pathway, which appears responsible for myofibrillar proteolysis and may also contribute to non-myofibrillar proteolysis; and (2) a lysosomal pathway, which appears to contribute only to non-myofibrillar proteolysis. In keeping with our results, two previous studies have shown chloroquine and leupeptin to be ineffective in curtailing the loss of prelabelled myofibrillar protein by cultured heart (Wildenthal *et al.*, 1980) and skeletal-muscle (Riebow & Young, 1980) cells. However, interpretation of these studies could be complicated by re-incorporation of label into myofibrillar protein. In addition, those investigations did not rule out involvement of a lysosomal pathway in myofibrillar proteolysis, and indeed it was suggested that myofibrillar proteins could initially be cleaved in an extralysosomal site, with final degradation to free amino acids occurring within the lysosome. Our results suggest exclusive degradation of myofibrillar proteins by a non-lysosomal pathway under both basal and stimulated conditions.

Another noteworthy finding in our study was that myofibrillar protein breakdown as assessed by N^{τ}-methylhistidine release was markedly augmented after only a brief starvation. A similar observation has been reported by Li & Wassner (1984), but in young (90 g) rats starved for 48 h. We also found a disproportionate increase in myofibrillar proteolysis with respect to total protein breakdown after brief starvation. This was especially evident in older rats, suggesting that the degradation of myofibrillar and non-myofibrillar proteins may be regulated independently and by separate pathways. Furthermore, since total protein breakdown was only minimally altered in older rats, despite the large increase in myofibrillar proteolysis, it would appear that total proteolysis in these animals predominantly is a measure of non-myofibrillar protein breakdown, a fact often neglected in previous studies.

In conclusion, the results suggest that, in rats aged 8 weeks and older, there is a marked increase in muscle proteolysis after brief starvation that affects predominantly myofibrillar proteins. They also suggest that myofibrillar protein breakdown in muscle is mediated in starved as well as in fed rats by a non-lysosomal pathway. Finally, our results suggest that therapies designed to inhibit lysosomal proteinases may prove ineffective in retarding muscle atrophy when it is due to an increase in myofibrillar protein breakdown. However, it is possible that the regulation of myofibrillar protein degradation in pathological states, as well as during more prolonged starvation, is different and can occur by a lysosomal pathway. This remains to be determined.

We gratefully acknowledge the expert technical assistance of Ms. Sally Coles and Ms. Eva Belur, the secretarial assistance of Ms. Gretchen Fox, and the American Red Cross Blood Services (Northeast Region, Needham, MA, U.S.A.) for providing human erythrocytes for perfusion studies. This study was supported in part by U.S. Public Health Service Grants AM 19514 and AM 19469 and Research Career Development Award AM 00652 (M.N.G.)

REFERENCES

Ballard, F. J. & Gunn, J. M. (1982) Nutr. Rev. **40**, 33–42
Bates, P. C. & Millward, D. J. (1983) Biochem. J. **214**, 587–592
Bird, J. W., Carter, J. H., Triemer, R. E., Brooks, R. M. & Spanier, A. M. (1980) Fed. Proc. Fed. Am. Soc. Exp. Biol. **39**, 20–25
Brooks, B. A., Goll, D. E., Peng, Y. S., Greweling, J. A. & Hennecke, G. (1983) J. Nutr. **113**, 145–158
Clark, A. S., Kelley, R. A. & Mitch, W. E. (1984) J. Clin. Invest. **74**, 888–897
Dean, R. T. (1975) Nature (London) **257**, 414–416

Fulks, R. M., Li, J. B. & Goldberg, A. L. (1975) J. Biol. Chem. **250**, 290–298
Goodman, M. N., McElaney, M. A. & Ruderman, N. B. (1981) Am. J. Physiol. **241**, E321–E327
Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1977) Biochem. J. **164**, 399–407
Jenkins, A. B., Whittaker, M. & Schofield, P. J. (1979) Biochem. Biophys. Res. Commun. **86**, 1014–1019
Li, J. B. & Goldberg, A. L. (1976) Am. J. Physiol. **231**, 441–448
Li, J. B. & Wassner, S. J. (1984) Am. J. Physiol. **246**, E32–E37
Li, J. B., Higgins, J. E. & Jefferson, L. S. (1979) Am. J. Physiol. **236**, E222–E228
Libby, P. & Goldberg, A. L. (1978) Science **199**, 534–536
Long, W. M., Chua, B. H. L., Munger, B. L. & Morgan, H. E. (1984) Fed. Proc. Fed. Am. Soc. Exp. Biol. **43**, 1295–1300
Mortimore, G. E. (1982) Nutr. Rev. **40**, 1–12
Neff, N. T., DeMartino, G. N. & Goldberg, A. L. (1979) J. Cell. Physiol. **101**, 439–458
Riebow, J. H. & Young, R. B. (1980) Biochem. Med. **23**, 316–323
Seglen, P. O., Grinde, B. & Solheim, A. E. (1979) Eur. J. Biochem. **95**, 215–225
Wassner, S. J. & Li, J. B. (1982) Am. J. Physiol. **243**, E293–E297
Wassner, S. J., Schlitzer, L. & Li, J. B. (1980) Anal. Biochem. **104**, 284–289
Whitaker, J. N., Bertorini, T. E. & Mendell, J. R. (1983) Ann. Neurol. **14**, 133–142
Wibo, M. & Poole, B. (1974) J. Cell Biol. **63**, 430–440
Wildenthal, K. & Crie, J. S. (1980) Fed. Proc. Fed. Am. Soc. Exp. Biol. **39**, 37–41
Wildenthal, K., Waleland, J. R., Ord, J. M. & Stull, J. T. (1980) Biochem. Biophys. Res. Commun. **96**, 793–798
Young, V. R. & Munro, H. N. (1978) Fed. Proc. Fed. Am. Soc. Exp. Biol. **37**, 2291–2300

Received 1 October 1985/25 November 1985; accepted 2 December 1985