Physiol. Res. 55: 221-225, 2006

Levels of Myosin Heavy Chain mRNA Transcripts and Content of Protein Isoforms in the Slow Soleus Muscle of 7-month-old Rats with Altered Thyroid Status

A. VADÁSZOVÁ, S. HUDECOVÁ[1], O. KRIŽANOVÁ[1], T. SOUKUP

Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic and [1]Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovak Republic

Received September 15, 2005
Accepted December 22, 2005

Summary

Skeletal muscles of small rodents contain four main fiber types, namely type 1, 2A, 2X/D and 2B fibers containing myosin heavy chain (MyHC) 1, 2a, 2x/d and 2b isoforms. Each of these MyHC isoforms is the product of a distinct gene and their expression is believed to be primarily transcriptionally controlled. In most rat muscles, messenger RNA (mRNA) transcripts for MyHC1, 2a, 2x/d and 2b and their corresponding protein products were found with the exception of the soleus muscle, where typically only MyHC1 and 2a transcripts and protein isoforms were demonstrated under normal conditions. Here we show the expression of all four MyHC1, 2a, 2x/d and 2b mRNA transcripts in the soleus muscle under normal conditions in euthyroid, as well as in experimental hypothyroid and hyperthyroid (with the exception of 2b MyHC transcript) 7-month-old female inbred Lewis rats. This is not matched, however, by the appearance of corresponding four isoforms, as we have found that 2x/d and 2b protein isoforms are not present at levels detectable by SDS-PAGE. We also show that the chronic hypothyroid and hyperthyroid status affects the expression of MyHC isoforms both at the mRNA and protein levels.

Key words

Rat soleus muscle • Myosin heavy chains • mRNA transcripts • Thyroid hormones • SDS-PAGE and RT-PCR

Introduction

The skeletal muscles of small rodents are composed of four main fiber types, namely type 1, 2A, 2X/D and 2B, containing corresponding myosin heavy chain (MyHC) 1, 2a, 2x/d and 2b isoforms. Each of the MyHC isoforms is the product of a distinct gene and they are believed to be primarily transcriptionally controlled, e.g. by the level of thyroid hormones. In many rat muscles, messenger RNA (mRNA) transcripts and corresponding MyHC1, 2a, 2x/d and 2b protein isoforms were found, the soleus muscle being the most obvious exception. The soleus is a slow antigravity muscle consisting of a majority of slow type 1 fibers in adult rats, supplemented with a small number of fast 2A fibers as demonstrated by histochemical, immunocytochemical, electrophoretic and immunoblotting methods under normal conditions. Similarly, only MyHC1 and 2a, but not 2x/d or 2b mRNA transcripts were usually found in the normal rat soleus muscle (Wright *et al*. 1997). Single

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

fiber mRNA analysis, however, revealed that a majority of type 1 fibers contained 2a mRNA and about half of these fibers contained 2x/d or 2x/d and 2b mRNA as well (Stevens *et al.* 1999). Furthermore, it was shown that the thyroid hormone induced the appearance of the MyHC2x/d isoform in fibers expressing predominantly the MyHC2a isoform (Schiaffino *et al*. 1990). The MyHC2x/d *mRNA* expression was also detected by *in situ* hybridization after one week of T_3 treatment (DeNardi *et al*. 1993). At present, we are studying within the European Muscle Network project (MYORES, Network of Excellence No. 511978, Multi-organismic Approach to Study Normal and Aberrant Muscle Development, Function and Repair) the influence of innervation and thyroid hormones on the diversification of muscle fiber phenotype in normal and regenerated muscles (Vadászová *et al*. 2004a,b, Zachařová *et al*. 2005). In this study we aim to ascertain if all four mRNA MyHC isoform transcripts are expressed in the rat soleus muscle and to reveal further details about the effect of altered thyroid status on MyHC gene expression. We have therefore studied mRNA levels and the MyHC isoform content in the slow soleus muscle of euthyroid, hypothyroid and hyperthyroid 7-month-old female inbred Lewis rats.

