

Fine architectonics and protein turnover rate in myofibrils of glucocorticoid caused myopathic rats

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Abstract

Objective: The purpose of this study was to assess the relationship between the synthesis and degradation rate of thick and thin myofilaments, and changes in fine architectonics of myofibrils of glycolytic muscle fibers of glucocorticoid caused myopathic rats.

Methods: Male Wistar rats receive dexamethasone to induce myopathy. Protein synthesis rate degradation and structure of myofibrils was measured.

Results: Myofibrils from glycolytic muscle fibers of 16-week-old male rats of the Wistar Strain were used for analysis. Myofibrils of glucocorticoid caused myopathic rats were thinner than in the control group ($1.10 \pm 0.05 \mu\text{m}^2$ and $2.56 \pm 0.08 \mu\text{m}^2$, respectively) and thick and thin myofilaments in myopathic muscle disappeared from $22.0 \pm 2.7\%$ of the myofibrils cross-section area. In the control group, myofilaments were absent from $1.8 \pm 0.5\%$. In the myopathic group, a positive correlation was found between the area of myofibrils where myofilaments disappeared and the myofibrillar protein degradation rate ($r=0.849$; $p<0.001$). A positive correlation was found between myofibrils cross-sectional area and thick myofilaments synthesis rate ($r=0.933$; $p<0.001$).

Conclusions: Increased myofibrillar protein degradation rate, decreased myosin heavy chain (MyHC) and actin synthesis rate and a decrease in MyHCIIb isoform relative content in myopathic glycolytic muscle are related to qualitative remodeling of the myofibrillar apparatus in myopathic glycolytic muscle fibers.

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INTRODUCTION

About four decades ago, it was shown that iatrogenic steroid myopathy as well as Cushing's disease cause selective atrophy of fast-twitch muscle fibers [1] and changes in the turnover rate of muscle proteins [2,3]. Further studies have shown that major changes take place in myofibrillar protein turnover [4,5,6]. Myofibrils of steroid caused myopathic IIB fibers show the disarray of thick myofilaments, increased lysosomal activity [7], a decreased synthesis rate of myosin and actin [6] and a decrease in myosin heavy chain (MyHC) IIB isoforms' relative content [8]. Sarcopenic muscles caused by both steroid and ageing have diminished capacity for regeneration [9]. It has been

shown that the effect of excessive glucocorticoids on the extracellular compartment does not differ significantly between slow- and fast-twitch muscle fibers [10].

This raises the question how substantially changes in myofibrillar apparatus are related to functional and structural modification in glucocorticoid caused myopathic muscle. We hypothesized that as in corticosteroid caused myopathic glycolytic fibers, thick and thin myofilaments synthesis rate would decrease, and degradation rate would increase, and this may be related to the disarray of myofilaments from myofibrils and subsequently to the destruction of filaments.

We aimed to assess the relationship between thick and

thin myofilaments synthesis and degradation rate, and changes in ultrastructural architectonics of myofibrils of glycolytic muscle fibers of glucocorticoid caused myopathic rats. The specific aim was to assess the relationship between the destruction of thick and thin filaments in myofibrils, their disappearance from the cross-section area (CSA), and their relationship with synthesis and degradation rate.

MATERIALS AND METHODS

The use of animals was in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes and all procedures used in this study were approved by the Animal Experiment Committee of the Estonian Ministry of Agriculture, Tallinn, Estonia.

Animals, dexamethasone treatment and anesthesia

The animals involved were 16-week-old male Wistar rats. All the animals were housed in identical conditions in polycarbonated type III cages, at 21°C. They received diet [SDS-RM-1(C) 3/8, Witham, Essex, UK] and water ad libitum. The rats were assigned to control (n=8) and dexamethasone treated group (n=8). Dexamethasone (Glucocortin-20, Interchemie, Holland) was diluted to 200 µg/ml with 0.15 M NaCl and administered intraperitoneally daily 100 µg/100 g body weight (bw) during 10 days. The control animals received respective amounts of 0.15 M NaCl. Twenty-four hours after the last Dexamethasone infusion, rats were anesthetized by an intraperitoneal injection of ketamin (Calycol, Gedeon Richter A.O. Budapest, Hungary) and diazepam (Lab Renaudin, France) and sacrificed.

Labeled amino acid infusion and separation of glycolytic muscle fibres

L-[4,5-3H] leucine (170 Ci/mmol) was infused intraperitoneally for 6 h, 250 µCi/100 g bw.

