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The effect of glucocorticoids on the myosin heavy chain isoforms' turnover in skeletal muscle

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Abstract

The purpose of this study was to find the effect of dexamethasone on the myosin heavy chain (MyHC) isoforms' composition in different skeletal muscles and glycolytic (G) fibres in relation with their synthesis rate and degradation of MyHC isoforms by alkaline proteinases. Eighteen-week-old male rats of the Wistar strain were treated with dexamethasone (100 μg/100 g bwt) during10 days. The forelimb strength decreased from 9.52 to 6.19 N ($P < 0.001$) and hindlimb strength from 15.54 to 8.55 N ($P < 0.001$). Daily motor activity decreased (total activity from 933 to 559 and ambulatory activity from 482 to 226 movements/h, $P < 0.001$). The degradation rate of muscle contractile proteins increased from 2.0 to 5.9% per day ($P < 0.001$), as well as the myosin heavy chain IIB isoform degradation with alkaline proteinase in fast-twitch (F-T) muscles ($12 \pm 0.9\%$; $P < 0.05$) and glycolytic muscle fibres ($15 \pm 1.1\%$; $P < 0.001$). The synthesis rate of MyHC type II isoforms decreased in Pla muscles ($P < 0.05$) and MyHC IIA ($P < 0.05$) and IIB in EDL muscle and G fibres ($P < 0.001$). The relative content of MyHC IIB isoform decreased in F-T muscles ($P < 0.001$) and in G fibres ($P < 0.01$), and the relative content of IIA and IID isoforms increased simultaneously. Dexamethasone decreased the MyHC IIB isoform synthesis rate and increased the sensibility of MyHC IIB isoform to alkaline proteinase, which in its turn led to the decrease of MyHC IIB isoform relative content in F-T muscles with low oxidative potential and G muscle fibres.
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Keywords: Glucocorticoid myopathy; MyHC isoforms relative content and synthesis rate; MyHC isoform degradation

1. Introduction

It is well known that iatrogenic steroid myopathy as well as Cushing's disease leads to a marked reduction in muscle mass, wasting of muscle, loss of strength and selective atrophy of fast-twitch (F-T) muscle fibres. It was shown that glucocorticoids increase the expression of myostatin, a negative regulator of skeletal muscle growth in polynucleated muscle fibres, expressing myosin heavy chain (MyHC) fast isoforms [1]. In glucocorticoid myopathic rats the turnover rate of MyHC was lower in the fast-twitch muscle fibres, myosin light chains (MyLCs) turned over more rapidly in all types of muscle fibres [2]. The turnover rate of MyHC in myopathic rats is related to the changes in MyHC isoform pattern. In myopathic glycolytic (G) muscle fibres of rats the relative content of MyHC IIB isoform decreased and IID increased [3]. The ultrastructural studies showed the disarray of thick myofilaments in glycolytic muscle fibres of glucocorticoid treated rats and increased lysosomal activity in these fibres and in the satellite cells [4]. There were only slight morphological changes in oxidative-glycolytic fibres and there were no structural changes in slow-twitch oxidative muscle fibres in glucocorticoid treated rats [4].

Although widely studied, the ultimate causes and pathophysiological nature of changes in isoforms, their synthesis and degradation rate in myopathic muscle are still unknown. However, this is necessary for the characterization of changes in kinetic criteria of glucocorticoid myopathic muscle.

The purpose of this study was to find the effect of dexamethasone on the MyHC isoforms' composition in different skeletal muscles and glycolytic fibres in relation with their synthesis rate and degradation of MyHC isoforms by alkaline proteinases.

We hypothesized that MyHC isoforms' composition is changing in fast-twitch skeletal muscles as a result of the decreased synthesis rate and increased degradation rate of MyHC isoforms.

2. Materials and methods

This experiment was approved by the Ethical Committee of the University of Tartu for the use of animals in research no. 2000/02 (17 April 2000).

