Effect of starvation or treatment with corticosterone on the amount of easily releasable myofilaments in rat skeletal muscles

Burkhardt DAHLMANN, Malte RUTSCHMANN and Hans REINAUER

Biochemische Abteilung, Diabetes Forschungsinstitut, Auf'm Hennekamp 65, D-4000 Düsseldorf, Federal Republic of Germany

Treatment of isolated myofibrils with an ATP-containing relaxing solution results in the dissociation of a preformed quantity of myofilaments called 'easily releasable myofilaments'. Van der Westhuyzen, Matsumoto & Etlinger [(1981) *J. Biol. Chem.* **256**, 11791–11797] presented experimental evidence that these myofilaments represent intermediate products in the turnover of myofibrillar proteins. To investigate further this question, we measured the size of the fraction of easily releasable myofilaments in three different species of skeletal muscles from rats subjected to well-defined catabolic conditions, namely starvation or chronic glucocorticoid administration. The results were as follows: (1) The amount of easily releasable myofilaments was transiently increased about 2–3-fold during both experiments, and thus paralleled the known alterations in the rate of overall muscle protein breakdown rather than in those of synthesis. (2) These changes were observed in muscles containing predominantly fast-twitch fibres, but not in slow-twitch soleus muscle, a muscle that is known to be more resistant to catabolic conditions. (3) The starvation-induced increase of the size of the fraction of easily releasable myofilaments could be significantly reduced by treatment of the starving animals with the proteinase inhibitor E-64. These results are compatible with the idea that easily releasable myofilaments are intermediates in the degradative pathway of myofibrillar proteins and that a proteolytic step may be involved in the conversion of myofilaments into easily releasable myofilaments.

INTRODUCTION

Owing to the various functions of proteins within the cell, a prominent feature of their metabolism is the heterogeneity of their turnover rates. These rates are high for proteins with metabolically important functions, whereas they are very often low for those proteins carrying out less important processes (Duque-Magalhães, 1984). Proteins of the latter group obviously are those responsible for motility of the organism. Since motility is a highly energy-consuming process, the contractile proteins are bundled to myofibrils for optimal generation of force. Despite the compact structure of the myofibril, the myofibrillar subunits turn over with quite different and distinct rates (Waterlow *et al.*, 1978), and this property is difficult to understand, since replacement of the contractile proteins occurs without any remarkable disorder of the function of the whole contractile machinery.

Millward (1980) has outlined a hypothetical scheme for myofibrillar protein turnover in which he assumes that contractile proteins exchange between myofilaments; those proteins which arrive at the periphery of the myofibril dissociate from the fibril to interact with a degrading system and they are replaced by newly synthesized proteins.

In an attempt to detect newly synthesized myofilaments, Etlinger *et al.* (1975) treated isolated myofibrils with an ATP-containing relaxing solution and observed a release of a small fraction of myofilaments from residual, intact, myofibrils. In experiments using radiolabelling techniques those authors found that these easily releasable myofila-

ments apparently contain newly synthesized proteins, whereas in more recent experiments those authors and others (van der Westhuyzen *et al.*, 1981; Sims *et al.*, 1978) presented evidence that, in addition, the fraction of easily releasable myofilaments contains intermediates of the degradative pathway of myofibrils.

To explain the rate-limiting step in his hypothetical scheme for myofibrillar protein turnover, Millward (1980) proposed a model (besides others) in which the rates of dissociation of all subunits from the myofibrils are assumed to be the same. This would result in a pool of dissociated myofibrillar proteins having the same composition as the residual myofibril. Easily releasable myofilaments contain thin and thick filaments and, with the exception of M-line and Z-disc proteins, they are composed of all contractile and regulatory proteins that are also found in the residual myofilaments (Etlinger *et al.*, 1976; van der Westhuyzen *et al.*, 1981). Thus it appears reasonable to suggest that easily releasable myofilaments may be intermediates on their way to enter the pool of myofibrillar proteins designated for degradation. Etlinger and co-workers (Etlinger *et al.*, 1980; van der Westhuyzen *et al.*, 1981) have also shown that factors which affect overall proteolysis in muscle, concomitantly affect the amount of easily releasable myofilaments. Since these experiments were performed *in vitro* with isolated skeletal and heart muscles, we were interested whether, under protein-catabolic conditions *in vivo*, the amount of easily releasable myofilaments also parallels the increase of overall muscle protein breakdown. Here, experimental conditions were chosen that are characterized by greatly depressed muscle protein-synthesis rates and, at least

transiently, increased muscle protein-breakdown rates, conditions which prevail during the starvation of young rats (Millward & Waterlow, 1978) as well as during administration of pharmacological doses of corticosterone to young rats (Odedra *et al.*, 1983).