For studies of the fast-twitch glycolytic (FG) fibers or type IIB fibers, the *m. quadriceps femoris* was dissected, liberated from fat and connective tissue and separated into a superficial white portion. This portion is consisting predominantly of FG fibers (MyHCIIb isoforms relative content 97.0±5.0% and MyHCIIId isoforms relative content 3.0±0.2%, cytochromes aa₃ concentration 9.20±0.80 ng/g muscle wet weight and myoglobin concentration 0.85±0.09 mg/g muscle wet weight). Cytochromes aa₃ and myoglobin were provided as described by us previously [6].

Ultrastructural studies

Muscle samples for ultrastructural studies were fixed in 2.5% glutaraldehyde, post-fixed in 1% sodium tetroxide, dehydrated in graded alcohol and embedded

in Epon-812. Ultra-thin sections were cut from longitudinally and transversely oriented blocks, stained with uranyl acetate and lead hydroxide, using 17 blocks from each animal. For analysis of myofibrils CSA and the area of disappeared myofilaments, the Imaging and analysis software (Cell* Soft Imaging System GmbH, Münster, Germany) was used. As shown in Fig. 1A and B, in myopathic glycolytic muscle myofibrils CSA, thick or thin filaments or both disappeared from some places.

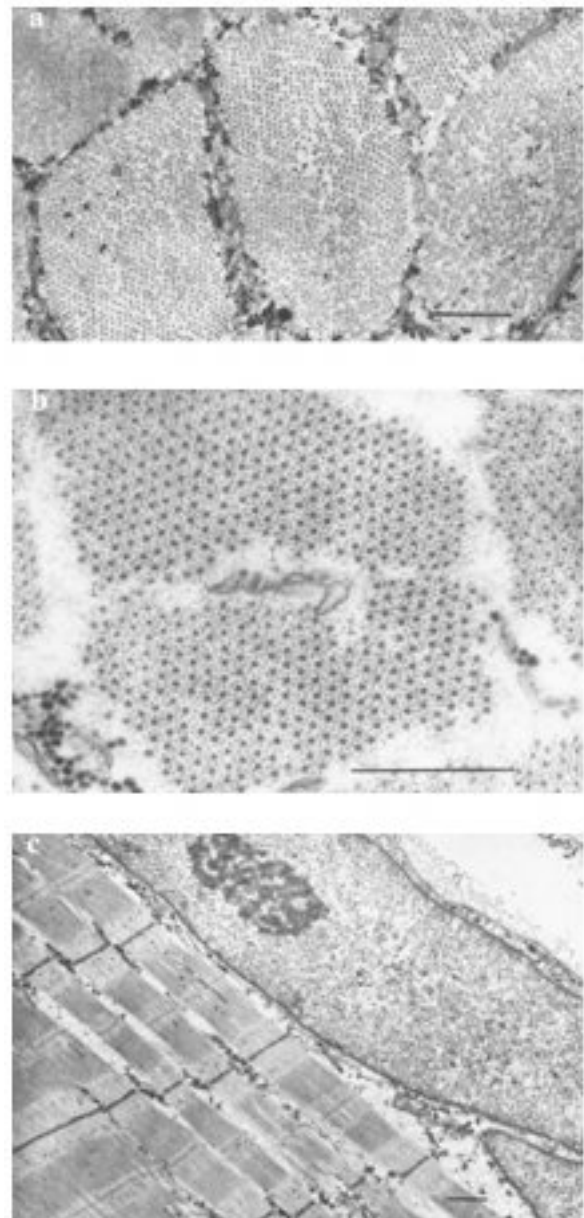


Figure 1. Electron micrographs of cross-section (a, b) and longitudinal section (c) of glycolytic muscle fibers of glucocorticoid treated rats. Disappearance of thick and thin myofilaments in some places of myofibrils cross-section area (Bar: 0.5 µm)

Separation of total muscle protein

The minced muscle samples were homogenized in a buffer containing: 50 mM KCl, 10 mM K_2HPO_4 , 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM dithiothreitol at pH=7.0 and analyzed as total protein fraction. The total muscle homogenate was dissolved in 0.3 M NaOH and was analyzed for radioactivity and protein.

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 of 100 mM sodium hydrophosphate, 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.

Estimation of 3-Methylhistidine (3-MeHis) in skeletal muscle and urine

The 3-MeHis in skeletal muscle and urine was used as an indicator for myofibrillar protein degradation. The determination was performed as described previously [7]. Briefly, total muscle protein was hydrolyzed in 6M HCl 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 with HPLC [7].