Table 1. Primer sequences, fragment size (bp) and annealing temperature used in the PCR reaction.

Gene	Primer sequence Sense (S) and antisense (A)	Fragment size	Annealing temperature
MyHC 1/β	S: 5′-ACA GAG GAA GAC AGG AAG AAC CTA C-3′ A: 5′-GGG CTT CAC AGG CAT CCT TAG-3′	288 bp	64 ºC
MyHC 2a	S: 5′-TAT CCT CAG GCT TCA AGA TTT G-3′ A: 5′-TAA ATA GAA TCA CAT GGG GAC A-3′	310 bp	59 ºC
MyHC 2x	S: 5′-CGC GAG GTT CAC ACC AAA-3′ A: 5′-TCC CAA AGT CGT AAG TAC AAA ATG G-3′	120 bp	55 ºC
MyHC 2b	S: 5′-CTG AGG AAC AAT CCA ACG TC-3′ A: 5′-TTG TGT GAT TTC TTC TGT CAC CT-3′	197 bp	59 ºC
GAPDH	S: 5′-AGA TCC ACA ACG GAT ACA TT –3′ A: 5′-TCC CTC AAG ATT GTC AGC AA –3′	309 bp	60 ºC

Material and Methods

Pregnant female inbred rats of Lewis strain rats were obtained from an authorized laboratory rat-breeding 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, Czech Republic. The hypothyroid status was induced and maintained with a 0.05 % solution of methimazole (2-mercapto-1-methylimidazole, Sigma) in the drinking water since fetal day 14, the hyperthyroid status was induced and maintained in 4-week-old rats by intraperitoneal injections of 3, 3',5-triiodo-L-thyronine (Sigma, sodium salt, T_3) 150 μg/kg body weight, three times a week throughout the whole experimental period. Muscles were excised from 7-month-old rats anesthetized with an intraperitoneal injection of Nembutal (sodium pentobarbital 40 mg/kg) and frozen in liquid nitrogen. They were stored at –80 ^{0}C until used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and reverse transcription-polymerase chain reaction (RT-PCR). Rats were sacrificed by an overdose of the anesthetic.

For SDS-PAGE, muscle samples were treated as described earlier (Zachařová *et al*. 2005). 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, previously described primers for each

MyHC isoform and GAPDH (Table 1, for details see Jaschinski *et al*. 1998). Amplified fragments were evaluated on 2 % agarose gels and intensity (i.e. optical density per mm^2) of each fragment was measured using PCBAS software (for details see Hudecová *et al*. 2004). 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).

Results and Discussion

Four mRNA transcripts encoding MyHC1, 2a, 2x/d and 2b were found in the soleus muscles of 7-month-old euthyroid and hypothyroid rats, whereas in hyperthyroid animals, the MyHC2b mRNA transcript was not detected (Table 2). The MyHC1 transcript increased by about 40.5 % in hypothyroid rats and decreased by about 33 % in hyperthyroid rats, compared to the euthyroid status. On the contrary, the MyHC2a transcript level slightly increased in hypothyroid and significantly increased in hyperthyroid rats compared to rats with euthyroid status. The values for the 2x/d and 2b transcripts did not significantly vary between euthyroid and hypothyroid rats, but were significantly lower in hyperthyroid rats (the 2b transcript was even not detected). At the protein level, we have detected in the same soleus muscles only MyHC1 and 2a isoforms using SDS-PAGE. Their levels were similar in euthyroid and hypothyroid rats, but the content of MyHC1 isoform was significantly decreased by 30.5 % in hyperthyroid animals, whereas MyHC2a content was significantly increased compared to euthyroid (and hypothyroid) rats (Table 2).

