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RAPID COMMUNICATION

Levels of Myosin Heavy Chain *mRNA* Transcripts and Protein Isoforms in the Fast Extensor Digitorum Longus Muscle of 7-Month-Old Rats with Chronic Thyroid Status Alterations

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Summary

We have studied the effect of chronic thyroid status alterations on the myosin heavy chain (MyHC) isoform composition (by SDS-PAGE) and on MyHC mRNA levels (by RT-PCR) in the fast extensor digitorum longus (EDL) muscle of 7-month-old inbred Lewis strain female rats and compared this with corresponding results of the previously studied slow soleus muscle. Our findings show that in the EDL muscle, all four types 1, 2a, 2x/d and 2b of MyHC mRNA transcripts and protein isoforms are present in euthyroid, hypothyroid and hyperthyroid rats, i.e. after chronic treatment with methimazole and T_3, respectively. This is in contrast with the soleus, where only MyHC1 and 2a protein isoforms are expressed under similar conditions. Except for 2x/d MyHC mRNA transcripts in the EDL muscles, there was always significant difference between hypothyroid and hyperthyroid rats both at mRNA and protein levels. From our results we can conclude that extended alteration of the thyroid status leads to typical changes in the expression of MyHC mRNA transcripts and MyHC protein isoforms in the fast EDL and the slow soleus muscles. These changes correspond to those described after shorter periods of altered thyroid status. The characteristic phenotype differences between soleus and EDL muscles remain, however, preserved even after 7 months of thyroid hormone status alteration.

Key words

Rat EDL muscle • Myosin heavy chains • *mRNA* transcripts • Thyroid hormones • SDS-PAGE • RT-PCR

It is well known that striated muscles are privileged targets for thyroid hormones that modulate MyHC gene expression and the MyHC isoform composition of mammalian skeletal muscles (d´Albis and Butler-Browne 1993, Soukup and Jirmanová 2000). In general, hyperthyroidism increases the expression of fast, whereas hypothyroidism of slow MyHC isoforms. For example, slow soleus muscle (SOL) in hyperthyroid rats, where the predominant isoform is the slow MyHC 1 isoform, the expression of 2a MyHC increases together with the contraction velocity of the muscle. On the other hand, in the fast extensor digitorum longus (EDL) muscle, which contains predominantly fast 2a, 2x/d and 2b MyHC isoforms, the faster 2x/d and 2b MyHC are favored. Myosin transitions that can be induced by changes in the levels of thyroid hormones (even after

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treatment lasting only a few weeks), thus follow a preferential sequence for the transformation of MyHC isoforms: 1↔2a↔2x/d↔2b (for review see Schiaffino and Reggiani 1996, Pette 2002).

At present, we are studying the influence of both innervation and thyroid hormones on the diversification of muscle fiber phenotypes in normal and regenerated muscles in long term experiments (Soukup *et al.* 2002, Hudecová *et al.* 2004, Říčný and Soukup 2006, Vadászová *et al.* 2004a,b, 2006a,b, Zachařová *et al.* 2005). The aim of the present study was therefore to analyze if similar changes in MyHC isoforms and their *mRNA* levels, as reported after shorter alterations of thyroid status, will also occur after chronic 7 months lasting hyperthyroid or hypothyroid status in EDL muscle of 7-month-old inbred female Lewis rats and to compare them with results previously reported for SOL muscle after the same chronic treatment (Vadászová *et al.* 2006a).

