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Regulation of total and myofibrillar protein breakdown in rat extensor digitorum longus and soleus muscle incubated flaccid or at resting length

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The present study characterized total and myofibrillar protein breakdown rates in a muscle preparation frequently used *in vitro*, i.e. incubated extensor digitorum longus (EDL) and soleus (SOL) muscles of young rats. Total and myofibrillar protein breakdown rates were assessed by determining net production by the incubated muscles of tyrosine and 3-methylhistidine (3-MH) respectively. Both amino acids were determined by h.p.l.c. Both total and myofibrillar protein breakdown rates were higher in SOL than in EDL muscles and were decreased by incubating the muscles maintained at resting length, rather than flaccid. After fasting for 72 h, total protein breakdown (i.e. tyrosine release) was increased by 73% and 138% in EDL muscles incubated flaccid and at resting length respectively. Net production of tyrosine by SOL muscle was not significantly altered by fasting. In contrast, myofibrillar protein degradation (i.e. 3-MH release) was markedly increased by fasting in both muscles. When tissue was incubated in the presence of 1 munit of insulin/ml, total protein breakdown rate was inhibited by 17–20%, and the response to the hormone was similar in muscles incubated flaccid or at resting length. In contrast, myofibrillar protein breakdown rate was not altered by insulin in any of the muscle preparations. The results support the concepts of individual regulation of myofibrillar and non-myofibrillar proteins and of different effects of various conditions on protein breakdown in different types of skeletal muscle. Thus determination of both tyrosine and 3-MH production in red and white muscle is important for a more complete understanding of protein regulation in skeletal muscle.

INTRODUCTION

Incubated extensor digitorum longus (EDL) and soleus (SOL) muscles of mice or young rats are frequently used for the study of protein turnover. Measurements of protein metabolism in this system *in vitro* have identified changes that occur in response to altered physiological or pathophysiological states of the animals, such as starvation [1,2], hyperthyroidism [3], injury [4] and sepsis [5,6]. The muscles are also responsive *in vitro* to different hormones and substances such as insulin [2,7], amino acids, particularly leucine [8,9], prostaglandins [10] and corticosteroids [11].

In most previous reports using incubated EDL and SOL muscles, protein breakdown was measured as release of tyrosine or phenylalanine into incubation medium. Although this method reflects total proteolysis, it does not distinguish between the breakdown of myofibrillar and non-myofibrillar proteins. Approx. 60% of skeletal-muscle protein is myofibrillar, but its rate of turnover is several times slower than that of non-myofibrillar proteins [12]. Consequently, determination of total protein breakdown rates may primarily reflect changes in non-myofibrillar proteolysis.

Since 3-methylhistidine (3-MH) is present only in actin and myosin, and is neither degraded nor reutilized for protein synthesis after its release during proteolysis [13], the production of 3-MH from muscle reflects myofibrillar protein breakdown. Previous attempts to measure myofibrillar protein degradation in individual incubated skeletal muscles were restricted by the lack of sensitive techniques to measure 3-MH at low concentrations. A sensitive h.p.l.c. method for the measurement of low concentrations of 3-MH has been described [14,15], and by using this assay it was possible to determine myofibrillar protein breakdown rates in perfused rat hindquarter preparations and incubated rat EDL muscles [14–16]. Results in those studies suggested that the breakdown of myofibrillar and non-myofibrillar proteins in skeletal muscle is regulated independently and by different pathways. Thus measurement of both total and myofibrillar proteolysis is useful to achieve a more complete understanding of muscle protein breakdown and its regulation.

Incubated EDL and SOL muscles of young rats offer several advantages in the study of protein metabolism. The muscles are thin, allowing for adequate diffusion of oxygen and substrates, although previous studies suggested that a hypoxic central core may develop during incubation [17,18]. The muscles can be incubated intact, without cut muscle fibres, and the tissue can be studied in a flaccid position or maintained at resting length, factors which are known to affect protein turnover both *in vivo* and *in vitro* [19,20]. Furthermore, since EDL is a white fast muscle and SOL is a predominantly slow red muscle [21], the response in different types of skeletal muscle to different conditions or substances can be compared.

Although incubated rat EDL and SOL muscles have been used in a large number of studies to assess total protein breakdown, myofibrillar protein degradation rates have not been defined in this system *in vitro*. In the present study we determined tyrosine and 3-MH release by rat EDL and SOL muscles, incubated flaccid or fixed at resting length. We also examined the influence of a catabolic condition, i.e. starvation, and of an anabolic hormone, i.e. insulin, on total and myofibrillar protein breakdown rates.

Abbreviations used: EDL, extensor digitorum longus; SOL, soleus; 3-MH, 3-methylhistidine.

