Age- and activity-related changes in three proteinase enzymes of rat skeletal muscle

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(Received 28 May 1985/28 June 1985; accepted 8 July 1985)

The specific activities of three proteinases, cathepsins B, D and H, were measured in two skeletal-muscle types as a function of age (i.e. from large foetal life to old age), and in muscles immobilized at various lengths for 3 days. The activities of the lysosomal endopeptidases B and D, but not H, consistently changed in parallel with previously determined rates of protein breakdown, indicating a good and potentially useful correlation between the two.

Several studies (Waterlow *et al.*, 1978; Lewis *et al.*, 1984; Kelly *et al.*, 1984) have indicated that in the rat the rapid pre- and post-natal growth of skeletal muscle is accompanied by a progressive decline in the fractional rates of protein synthesis and protein breakdown. The ready availability of reliable techniques for measuring average rates of protein synthesis *in vivo*, coupled with the magnitude of the developmental changes, leave little doubt about the age-related decline in the synthetic rate (Waterlow *et al.*, 1978; Kelly *et al.*, 1984). However, the same confidence cannot be attached to the less marked fall in the rate of protein degradation (Lewis *et al.*, 1984; Kelly *et al.*, 1984). Technical problems, such as the non-exponential decay of pre-labelled proteins and the efficient reutilization of tracer amino acids (Waterlow *et al.*, 1978), prevent the acquisition of accurate measured rates of degradation *in vivo*. In consequence, indirect rates of breakdown have often been used, these being calculated as the difference between the measured rates of protein synthesis and muscle growth (i.e. protein accretion).

Since muscle proteinases are believed to participate in the normal turnover of intracellular proteins (Pennington, 1977; Bird & Carter, 1980), age-related changes in the activities of such enzymes might be expected to parallel the developmental changes in protein degradation. In the present study we set out to determine if a correlation exists between the age-related changes in the activities of three lysosomal protein hydrolases, i.e. cathepsins B, D and H, and previously determined indirect rates of protein breakdown (Lewis *et al.*, 1984; Kelly *et al.*, 1984). In addition, a similar relationship was sought in two muscle

types, and in immobilized muscles, where the rates of protein degradation are known to increase in response to being rendered less active (Goldspink, 1977).

Methods

In all investigations, male albino rats (CD strain) were used from our own breeding colony, stock having previously been derived from Charles River U.K. Ltd. (Manston, Kent, U.K.). All animals were fed *ad libitum* on a standard cubed diet, containing a minimum of 21% (w/v) protein, and housed at 22°C with a 12h-light (06.00–18.00h)/12h-dark cycle.

In the developmental study the activities of cathepsin B, D and H were measured in the extensor digitorum longus (a fast-twitch muscle) and the soleus (a slow-twitch muscle) muscles of the rat between the 21-day foetus and 2.5 years of age. In addition, the same enzymes were assayed in both muscle types of 55g rats (approx. 4 weeks of age), 3 days after one hind limb had been immobilized in plantar flexion by using plaster of Paris casts (Goldspink, 1977). Total cathepsin activities were measured in crude homogenates derived by homogenizing freshly dissected, and finely chopped, muscles in distilled water (1:50, w/v) with a ground-glass homogenizer. Proteinase activities were expressed in appropriate units (see below) either per whole muscle or per mg of protein. Muscle protein was measured throughout by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Cathepsin B and H activities were measured after 10min incubation, at 37°C, with the synthetic substrate (5 μ M) Z-Phe-

Arg-7-aminomethylcoumarin and L-Arg-7-aminomethylcoumarin (Sigma) in $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (100mM), at pH 6.0 and 6.8 respectively (Barrett, 1980). Incubation was stopped by the addition of an equal volume of 100mM-sodium chloroacetate in a buffer containing sodium acetate (30mM) and acetic acid (70mM), pH 4.3. In both cases one unit of activity is defined as the quantity of enzyme capable of releasing $1\text{ }\mu\text{mol}$ of aminomethylcoumarin/min; this end product was measured fluorimetrically (Barrett, 1980). Cathepsin D activity was measured after 1 h at 37°C against acid-denatured haemoglobin in 75mM - acetate buffer, pH 3.5. Incubation of the reaction mixture (0.2ml) was stopped by the addition of 0.9ml of 6.7% (v/v) HClO_4 and standing it on ice for 15 min. After centrifuging, the A_{224} of the acid-soluble peptides in the supernatant was measured (Pennington, 1977). Enzyme activity (in arbitrary units) was determined as the difference in values between incubated tests (i.e. enzyme present throughout) and reagent blanks (i.e. enzyme added only at end of incubation).