Pregnant female inbred Lewis strain rats were obtained from the rat breeding laboratory unit of the Institute of Physiology. The maintenance and handling of experimental animals was in accordance with EU Council Directive (86/609EEC) and the investigation was approved by the Expert Committee of the Institute of Physiology AS CR, Prague. Hypothyroid status was induced with a 0.05 % solution of methimazole (2-mercapto-1-methylimidazole, Sigma) in the drinking water of mothers, beginning at fetal day 14 and after the postnatal day 23 (i.e. after weaning) in the drinking water of pups. This status was maintained up to the 7th month of age. The hyperthyroid status was induced in 4-week-old rats by intraperitoneal injections of 3, 3',5-triiodo-L-thyronine (Sigma, sodium salt, T3) 150 μg/kg body weight, 3 times a week and maintained up to the 7th month of age. The SOL and EDL muscles were excised from 7-month-old rats anesthetized with an intraperitoneal injection of Nembutal (sodium pentobarbital 40 mg/kg), frozen in liquid nitrogen and used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), RT-PCR and cryostat sectioning (results of quantitative morphometry of fiber type composition will be published separately elsewhere). Rats were euthanized by an overdose of the anesthetic.

Muscle samples were treated as described earlier for SDS-PAGE (Zachařová *et al.* 2005) and RT-PCR (Hudecová *et al.* 2004, Vadászová *et al.* 2006a). In brief, for SDS-PAGE the cut muscle samples were homogenized in a glass tissue grinder, incubated for 10 min at 95 ºC and 4 μl of the homogenate were loaded onto the gel. MyHC isoforms were separated according to Talmadge and Roy (1993) by SDS-PAGE carried out at constant voltage (70 V) for 30 h at 4 ºC, the gels were silver-stained (Blum *et al.* 1987) and the individual MyHC isoforms were densitometrically evaluated using AIDA 3.28 computer program (Advanced Image Data Analyzer, Germany). The *mRNA* levels of MyHC isoforms were quantified using RT-PCR approach relatively to the housekeeper glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reverse transcription was performed using Ready-To-Go You-Prime First Strand Kit (Amersham Biotech) and pd(N)6 primer. Polymerase chain reaction (PCR) was performed using specific, primers previously described for each MyHC isoform and GAPDH (for further information see Jaschinski *et al.* 1998, Vadászová *et al.* 2006a). Amplified fragments were evaluated on 2 % agarose gels and the intensity (i.e. optical density per mm^2) of each fragment was measured using PCBAS software (for details see Hudecová *et al.* 2004, Vadászová *et al.* 2006a). The data were expressed as means ± S.D. and the significance of differences was evaluated using Student's t-test and Statgraphics 5.1 (Statpoint Inc., USA).

Four *mRNA* transcripts and MyHC isoforms MyHC1, 2a, 2x/d and 2b were found in the EDL muscles of 7-month-old euthyroid, hypothyroid and hyperthyroid rats and their levels were affected by the altered thyroid

Table 1. *mRNA* expression (a.u. x 10) and MyHC protein isoforms (%) in the EDL muscles of 7-month-old euthyroid (EU), hypothyroid (HY) and hyperthyroid (TH) rats.

		***mRNA* (a.u.)**	**Protein (%)**
MyHC 2b	TH	4.0±3.6#*	45.6±5.1*
	HY	8.2±0.5#	34.9±5.2#
	EU	13.2±3.4	48.6±7.3
MyHC 2x/d	TH	5.1±1.3#	39.8±3.9#*
	HY	6.1±2.1#	31.1±4.7
	EU	11.5±3.0	33.9±6.4
MyHC 2a	TH	2.6±2.1#*	12.5±6.8*
	HY	9.0±1.6	23.5±5.0#
	EU	7.8±0.6	13.8±2.4
MyHC 1	TH	0.1±0.1*	2.1±2.2*
	HY	8.4±0.7	10.2±3.1#
	EU	4.5±4.5	3.9±2.5

Number of measurements was 2-4 for *mRNA* and 5-10 for protein isoforms; #significant difference $p<0.05$ of HY or TH against EU rats, *significant difference $p<0.05$ between HY and TH rats.