EXPERIMENTAL

Materials

Corticosterone and MgATP salt were obtained from Sigma Chemie G.m.b.H., München, Germany. Tween 80, carboxymethylcellulose (Servacel CM-80), EDTA, glycerol, Triton X-100, DTT, Serva Blue R and all chemicals for SDS/polyacrylamide-gel electrophoresis were from Serva Feinbiochemica, Heidelberg, Germany, except for SDS, which was from Pierce Chemical Co., Rockford, IL, U.S.A.. E-64 was purchased from Cambridge Research Chemicals, Cambridge, U.K. Sesame oil was from Caesar and Loretz, Hilden, Germany. Gel-filtration-column Ultropak TSK G 4000SW was obtained from LKB, Bromma, Sweden. All other chemicals of analytical grade were purchased from Merck, Darmstadt, Germany.

Animals

Male Wistar rats were obtained from Winkelmann, Borcheln, Germany. Four to six animals were housed together in cages and were fed on standard diet (SsniffR; Intermast, Soest, Germany) with water *ad libitum*. The rats to be starved had their food removed at 4 p.m. Subcutaneous administration of corticosterone to rats was performed exactly as described by Tomas *et al.* (1979). Animals of the control group were treated with vehicle solution only. Proteinase inhibitor E-64 was dissolved in sesame oil and injected subcutaneously, whereas control rats received an injection of sesame oil only.

Isolation and quantitative determination of easily releasable myofilaments

Rats were killed by cervical dislocation under diethyl ether anaesthesia at 8.00–9.00 h and the muscles (gastrocnemius, EDL, soleus) were quickly removed. Unless otherwise stated, for further processing, each left and right EDL or soleus muscles of one animal were pooled, whereas gastrocnemius muscles were processed separately. Myofibrils and easily releasable myofilaments were isolated as described by van der Westhuyzen *et al.* (1981).

The fraction of easily releasable myofilaments obtained from EDL as well as from soleus muscles were concentrated by centrifugation in Centricon 10 micro-concentrators (Amicon G.m.b.H., Witten, Germany) to a volume of about 0.1 ml before 0.2 ml of a solution containing 2% (w/v) SDS/0.02% (w/v) NaN_3 /2% (v/v) 2-mercaptoethanol/0.2 M- NaH_2PO_4 /NaOH, pH 6.8, was added and the mixture boiled at 100 °C for 30 min. The fraction of easily releasable myofilaments isolated from gastrocnemius muscle was concentrated to a volume of about 0.6 ml and then heated for 30 min at 100 °C after addition of 0.2 ml of 10% (w/v) SDS/0.02% (w/v) NaN_3 /10% 2-mercaptoethanol/0.2 M- NaH_2PO_4 , pH 6.8.

The pellets of the residual myofibrils isolated from EDL and soleus as well as from gastrocnemius muscles were dissolved in 1 ml and 4 ml respectively of a solution of 2% (w/v) SDS/0.02% (v/v) NaN_3 /2% (v/v) 2-mercapto-

ethanol/0.2 M- NaH_2PO_4 /NaOH, pH 6.8, and were then boiled for 60 min. The final volumes of easily releasable myofilament as well as of residual myofibril solutions were determined and portions were then subjected to chromatography on Ultropak TSK G 4000SW. The column was equilibrated with 0.1% (w/v) SDS/0.1 M- NaH_2PO_4 /NaOH, pH 6.8, and was run at a flow rate of 0.6–1.0 ml/min. Eluted proteins were detected by measurement of A_{206} and the area of the myosin heavy-chain peak and of actin respectively was integrated by use of a Hewlett–Packard 3390A integrator. A linear relationship was obtained between the amount of protein loaded on to the column and the area of the absorption peak after elution, as tested with phosphorylase *b* and serum albumin. On the basis of the total amount of these proteins present in the fraction of easily releasable myofilaments and in residual myofibrils respectively, the concentration of easily releasable myofilaments is expressed as the percentage of myosin heavy chain (or actin) determined in the fraction of easily releasable myofilaments divided by the amount of these proteins measured in the myofibril plus easily-releasable-myofibril fraction.