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MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats weighing 40–60 g were obtained from Zivic Miller (Allison Park, PA, U.S.A.). Animals were housed in groups of five in ordinary rat cages and had free access to food and water. Fasted animals had food removed 72 h before muscles were harvested. The animal cages were cleaned each day to minimize coprophagia.

Muscle incubations

The left and right EDL and SOL muscles were dissected with intact tendons under diethyl ether anaesthesia. Either muscles were incubated in a flaccid state, or the tendons were tied to stainless-steel supports with 3-0 silk sutures at approximate resting length. Muscles were weighed and preincubated for 30 min in 3 ml of a medium consisting of Krebs–Henseleit [21a] bicarbonate buffer (pH 7.4) and glucose (10 mM). The medium was vigorously gassed with O_2/CO_2 (19:1) for 15–20 min immediately before use. After addition of the muscles, the incubation flasks were flushed with O_2/CO_2 (19:1), re-stoppered and placed in a shaking water bath at 37 °C. After preincubation, one EDL and one SOL muscle were transferred to ice-cold 3% (w/v) $HClO_4$ for determination of '0 h' tissue tyrosine and 3-MH (see below). The contralateral muscles were individually transferred to 3 ml of fresh medium containing cycloheximide (0.5 mM) and incubated for 2 h. The vials were gassed with O_2/CO_2 (19:1) at the start and after 1 h of incubation. In experiments in which the effect of insulin (pig; Sigma, St. Louis, MO, U.S.A.) of leupeptin (Sigma) was tested, the substance was present in the incubation medium during both preincubation and incubation. After incubation, muscles were blotted and transferred to ice-cold 3% $HClO_4$. The muscles and incubation media were stored at −20 °C until analysed.

Although previous studies have shown that incubated muscles become less catabolic if amino acids, especially leucine, are added to the incubation medium [22], unsupplemented medium was used in the current experiments. The main reason for this was that in preliminary experiments we observed that amino acids, in particular leucine, interfered with the 3-MH assay, giving rise to falsely low 3-MH readings (P.-O. Hasselgren, J. H. James & J. E. Fischer, unpublished work). Since the present system *in vitro* is used to study relative changes of protein turnover rates in different pathophysiological conditions, rather than absolute protein turnover rates, and since incubated muscles remain in a catabolic state even if the medium is supplemented with amino acids (and/or insulin), we considered it justified to omit amino acids from the incubation medium in the present experiments. One concern is that viability may be affected when muscles are incubated in an unsupplemented medium. Control experiments were therefore performed in which muscle ATP levels were determined (as described below) *in vivo* and after incubation for 2 h. ATP in EDL was 7.22 ± 0.18 μmol/g wet wt. *in vivo* ($n = 4$) and 6.54 ± 0.28 μmol/g after 2 h incubation at resting length ($n = 4$) and 4.63 ± 0.61 μmol/g after 2 h incubation in a flaccid state ($n = 4$). The corresponding values for SOL were 4.98 ± 0.08 μmol/g *in vivo* ($n = 4$), 5.01 ± 0.65 μmol/g after 2 h incubation at resting length ($n = 4$), and 2.78 ± 0.18 μmol/g after 2 h incubation flaccid ($n = 4$). These results, both absolute energy levels and maintenance of ATP levels in muscles incubated at resting length and somewhat decreased ATP levels in muscles incubated flaccid, are almost identical with those of other studies in which muscles were incubated in a medium supplemented with both amino acids and insulin [20].

Determination of 3-MH and tyrosine

The muscles were homogenized in 2 ml of ice-cold 3% $HClO_4$, and after centrifugation (2000 *g*, 20 min, 4 °C) the supernatant was neutralized with 0.28 ml of 2 M-KOH containing 0.5 M-triethanolamine. The precipitated $KClO_4$ was removed by centrifugation (2000 *g*, 5 min, 4 °C). 3-MH in $HClO_4$ extracts of tissue and in incubation-medium samples was determined by h.p.l.c. as described by Lowell *et al.* [15] with minor modifications. Briefly, 500 μl of 0.2 M-sodium borate (pH 10.0) was added to a small glass screw-cap tube containing 500 μl of 3-MH standard, medium sample or $HClO_4$ extract of muscle. With continuous vortex-mixing, 500 μl of fluorescamine in acetonitrile (1.6 mg/ml) was added slowly. After 5 min 175 μl of 60% $HClO_4$ was added, and the tubes were heated to 80 °C for 1 h. After cooling, the mixtures were neutralized with 500 μl of 3.0 M-NaOH, containing 0.5 M-Mops reagent.

