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THE CONTENT OF MYOSIN HEAVY CHAINS IN HINDLIMB MUSCLES OF FEMALE AND MALE RATS

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The aim of the study was to test whether the considerable differences in the hindlimb muscles mass, the number and diameter of muscles fibers were connected with differences in the myosin heavy chain isoform content (expressed as the percentage of the given isoform in respect to total myosin heavy chains). Therefore, the content of myosin heavy chain (MHC) isoforms was studied in four hindlimb muscles: flexor digitorum brevis, soleus, tibialis anterior and gastrocnemius medialis of female and male rats by means of polyacrylamide gel electrophoresis supplemented with densitometric analyses. Muscles were isolated and homogenized prior to electrophoretic analysis. The most interesting result concerned considerably different composition of myosin isoforms for male and female subjects in the slow soleus muscles, which contained predominantly slow MHC isoform (MHC I). However, in the male muscle about 13% of IIa isoform (MHC IIa) was also detected; this isoform was not found in the majority of the studied female muscles (81% of muscle samples). This dimorphic difference was further confirmed by immunofluorescence stainining for slow and fast skeletal myosin isoforms and by assessment of the fiber ATPase activity. For the three remaining fast muscles (flexor digitorum brevis, tibialis anterior and gastrocnemius medialis) all four MHC isoforms were detected with the fast isoforms being dominant ones. However, there were not statistically significant differences observed between males and females, with the exception of IIx isoform, which was more frequent in male tibialis anterior muscle.

Key words: *electrophoresis, immunofluorescence staining, mATPase staining, muscle fiber, myosin heavy chain, sexual dimorphism, skeletal muscle, fast-twitch glycolytic muscle*

INTRODUCTION

The diversity of skeletal muscle properties can be attributed to heterogeneous characteristics of the individual muscle fibers and the mosaic composition of the numerous fiber types (1). Myosin, the major motor protein found skeletal muscle, is composed of a pair of heavy chains (MHC) and two pairs of light chains (MLC) (2). In skeletal muscles from adult rats, four types of the MHC isoforms are expressed, namely IIa, IIx, IIb and I (3). According to the classical study of Stein and Padykula (4), muscle fibers can be classified into three main physiological types: fast-twitch glycolytic (FG), fast-twitch oxidative glycolytic (FOG) and slow-twitch oxidative (SO). However, another classification has been proposed based on the type of MHC isoform the muscle fibers contain, I, IIA, IIB and IIX, respectively (5). Therefore, as might be expected, there are some discrepancies between these two classifications of muscle fibers. The differences can be explained partly by the existence of pure (containing only one MHC isoform) and hybrid (containing multiple isoforms) fiber types (6-9). Since the discovery of the variability of myosin isoforms and the resulting molecular heterogeneity of muscle fibers, the dominant MHC isoform has been considered to be responsible for functional differences of skeletal muscles. The plasticity of muscle phenotype in response to different factors was documented in numerous studies. The content of myosin isoforms has been studied in many publications addressing the effects of androgen and estrogen hormones on the expression of MHC in different slow and fast skeletal muscles (10-12). In experiments performed on the masseter muscle studied in castrated rabbits but later treated with testosterone, it was observed that testosterone administration resulted in a change of muscle fiber phenotype proportions. The authors have explained their observation in terms of the induction of changes evoked by testosterone binding to muscle fibers receptors in their allotype such that the fibers responded in a different way to neuronal activity than they had done before an exposure to the androgen (12). These authors suggested that this mechanism might result in a different myosin content in male and female masseter. One of the most important mechanisms of skeletal muscle plasticity is a change in contractile protein content. Therefore, the expression levels of MHC isoforms in a single muscle fiber under the influence of hindlimb suspension (13-15), unloading (16, 17) and microgravity (18) have also been studied. In rats, Novak *et al*. (19) reported age-related differences in fiber type composition in fast (extensor digitorum longus) and slow (soleus) muscles using myofibrillar adenosine triphosphatase (mATPase) staining after pre-incubation at pH 10.3, 4.5 and 4.3. However, they did not find significant differences between females and males for the age groups studied.