Experimental protocols

Starvation. From a group of 22 male rats with 137 ± 7 g initial body wt., 19 animals were starved and each day after starting the experiment three to four rats were used as a source of muscle. The experiment lasted for 5 days and three fed animals were used as controls, 2 days before starting the starvation period.

Refeeding. From a group comprising 24 male rats with 109 ± 7 g body wt., four rats were used as controls and the remaining 20 animals were starved for 3 days. After the third day, four rats were used for taking skeletal muscles and the remaining 16 animals were refed. At each day after starting the refeeding, four animals were killed and skeletal muscles were excised.

Administration of corticosterone. From a group of 42 male rats with 85 ± 4 g body wt. 30 animals were treated daily with 10 mg of corticosterone/100 g body wt. During days 1–5 after starting the glucocorticoid injections, six rats were killed/day and used for taking skeletal muscles. One gastrocnemius muscle from each animal as well as four pooled soleus muscles from two animals were used to isolate easily releasable myofilaments. The controls consisted of the respective muscles from six untreated rats, killed 2 days before the start of the experiment, as well as of those from six rats daily injected with vehicle solution only during 5 days.

Effect of proteinase inhibitor E-64. Ten female rats with a body wt. of 120–130 g were randomly divided into groups of four fed control animals, three rats starved for 3 days, and three rats starved for 3 days and receiving a daily injection of 20 mg of E-64/kg body wt. At the end of the experiment, gastrocnemius muscles from all animals were taken out and used to measure the amount of easily releasable myofilaments.

Statistics

Mean values, S.D. and S.E.M. were calculated, and for comparison of the means, Student's *t* test was used.

RESULTS

The values of the amounts of easily releasable myofilaments measured within each of the muscle species of control rats used in the present experiments were not statistically different. For 26 gastrocnemius muscles with wet wts. of 434–741 mg from animals with body weights of 85–132 g, the amount of easily releasable myofilaments was $6.64 \pm 0.87\%$. The correlation coefficient of muscle weights and the amount of easily releasable myofilaments was $r = 0.24$, indicating that there is no significant correlation of the percentage of easily releasable myofilaments and muscle weight within the range tested. The percentage of easily releasable myofilaments in EDL muscles (muscle wts. 31.2–58 mg) was found to be $2.55 \pm 0.86\%$, and in soleus muscle (muscle wts. 23.5–45 mg) to be $1.48 \pm 0.23\%$.

Starvation and refeeding

During the 5-day starvation period the body weight of the animals decreased from 137 ± 7 g to 93 ± 5 g. The wet weights of the gastrocnemius and EDL muscles decreased by about 20%, whereas for soleus muscle the loss in weight was only 8% during this period. The percentage of easily releasable myofilaments in gastrocnemius muscle continuously increased after removal of food, and reached a maximum of 11.5% at days 3 and 4. Although on the fifth day of starvation the amount of easily releasable myofilaments declined again, the mean value of 9.5% was still significantly higher than that in control rats (Fig. 1). Although the same course of alterations was observed when actin was used to calculate the percentage

Fig. 1. Percentage of easily releasable myofilaments in gastrocnemius muscle of starving rats

Easily releasable myofilaments were measured by quantitative determination of myosin heavy chain as well as of actin. Each value represents the mean $\pm$ S.D. from separate measurements of six to eight muscles from three to four starved animals ($\bullet$). *P* values representing the significance of difference as compared with the control value for fed animals ($\circ$) are indicated. Abbreviations used: NS, not significant ($P > 0.05$).

Fig. 2. Percentage of easily releasable myofilaments in EDL and soleus muscle of starving rats

Easily releasable myofilaments were measured by quantitative determination of myosin heavy chain. Each value represents the mean $\pm$ S.D. from separate measurements of pooled left and right hind-leg muscles from three to four starved animals ($\bullet$). *P* values representing the significance of difference as compared with the control value for fed animals ($\circ$) are indicated. Abbreviations used: NS, not significant ($P > 0.05$).

of easily releasable myofilaments, these values are lower than those based on myosin content (Fig. 1).