status compared to euthyroid littermates (Table 1). The levels of the MyHC1 and MyHC2a *mRNA* transcripts increased in hypothyroid and decreased in hyperthyroid rats. At the protein level, the MyHC1 isoform content increased in hypothyroid and decreased in hyperthyroid, MyHC2a isoform content increased in hypothyroid, but did not change in hyperthyroid animals. Levels of MyHC 2x/d and 2b *mRNA* transcripts were at 7 months after the onset of experiment, lower not only in hypothyroid (as expected), but also in hyperthyroid rats. On the other hand, 2x/d and 2b protein isoforms decreased in hypothyroid and 2x/d increased in hyperthyroid rats. These results show that the changes at *mRNA* and protein levels were equal in case of MyHC1 (increase in hypo-, decrease in hyperthyroid) and 2b isoform in hypothyroid rats (decrease). On the other hand, methimazole treatment led to significant increase of 2a isoform only at protein level, while T3 application decreased only *mRNA* transcript level. Both treatments lead to a decrease of 2x/d *mRNA* and T3 treatment lead to a significant increase of 2x/d protein isoform. With the only exception of *mRNA* 2x/d transcripts, there were, however, always significant differences between hypo- and hyperthyroid rats, as these changes were in opposite direction according to the suggested transformation scheme: 1↔2a↔2x/d↔2b. In the SOL muscle, the same effect of altered thyroid status was observed for MyHC1 and 2a *mRNA* transcripts and protein isoforms, while no significant changes were observed in expression of 2x/d and 2b *mRNA*. This makes sense since 2x/d and 2b transcripts are generally not translated into the protein form in the SOL muscle.

Our studies demonstrate that all four MyHC1, 2a, 2x/d and 2b isoform *mRNA* transcripts are present in the SOL and EDL muscles, which indicates that all four MyHC genes are active and their *mRNA* is copied from the DNA in both SOL and EDL muscles. The reason why MyHC2x/d and 2b transcripts are not translated into detectable protein isoforms in the SOL muscle is not clear. This might be due to their short half-time, which would indicate a possible involvement of post-transcription regulation of their translation efficiency, or may also be due to a cross-reactivity of primers. Cross reactivity is not probable, as these primers were previously well characterized and frequently used with success (Jaschinski *et al.* 1998; for more detailed discussion see Vadászová *et al.* 2006a). Nevertheless, we have performed analyses by MALDI-TOF and LC/MS Ion Trap mass spectrometer techniques of rat 2x/d and 2b MyHC isoforms. We have proposed new pairs of primers specific for all four MyHC isoforms designed with respect to their equal Tms and lengths of PCR products and matching to separate exons. This will make the organization of the next RT-PCR experiment more simple. In our hands, each pair of primers gave specific products under equal conditions (Žurmanová *et al.* 2006).

Using our current methods, we cannot clearly demonstrate whether individual MyHC isoforms are localized in pure muscle fiber types or in transitional (hybrid) fibers containing various combinations of MyHC isoforms (for review, see Pette 2002). Coexpression of MyHC *mRNA* transcripts in a single fiber was demonstrated both in rat (DeNardi *et al.* 1993, Stevens *et al.* 1999) and human muscles (Smerdu *et al.* 1994, Andersen and Schiaffino 1997). The recent study of muscle phenotype composition (Zachařová *et al.* 2005) revealed coexpression of MyHC1 and 2a isoforms determined in serial cross sections of the SOL and of all four MyHC isoforms in the EDL muscles immunostained with specific anti MyHC monoclonal antibodies. Coexpression was especially high in the hyperthyroid SOL and hypothyroid EDL muscles (Vadászová 2005).

Our findings suggest that as a result of chronic long-lasting alteration of thyroid status, both slow SOL and fast EDL muscles undergo characteristic changes in the expression of MyHC *mRNA* transcripts and MyHC protein isoforms. These changes, however, correspond to changes described after shorter intervals of thyroid status alterations. Our results also suggest that the SOL is more influenced by hyperthyroid status, whereas EDL seems to respond to both hypothyroid and hypothyroid statuses.

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