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2.1. Animals and dexamethasone treatment

The used animals were 18-week-old (at the beginning of the experiment) 20 male rats of the Wistar strain (National Laboratory Animal Centre, Kuopio, Finland). All the animals were housed in identical environmental conditions in polycarbonate type III cages, at 21 °C, two per cage at 12/12 h light/dark period. They received diet (SDS-RM1 (C) 3/8, Witham, Essex, England) and water ad libitum.

The force and hindlimb grip strength was measured before and after dexamethasone treatment with Grip Strength Meter 0167-004L (Columbus Instruments) and the screening of the amounts of ambulatory and total (stereotypic) movements were measured with Opto-Varimex-Mini (Columbus Instruments).

The rats were assigned to control ($n = 10$) and dexamethasone treated ($n = 10$). Dexamethasone (Clucocortin-20, Interchemie, Holland) was diluted to 200 μg/ml with 0.15 M NaCl and administered intraperitoneally 100 μg/100 g bwt during 10 days. The control animals received appropriate amounts of 0.15 M NaCl.

2.2. Labelled amino acid infusion and muscle removal

L-[4.5–3H] leucine (170 Ci/mmol) was infused intraperitoneally for 6 h, 250 μCi/100 g bwt.

The animals were anesthetized by the intraperitoneal injection of ketamin (Calysol, Gedeon Richter A.O. Budapest, Hungary) and diazepam (Lab Renaudin, France) and sacrificed. The *plantaris* (PLA), *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles were quickly removed, trimmed clean of visible fat and connective tissue, weighed, frozen and stored in liquid nitrogen pending further processing. Glycolytic fibres were separated as described by us previously [3,5].

2.3. Separation of myofibrillar protein

Frozen muscles were thawed on ice, cut into small pieces, and washed with five volumes 20 mM NaCl, 5 mM sodium phosphate, 1 mM EGTA (pH 6.5). Myosin was extracted with three volumes 100 mM sodium pyrophosphate, 5 mM EGTA, 1 mM dithiothreitol (pH 8.5); after 30 min of gentle shaking myofibrillar fraction was diluted with one volume glycerol and stored at −20 °C.

2.4. Alkaline proteinase

Alkaline proteinase separation and activity measurement was provided by Dahlmann et al., as described by us earlier [3]. Incubation of myofibrils (5 mg/ml) in vitro conditions with alkaline proteinase (0.7 u/ml) was provided in 50 mM Tris–HCl, pH 8.5 at 37 °C during 20 min, mixed (1/10, v/v) with buffer (62.5 mM, Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% SDS, 5% β-merkaptoethanol) and incubated 2 min at 100 °C.

2.5. Estimation of 3-methylhistidine (3-MeHis) in skeletal muscle and urine

Total muscle protein for getting 3-MeHis was hydrolyzed in 6 M HCl (10 mg/ml acid) for 20 h at 110 °C in vacuum-sealed flasks. HCl was removed by evaporation, and the hydrolysate was dissolved in 0.2 M pyridine to achieve a concentration of 10–20 mg/ml. 3-MeHis in the urine and muscle tissue was estimated as described by us earlier [5]. 3-MeHis pool excreted was expressed as percentage per day.

2.6. Protein: DNA and RNA: protein

Protein: DNA and RNA: protein in skeletal muscle were determined as described by Millward and Waterlow [6].

2.7. Recovery and hydrolysis MyHC amino acid analysis

The MyHC was electroeluted from 12.5% SDS-PAGE [7] according to Hunkapiller et al. [8]. After staining with Coomasssie Brilliant Blue R-250 and detection on 12.5% SDS-PAGE the protein band was sliced and minced with razor blade, and rinsed with water. After soaking the gel in elution buffer (0.1% SDS in 0.05 M Tris–acetate, pH 7.8) for 5 min and in soaking buffer (2% SDS 0.4 M NH_4HCO_3) for 1.0 h, the electroelution went on in dialyzing bag, using horizontal electrophoresis cell (Gel Electrophoresis Apparatus GNA-100, Pharmica, Sweden). The running conditions for the elution cell were power supply 70 V (constant voltage) and current 7 mA for 1.5 h. After elution the samples were collected (1000 μl) and the gel pieces were removed by centrifugation (14,000 × *g*). Residual SDS was removed by the following dialyzing in 1 ml dialysis buffer (0.02% SDS in 0.01 M NH_4HCO_3) and rinsing the samples twice with 1.0 ml deionized water by centrifugation in microconcentration tube (10,000 Da) and MyHC, and actin was washed from filter with 800 μl deionized water.