Muscle dimorphism has only been reported in a few studies, initially for the flexor digitorum brevis (20) and masseter (21) muscles. Based primarily on studies of the influence of male hormones, it is generally known that gender differences concerning skeletal muscles result from the effect of androgens in males. For example, testosterone has been shown to have a significant effect on fiber type composition of the temporalis muscle in guinea pigs (22, 23). Although the differences in muscle mass in the two genders are evident, experimental data concerning muscle dimorphism are very limited and concern only certain muscles. The majority of studies have focused on the masseter muscle, in rabbits (12, 21, 24, 25). A higher number of IIA phenotype muscle fibers (about 80%) and larger diameter fibers were observed in males compared to females. Also, in the mouse masseter muscle, females contained twice as many fibers containing the IIa myosin isoform, while males contained twice as many fibers containing the IIb myosin isoform (26). For flexor digitorum brevis, larger muscle fiber diameter and larger motoneurons (about 10%) in males were noted (20). In levator ani ten times more fibers with seven times greater cross-sectional areas were found in male compared to female rats (27). Finally, studies of functionally isolated motor units of the rat gastrocnemius medialis showed that composition of the three types of motor units, their contractile properties and the number of motor units in the muscle differ between genders (28, 29). However, it should be pointed out that dimorphism in relation to the content of MHC isoforms, especially in hindlimb rat muscles, remains practically unknown. Therefore, the aim of the present study was to clarify the gender differences of the MHC isoforms in fast and slow hindlimb muscles of the young adult rats.

MATERIALS AND METHODS

The experiments were performed on ten 6–9-month old male Wistar rats (mass 460–600 g) and ten 6–9-month old female Wistar rats (mass 240–280 g). All the procedures were approved by the Local Bioethics Committee and followed European Union guidelines on animal care as well as Polish Law on the Protection of Animals. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). At the end of the experiment the animals were killed by an overdose of sodium pentobarbital (180 mg/kg i.p.). In rats of both genders, the medial head of the gastrocnemius muscle, tibialis anterior, soleus and flexor digitorum brevis muscles of both their right and left hindlimbs were exposed, isolated from surrounding tissues and excised.

Preparation of muscle homogenates

For eight males and eight females, the four types isolated muscles were homogenized in 10 volumes (w/v) of ice-cold 20 mM phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.2 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). To evaluate myosin heavy chain to actin (M:A) ratios, the homogenates were subjected to electrophoretic analysis in 10% acrylamide gel according to Laemmli (30).

Preparation of myofibrils

The procedure was based on the method described by Jakubiec-Puka *et al*. (31). Briefly, muscle homogenates were spun for 20 min at 10,000×g at 0°C, the pellets were washed twice with ice-cold buffer, and then thoroughly suspended in 2% sodium dodecyl sulphate (SDS). After 10-min boiling followed by 20-min centrifugation at 10,000×g at 25°C, supernatants containing myofibril proteins were collected and subjected to further analysis.

Quantification of myosin heavy chain isoforms

Myofibrils were subjected to electrophoretic separation in 8% polyacrylamide gels according to Talmadge and Roy (32). Usually 3-5 µg of protein was loaded. The bands corresponding to myosin heavy chains (MHC) of soleus and extensor digitorum longus muscles served as the isoform markers. Relative amounts of MHC isoforms in the analyzed muscles were estimated by comparing the degree of staining intensity with Coomassie brilliant blue. The content was presented as percentage of the given isoform in the total MHC sample.

Densitometric analysis

Gels were photographed using G:Box system from SynGene (Cambridge, UK) equipped with Gene Snap and GeneTools software. The ratio of myosin heavy chains to actin (M:A) was estimated by densitometric analysis of protein bands stained with Coomassie brilliant blue, corresponding to the relevant proteins. Due to statistical differences in myosin content concerning soleu*s* muscles, four soleus muscles (left and right) taken from additional two female and male rats were also studied with two techniques: immunofluorescence staining for fast and slow myosin isoforms and fiber ATPase staining.