Table 2. mRNA expression (arbitrary units - a.u. x 10) and MyHC protein isoforms (%) in the soleus muscles of 7-month-old euthyroid (EU), hypothyroid (HY) and hyperthyroid (TH) rats.

	mRNA (a.u. x 10)			Protein isoforms (%)		
	EU	**HY**	**TH**	**EU**	**HY**	**TH**
MyHC 1	11.6±0.7	16.3±1.8$^{\#\#}$	7.8±4.6*	98.6±3.2	98.2±5.3	68.1±15.5$^{\#\#**}$
MyHC 2a	1.9±1.7	3.1±2.4	10.7±2.3$^{\#\#**}$	1.4±3.2	1.8±5.3	31.9±15.5$^{\#\#**}$
MyHC 2x/d	8.6±0.3	11.4±2.4	3.7±1.5$^{\#\#**}$	n.d.	n.d.	n.d.
MyHC 2b	3.9±3.0	4.8±3.9	n.d.	n.d.	n.d.	n.d.

The number of measurements was 3-4 for the PCR and 6-10 for the SDS-PAGE analysis; n.d. = not detected, $^{\#}p<0.05$, $^{\#\#}p<0.01$ = significant difference HY or TH against EU rats, $^{*}p<0.05$, $^{**}p<0.01$ = significant difference between TH against HY rats.

Our study demonstrates that in the slow soleus muscles all four MyHC1, 2a, 2x/d and 2b isoform mRNA transcripts are present, similarly as e.g. in the fast EDL muscle (results not shown). The presence of all four MyHC transcripts indicates that all four MyHC genes are active and their mRNA is copied from the DNA. The reason why MyHC2x/d and 2b transcripts are not translated into protein isoforms in the soleus muscle is not clear. This could be due to their short half-time, which would indicate possible involvement of post-transcription regulation of their translation efficiency. A similar mechanism was suggested to explain the high level of β-F_1-ATPase mRNA and low amounts of protein ATPase in brown adipose tissue mitochondria (Houštěk *et al*. 1991). Another possibility is a cross-reactivity of the primers used. We have performed, however, the PCR reaction with previously well established primers (cf. Table 1, Jaschinski *et al*. 1998). As usual for MyHC isoforms, we have not sequenced the PCR amplicons, as the reverse primers (3'-5') are designed to the 3' untranslated regions of the respective mRNA, which means that each reverse primer is quite unique. Therefore, we strongly believe that it is very unlikely that the amplicons can be non-specific. It is even true if the forward primers (5' - 3') can cross-hybridized, as it is very difficult to find the position in MyHC mRNAs in which the whole primer sequence would be unique for the particular MyHC mRNA. What is the exact mechanism which prevents translation of mRNA 2x/d and 2b transcripts will thus require further studies.

Besides the basic muscle fiber types, transitional (hybrid) fibers containing various combinations of MyHC isoforms can also be identified (for review see Pette 2002). Coexpression of MyHC mRNA transcripts in a

single fiber was demonstrated both in the rat (DeNardi *et al*. 1993, Stevens *et al*. 1999) and human muscles (Smerdu *et al*. 1994, Andersen and Schiaffino 1997). A recent study of the phenotype composition revealed coexpression of MyHC isoforms determined in serial cross sections of the soleus and EDL muscles immunostained with specific anti-MyHC monoclonal antibodies (Zachařová *et al*. 2005). Coexpression was especially high in the hyperthyroid soleus and hypothyroid EDL muscles (Vadászová, PhD Thesis, 2005). This supports the suggestion that the 2x/d and 2b MyHC mRNA transcripts in our sample are also "co-expressed" with 2a and/or type1 mRNA transcripts in the 2A and type 1 fibers or in hybrid fibers containing both MyHC1 and 2a isoforms. They are, however, not translated into corresponding protein isoforms at sufficient levels detectable by the SDS-PAGE technique and to be manifested in the muscle fiber phenotype.