Samples (50 μl) of the fluorescamine derivatives were separated isocratically on a Waters (Milford, MA, U.S.A.) Bondapak 15 cm C-18 column with as mobile phase 23% (v/v) acetonitrile in water at a flow rate of 1.1 ml/min; 3-MH was eluted after 6 min. Concentrations of 3-MH were determined by comparing peak heights of samples with that of an external standard (0.1 nmol/ml). A Shimadzu (Kyoto, Japan) fluorimeter RF-535 with an 8 μl flow cell was used for detection. Excitation and emission wavelengths were 330 and 455 nm respectively. Under these conditions, the minimum concentration of 3-MH that could be reliably detected in incubation medium was around 0.002 nmol/ml. The assay was linear over a range of 3-MH concentrations of 0.005–0.40 nmol/ml ($r = 0.9996$). The within-run ($n = 5$) coefficient of variability (at the indicated concentrations) was 2.86% (0.050 nmol/ml), 3.93% (0.025 nmol/ml) and 6.86% (0.005 nmol/ml).

Tyrosine levels were also determined fluorimetrically by h.p.l.c. using a Waters Bondapak C-18 column (7.5 cm) and a mobile phase consisting of 0.1 M-KH_2PO_4 and 1.4 mM-sodium octyl sulphate, pH 2.9, delivered at a rate of 0.9 ml/min. Medium or supernatant of muscle homogenate was diluted 1:5 (v/v) with mobile phase before injection of 50 μl into the chromatograph. Concentrations of tyrosine were determined by comparing peak heights with that of an external standard (4.0 nmol/ml). Excitation and emission wavelengths were 275 and 310 nm respectively. Under these conditions, the minimum concentration of tyrosine that could be reliably detected in the medium was about 0.1 nmol/ml. The assay was linear over tyrosine concentrations of 0.20–10.0 nmol/ml ($r = 0.99993$). The within-run ($n = 10$) coefficient of variability was (at the indicated concentrations) 0.60% (4.0 nmol/ml), 0.84% (0.80 nmol/ml), 1.69% (0.40 nmol/ml) and 3.97% (0.20 nmol/ml). For both the 3-MH and tyrosine assays, coefficients of variability at the lowest concentration were smaller for peak-height data measured from a strip-chart recorder as compared with peak-area data measured by an automatic integrator. Peak-height data were therefore used throughout.

Determination of ATP in muscle tissue

For determination of ATP levels *in vivo*, muscles were clamped *in situ* with tongues pre-cooled in liquid N_2, excised, and immediately immersed in liquid N_2. In other experiments, muscles were frozen in liquid N_2 after incubation for 2 h. Tissue was stored at −70 °C until analysis. ATP was determined fluorimetrically as described previously [23].

Statistics

Results are presented as means ± S.E.M. Student's *t* test or analysis of variance followed by Tukey's test was used for statistical comparisons.

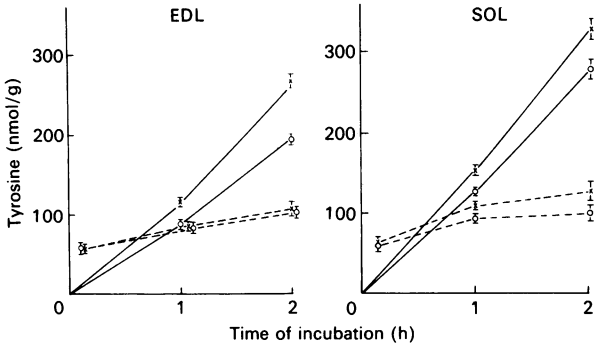

Fig. 1. Tyrosine in incubation medium (——) and in tissue (----); fed rats

Muscles from fed rats were incubated flaccid (×) or fixed at resting length (○). Each point represents the mean ± S.E.M. of 5 or 6 muscles.

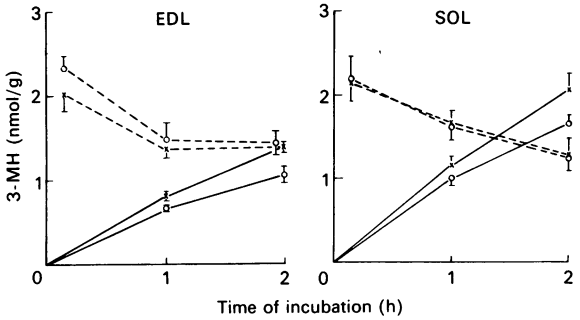

Fig. 2. 3-MH in incubation medium (——) and in tissue (----); fed rats

Muscles from fed rats were incubated flaccid (×) or fixed at resting length (○). Each point represents the mean ± S.E.M. of 5 or 6 muscles.