Fractional degradation of muscle protein

The degradation rate of myofibrillar protein was calculated as follows: 3-MeHis excretion ($\mu\text{mol/day} \times 0.75 \times 100$) divided by selected muscle myofibrillar protein ($\text{g} \times 3\text{-MeHis} (\mu\text{mol/g/muscle})$) and expressed as % per day.

Quantitation of MyHC and actin

12.5% SDS-polyacrylamide gel electrophoresis was used for the identification and further purification of proteins [11]. The gels were stained by Coomassie Brilliant Blue

R-250. The MyHC and actin bands of the sample and their identification and assignment were obtained by the comparison of the electrophoretic mobility of marker proteins. The molecular weight of the MyHC and actin was determined by calibrating with marker proteins (Bio-Rad molecular weight standard).

Recovery and hydrolysis of MyHC and actin amino acid analysis

MyHC and actin was electroeluted from 12.5% SDS-PAGE as described earlier [8]. Shortly after staining and detection in 12.5% SDS-PAGE, bands were sliced and minced with a razor blade, and then 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 soaking buffer (2% SDS 0.4 M NH_4HCO_3) for 1.0 h, the electroelution proceeded in a dialyzing bag using the horizontal electrophoresis cell, and further procedures were carried out as described earlier [8].

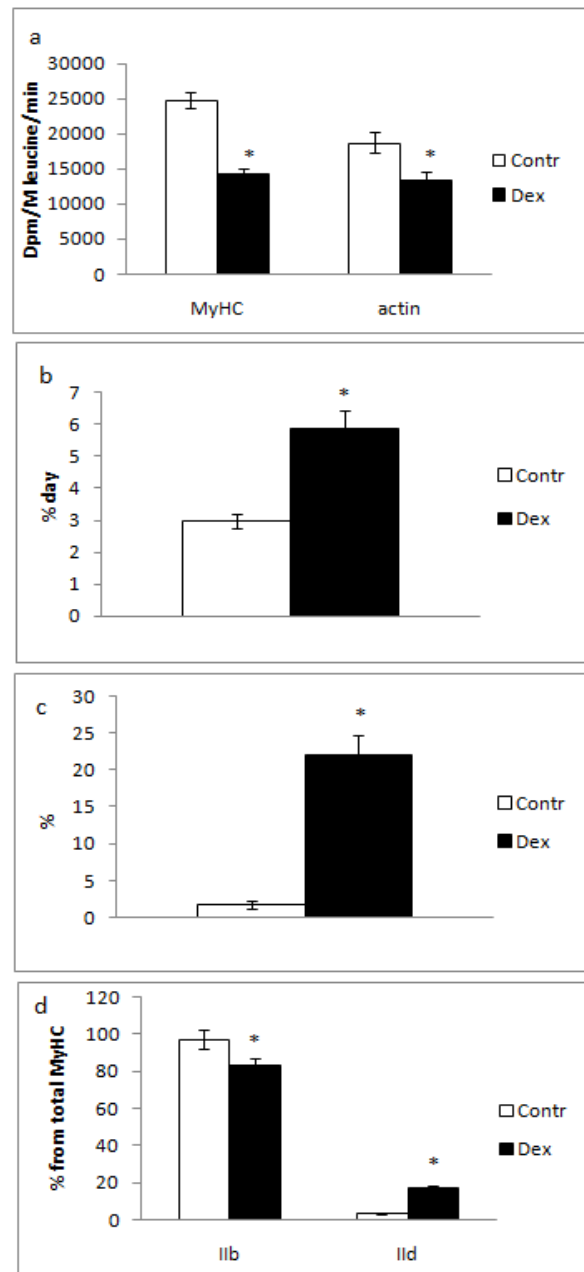


Figure 2. The effect of dexamethasone on the MyHC and actin synthesis rate (a), on the degradation of myofibrillar proteins (b), on the disappearance of actin and myosin filaments from the cross-sectional area of myofibrils (c), on the MyHC isoforms composition (d).

