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Comparison of Myosin Heavy Chain mRNAs, Protein Isoforms and Fiber Type Proportions in the Rat Slow and Fast Muscles

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Summary

We studied the expression of myosin heavy chain isoforms at *mRNA* and protein levels as well as fiber type composition in the fast extensor digitorum longus (EDL) and slow soleus (SOL) twitch muscles of adult inbred Lewis strain rats. Comparison of the results from Real Time RT-PCR, SDS-PAGE and fiber type analysis showed corresponding proportions of *MyHC* transcripts (*MyHC-1, -2a, -2x/d, -2b*), protein isoforms (MyHC-1, -2a, -2x/d, -2b) and fiber types (type 1, 2A, 2X/D, 2B) in both muscles. Furthermore, we found that slow *MyHC-1* mRNA expression in the SOL was up to three orders higher than that of fast *MyHC* transcripts. This finding can explain the predominance of MyHC-1 isoform and fiber type 1 and the absence of pure 2X/D and 2B fibers in the SOL muscle. Based on our data presenting quantitative evidence of corresponding proportions between *mRNA* level, protein content and fiber type composition, we suggest that the Real Time RT-PCR technique can be used as a routine method for analysis of muscle composition changes and could be advantageous for the analysis of scant biological samples such as muscle biopsies in humans.

Key words

Rat • Soleus • Extensor digitorum longus • Myosin heavy chain isoforms • Muscle gene expression • Real Time RT-PCR • SDS-PAGE • Fiber type analysis

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Introduction

It is generally accepted that skeletal muscle fiber types are defined by myosin heavy chain (MyHC) isoform content, which is dependent on the level of expression of the specific mRNAs. Both fiber type composition, MyHC isoform content and mRNA expression can be determined by appropriate (semi) quantitative methods. The fiber type composition can be estimated e.g. by stereological analysis (Zacharova and Kubinova 1995), MyHC isoform content by SDS-PAGE (Talmage and Roy 1993), though some attempts to employ other approaches have been published (Říčný and Soukup 2011). *MyHC* transcript level determination was performed by *in situ* hybridization or by various modifications of RT-PCR (DeNardi *et al.* 1993, Esser *et al.* 1993, Lieber *et al*. 1993, Smerdu *et al.* 1994, Peuker and Pette 1995, 1997, Jänkälä *et al*. 1997, Wright *et al*. 1997, Jaschinski *et al*. 1998, Jung *et al*. 1998, Stevens *et al*. 1999a,b, Weiss *et al.* 1999, Huey *et al.* 2001, Serrano *et al*. 2001, Smerdu and Eržen 2001, Caiozzo *et al.* 2003, Eizema *et al.* 2003, Sakuraba *et al*. 2005, Vadászová *et al*. 2006a,b, Vadászová-Soukup and Soukup 2007, Smerdu and Soukup 2008, for review see Pette *et al.* 1999) until Real Time RT-PCR (qRT-PCR) became generally accepted (for review see Pfaffl 2004) and routinely used for MyHC analyses in rats (Pattison *et al.* 2003, Zurmanova *et al*. 2008, Clause *et al.* 2012). It was found that the extensor digitorum longus (EDL) in Lewis rats, as well as in other strains (Novák *et al.* 2010), contains four fiber types (slow type 1, fast 2A, 2X/D and 2B fibers). The fiber types are related to four MyHC isoforms (MyHC-1, -2a, -2x/d and -2b) and four mRNA

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transcripts (*MyHC-1, -2a, -2x/d* and *-2b*) encoded by specific genes (for review see Schiaffino and Reggianni 1996). The soleus (SOL) muscle also expresses four mRNAs isoforms, however, only the two MyHC-1 and -2a protein isoforms, as well as type 1 and 2A (2C) fibers are usually detected under physiological conditions (Soukup *et al.* 2002, 2009, Zacharova *et al.* 2005, Vadászová *et al*. 2006a,b, Vadászová-Soukup and Soukup 2007, Zurmanova *et al*. 2007, Smerdu and Soukup 2008, Novák and Soukup 2011, Soukup *et al.* 2012, for review see Pette and Staron 1990, Schiaffino and Reggianni 1994, Soukup and Jirmanová 2000, Schiaffino 2010).

The main purpose of the present study was to compare the relative proportions of *MyHC* transcripts and translated MyHC isoform protein levels and fiber type composition using qRT-PCR, SDS-PAGE and stereological fiber type analysis of immunochemically stained muscle cross sections in the slow SOL and fast EDL muscles of adult Lewis rats. The second aim was to determine the quantitative differences between slow *MyHC-1* and fast *MyHC-2a, -2x/d* and *-2b* transcripts in an attempt to explain the predominance of MyHC-1 isoform and type 1 fibers in the SOL muscle in adult Lewis rats.