Eluent fraction containing MyHC (200 μl) was evaporated with nitrogen stream, and protein-bound amino acids from 10 μg samples were liberated by hydrolyzation at 110 °C for 18 h in 200 μl 6 N HCl in nitrogen area and HCl was evaporated with nitrogen. Leucine quantity in MyHC hydrolysate was determined by using an ultra rapid and sensitive HPLC method for measuring individual free amino acids in biological fluids by Graser et al. [9], employing precolumn derivatization with *o*-phtalaldehyde/3-mercaptopropionic acid and using 3 μm particle-size reversed-phase columns (Hyperchrome, Spherisorb ODS II, 3 μm, 125 × 4.6 with guard columns 10 mm × 4.6 mm, 5 μm; Leonberg, Germany). Resolution of the amino acid derivatives was accomplished with an acetonitrile gradient in 12.5 mM sodium phosphate buffer, pH 7.2 [9].

Table 1
The effect of dexamethasone on the body weight, muscle strength, motor activity and muscle protein degradation

	Body weight (g)	Grip strength (N)		Daily motor activity (movements/h)		Daily 3-MeHis pool excreted (%)
		Forelimb	Hindlimb	Total	Ambulatory	
Before ($n = 10$)	253.0 ± 5.6	9.52 ± 0.40	15.54 ± 0.86	933 ± 42	482 ± 23	2.0 ± 0.12
After ($n = 10$)	200.0 ± 5.1***	6.19 ± 0.35***	8.55 ± 0.70***	559 ± 31***	266 ± 18***	5.90 ± 0.31***

Before: before dexamethasone treatment. After: after 10 days dexamethasone treatment. 3-MeHis: daily 3-methylhistidine pool excreted was used for characterization of contractile proteins degradation. Grip strength of forelimb and hindlimb and daily motor activity of rats were used for characterization of development of glucocorticoid caused myopathy. Ambulatory activity characterizes movemental activity of animals, total activity includes stereotypic (scratching, grooming, digging ...) non-ambulatory movements.