Regulation of the MyHC gene family is complex and the same MyHC gene can be differently regulated by the thyroid hormone in various muscles. Hyperthyroidism (in contrast to hypothyroidism) increased the contractile velocity in the rat soleus and decreased the proportion of slow myosin from 93 % to 69 % (Caiozzo *et al*. 1991), while it concomitantly increased the expression of the fast MyHC2a gene (Izumo *et al*. 1986). Similarly in our sample of inbred Lewis female rats, elevated levels of T_3 increased the MyHC2a isoform content in the soleus muscle and decreased the MyHC1 isoform content from 98 % to 69 %. Hyperthyroid status thus increased the expression of the fast MyHC2a gene and decreased the expression of the MyHC1 gene. On the other hand, decreased T_3 levels in 7-month-old hypothyroid rats had practically no effect on MyHC mRNA transcript and protein isoform composition in the soleus muscle. Although our results were obtained in female inbred Lewis rats, it is feasible to suppose that similar results would be obtained in male Lewis rats or in rats of other strains even if MyHC composition of the soleus muscle differs among various rat (Soukup *et al.* 2002) or mouse strains (Asmussen *et al.* 2003).

It is not fully understood why the MyHC2b transcript is not expressed in the hyperthyroid status. It is possible that the specific positive T_3 regulation of fast isoforms is overwhelmed by the well known "unspecific" effect of T_3, generally accelerating the formation of adult tissue phenotype. The "unspecific" effect of T_3 together with a slow tonic impulse pattern can lead, in the case of the soleus muscle, to a decrease of 2x/d or the extinction of 2b mRNA transcripts, which are functionally not effective, as they are not utilized for protein isoform synthesis. The MyHC2b transcript seems to be more affected than the 2x/d one. Interestingly, direct stimulation of the soleus in intensity and frequency typical for normal motor unit activity in EDL muscle induced MyHC2x/d expression, but was unable to induce MyHC2b expression (Ausoni *et al*. 1990).

The differences between the expression of mRNA and the synthesis of corresponding protein isoforms and the lack of parallel changes caused by thyroid hormones in the rat soleus muscle indicates possible involvement by other than merely transcriptional regulation control. We can suppose that the basic role of innervation and the muscle activity pattern on the regulation of MyHC isoform expression is affected or even activated not only by thyroid hormones, but also by hitherto unknown retrograde signal(s) from muscle to motoneurons. This seems likely and was previously demonstrated for the fast-to-slow transition in the soleus muscle, as accelerated by intramuscular injections of recombinant neurotrophin NT-4/5 in neonatal rats (Carrasco and English 2003).

We can thus conclude that the rat soleus muscle contains MyHC1, 2a, 2x/d and 2b mRNA transcripts, but only MyHC1 and 2a mRNA transcripts are translated into the protein isoforms. Our findings also confirm that regulation of MyHC expression is complex and suggest the involvement of this regulation at transcriptional and posttranscriptional levels.

Acknowledgements

The authors wish to thank Ms Věra Semelová and Ms Marta Šírová for the technical assistance. Supported by grants from the Grant Agency of the Czech Republic (305/03/H148 and 304/05/0327), VEGA (2/6078), NATO (979876), APVT-51-027404, MYORES 511978 and Research project AV0Z 50110509.

References

ANDERSEN JL, SCHIAFFINO S: Mismatch between myosin heavy chain mRNA protein distribution in human skeletal muscle fibers. *Am J Physiol* **272**: C1881-C1889, 1997.

ASMUSSEN G, SCHMALBRUCH I, SOUKUP T, PETTE D: Contractile properties, fiber types and myosin isoforms of fast and slow muscles of hyperactive Japanese waltzing mice. *Exp Neurol* **184**: 758-766, 2003.

AUSONI S, GORZA L, SCHIAFFINO S, LØMO T: Expression of myosin heavy chain isoforms in stimulated fast and slow rat muscles. *J Neurosci* **10**: 153-160, 1990.

CAIOZZO VJ, HERRICK RE, BALDWIN KM: Influence of hyperthyroidism on maximal shortening velocity and myosin isoform distribution in skeletal muscle. *Am J Physiol* **261**: C285-C295, 1991.

CARRASCO DI, ENGLISH AW: Neurotrophin 4/5 is required for the normal development of the slow muscle fiber phenotype in the rat soleus. *J Exp Biol* **206**: 2191-2200, 2003.