Immunofluorescence studies

Monoclonal antibodies against rabbit fast (MY-32) or human slow (NOQ7.5.4D) myosin heavy chains isoforms (Abcam, United Kingdom), ToPro3 and goat anti-mouse IgG conjugated with AlexaFluor-546 (Invitrogen, USA) were used. Distribution of myosin heavy chain isoforms in the rat soleus male and female muscles was examined by indirect immunocytochemistry. Muscle cross-sections were fixed in 4% paraformaldehyde for 10 min. The fixed specimens were thoroughly washed in phosphate-buffered saline (PBS) and treated for 30 min with solution of 5% normal goat serum and 0.2% Triton X-100 in PBS. Subsequently, muscle slices were incubated overnight at 4°C with anti-myosin heavy chain antibodies at a dilution of 1:50 and followed by incubation with Alexa 546-conjugated anti-mouse secondary antibody at a dilution of 1:1000 for 60 min RT. For assessment of nuclei distribution muscles were stained at room temperature with ToPro3 dye following the manufacturer's instructions. The specimens were visualized using Leica TCS SP5 spectral confocal microscope equipped with an HCX PL APO 40x/1.25-0.75 Oil Cs objective.

Estimation of the number of muscle fibers stained with the antibodies was performed for soleu*s* muscles of two rats of each gender. The quantification was based on manual counting of fibers stained with the relevant antibodies in six randomly chosen fiber areas of each of the studied muscle cross-sections (at least 290 fibers for each muscle cross-section was analyzed). The results were presented as a percentage of fibers interacting with a given antibody with respect to the total number of visible fibers (fibers were identified based on ToPro3 staining).

ATPase staining

ATPase activity of male and female soleus rat muscles was determined in fiber cross-sections based on the staining after 15-min preincubation at pH 9.4 in the following buffer: 20 mM sodium barbital, 18 mM $CaCl_2$, pH 9.4 (33). After preincubation, the sections were incubated for 30 min in 20 mM sodium barbital, pH 9.4, containing 9 mM $CaCl_2$ and 2.7 mM ATP; rinsed in 3 changes of 1% $CaCl_2$ (3 min each); immersed for 3 min in 2% $CaCl_2$; and rinsed in 10-12 changes of tap water.

After staining for 30 sec in 1% $(NH_4)_2S$ the sections were washed with several changes of tap water, dehydrated with ethanol and cleared in xylene.

Fibers containing fast myosin heavy chain isoform (dark staining) of the rat female and male soleus muscles (two rats of each gender) were calculated manually on the muscle cross-sections taken in the middle part of muscle belly and presented as the percentage of total number of muscle fibers.

Statistical analysis

Mann-Whitney U-test and Student's t-test were used to evaluate the quantitative data. $P<0.05$ was considered significant.

RESULTS

Myosin heavy chain isoform expression probed with electrophoretic analysis

The ratio of MHC to actin (M:A) in homogenates obtained from all four studied muscles did not significantly differ between female and male samples (*Fig. 1*), despite noticeable differences in the mass of the analyzed female and male muscles (*Fig. 2*).

Representative electrophoregrams of myosin heavy chain isoforms in the studied muscles: flexor digitorum brevis (FDB), soleus (SOL), tibialis anterior (TA) and gastrocnemius medialis (GM) are presented in *Fig. 3*. There were no major differences observed in the MHC isoform content between female and male flexor digitorum brevis, tibialis anterior and gastrocnemius medialis muscles. Myofibrils contained four isoforms: I, IIa, IIb and IIx, and the fraction of each of the isoform in the particular muscle type was similar for females and males. Quantitative analysis of the protein bands corresponding to the isoforms also did not demonstrate statistically significant differences in the MHC content in the studied muscles with one exception, IIx myosin in tibialis anterior (*Table 1*). However, a pronounced difference between the female and male soleus muscles was observed. In 13 out of 16 female muscles only MHC isoform type I was detected, while the remaining three muscles contained additionally 2 to 12% of fast MHC IIa isoform. In contrast, all soleus muscles from the male rats contained two MHC isoforms, type I and type IIa (1–24%). For male soleus the mean content of MHC IIa isoform was about 13%, whereas for female soleus it was about 2% (*Table 1*). Note that this difference was statistically significant at $p<0.001$ (Mann-Whitney U test).