In EDL muscle, the amount of easily releasable myofilaments is also increased after food removal; however, the maximum was reached at days 2 and 3 of the starvation period. Interestingly, these values returned to control levels at day 4, before a further substantial increase could be measured on day 5 (Fig. 2). Thus, in gastrocnemius and EDL muscle, starvation resulted in a transient increase of easily releasable myofilaments, whereas the alterations measured in soleus muscle were not statistically significant, with the exception of the increase at day 5 (Fig. 2).

In the second experiment we investigated the effect of refeeding of starved animals. Table 1 shows the amount of easily releasable myofilaments in gastrocnemius muscle was significantly increased in rats starved for 3 days, but these values rapidly fell upon refeeding of the animals. On the second day of the refeeding period this amount was of the same magnitude as that measured in control rats.

Administration of corticosterone

With daily injections of corticosterone/100 g body wt., growth of the animals was arrested (97.1 ± 4.2 g body wt. at the first day of the experiment; 93.2 ± 7.9 g after 5 days of corticosterone administration), and the same effect was observed with respect to muscle weights. These were, at day 1 and day 5 of the experiment: gastrocnemius, 498 ± 44 mg, 461 ± 55 mg; EDL, 43 ± 5 mg, 41 ± 4 mg; soleus, 40 ± 5 mg, 39 ± 3 mg. Control animals receiving only vehicle solution for 5 days gained body weight up to 114 ± 7 g.

The amount of easily releasable myofilaments in the gastrocnemius muscle was significantly increased as early as the first day after corticosterone administration,

Table 1. Effect of starvation and refeeding on the body weight, gastrocnemius muscle weight and amount of easily releasable myofilaments in gastrocnemius muscle

Means $\pm$ S.D. are given for four animals at each time point. *P* values are given for the differences of the amount of easily releasable myofilaments from starved and refed rats as compared with fed controls. Abbreviation used: NS, not significant ($P > 0.05$).

	Gastrocnemius			
	Body wt. (g)	Wet wt. (mg)	Easily releasable myofilaments (%)	<i>P</i>
Fed controls	106.0 $\pm$ 0	531.0 $\pm$ 20.0	6.0 $\pm$ 0.5	
3 days starved	77.0 $\pm$ 3.6	407.8 $\pm$ 2.9	9.3 $\pm$ 0.7	< 0.001
1 day refed	99.0 $\pm$ 1.0	437.5 $\pm$ 29.3	8.3 $\pm$ 0.4	< 0.001
2 days refed	105.3 $\pm$ 5.6	491.0 $\pm$ 22.1	6.0 $\pm$ 0.3	NS
3 days refed	116.0 $\pm$ 5.5	525.5 $\pm$ 41.0	6.1 $\pm$ 0.5	NS
4 days refed	114.0 $\pm$ 6.9	536.5 $\pm$ 42.9	5.9 $\pm$ 0.3	NS

Fig. 3. Percentage of easily releasable myofilaments in gastrocnemius muscle from rats treated with corticosterone

Male rats with an initial body wt. of about 85 g received a daily dose of 10 mg of corticosterone/100 g body wt. On the days indicated, the concentration of easily releasable myofilaments was measured in gastrocnemius muscle by quantitative determination of myosin heavy chain. The values are means $\pm$ S.D. of six muscles from six steroid-treated rats (●). The means for the two groups of control rats (○), i.e. untreated animals at day 0 and animals treated with vehicle solution for 6 days, are not statistically different. *P* values representing the significance of difference as compared with the mean for the control group treated with vehicle only are indicated. Abbreviation used: NS, not significant ($P > 0.05$).

Fig. 4. Percentage of easily releasable myofilaments in EDL and soleus muscle from rats treated with corticosterone

Muscles were taken from the same animals used in the experiment described in the legend to Fig. 3. The values are means $\pm$ S.D. from separate determinations (measurement of myosin heavy chain) each using four pooled muscles from two steroid-treated animals (●). *P* values representing the significance of difference as compared with the mean of the control group of untreated rats (○) are indicated. Abbreviation used: NS, not significant ($P > 0.05$).

reached a maximum 3 days after starting the steroid injections (equivalent to a 100% increase with respect to the controls) and then decreased during the following 2 days to nearly control values (Fig. 3).