*** $P < 0.001$ in comparison with before dexamethasone treatment.

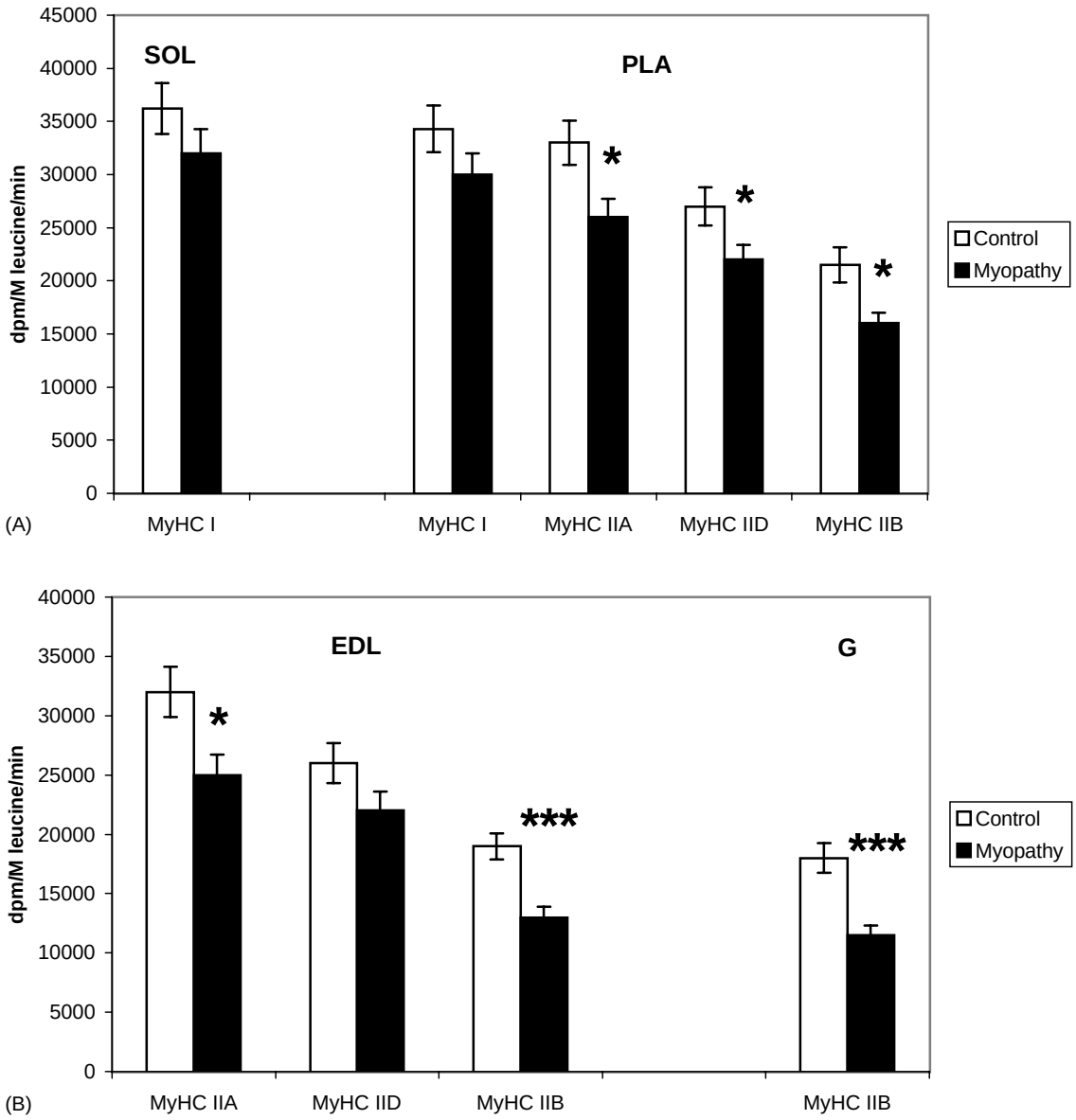

Fig. 1. Changes in MyHC isoforms synthesis rate in different muscles in glucocorticoid myopathy. SOL: *soleus* muscle; PLA: *plantaris* muscle; EDL: *extensor digitorum longus* muscle; G: fast-twitch glucolytic muscle fibres from *quadriceps femoris* muscle. * $P < 0.05$ and *** $P < 0.001$ in comparison with subsequent control group.

2.8. Determination of whole muscle MyHC isoform composition

MyHC isoforms were separated by 7.2% SDS-PAGE using 0.75 mm thick gel. Myofibrils containing 0.5 μg of protein were loaded on the gel after being incubated for 10 min at 65 °C in sample buffer containing 62.5 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.0% SDS, 0.05% bromphenol blue. Electrophoresis lasted for 24 h at 120 V [10]. Gels were silver-stained by the method of Oakley et al. [11]. Protein isoform bands were analysed densitometrically by Image Master® 1 D program, Version 3.0 (Amersham Pharmacia Biotech) and the percentage distribution of the various isoforms was evaluated. MyHC isoforms' fractions were analyzed for radioactivity and expressed as dpm/M leucine/min.

2.9. Protein assay

Total muscle protein and myofibrillar protein was assayed by using the technique described by Bradford [12].

2.10. Statistics

Means, standard errors of means were calculated from individual values by standard procedures. The two-tailed independent *t*-test was used for comparisons of two populations. Differences were considered significant at $P < 0.05$. Regression analyses was performed to determine the correlation between the measured parameters.