Myosin heavy chain isoform expression probed with immunofluorescence microscopy and muscle ATPase staining

To confirm the observed differences between male and female soleus muscles in the MHC isoform content, immunofluorescence staining using specific anti-myosin antibodies (*Fig. 4*) was performed. As shown in *Fig. 4A*, which

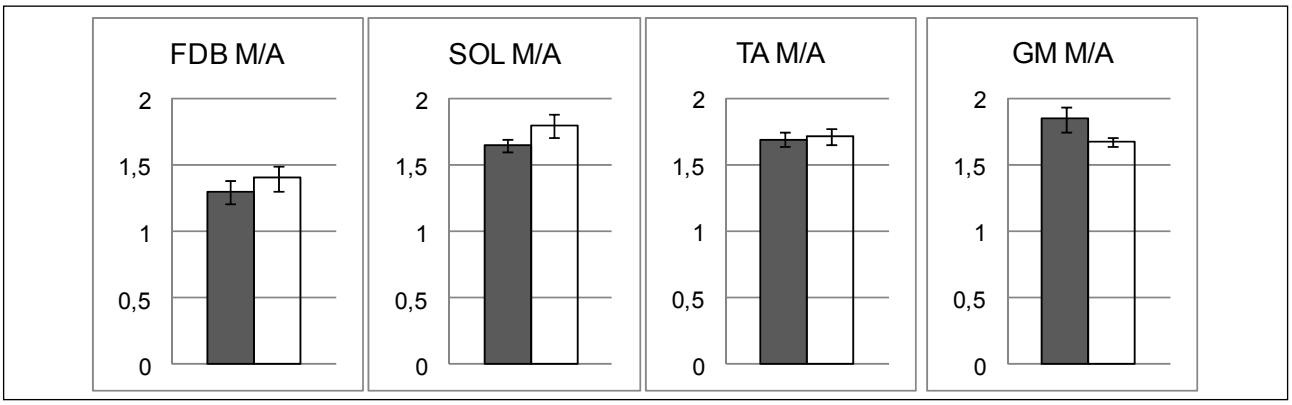

Fig. 1. The mean values and standard deviations for the ratio of myosin heavy chains to actin (M:A) in homogenates of flexor digitorum brevis (FDB), soleus (SOL), tibialis anterior (TA) and gastrocnemius medialis (GM) muscles. Dark grey bars, female muscles; empty bars, male muscles. Usually about 30 µg of homogenates was loaded onto a 10% polyacrylamide gel and electrophoresis was performed according to Laemmli (30).

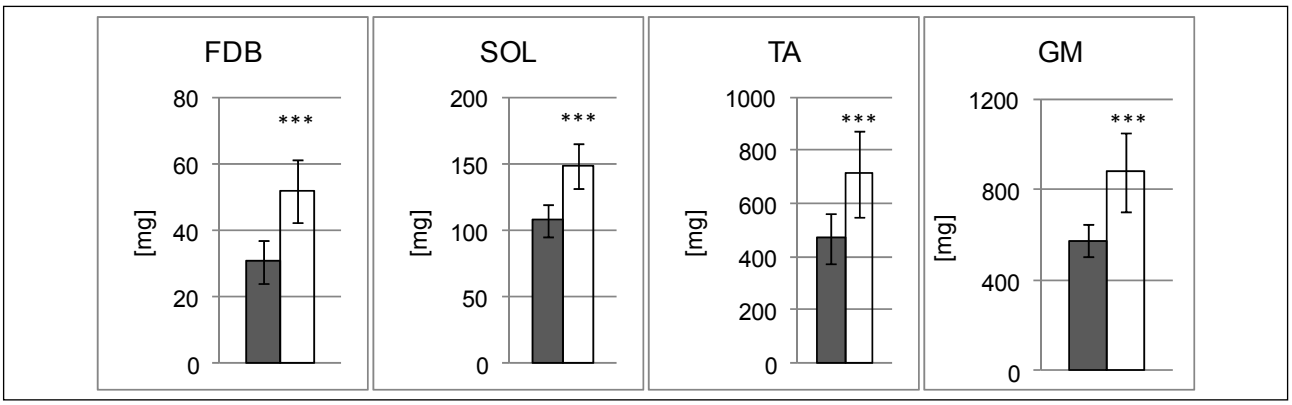

Fig. 2. The mean values and standard deviations for female and male muscles masses. Dark grey bars, female muscles; empty bars, male muscles. *** - the difference significant at $p<0.001$ (Student's t-test).