Contr – control group

Dex – dexamethasone treated group

*p=0.000155 according to Wilcoxon rank sum (Mann-Whitney U) test

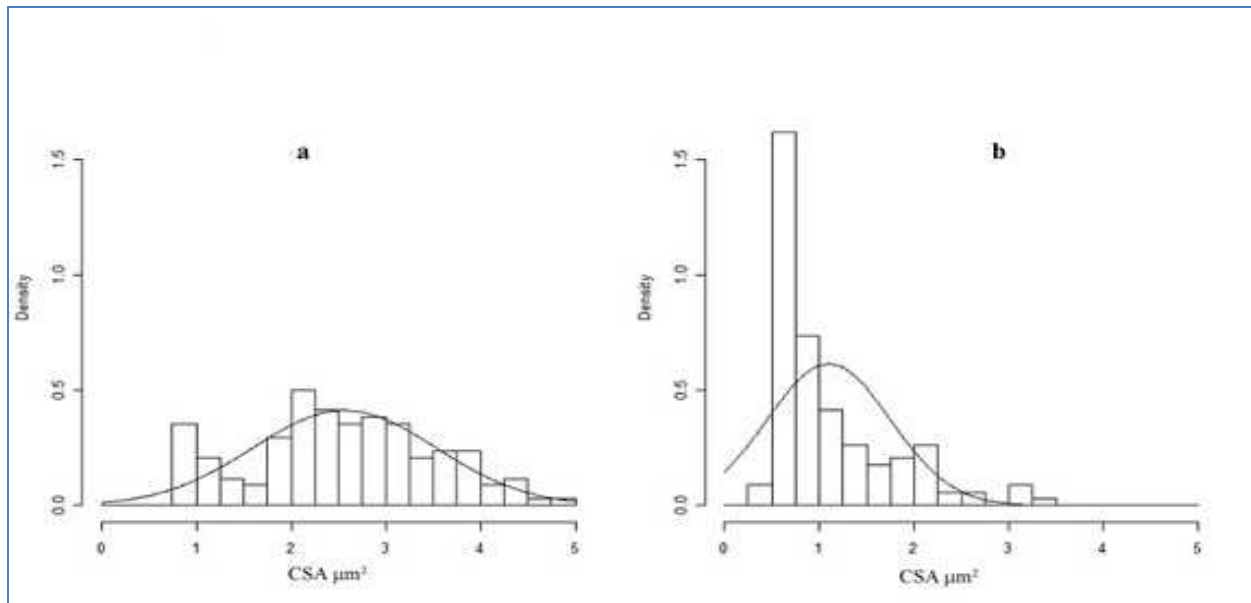


Figure 3. Histogram of distribution of myofibrils cross-sectional area in glycolytic muscle (fibers) of control (a) and dexamethasone treated rats (b)
CSA - cross-section area

MyHC isoforms separation

MyHC isoforms were separated by 7.2% SDS-PAGE using 0.75 mm thick gels. Myofibrils containing 0.5 μ g 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 [12]. Gels were silver-stained by the method of Oakley et al. [13]. Protein isoform bands were analyzed densitometrically by Image Master® 1D program, Version 3.0 (Amersham Pharmacia Biochem, USA) and the percentage distribution of the various isoforms was evaluated.

Protein assay

Total muscle protein and myofibrillar protein was assayed by using the technique described by Bradford [14].

Statistics

Means and standard errors of means were calculated from individual values using standard procedures of Excel. The data were analyzed by R 2.12.2. [15]. Pearson correlation coefficients were used for describing relationships between variables. Differences between groups were analyzed by the Wilcoxon rank sum (Mann-Whitney U) test. Probability distributions were compared using the Kolmogorov-Smirnov test. Differences were considered significant at $p < 0.05$.

RESULTS

In the control group CSA of myofibrils composed $1.8 \pm 0.5\%$ (0.2-3.5%), whereas in the myopathic group myofilaments disappeared from $22.0 \pm 2.7\%$ (13.2-38.4%) of the CSA of myofibrils (Fig. 2C). The average CSA of myofibrils in control group is $2.56 \pm 0.08 \mu\text{m}^2$ and in myopathic group $1.10 \pm 0.05 \mu\text{m}^2$ ($p < 0.001$). The distribution of myofibrils CSA of control and myopathic group, as it is shown in the histogram (Fig. 3), is significantly different according to Kolmogorov-Smirnov test ($p < 0.001$). In myopathic group, there are positive correlations between the area of myofibrils where myofilaments disappeared and myofibrillar protein degradation rate ($r = 0.849$, $p = 0.008$) and a negative correlation between myofibrils CSA and the area where myofilaments disappeared ($r = -0.787$, $p = 0.02$). There is also a negative correlation between the CSA of myofibrils and myofibrillar protein degradation rate ($r = -0.971$, $p < 0.001$) and in control group ($r = -0.781$, $p = 0.022$). A positive correlation was found between myofibrils CSA and thick myofilaments synthesis rate ($r = 0.933$, $p < 0.001$) and a negative correlation between thick myofilaments synthesis rate and the area of myofibrils where myofilaments disappeared ($r = -0.784$, $p = 0.021$). Both, the synthesis rate of MyHC and actin in myopathic group were significantly slower than in control group ($p < 0.001$ and $p < 0.001$, respectively, Fig. 2A). Myofibrils of myopathic glycolytic muscle fibers are thinner and have wide distances between myofibrils (Fig. 1C). Thin myofibrils are a result of intensive degradation rate of