3. Results

Basic characteristics of glucocorticoid caused myopathy like the decrease in the body weight, muscle strength, motor activity and increase in the degradation of the muscle protein are presented in Table 1. Table 2 is characterizing changes in different muscles during the glucocorticoid treatment. The decrease in the fast-twitch muscle weight, DNA synthesis and muscle size were determined by the number of DNA units. The increase in DNA unit size shows the decrease in the total muscle protein synthesis, and the decrease in RNA unit size shows the decrease in RNA concentration in fast-twitch muscles in glucocorticoid treated rats.

Table 2
The effect of dexamethasone on the muscle weight and content of DNA and RNA in different muscles

Group	*Extensor digitorum longus* muscle	*Plantaris* muscle	*Soleus* muscle
Control ($n = 10$)			
Weight (mg)	98.0 ± 3.2	192.0 ± 5.6	86.0 ± 2.9
DNA unit number	39.2 ± 1.9	101.8 ± 3.1	67.9 ± 2.8
DNA unit size	523 ± 32	396.0 ± 25	268.0 ± 22
RNA unit size	4.31 ± 0.28	4.29 ± 0.19	6.38 ± 0.33
RNA/DNA	2.30 ± 0.2	1.70 ± 0.1	1.71 ± 0.1
Myopathic ($n = 10$)			
Weight (mg)	57.0 ± 2.9***	126.0 ± 3.8***	81.0 ± 3.4
DNA unit number	17.1 ± 0.8***	50.4 ± 2.4***	61.6 ± 3.7
DNA unit size	702.0 ± 53**	526.0 ± 26**	291.6 ± 21
RNA unit size	2.85 ± 0.2***	3.09 ± 0.2***	6.05 ± 0.3
RNA/DNA	2.60 ± 0.2	1.62 ± 0.1	1.76 ± 0.2

DNA unit number: DNA content μg/muscle. DNA unit size: protein/DNA (mg/mg). RNA unit size: RNA/protein (μg/mg). RNA/DNA: RNA/DNA (μg/μg). DNA: unit is imaginary volume of cytoplasm managed by a single nucleus; DNA: unit is therefore only an operational term. RNA unit size expresses muscle RNA concentration. $^{**}P < 0.01$ and $^{***}P < 0.001$ in comparison with subsequent control group.

Changes in MyHC isoforms' synthesis rate in glucocorticoid myopathic skeletal muscles are shown in this study for the first time. The synthesis rate of MyHC I isoforms does not depend on the twitch characteristics of the skeletal muscle (Fig. 1). In both studied fast-twitch muscles the synthesis rate of MyHC type II isoforms decreased, as well as in fast-twitch glycolytic muscle fibres (Fig. 1). Therefore, the synthesis rate of MyHC IIB isoforms decreased more significantly than the rate of IIA and IID (Fig. 1). In SOL muscle MyHC isoform composition did not change significantly as well as in PLA muscle (Fig. 2). As seen in Figs. 2 and 3, the relative content of MyHC IIB isoform decreased in both studied muscles and in glycolytic muscle fibres, and

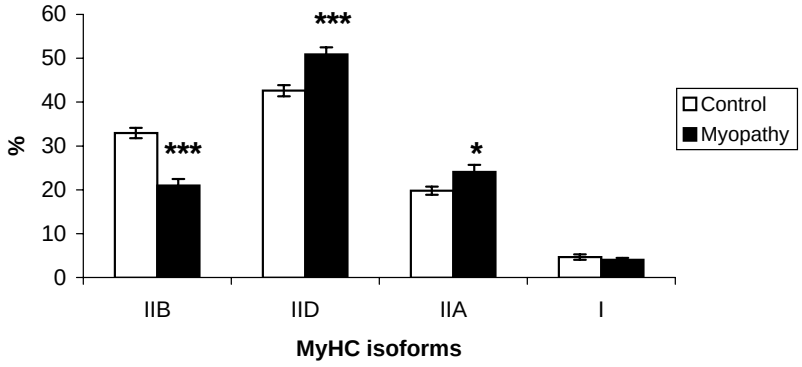

Fig. 2. Changes in MyHC isoforms relative content in *plantaris* muscle in glucocorticoid myopathy. $^{*}P < 0.05$ and $^{***}P < 0.001$ in comparison with subsequent control group.