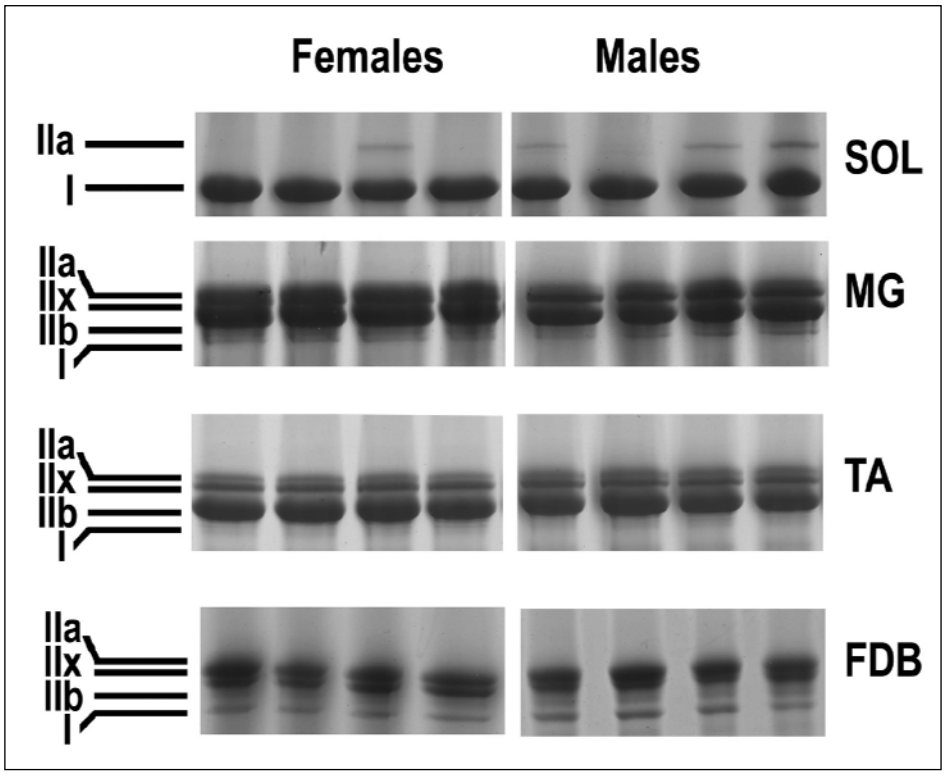

Fig. 3. Electrophoregrams of myosin heavy chain isoforms from the female and male rat muscles: flexor digitorum brevis (FDB), soleus (SOL), tibialis anterior (TA) and gastrocnemius medialis (GM). Usually about 3-5 µg of myofibril proteins were loaded onto 8% polyacrylamide gel and electrophoresis was performed according to Talmadge and Roy (32).