myofibrillar proteins.

The degradation rate of myofibrillar proteins in myopathic group was about twice more intensive than in control group ($p < 0.001$, Fig. 2B). MyHCIIb isoform relative content in glycolytic muscle decreased and IId isoform increased in myopathic muscle in comparison with control group ($p < 0.001$ and 0.001 , respectively, Fig. 2D).

DISCUSSION

Type II muscle fiber atrophy is a typical abnormality in glucocorticoid mediated myopathy and it is related to the inhibition of insulin-like growth factor-1 (IGF-1) [16]. The mechanism of glucocorticoid induced muscle atrophy is related to upregulation of two genes, myostatin and glutamate synthase [17]. Glucocorticoids have shown to have a potent effect on muscle contractile proteins degradation, particularly MyHCIIb isoforms [18]. Results of the present study show that the myofibrils of glucocorticoid caused myopathic glycolytic muscle fibers are thinner and from about one fifth of their area myofilaments disappeared. The results of the present study explain the reason of reduced muscle strength and motor activity of glucocorticoid caused myopathic rats, which we have registered earlier [8,9]. Myofibrils in these muscle fibers are thinner due to the slower myofibrillar protein synthesis rate and more intensive myofibrillar protein degradation rate. A decrease in MyHCIIb isoform relative content and an increase in MyHCIIId isoform show that the above mentioned quantitative changes in myofibrils are significantly related to qualitative remodeling of thick myofilaments in myopathic glycolytic muscle fibers. As glucocorticoid myopathy does not affect all muscle fibers, but only the fast-twitch ones and particularly glycolytic fibers [2,4,6,19,20] and their myofibrillar apparatus [4,5,21,22], it is important to know how substantially changes in myofibrils fine architectonics in myopathic glycolytic fibers are related to the functional modification of these muscle fibers. It was shown that skeletal muscles with relatively high oxidative capacity are less sensitive to the catabolic action of dexamethasone [8]. This may explain why type IIA muscle fibers are less sensitive to action of glucocorticoids. It was previously mentioned that the terms catabolic and myopathic are misleading descriptions of the actions of corticoids on the level of muscle tissue, because the response to hormone within the different muscle fiber types varied [7,21]. From this viewpoint, thinner myofibrils, disappearance of myofilaments from about one fifth of their CSA, decreased synthesis rate of filaments and intensified

myofibrillar protein degradation in glycolytic muscle fibers indicate that the contribution of these fibers to the structural and functional rearrangement in myopathic muscle is impossible to overestimate. Regeneration capacity of glucocorticoid caused myopathic skeletal muscle is decreased and is in good correlation with decreased numbers of satellite cells [9]. Our previous work has shown that in myopathic muscle destructive changes in satellite cells on the ultrastructural level are similar to mother cell damages [7]. A significant decrease of MyHCIIb isoform relative content and an increase of MyHCIIId isoform in myopathic glycolytic muscles confirm the process of qualitative rearrangement in glycolytic muscles during glucocorticoid excess. These rearrangements determine the structural and functional homogeneity of myopathic glycolytic muscle fibers and subsequently cause a decrease in muscle strength and motor activity of myopathic rats.

In conclusion, the disappearance of thick and thin myofilaments from about one fifth of CSA of myofibrils from myopathic glycolytic muscle fibers explains the reduced muscle strength and motor activity during glucocorticoid excess. Increased myofibrillar protein degradation rate, decreased MyHC and actin synthesis rate, and a decrease in MyHCIIb isoform relative content in myopathic glycolytic muscle are related to qualitative remodeling of myofibrillar apparatus in myopathic glycolytic muscle fibers.

The authors declare that they have no conflict of interest.

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