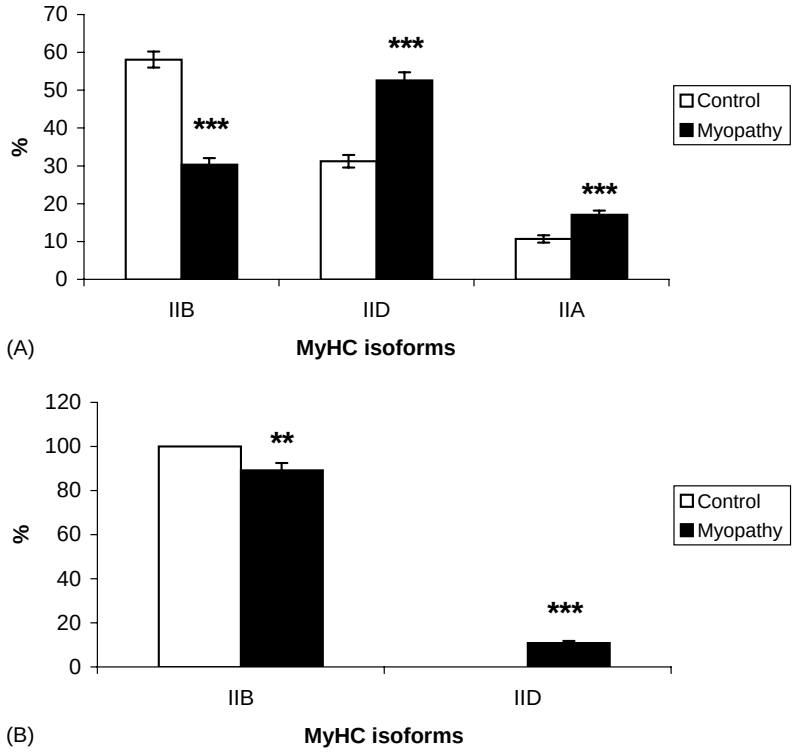

Fig. 3. Changes in MyHC isoforms relative content in *extensor digitorum longus* muscle (A) and glycolytic muscle fibres (B) in glucocorticoid myopathy. $^{**}P < 0.01$ and $^{***}P < 0.001$ in comparison with subsequent control group.

the relative content of IIA and IID isoforms increased at the same time. Incubation of myofibrils from all the studied muscles with alkaline proteinase shows that the sensibility of MyHC isoforms under in vitro conditions depends on muscle twitch and metabolic characteristics as well as on the type of isoform itself. In PLA muscle MyHC IIB isoform decreased during incubation with alkaline proteinase $9 \pm 0.9\%$, changes in MyHC IID, IIA and I isoform were not significant. In EDL muscle MyHC IIB isoform content decreased $12 \pm 0.9\%$, changes in other MyHC isoforms in this muscle were statistically insignificant. In the glycolytic muscle fibre the content of MyHC IIB isoform decreased during incubation with alkaline proteinase $15 \pm 1.1\%$. The comparison of MyHC IIB isoform decrease in PLA and EDL muscle ($P < 0.05$) and in glycolytic muscle fibres ($P < 0.001$) shows that sensibility of MyHC IIB isoforms to alkaline proteinase depends on the muscle oxidative potential.

Changes in MyHC I isoforms in PLA and SOL muscles during incubation were about 2% and statistically insignificant.

4. Discussion

Contractile properties of skeletal muscle depend on the composition of MyHC isoform in the muscle. Unfortunately, changes in MyHC isoforms' composition and turnover rate during glucocorticoid myopathy have been poorly studied.