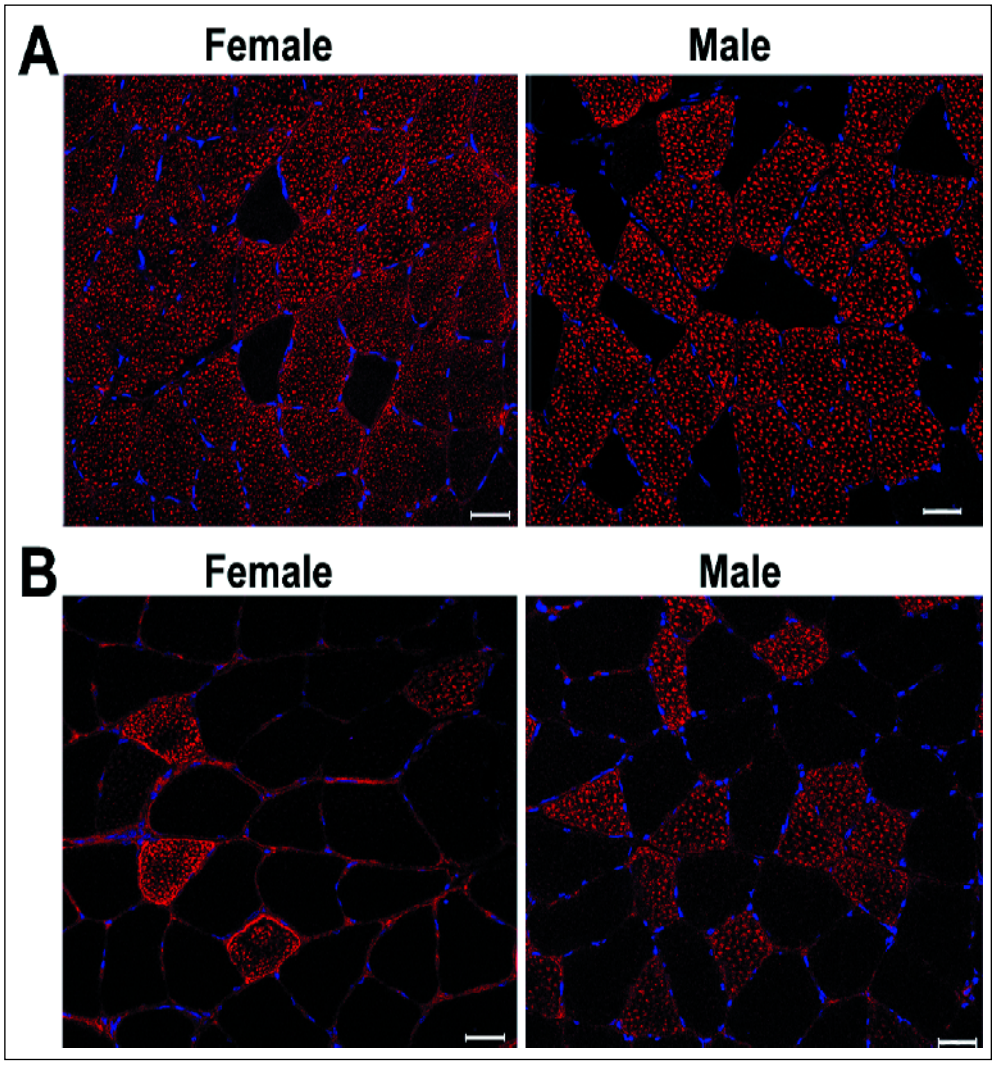

Fig. 4. Immunofluorescence staining for muscle myosin isoforms. Soleus muscles of female and male rats were stained for slow (A) and fast (B) myosin isoforms. Anti-myosin monoclonal antibodies were detected with secondary antibody conjugated with Alexa 546 (red) and nuclear chromatin was visualized with ToPro3 dye (blue). The confocal 0.4-µm images of the slice center were attained with Leica confocal microscope. Bars, 30 µm.

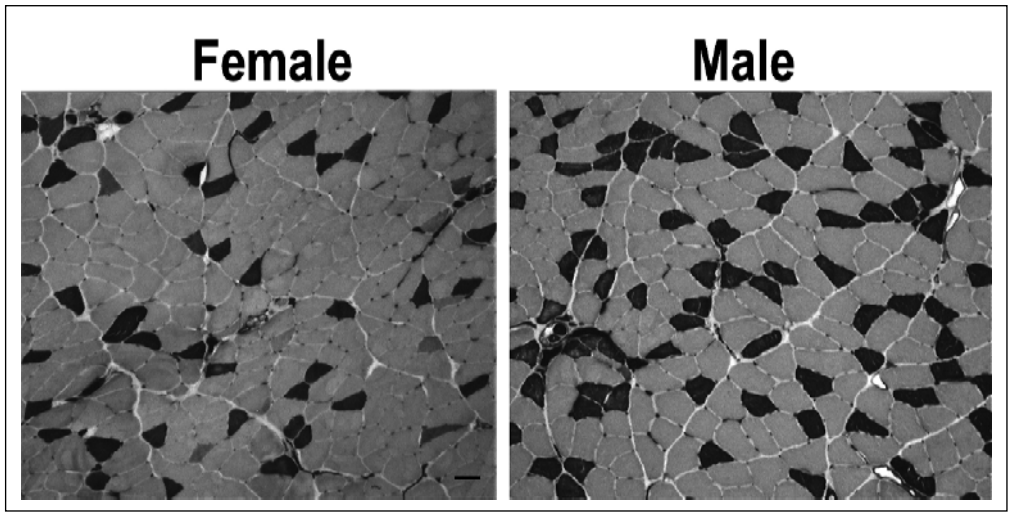

Fig. 5. Myosin ATPase staining. Female and male rat soleus muscles were stained for ATPase activity after preincubation at pH 9.4. The images were taken using a Nikon inverted microscope equipped with a 10× objective. Bar, 30 μm.