A very similar pattern of changes of the amount of easily releasable myofilaments was observed in EDL muscle, but the maximum was reached on the second day of the experiment (Fig. 4), whereas the changes measured in the soleus muscle were statistically not significant (Fig. 4).

Effect of proteinase inhibitor E-64

In another experiment we tested whether treatment of starving rats with a proteinase inhibitor could prevent the increase of the amount of easily releasable myofila-

Table 2. Effect of proteinase inhibitor E-64 on body weight, gastrocnemius muscle weight and amount of easily releasable myofilaments in gastrocnemius muscle of 3-day-starved rats

Subcutaneous injections of 20 mg of E-64/kg body wt. were given daily. Values are means $\pm$ S.D. from three rats in each group.

Parameter	Control	3 days starved	3 days starved + E-64
Body wt. (g)	135 $\pm$ 4.8	86.7 $\pm$ 2.9	87.3 $\pm$ 2.1
Gastrocnemius muscle			
Wet wt. (mg)	681 $\pm$ 22	457 $\pm$ 23	442 $\pm$ 30
Easily releasable myofilaments (%)	7.75 $\pm$ 0.6	11.3 $\pm$ 0.3	9.6 $\pm$ 0.4
Significance (<i>P</i>)			
Comparison with control		< 0.001	< 0.01
Comparison with 3 day-starved			< 0.02

ments. As shown in Table 2, we found that daily administration of 20 mg of E-64 to starving rats significantly suppressed the elevation of the level of easily releasable myofilaments by about 48% as compared with starved rats.

DISCUSSION

When a mammalian organism is completely restricted from food, the time course of the resulting metabolic alterations depends on the available energy stores of the organism (Goodman & Rüderman, 1980; Goodman *et al.*, 1980). A very early event is the substantial decrease in the rate of protein synthesis (Millward, 1970; Garlick *et al.*, 1975; Millward *et al.*, 1976; Li & Goldberg, 1976; Frayn & Maycock, 1979; Li *et al.*, 1979), which, in muscle tissue, particularly affects the synthesis of long-lived myofibrillar proteins (Bates *et al.*, 1983). In addition to this decreased protein synthesis, an acceleration of protein breakdown was found after food restriction (Goodman *et al.*, 1984). In rats having an initial body wt. of about 100 g, substantially increased rates of protein breakdown in skeletal-muscle tissue were measured at days 3 and 4 after food removal (Millward & Waterlow, 1978) and, according to Li & Wassner (1984), the increased protein catabolism affects the myofibrillar more than the sarcoplasmic proteins.

Our results obtained on changes in the amount of easily releasable myofilaments in gastrocnemius muscle also showed a maximum increase in concentration of these myofilaments 3 and 4 days after onset of starvation, although at days 1 and 2 of the experiment the concentration was also higher than in control rats (Fig. 1). With EDL the biggest change was observed at day 2 of the starvation period, whereas in soleus muscle the alterations at day 2 and 3 were not statistically significant (Fig. 2). Thus the alteration of easily-releasable-myofilament concentration observed in skeletal muscle of rats during starvation parallels the known changes of the rate of protein breakdown rather than that of protein synthesis.

Refeeding of starved animals results in an immediate increase in protein synthesis and a decrease in protein breakdown (Nishizawa *et al.*, 1978; Li & Wassner, 1984). Again, in our experiment (Table 1), the initially larger fraction of myofilaments easily released by extraction with ATP decreased after refeeding and thus parallels the change of the breakdown rate.

Although it is generally accepted that glucocorticoids

have a repressive effect on muscle protein synthesis, the effect of these hormones on muscle protein breakdown has been a matter of debate [for references, see McGrath & Goldspink (1982)] until such time as Santidrian *et al.* (1981) found that increased muscle-protein-breakdown rates in young rats can be observed only after repeated subcutaneous injections of supraphysiological concentrations of glucocorticoids. By daily administration of catabolic doses (10 mg/100 g body wt.) to young rats, Odedra *et al.* (1983) observed a constant fall in the overall muscle-protein-synthesis rate and a disproportionate decrease in the rate of overall protein synthesis by about 70% as compared with the initial rates. On the other hand, the rate of protein degradation transiently increased at days 2 and 3 to about 193% and 203% respectively. After 5 days of glucocorticoid administration the fractional rate of protein synthesis still remained at a level as low as 39%, and the rate of protein breakdown was again decreased to 75%.