RESULTS

Release of tyrosine into incubation medium by EDL and SOL muscles from normal fed rats was linear during 2 h incubation, when muscles were incubated either flaccid or at resting length (Fig. 1). Tissue levels of free tyrosine increased by 70–100% in both EDL and SOL muscles during incubation (Fig. 1), and from these data it could be calculated that, if total protein breakdown rates were determined only from the tyrosine released into incubation medium, the proteolytic rates would be underestimated by approx. 15–20%. Also, 3-MH was released by incubated EDL and SOL muscles from normal fed rats at an almost linear rate, but, in contrast with tyrosine, tissue levels of 3-MH decreased by approx. 40% during incubation (Fig. 2), and after 2 h, 50–70% of the 3-MH found in the incubation medium could be accounted for by decreased tissue levels of the amino acid.

Tissue levels of tyrosine and 3-MH behaved somewhat differently when muscles from 72 h-starved rats were incubated. Thus tyrosine levels were relatively stable during incubation in both EDL and SOL, although at a higher level than in muscles from fed rats (Fig. 3). 3-MH levels were approx. 3 times higher in muscles from starved rats than in muscles from fed animals, and decreased during incubation in both EDL and SOL (Fig. 4). The relative decrease of 3-MH in EDL from fasted rats was less pronounced than in muscles from fed animals, whereas the changes in tissue levels of 3-MH were similar in SOL muscles from fed and starved animals (Figs. 2 and 4).

These results illustrate the importance of taking changes in

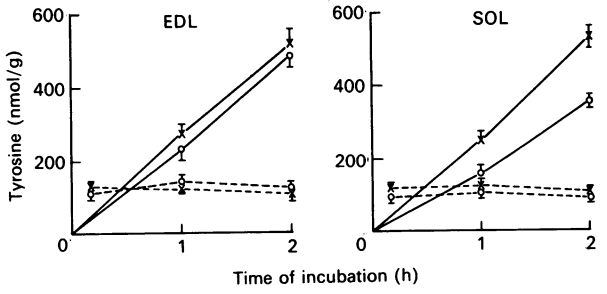

Fig. 3. Tyrosine in incubation medium (——) and in tissue (----); 72 h-fasted rats

Muscles from fasted (72 h) rats were incubated flaccid (×) or fixed at resting length (○). Each point is the mean ± S.E.M. from 5 or 6 muscles.

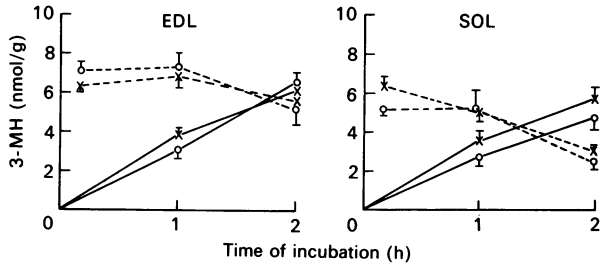

Fig. 4. 3-MH in incubation medium (——) and in tissue (----); 72 h-fasted rats

Muscles from starved (72 h) rats were incubated flaccid (×) or fixed at resting length (○). Each point represents the mean ± S.E.M. of 5 or 6 muscles.

tissue concentrations of tyrosine and 3-MH into account when total and myofibrillar proteolytic rates in incubated muscles are determined. In subsequent experiments, therefore, two EDL and two SOL muscles were harvested from each rat; one muscle was used for determination of tissue concentration of tyrosine and 3-MH at the onset of incubation (i.e. after 30 min preincubation) and the other muscle was incubated for 2 h, and both tissue and medium concentrations of the amino acids were measured.

Total protein breakdown rate (i.e. net production of tyrosine) was higher in SOL than in EDL ($P < 0.01$; Table 1), in line with previous reports [1]. When muscles were incubated fixed at resting length, total protein breakdown rate was decreased by 24% in EDL and by 19% in SOL (Table 1), in good agreement with a report by Baracos & Goldberg [20]. Also, myofibrillar protein breakdown rate (i.e. net production of 3-MH) was higher in SOL than in EDL ($P < 0.05$) and was decreased by incubating the muscles at resting length (Table 1). The effect of incubating tissue at resting length seemed to be more pronounced on myofibrillar than on total protein breakdown rates.

The molar ratio of tyrosine to 3-MH in isolated myofibrillar protein from pooled muscles of rat hindquarter was approx. 31:1 in a recent study [15]. Although this ratio may not be the same in EDL and SOL muscles, we used it to compute the amount of tyrosine released from both myofibrillar and non-myofibrillar proteins. To obtain myofibrillar tyrosine release, the mean values of 3-MH net production in Table 1 were multiplied by 31, and, by subtracting myofibrillar from total tyrosine release, the tyrosine released from non-myofibrillar proteins could be estimated. The results of these computations are shown in Table 2 and suggest that the relative changes in non-myofibrillar protein breakdown rates, induced by maintaining muscles at

Table 1. Tissue levels and release of tyrosine and 3-MH (nmol/g) during 2 h incubation of rat EDL and SOL muscles, incubated flaccid or at resting length

$n = 6$ or 7 in each group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus flaccid.