Results and discussion

Cathepsin B, H and D activities were initially measured in two muscle types as a function of age. Throughout, all proteinase activities per mg of protein were age for age higher in the slow-twitch soleus muscle (Table 1), a feature consistent with the higher rate of turnover in this muscle (Waterlow *et al.*, 1978; Kelly *et al.*, 1984) and with

previous reports on cathepsins B and C at a few specific post-natal ages (Pluskal & Pennington, 1973; Pennington, 1977). Because of the large increases in muscle size, all three peptide hydrolase activities increased when expressed per whole muscle. However, when such differences in tissue size were eliminated by the use of specific activities (i.e. per mg of protein) a different picture emerged (Table 1).

In general agreement with the previously reported developmental fall in the calculated rates of protein breakdown (Kelly *et al.*, 1984), an age-related fall in the specific activities of all three cathepsins was apparent in both muscle types (Table 1). However, within this broad and wide-ranging developmental trend, interesting differences emerged between the three endopeptidases, these being consistently expressed in both muscle types. Cathepsin B specific activity progressively decreased (i.e. 15–30-fold) from late foetal life right through to old age (130 weeks). Although the specific activity of cathepsin D also decreased sharply up to weaning (i.e. 3 weeks), it changed little thereafter. In contrast, cathepsin H appeared to increase slightly up to 3–4 weeks after birth, before falling by some 40–55% into old age (Table 1). Despite these interesting variations, when viewed from one extreme of life to the other all three peptide hydrolases exhibited a substantial developmental decline in their specific activities (Table 1). A similar decline in the specific activities of cathepsins A, B, C and D has been reported for chicken breast muscle, as investigated

Table 1. *Developmental changes in the specific activities of cathepsins B, H and D in two skeletal muscle types*
Specific activities of cathepsins B, H and D were measured in muscle homogenates derived from a minimum of five extensor digitorum longus or soleus muscles at each age (i.e. means $\pm$ S.E.M.). No soleus muscles could be isolated at 3 weeks of foetal life, because of their extremely small size. A combined preparation of extensor digitorum longus and tibialis anterior muscles was, however, obtained at this age. Cathepsin D was measured against denatured haemoglobin (Pennington, 1977) and activity expressed as the change in A_{224} in HClO_4 -soluble material. Cathepsins B and H were respectively assayed against the synthetic substrates Z-Phe-Arg-NMec and Arg-NMec (Barrett, 1980); in these cases one unit of activity represents the amount of enzyme capable of releasing $1\text{ }\mu\text{mol}$ of aminomethylcoumarin (Mec) min.

		Activity (per mg of protein)						
		Foetal	Postnatal					
Age (weeks)...		3	1	3	4	52	130	
Muscle	Cathepsin							Non-Ambulatory
Extensor digitorum longus	B (μ units)	664 $\pm$ 62	465 $\pm$ 20	159 $\pm$ 15	—	48 $\pm$ 6	20 $\pm$ 2	57 $\pm$ 10
	H (μ units)	320 $\pm$ 7	372 $\pm$ 23	403 $\pm$ 25	—	254 $\pm$ 13	242 $\pm$ 16	285 $\pm$ 15
	D (A_{224}/h)	0.83 $\pm$ 0.04	0.70 $\pm$ 0.01	0.36 $\pm$ 0.02	—	0.47 $\pm$ 0.01	0.42 $\pm$ 0.01	0.73 $\pm$ 0.04
Soleus	B (μ units)	—	515 $\pm$ 40	227 $\pm$ 11	210 $\pm$ 11	96 $\pm$ 2	34 $\pm$ 3	100 $\pm$ 9
	H (μ units)	—	535 $\pm$ 19	606 $\pm$ 43	612 $\pm$ 39	236 $\pm$ 20	272 $\pm$ 10	274 $\pm$ 15
	D (A_{224}/h)	—	0.90 $\pm$ 0.02	0.43 $\pm$ 0.03	0.42 $\pm$ 0.04	0.48 $\pm$ 0.02	0.40 $\pm$ 0.01	0.75 $\pm$ 0.06