Table 1. Myosin heavy chain content in female and male hindlimb muscles. The data are presented as mean values ± standard deviations and variability ranges in the myosin isoforms content in the following muscles: flexor digitorum brevis (FDB), soleus (SOL), tibialis anterior (TA) and gastrocnemius medialis (GM). The statistically significant differences between content of myosin isoforms in males and females are indicated by asterisks: ***, the difference significant at p<0.001; *, the difference significant at p<0.05; N.S., the difference non significant, P>0.05 (U Mann-Whitney test).

	IIa [%]	**IIx** [%]	**IIb** [%]	**I** [%]
FEMALES				
n=16				
FDB	49.19±6.78	53.34±6.52	5.00±1.02	10.59±1.48
	38.83-62.39	24.62-46.63	3.43-6.43	8.00-13.67
	N.S.	N.S.	N.S.	N.S.
SOL	2.34±4.23	0.00	0.00	98.68±3.31
	0.00-11.58	-	-	88.42-100.00
	***			***
TA	9.13±2.78	23.22±1.44	66.55±3.32	1.07±1.19
	5.45-14.66	20.96-25.88	60.70-71.09	0.00-3.30
	N.S.	*	N.S.	N.S.
GM	7.45±2.57	25.66±4.02	60.35±6.36	6.52±1.15
	4.04-11.46	19.33-33.15	51.67-69.77	3.72-7.92
	N.S.	N.S.	N.S.	N.S.
MALES				
n=16				
FDB	46.05±8.65	37.17±9.43	4.86±0.99	11.86±1.77
	33.58-60.51	20.49-50.41	3.08-7.12	9.25-15.99
SOL	12.97±8.06	0.00	0.00	87.03±8.07
	1.36-24.37	-	-	75.62-98.64
TA	10.44±3.98	24.48±2.26	64.16±6.47	0.79±0.85
	5.33-16.54	20.06-28.36	56.74-73.66	0.00-2.03
GM	8.01±2.97	28.17±4.35	57.89±5.98	5.93±1.46
	3.92-11.54	21.7-35.53	47.95-66.30	3.09-8.38

demonstrates staining for the slow myosin isoform, the vast majority of fibers from female muscles were detected by the antibody (91.1±4.4%), compared to males (69.9±4.9%). Also, staining for the fast skeletal myosin isoform revealed the presence of positive fibers (17.1±3.9%) in the female muscles while a substantially higher amount was found in male muscles (27.6±5.8%; *Fig. 4B*).

Staining for myofibrillar ATPase performed after pre-incubation in alkaline conditions was consistent with the results obtained using immunofluorescence. There was a significantly higher amount of dark-stained fibers in the soleus muscles of males compared to females indicating a higher number of fast muscle fibers in male muscles (*Fig. 5*). Quantification of the dark-stained fibers within the entire muscle revealed that 10.5±2.5% of fast type fibers were detected in female muscles and 35.5±1.5% in the male ones.

DISCUSSION

The present study has addressed for the first time the dimorphism of a content of myosin heavy chain (MHC) isoforms in four hindlimb muscles of adult female and male rats. The most striking result of the analysis was a difference concerning soleus muscle showed by electrophoretic analysis and confirmed by immunofluorescence technique as well as by muscle ATPase staining. It should be pointed out that the three applied techniques revealed the same qualitative tendency in the gathered results, *i.e.* higher content of fast myosin or fibers in male soleus. The quantitative differences observed between these techniques (particularly between the electrophoretic analysis and both staining methods) are of methodological nature.