		Tyrosine				3-MH			
		Tissue level				Tissue level			
		0 h	2 h	Medium	Net production	0 h	2 h	Medium	Net production
EDL	Flaccid	55±5	106±7	265±9	316±9	2.02±0.19	1.37±0.06	1.39±0.06	0.74±0.06
	Resting length	56±5	104±5	193±6	241±6*** (−24%)	2.17±0.10	1.44±0.06	1.06±0.09	0.33±0.09** (−55%)
SOL	Flaccid	63±7	127±10	323±15	387±15	2.15±0.17	1.29±0.21	2.06±0.19	1.20±0.19
	Resting length	62±6	100±8	275±12	314±10** (−19%)	2.19±0.26	1.28±0.12	1.66±0.11	0.75±0.11* (−38%)

Table 2. Calculated tyrosine release from myofibrillar and non-myofibrillar proteins by rat EDL and SOL muscles, incubated flaccid or at resting length

		Tyrosine release (nmol/2 h per g)		
		Total	Myofibrillar	Non-myofibrillar
EDL	Flaccid	316	23	293
	Resting length	241 (−24%)	10 (−56%)	231 (−21%)
SOL	Flaccid	387	37	350
	Resting length	314 (−19%)	23 (−38%)	291 (−17%)

Table 3. Net production of tyrosine and 3-MH by EDL and SOL muscles incubated in normal medium (containing 2.5 mM-Ca^{2+}), Ca^{2+}-free medium, or normal medium containing 30 μM-leupeptin

Results are from 14 muscles in each group, except for experiments in which leupeptin was present in incubation medium, when 21 muscles were used in each group. * $P < 0.05$ versus resting length by ANOVA followed by Tukey's test; N.S., not significant.

			Production (nmol/2 h per g)		
Amino acid	Muscle		Normal medium	Ca^{2+}-free medium	Leupeptin
Tyrosine	EDL	Resting length	219±12	168±7	160±6
		Flaccid	325±9* (+48%)	243±7* (+45%)	236±15* (48%)
	SOL	Resting length	294±16	267±11	249±12
		Flaccid	427±20* (+45%)	335±17 (N.S.)	321±21 (N.S.)
3-MH	EDL	Resting length	0.30±0.14	0.13±0.06	0.38±0.12
		Flaccid	0.93±0.23* (+210%)	0.25±0.12 (N.S.)	0.32±0.09 (N.S.)
	SOL	Resting length	0.43±0.23	0.34±0.09	0.45±0.09
		Flaccid	1.60±0.50* (+272%)	0.37±0.13 (N.S.)	0.55±0.11 (N.S.)

resting length, were well reflected by changes in total protein breakdown rates. This in turn reflects the fact that in these muscles (from fed rats) non-myofibrillar protein breakdown rates were 10–15 times higher than myofibrillar protein breakdown rates and, consequently, most of the total tyrosine originated from non-myofibrillar protein degradation.

In order to study possible mechanisms by which protein breakdown is altered when muscles are incubated flaccid or at resting length, muscles were incubated in normal medium (containing 2.5 mM-Ca^{2+}), in Ca^{2+}-free medium, or in normal medium containing leupeptin (30 μM). The difference in tyrosine production between muscles incubated flaccid or at resting length was almost identical in EDL incubated in normal or Ca^{2+}-free medium or in medium with leupeptin, whereas the increase in tyrosine release by flaccid SOL muscle was attenuated in the absence of Ca^{2} or in the presence of leupeptin (Table 3). The

Table 4. Tissue levels and release of tyrosine and 3-MH (nmol/g) during 2 h incubation of muscles from fed or fasted (72 h) rats

$n = 7$ in each group; * $P < 0.001$ versus fed.

Muscle		Rats	Tyrosine				3-MH			
			Tissue level			Net production	Tissue level			Net production
			0 h	2 h	Medium		0 h	2 h	Medium	
EDL	Flaccid	Fed	67±4	149±5	244±8	326±7	2.64±0.10	1.59±0.12	1.90±0.15	0.98±0.22
		Fasted	117±5	135±7	545±11	564±14* (+73%)	5.02±0.34	3.52±0.32	11.03±0.30	9.53±0.53* (+872%)
	Resting length	Fed	77±8	114±5	171±9	208±9	2.34±0.08	1.43±0.09	1.35±0.09	0.50±0.08
		Fasted	109±9	155±9	450±24	496±38* (+138%)	4.85±0.35	3.52±0.30	9.02±0.35	7.69±0.67* (+1438%)
SOL	Flaccid	Fed	52±4	150±14	349±26	447±36	2.64±0.15	1.51±0.16	2.44±0.15	1.30±0.21
		Fasted	100±6	118±11	452±35	469±38	4.92±0.85	2.84±0.43	8.97±1.17	6.88±1.15* (+429%)
	Resting length	Fed	61±5	100±3	271±9	309±11	2.29±0.14	1.27±0.08	1.89±0.12	0.86±0.10
		Fasted	89±7	101±4	331±14	343±13	4.06±0.47	2.25±0.20	6.46±0.53	4.66±0.42* (+442%)