DENARDI C, AUSONI S, MORETTI P, GORZA L, VELLECA M, BUCKINGHAM M, SCHIAFFINO S: Type 2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene. *J Muscle Res Cell Motil* **10:** 197-205, 1993.

HOUŠTĚK J, TVRDÍK P, PAVELKA S, BAUDYŠOVÁ M: Low content of mitochondrial ATPase in brown adipose tissue is the result of post-transcriptional regulation. *FEBS Lett* **294**: 191-194, 1991.

HUDECOVÁ S, VASDÁSZOVÁ A, SOUKUP T, KRIŽANOVÁ O: Effect of thyroid hormones on the gene expression of calcium transport systems in rat muscle. *Life Sci* **75**: 923-931, 2004.

IZUMO S, NADAL-GINARD B, MAHDAVI V: All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science* **231:** 597-600, 1986.

JASCHINSKI F, SCHULER M, PEUKER H, PETTE D: Changes in myosin heavy chain mRNA and protein isoforms of rat muscle during forced contractile activity. *Am J Physiol* **274:** C365-370, 1998.

PETTE D: The adaptive potential of skeletal muscle fibers. *Can J Appl Physiol* **27**: 423-448, 2002.

SCHIAFFINO S, GORZA L, AUSONI S, BOTTINELLI R, REGGIANI C, LARSSON L, EDSTROM L, GUNDERSEN K, LØMO T: Muscle fiber types expressing different myosin heavy chain isoforms: their functional properties and adaptive capacity. In: *The Dynamic State of Muscle Fibers*. D. Pette (ed.), Walter de Gruyter, New York, 1990, pp 329-341.

SMERDU V, KARSCH-MIZRACHI I, CAMPIONE M, LEINWAND L, SCHIAFFINO S: Type IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal muscle. *Am J Physiol* **267:** C1723-C1728, 1994.

SOUKUP T, ZACHAŘOVÁ G, SMERDU V: Fibre type composition of soleus and extensor digitorum longus muscles in normal female inbred Lewis rats. *Acta Histochem* **104**: 399-405, 2002.

STEVENS L, GOHLSCH B, MOUNIER Y, PETTE D: Changes in myosin heavy chain mRNA and protein isoforms in single fibers of unloaded rat soleus muscle. *FEBS Letts* **463**: 15-18, 1999.

VADÁSZOVÁ A: *Influence of Thyroid Hormones on MyHC Isoform Expression in Skeletal Rat Muscles*. Ph.D. Thesis. Institute of Physiology AS CR and Faculty of Science, Charles University, Prague, 2005.

VADÁSZOVÁ A, ZACHAŘOVÁ G, MACHÁČOVÁ K, JIRMANOVÁ I, SOUKUP T: Influence of thyroid status on the differentiation of slow and fast rat muscle phenotypes. *Physiol Res* **53** (Suppl 1): S57-S61, 2004a.

VADÁSZOVÁ A, ZACHAŘOVÁ G, ASMUSSEN G, HUDECOVÁ S, KRIŽANOVÁ O, SOUKUP T: Thyroid hormones regulate differently muscle MHC isoforms and their mRNA levels. *Physiol Res* **53**: 42P, 2004b.

WRIGHT C, HADDAD F, QIN AX, BALDWIN KM: Analysis of myosin heavy chain mRNA expression by RT-PCR. *J Appl Physiol* **83**: 1389-1396, 1997.

ZACHAŘOVÁ G, VADÁSZOVÁ A, SMERDU V, ASMUSSEN G, SOUKUP T: The effect of a unilateral muscle transplantation on the muscle fiber type and the MyHC isoform content in unoperated hind limb slow and fast muscles of the inbred Lewis rats. *Physiol Res* **54**, 691-696, 2005.

Reprint requests

T. Soukup, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague, Czech Republic, Fax: +420 24106 2488, E-mail: tsoukup@biomed.cas.cz