for several months beyond hatching (Iodice *et al.*, 1972). These alterations in enzyme activity were consistent with changes in the autolytic activity of the chicken muscle homogenates when measured at pH 4.4 (Iodice *et al.*, 1972). To our knowledge no similar comprehensive study has previously been undertaken with mammalian muscles. A few studies have, however, compared cathepsin, and other lysosomal enzyme, activities in muscles of mature adult and senescent rodents (Hajek *et al.*, 1965; Pilstrom *et al.*, 1978). In all instances higher enzymic activities were found in the older group of muscles. It is, however, clear that this late phase of post-natal life represents a stage where enzymic changes are relatively small, compared with earlier developmental changes (Table 1).

In the group of senile rats (i.e. 130 weeks) an additional point of interest emerged (Table 1). Whereas some of these animals were fully ambulatory, others possessed only limited mobility, dragging their hind legs in extension behind them. That is, in these particular animals both hind limbs were almost permanently held in plantar flexion, in contrast with the usual dorsi-flexed standing position. Although old-age atrophy was evident in both groups of senile rats (body and muscle weights decreasing relative to values at the earlier age of 52 weeks), the wasting was more pronounced in the rats with only limited mobility. For example, the extensor digitorum longus and soleus muscles weighed 107 ± 7 and 76 ± 13 mg respectively; these on average were 50 and 40% of the weights in the fully ambulatory animals at the same age. The precise nature of this defect is unknown, but may be linked to a greater incidence of end-plate degeneration and loss of motor units in aging

muscle (Gutmann, 1977). However, it is clear that, in association with the decreased muscle activity, the specific activities of cathepsin B and D, but not H, significantly ($P < 0.01$) increased relative to the values in the age-matched control muscles (i.e. ambulatory group; Table 1). Although we were unable to measure the rates of muscle protein breakdown in these senile rats, the changes in the limb position (i.e. in the non-ambulatory animals) could be mimicked by immobilization in plantar flexion (Goldspink, 1977). Under these circumstances extensor muscles such as the soleus are permanently immobilized in a fully shortened state and undergo appreciable atrophy as a result of protein synthesis decreasing and protein degradation increasing (Goldspink, 1977). Such changes in protein turnover appear to be directly related to the decreased muscle activity. Electromyographic studies have revealed that less than 10% of the normal activity remains in muscles when immobilized in this position (Hnik *et al.*, 1985). A rapid and significant ($P < 0.001$) increase in the specific activities of cathepsins B (40%) and D (85%) was apparent in the soleus muscle when intentionally immobilized in plantar flexion (Table 2a), and in non-ambulatory rats where this muscle would be similarly held in a shortened position (Table 1). As such, the increases in the endopeptidase activities are consistent with reported changes in the specific activities of other peptide hydrolases when measured in 7-day-immobilized muscles (Miyazawa *et al.*, 1983). All of these changes correlate with the increased rates of protein breakdown when measured as a function of time in muscles rendered less active either by plaster-cast immobilization (Goldspink, 1977) or under

Table 2. Specific activities of cathepsins B, H and D activities in muscles immobilized for 3 days in either a shortened or fully lengthened state

One hind limb of six rats was immobilized in plantar flexion, thereby restraining the soleus muscle (a) in a shortened state and the extensor digitorum longus (b) in a lengthened, stretched, state (Goldspink, 1977). The three cathepsins were measured in muscle homogenates prepared from either the immobilized muscles or controls derived from the unrestrained contralateral limb. Proteinase activities are expressed in the same units as Table 1, each value representing the mean $\pm$ S.E.M. of measurements on a minimum of five muscles. Statistical significance levels (* $P < 0.01$; ** $P < 0.001$) were determined by using Student's *t* test.