Table 5. Calculated tyrosine release from myofibrillar and non-myofibrillar proteins by incubated EDL muscles from fed and fasted (72 h) rats

EDL	Rats	Tyrosine release (nmol/2 h per g)		
		Total	Myofibrillar	Non-myofibrillar
Flaccid	Fed	326	30	296
	Fasted	564 (+73%)	295 (+883%)	269 (−9%)
Resting length	Fed	208	16	192
	Fasted	496 (+138%)	238 (+1388%)	258 (+34%)

Table 6. Tissue levels and release of tyrosine and 3-MH (nmol/g) during 2 h incubation of muscles incubated in the absence or presence of insulin (1 munit/ml)

Key: −, no insulin; +, 1 munit of insulin/ml. $n = 6$ or 7 in each group; * $P < 0.05$, ** $P < 0.01$ versus no insulin.

			Tyrosine					3-MH				
			Tissue level			Net production		Tissue level			Net production	
			0 h	2 h	Medium			0 h	2 h	Medium		
EDL	Flaccid	−	68±4	122±5	266±9	320±9	−	3.45±0.22	2.30±0.17	3.45±0.13	2.30±0.09	
		+	56±4	87±8	226±15	257±19** (−20%)	+	3.43±0.21	2.48±0.18	3.79±0.19	2.84±0.20	
	Resting length	−	60±5	103±5	210±12	252±12	−	4.99±0.32	3.07±0.20	2.68±0.18	0.77±0.42	
		+	66±5	99±5	176±7	209±10* (−17%)	+	4.87±0.24	3.36±0.20	2.84±0.17	1.33±0.31	
SOL	Flaccid	−	70±5	140±7	396±10	466±12	−	3.88±0.25	2.62±0.18	4.78±0.22	3.52±0.34	
		+	57±5	123±10	322±25	389±32* (−17%)	+	3.87±0.28	2.51±0.24	4.63±0.30	3.36±0.43	
	Resting length	−	70±6	116±7	301±12	347±14	−	5.81±0.27	3.46±0.35	3.70±0.16	1.34±0.37	
		+	67±5	113±8	239±8	285±12** (−18%)	+	4.87±0.39	2.99±0.21	3.57±0.17	1.69±0.48	

increase in myofibrillar protein breakdown in flaccid muscles was abolished in Ca^{2+}-free medium and by the presence of leupeptin in both EDL and SOL (Table 3).

Total protein breakdown rate was higher in EDL muscles from fasted than from fed rats (Table 4), similar to other studies [1], and the difference was most pronounced in EDL muscles incubated at resting length. Total protein breakdown in SOL muscle was not significantly altered by fasting. The effect of fasting on myofibrillar protein breakdown rate was dramatic, with a 9–14-fold increase in flaccid and stretched EDL, respectively, and an approx. 4-fold increase in SOL muscle (Table 4). The elevated tissue levels of tyrosine and 3-MH in muscles from fasted rats may reflect altered protein synthesis rate or amino acid transport in addition to accelerated protein breakdown rates. When myofibrillar and non-myofibrillar tyrosine release was calculated as described above, by using a tyrosine:3-MH ratio of 31:1, results showed that the increase in total protein breakdown noted in EDL muscles from fasted rats was caused almost exclusively by increased myofibrillar protein breakdown (Table 5).

When muscles were incubated in the presence of 1 munit of insulin/ml, total protein breakdown was inhibited by 17–20% and the response to the hormone was similar in EDL and SOL muscles incubated flaccid or at resting length (Table 6). In contrast, myofibrillar protein breakdown rate was not significantly altered by insulin in any of the muscle preparations (Table 6).

When comparing protein breakdown rates in Table 6 with those in Tables 1, 3 and 4, it is obvious that myofibrillar protein breakdown in control muscles was higher in Table 6. We can offer no definitive explanation for this variability at present. It is possible that the different degradation rates reflected seasonal variation, since the insulin experiments were performed approx. 3 months apart from the experiments shown in Tables 1, 3 and 4. The fact that total protein breakdown rates were similar in the different experiments, however, would argue against that explanation. It is important to point out that the different groups within each experiment were always studied on the same day. Thus, when muscles were incubated flaccid or at resting length (Table 1), when muscles were from fed or fasted rats (Table 4), and when muscles were incubated in the absence or presence of insulin (Table 6), differences between the experimental groups were not influenced by seasonal or day-to-day variation.