Chamberlain and Lewis (34) studied the soleus muscle in males, and found that it was composed exclusively of slow motor units (MUs). Betto *et al*. (35) reported that male rat soleus muscles were composed mainly of type I muscle fibers but they also contained type IIA fibers as well as hybrid slow fibers composed of MHC I and MHC IIa isoforms. Considerable interspecies differences in the proportion of myosin isoforms were revealed for rat and mouse. Nevertheless, Hartman *et al*. (36) found that female mouse soleus muscle also contained a higher percentage of MHC I isoform (55%) than in males (41%). Additionally, in male muscles about 8% of MHC IIb myosin was detected whereas this myosin isoform was not detected in females. Staron *et al*. (37) showed that soleus muscles isolated from male Fisher rats were composed of 93% of MHC I and about 7% of MHC IIa isoforms. However, Ishihara *et al*. (38) revealed no differences in male and female rat soleus muscle with respect to ATPase-based fiber type (pH 10.3, 4.3). We have shown (*Table 1*) that in the soleus muscle of male rats the MHC IIa isoform constituted about 13% of the heavy chain content and was several times higher than in females (about 2%). We have further confirmed these elecrophoretic data with microscopy techniques. These observations, together with the

results of Betto *et al*. (35), indicate mostly slow muscle fibers are formed in the soleus muscle of females, and therefore nearly exclusively slow motor units exist. On the other hand, the presence of MHC IIa isoform in each of the studied male soleus muscles corresponds to the existence of pure IIA muscle fibers and suggests a presence of fast resistant to fatigue MUs. However, the lack of differences in muscle fiber type content determined by ATPase activity studied at pH 10.3 and 4.3 between the two genders reported by Isihara *et al*. (38) and Novak *et al*. (19) suggests that there is no simple relationship between these two methods of muscle fiber classification and/or that the differences can be due to methodological differences.

Interestingly, dimorphic differences in the content of MHC isoforms were also described for another muscle predominantly composed of slow fibers, rabbit and mouse masseter. In the female rabbit muscles, a half of its muscle fibers contained only MHC IIa isoform, while the remaining half contained also the slow isoform (11, 21). In males, nearly 80% of fibers contained only MHC IIa isoform (11). In the case of adult mouse masseter, it was shown the male muscles had a higher number of fibers containing MHC IIb isoform than the female ones, while female muscles contained more fibers of IIA phenotype than the male ones (26).

Notably, of the three fast muscles studied, namely flexor digitorum brevis, tibialis anterior and gastrocnemius medialis there was no major difference in the expression of MHC isoforms between female and male rats. However, it is known that contractile properties of rat gastrocnemius medialis muscle differ between two genders. Several studies have shown sex-dependent differences in the basic contractile properties, such as duration and force of contraction of the gastrocnemius medialis muscle, as well as, differences in muscle composition of the three main types of motor unit with a greater proportion of FF units found in male muscles. Previous studies (28, 29) showed that motor units of the rat gastrocnemius medialis muscle were dimorphic. The basic contractile properties such as the contraction time and the twitch force of the three physiological types of motor units of female and male muscle were different (28). Also, different proportions of the three main types of motor unit were found (29). Male muscles contained a higher proportion of fast fatigable (FF) motor units and smaller proportion of slow (S) motor units than in females. Notably, each of the three types of motor units contains one type of muscle fiber, *i.e*., FF motor units contain fast glycolytic (FG) muscle fibers, fast resistant (FR) motor units contain fast oxidative glycolytic (FOG) muscle fibers and S motor units contain slow oxidative (SO) muscle fibers (39, 40). Moreover, male gastrocnemius muscles had approximately 10% more motor units than females muscles (29) and these units generated greater force (28), thus suggesting that they were composed of a higher number of muscle fibers. The number of muscle fibers in female and male muscles was directly calculated in a separate study (41), which revealed about 47% more fibers in males. However, the results presented herein have not revealed significant differences between the two genders in the composition of MHC isoforms in gastrocnemius medialis muscle. This seems to correspond to another observation, concerning that the mean total values of forces generated by groups of FF, FR and S motor units in male and female muscles, which were similar and were estimated to constitute 65, 30, 5% in males and 63, 27, 10% of the muscle output force in females, respectively (29).

In conclusion, in soleus a significantly higher content of the fast MHC IIa isoform and higher percentage of fast fibers, revealed by ATPase staining, was found in males compared to female rats. In contrast, no sex difference in MHC isoforms was observed for the fast muscles: flexor digitorum brevis, gastrocnemius medialis and tibialis anterior (except the IIx isoform). This study indicates that the dimorphic content of MHC isoforms in hindlimb muscles concerns predominantly slow muscles. Further studies are required to determine whether other factors such as for example physical activity (42), and ageing or different supplementation (43) influence muscle dimorphism in relation to the content of MHC isoforms.

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