	Cathepsin activities (per mg of protein)		
	B (μ units)	H (μ units)	D (A_{224}/h)
(a) Soleus			
Control	227 ± 11	606 ± 43	0.43 ± 0.03
Immobilized (shortened)	319 ± 11	676 ± 44	0.79 ± 0.06
Change (%)	+41*	+12	+82**
(b) Extensor digitorum longus			
Control	157 ± 12	403 ± 25	0.36 ± 0.03
Immobilized (stretched)	224 ± 11	532 ± 18	0.46 ± 0.03
Change (%)	+43*	+32*	+28*

weightless conditions (D. F. Goldspink, A. J. Morton, P. Loughna & G. Goldspink, unpublished work).

Although both muscle types in the non-ambulatory senile rats exhibited appreciable fibre atrophy and degeneration (with some limited compensatory hypertrophy in a few fibres), these changes were much more pronounced in the soleus than in the extensor digitorum longus muscle (C. A. G. Boreham & D. F. Goldspink, unpublished work). In keeping with these pathological changes, the degree of atrophy and the increases in cathepsins B and D (Table 1) were also more marked in the slow-twitch muscle. It is possible that the extensor digitorum longus muscle in these non-ambulatory animals may be partially protected from the full extent of wasting by being held at a longer length (i.e. in plantar flexion) than usual. The known anabolic influences of stretch appear to be mediated by increased rates of protein synthesis (Goldspink, 1977; Watson *et al.*, 1984). However, because muscles permanently immobilized in this position also exhibit less activity than their contralateral controls (Hnik *et al.*, 1985), breakdown in also increased (Goldspink, 1977). In keeping with this increase in protein degradation, the three proteinase specific activities (now including cathepsin H) were significantly enhanced by 30–40% in muscles immobilized in a stretched state by using plaster casts (Table 2b). These acute changes (Table 2b) are probably akin to the more prolonged events in the extensor digitorum longus of non-ambulatory animals, where this muscle will be restrained in an equivalent stretched state in the dragged hind limb of those aged animals (Table 1).

It seems probable therefore that the significantly increased proteinase activities (B and D) in the muscles of the non-ambulatory animals arise from a combination of decreased muscular activity (Tables 2a and 2b) and denervation (Goldspink *et al.*, 1970; Pennington, 1977). Such observations (Table 1), and variable degrees of mobility, denervation and macrophage infiltration in muscles of aged animals, may explain the previous findings of higher specific activities of some proteinases when compared with enzyme activities in muscles of younger adult animals (Hajek *et al.*, 1965; Pilstrom *et al.*, 1978). In all of the situations cited above (Tables 1, 2a and 2b), the alterations in cathepsin B and D specific activities paralleled the measured (Goldspink, 1977) or calculated (Kelly *et al.*, 1984; Lewis *et al.*, 1984) changes in protein breakdown. An interesting partial exception to this was cathepsin H. Although similarly believed to be lysosomal in origin, this protein hydrolase appeared considerably more resistant to changes in its specific activity. Some difficulties do arise, however, when directly comparing the changes in averages rates of protein breakdown

and individual proteinase activities as measured in muscle homogenates. For example, both plasma and intracellular inhibitors (Pennington, 1977 Bird & Carter, 1980) are known to be capable of modifying the activities of cysteine proteinases (e.g. cathepsins B and H) in crude homogenates after cell membranes have been disrupted. It is also conceivable that the amount and/or activity of such inhibitors, as well as the enzymes themselves, might change with age. Nonetheless, both the degradative rates and the specific activities of cathepsins B and D consistently changed in parallel. Hence measurements of the activities of these two muscle proteinases should be helpful in supporting, or verifying, changes in the indirect rates of protein breakdown in situations where direct rates cannot be meaningfully obtained.

We are grateful to Paul Anderson, B.Sc., for his technical assistance, and to The Royal Society and the British Heart Foundation for their financial support of this work.

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