DISCUSSION

The technique of using incubated rat skeletal muscles for the study of protein turnover and its regulation under different conditions has been well described and characterized by Goldberg and co-workers [1,20,22,24], and has been used in many studies from other laboratories as well. Since in most previous studies protein breakdown rates were assessed as release of tyrosine, little is known about the regulation of myofibrillar protein breakdown in this muscle preparation *in vitro*. In the present study, therefore, total and myofibrillar protein breakdown rates in incubated rat EDL and SOL muscles were assessed by determining net production of free tyrosine and 3-MH respectively. Results suggest that both total and myofibrillar protein breakdown rates are higher in SOL than in EDL. Higher total protein breakdown rate in SOL than in EDL was reported previously [1], but the difference in myofibrillar protein degradation rate between red (slow) and white (fast) muscle is a new finding. Since 3-MH is only present in actin in red muscle, but in both actin and myosin in white muscle [13], the difference in myofibrillar protein breakdown rate between the two muscles may be even greater than suggested by the differences in 3-MH production. Although inhibited tyrosine release by muscles incubated at resting length has been described previously [20], decreased 3-MH release, as observed here, has not been reported previously.

The finding that tissue levels of tyrosine increased during incubation of muscles from normal fed rats was unexpected, since stable tissue levels of the amino acid during incubation of normal muscles have been reported previously [1]. Although there is an accumulation of tyrosine in tissue during incubation, the error introduced by using only tyrosine in medium for assessment for total proteolysis is rather small. The current data showed that total protein breakdown rate in normal muscle would have been underestimated by 15–20% if changes in tissue levels of tyrosine had not been taken into account. In the study by Baracos & Goldberg [20], tissue tyrosine levels increased in EDL and SOL muscles incubated in a flaccid state, and it was calculated that protein breakdown rate was underestimated by 10–20% when tyrosine in medium alone was used to assess proteolytic rate. In the same study [20], tissue levels were reported to stay constant when muscles were incubated fixed at resting length. This is in contrast with the present report, in which tissue tyrosine increased during incubation in muscles incubated either flaccid or at resting length.

The necessity to take amino acid tissue levels into account was more obvious when myofibrillar protein breakdown rates were determined, since 50–70% of the 3-MH found in the medium after incubation of normal muscles for 2 h could be accounted for by decreased tissue levels. In the present study, the decrease in free tissue 3-MH during incubation was substantial in both flaccid and stretched muscles from fed animals, whereas tissue levels of 3-MH were more stable in EDL from starved rats. Goodman [16] reported that tissue 3-MH remained stable in incubated rat EDL muscles from both fed and overnight-starved rats. Muscle concentrations of 3-MH were not significantly altered during 3 h perfusion of hemicorpus from fed rats, whereas in preparations from food-deprived animals tissue concentrations decreased by about 12% [25]. We recently observed relatively stable tissue levels of 3-MH during incubation of EDL and SOL muscles from septic rats [26]. Thus the tissue pool of free 3-MH, and tyrosine as well, may behave differently in different types of muscle and under different conditions.

One important implication of the present results is that a linear release of amino acids into incubation medium does not necessarily mean that the proteolytic rate is constant during incubation. For example, when EDL muscles from fed rats were incubated, release of 3-MH into medium during the first 1 h of incubation was almost balanced by a loss from tissue pools (Fig. 2), suggesting that myofibrillar protein breakdown rate in this muscle preparation was very low during the initial part of incubation. Thus net production of tyrosine or 3-MH at the end of the 2 h incubation reflects the average proteolytic rate over that period of time. On the other hand, our results suggest that proteolytic rates are indeed constant during incubation under most circumstances, since tissue levels were either relatively stable or changed at an almost linear rate in the other muscle preparations.

The present result of higher non-myofibrillar than myofibrillar protein turnover rate is in line with previous reports, although smaller differences were observed by others [12,27]. Possible reasons for the unexpectedly high ratio between non-myofibrillar and myofibrillar protein breakdown rates observed here may be that protein breakdown was measured *in vitro* in muscles from young growing rats. When myofibrillar protein turnover rates were assessed by measuring protein synthesis rates *in vivo*, the ratio of sarcoplasmic to myofibrillar protein synthesis was approx. 2:1 [12]. When total and myofibrillar protein breakdown rates were determined in perfused hindquarters of rats of different

age groups, the ratio of non-myofibrillar to myofibrillar protein degradation (based on calculations using the tyrosine:3-MH ratio of 31:1 described above) was 2.9 in rats 24 weeks old and 5.2 in rats 4 weeks old (weighing approx. 100 g) [27].