Skeletal muscle weakness in case of glucocorticoid myopathy has been caused by lesions of the myofibrillar apparatus and by changes in the neuromuscular synapses, particularly in fast-twitch glycolytic muscle fibres [3,4,13]. Unfortunately, it is also unclear how the catabolic effect of glucocorticoids, particularly selective degradation of contractile proteins, including MyHC isoforms, realise in the skeletal muscle. It is well established that lysosomal and non-lysosomal pathways exist in the skeletal muscle to account for the degradation of their intracellular proteins. As the content of the lysosomes in the skeletal muscle, particularly in fast-twitch muscles, is relatively low, the non-lysosomal pathway makes a particularly significant contribution and may be of special importance in the initial rate-limiting steps in the catabolism of myofibrillar proteins.

At the same time the synthesis rate of contractile proteins, particularly in fast-twitch muscles and glycolytic fibres, decreases. It was shown that glucocorticoid treatment decreases the synthesis rate of MyHC [2,14]. The synthesis of MyHC may not reflect changes in the synthesis rate of different MyHC isoforms, as well as the synthesis of the mixed muscle protein may not reflect the changes occurring in individual proteins [15]. The results of this study support the abovementioned standpoint. The changes in the synthesis rate of different MyHC isoforms in glucocorticoid myopathic skeletal muscle are shown in this study for the first time. The synthesis rate of MyHC I isoform does not change in myopathic soleus muscle where this isoform makes up

about 99% of MyHC, as well as in plantaris muscle where it forms about 5% of MyHC. In myopathic muscles the synthesis rate of type II MyHC isoforms decreases. In glycolytic fibres the expression of MyHC IID isoform synthesis was initiated in myopathic muscles. It was also shown in the diaphragm that dexamethasone treatment decreased MyHC IIB isoform expression [16]. It appears from our experiment that the synthesis rate of MyHC isoforms in myopathic muscles does not depend so much on metabolic and twitch characteristics of the muscle, but mainly on the type of MyHC.

It was shown by us about two decades ago that MyHC are more sensitive to the action of alkaline proteinases than MyLC, at least under in vitro conditions [17]. The glucocorticoid treatment increased the activity of alkaline proteinase in the skeletal muscle [18,19]. Alkaline proteinases are synthesized in mast cells [2]. After degranulation the enzyme enters the muscle fibre. Upon administration of large doses of glucocorticoids there is an increase in the number of mast cells in the perivascular porous connective tissue of the muscle fibres [2]. The degranulation of mast cells is clearly expressed around the fast glycolytic muscle fibres [20]. The lymph nodes are the sources of the muscle mast cells, which may be migrated from there. In myopathic muscles, where the alkaline proteinase activity is high, the glycolytic fibres separate from the adjacent ones, bend and lyse [3,4]. As the results of the present study show, the incubation of myofibrils from the muscles of different metabolic and twitch characteristics with alkaline proteinases, the sensibility of MyHC IIB isoforms depend to some extent on the abovementioned characteristics of the muscle. In fast-twitch muscles, where the oxidative potential is higher, the sensibility of MyHC IIB isoform to the alkaline proteinase activity is diminished.

Taking into account the synthesis rate of MyHC isoforms and their sensibility to alkaline proteinases, changes in the relative content of MyHC isoforms in our study in different muscles seem logical enough. As a result of the decrease in the synthesis rate of MyHC IIB isoform and its increased degradation, the relative content of this MyHC IIB isoform decreased in myopathic skeletal muscle.

In conclusion, the synthesis rate of MyHC IIB isoform in case of glucocorticoid-caused myopathy decreases in skeletal muscles in which the oxidative potential is low. At the same time the sensibility of MyHC IIB isoform to the action of alkaline proteinases in the muscles increases. As a result, in myopathic muscles the relative content of MyHC IIB isoform decreases, the relative content of MyHC IIA and IID increases and the twitch characteristics of the skeletal muscle change.

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