In the present study, our experimental set-up for corticosterone administration to rats was identical with that described by Odedra *et al.* (1983), and we have found that, in gastrocnemius muscle, the concentration of easily releasable myofilaments increased to reach a maximum after 3 days of steroid administration before decreasing again, to almost control values, within 2 further days. In EDL muscle the peak was observed 2 days after starting the experiment, whereas no significant changes were found in soleus muscle. Similarly to the results of the starvation and the starvation-refeeding experiment, the changes in easily-releasable-myofilament concentration in muscles of rats treated with corticosteroids again parallel the alterations in protein degradation rates as described by Odedra *et al.* (1983).

Since in both the starvation and the glucocorticoid experiments the rates of protein synthesis are known to be substantially lowered, the increase of the concentration of easily releasable myofilaments in parallel with the acceleration of the protein breakdown suggests that this fraction of myofilaments is involved in the degradative pathway rather than in the synthetic pathway of myofibrils. The small changes in the concentration of easily releasable myofilaments in soleus muscle, when compared with EDL or gastrocnemius muscle, as demonstrated in our study, are in keeping with findings of Li & Goldberg (1976), Frayn & Maycock (1979), Goldberg & Goodman (1969) and Goodman *et al.* (1984), in that the tonic slow-twitch soleus muscle is more resistant to the catabolic effect induced by starvation or

glucocorticoid administration than muscles containing phasic fast-twitch fibres like EDL and gastrocnemius muscle.

By incubating isolated muscles with the proteinase inhibitor leupeptin, van der Westhuyzen *et al.* (1981) found that the amount of easily releasable myofilaments was decreased. In our experiment, administration of the proteinase inhibitor E-64 to fasting rats resulted in a significant suppression of the amount of easily releasable myofilaments compared with starved rats not treated with the proteinase inhibitor. E-64 is known to be a powerful inhibitor of the lysosomal cysteine proteinase cathepsin B, H and L (Barrett & Kirschke, 1981; Hashida *et al.*, 1980) but, similarly to leupeptin, it has been shown to inhibit the calcium-activated proteinase, calpain, *in vitro* and *in vivo* (Sugita *et al.*, 1980). Since the activity of calpain was reported to be increased in skeletal muscles of fasting rats (Arakawa *et al.*, 1983), and since increased intracellular calcium concentrations lead to a higher concentration of easily releasable myofilaments (van der Westhuyzen *et al.*, 1981), both E-64 and leupeptin may interact with calpain or possibly another proteinase, resulting in a diminution of the fraction of easily releasable myofilaments. It is therefore conceivable that the mechanism by which myofilaments become easily releasable involves a step of limited proteolysis leading to a disassembly of those structures that keep the filaments in register, as has been observed in muscle of starving marine teleost fish (Johnston, 1981; Beardall & Johnston, 1983).

In the control rats of all our experiments we found the percentage of easily releasable myofilaments to be higher in EDL than in soleus muscle. Although this does not correlate with the finding that basal overall protein turnover as well as actomyosin turnover is higher in slow-twitch muscles than in fast-twitch muscles (Waterlow *et al.*, 1978), we think that the positive correlation between changes of the rate of muscle protein breakdown and the size of the pool of easily releasable myofilaments support the idea (Sims *et al.*, 1978; van der Westhuyzen *et al.*, 1981) that this fraction of myofilaments contains intermediates of the degradative pathway for myofibrillar proteins.

We gratefully acknowledge the technical assistance of Mrs. R. Schwitte and thank Dr. L. Kuehn for valuable comments on the manuscript. This work was supported by the 'Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen', Düsseldorf, by the 'Bundesministerium für Jugend, Familie und Gesundheit', Bonn, by a grant (SFB 113, Projekt G4) from the 'Deutsche Forschungsgemeinschaft', Bonn, and by a NATO research grant.

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