The exact mechanism of accelerated proteolysis in flaccid muscle is not known, but the catabolism in these muscles probably reflects shortening of the tissue during incubation. In a previous study, flaccid SOL and EDL shortened to 25% and 44% of their resting length, respectively, during incubation for 2 h [20]. The decrease in length was associated with an increased diameter, most pronounced in SOL, the thickness of which increased from 1.5 mm to 5 mm during incubation. The risk of development of a central hypoxic core in the incubated tissues is greater in thicker muscles, which probably explains why energy levels were less well maintained in muscles incubated flaccid than at resting length [20].

The present study supports a role of Ca^{2+} and lysosomal proteases for increased total protein breakdown in flaccid SOL muscle, whereas the difference in tyrosine release between flaccid and stretched EDL was not influenced by the absence of Ca^{2+} or the presence of leupeptin. This is in contrast with the study by Baracos & Goldberg [20], in which the increase in total protein breakdown in flaccid muscle was attenuated by Ca^{2+}-free medium and thiol protease inhibitors in both EDL and SOL.

The finding that the increase in myofibrillar protein breakdown in flaccid EDL and SOL muscles was abolished by the absence of Ca^{2+} or presence of leupeptin was surprising, since previous studies found no evidence that myofibrillar protein degradation is regulated by Ca^{2+} or lysosomal proteases [16,27]. However, other studies suggested that calpain may play an important role in degradation of myofibrillar proteins and contribute to muscle wasting in certain types of disease [28–30]. A possible role of Ca^{2+} for myofibrillar protein breakdown was further supported by the finding that the thiol reagent mersalyl, which inactivates calpain [31], decreased myofibrillar protein breakdown in incubated muscle [32]. It is possible that Ca^{2+} regulates only basal, but not stimulated, myofibrillar protein degradation, since we recently found that Ca^{2+} at a concentration of 2.5 or 5 mM did not affect 3-MH release by incubated SOL muscles of rats which were septic or which had undergone laparotomy and a 16 h fast, conditions that result in a pronounced increase in myofibrillar protein breakdown [33].

Accelerated protein breakdown in skeletal muscle during starvation is in line with several previous reports [1,15,22,25]. To the best of our knowledge, however, the current study is the first in which the effects of starvation on both myofibrillar and total protein degradation rates in two different types of skeletal muscle (i.e. the red SOL and the white EDL) were directly compared. The present finding that starvation stimulated tyrosine release by EDL, but not by SOL, is in line with a previous report by Li & Goldberg [1]. Our experiments extended previous observations in demonstrating that the effect of starvation on myofibrillar protein breakdown also was more pronounced in EDL than in SOL. The mechanism of the greater response to starvation in white than in red muscle and of the predominant increase in myofibrillar proteolysis is not known, but this type of response does not seem to be exclusive for starvation, since other catabolic conditions, such as sepsis [26], also mainly stimulate myofibrillar protein breakdown in white muscle.

Previous studies demonstrated that insulin *in vitro* increased muscle protein synthesis and decreased protein breakdown, measured as release of tyrosine or phenylalanine [2,7,22]. A previous report suggested that the anabolic effect of insulin *in vivo* mainly reflects inhibition of protein breakdown [34]. In the present study, insulin decreased total protein breakdown rate in both EDL and SOL, and the effect was similar in muscles incubated flaccid or at resting length. In previous investigations, protein degradation in incubated SOL muscle was unchanged [2,7] or inhibited by insulin [4,35], whereas the effect of the hormone on protein breakdown in EDL and other white muscles is more consistent. The reason for these varying results is not known, but may reflect different insulin concentrations, different conditions *in vitro*, i.e. different compositions of incubation medium and muscles incubated flaccid or at resting length, and different conditions *in vivo*, i.e. muscles from fed or fasted animals.

Insulin did not affect 3-MH release from incubated EDL or SOL muscle, suggesting that myofibrillar protein breakdown is not regulated by the hormone in either type of skeletal muscle. This result is similar to previous reports in which 3-MH release from perfused hemicorpus was not affected by insulin, not even at concentrations as high as 20–25 munits/ml [15,25,27]. In the present study, the effect of insulin was only tested in muscles from fed rats. In previous reports, myofibrillar protein breakdown was unresponsive to the hormone also in muscles from rats which had been fasted for up to 5 days [15,27].

Although the present data are mainly descriptive, they are important, since they provide a systematic characterization of total and myofibrillar protein breakdown rates in a frequently used muscle preparation *in vitro*, i.e. incubated EDL and SOL muscles of young rats. The study also gives new insight into the regulation of myofibrillar protein breakdown in different types of skeletal muscle incubated flaccid or at resting length. Since substantial changes in the myofibrillar protein pool can go undetected if only total protein breakdown rate is measured, as was observed in SOL muscle from fasted rats, the simultaneous determination of tyrosine and 3-MH release is important for a more complete understanding of protein regulation in skeletal muscle.

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