Materials and Methods

Animals

Experiments were performed on 12 adult (13.8 to 16.8 months old) female inbred Lewis strain rats obtained from the authorized laboratory rat-breeding unit of the Institute of Physiology, Academy of Science of the Czech Republic, v. v. i., Prague, (Accreditation No. 1020/491/A/00). They were housed at 23±1 °C and at 12-hour light-dark cycle periods with *ad libitum* access to water and a complete laboratory diet. The maintenance and handling of experimental animals were in accordance with the EU Council Directive (86/609EEC) and the investigation was approved by the Expert Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic, v. v. i., Prague. The animals were anesthetized with intraperitoneal injections of 1 ml (100 mg) of Narketan (Ketaminum ut hydrochloridum) per 1000 g of body weight, followed by 0.5 ml (10 mg) of the myorelaxant Xylapan (Xylazinum ut hydrochloridum) per 1000 g of body weight (Vetoquinol S.A. France and Vetoquinol Biowet Poland, respectively) and sacrificed by an overdose of the anesthetic. Both EDL and SOL muscles were excised from left and right hind limbs, the left muscles were immediately frozen in liquid nitrogen until used for quantitative RT-PCR, the right muscles were frozen as well and the middle portion was used immediately for preparation of cross cryosections followed by myofibrillar adenosine-triphosphatase (mATPase) reaction and immuno-staining, the other portions were stored at –80 °C until used for SDS-PAGE analysis.

Quantitative Real Time RT-PCR

The primers against four *MyHC* isoform transcripts (described in detail in Zurmanova *et al.* 2008) were designed using the Gene Runner program (Hastings Software).

RNA isolation and RT-PCR

Total cellular RNA was extracted from each muscle sample using the TRIZOL Reagent (Invitrogen). The purity and integrity of the RNA preparations was checked spectroscopically and by agarose gel electrophoresis. One µg of total RNA was converted to cDNA using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) using oligo (dT) primers according to the manufacturer's instructions. Samples of cDNA (1 µl) were amplified in 25 µl of PCR reaction mixture containing iQ^{TM} SYBR Green Supermix (Bio-Rad) plus 1 µM of each MyHC isoform-specific primers according to the manufacturer's instructions.

PCRs were performed on a Light Cycler (Roche Ltd.) as described previously (Waskova-Arnostova *et al.* 2013) using the following temperature profile: initial denaturation at 95 °C for 2 min, followed by 38 cycles consisting of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and elongation at 72 °C for 20 s. Fluorescence was acquired in each cycle after heating the samples to 80 °C eliminating the noise of primer dimers (Pfaffl 2001). At the end of each run, melting curve analysis was performed to ascertain the presence of a single amplicon. The data used for calculation were the mean of crossing point (Cp) values obtained from qPCR performed in triplicates. We verified that the variation of triplicates did not exceed 0.5 Cp. Standard curves were generated for each pair of primers using 3-fold serial dilution of cDNA. The efficiency of the PCR amplification for each primer pair was then calculated from the standard curve to state precisely the relative expression. The level of analyzed transcripts was normalized to the level of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts with regard to the specific PCR efficiency (E) for each gene as follows:

Normalized amount = $(1+E)^{Cp\ reference\ transcript}/(1+E)^{Cp\ target\ transcript}$

Non-template and non-RT reactions were performed as controls. The quantitative data are the means of four measurements and these are presented as the ratio of values for individual mRNAs.

Immunocytochemistry

Muscle fiber types were determined on fresh frozen muscle cross sections 10 μm thick incubated with different mouse monoclonal antibodies (mAbs) specific for rat MyHC isoforms BA-D5 (MyHC-1), SC-71 (MyHC-2a), F-35 (all MyHC except -2x/d) and BF-F3 (MyHC-2b) (cf. Schiaffino *et al.* 1986, Developmental Studies Hybridoma Bank). Additionally, mAbs anti Slow (MyHC-1) and anti-Fast (MyHC-2), both provided by Biotrend or Medac/Novocastra, were used to further distinguish slow and fast MyHC isoforms. Primary antibody binding was revealed using donkey secondary antibody conjugated with HRP (Jackson Immunoresearch Laboratories, USA). (For a detailed description see Smerdu and Soukup 2008, Soukup *et al.* 2009).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, the muscle samples (approximately 30 mg) were thoroughly cut using scissors in 5 volumes of ice-cold washing buffer (5 mM Na-phosphate pH 7.0, 20 mM NaCl, 1 mM EGTA), centrifuged 5 min/12000 x g. The pellet was re-suspended in 3 volumes of extraction buffer (100 mM pyrophosphate pH 8.4, 5 mM EGTA, 1 mM dithiothreitol), extracted for 30 min on ice by shaking and centrifuged 5 min/12000 x g. The supernatant was diluted 1:4 with the sample buffer (125 mM TRIS-HCl pH 6.8, 1 mM EDTA, 5 % SDS, 5% mercaptoethanol, 0.1 % bromophenol blue, 20 % glycerol) and 5 μl of the sample (1 μg of protein per well) were loaded onto the gel. MyHC isoforms were separated by SDS-PAGE (Talmadge and Roy 1993) using MiniPROTEAN3 Cell (Bio-Rad Ltd.) at a constant voltage (100 V) for 18-19 h at 4 °C. After MyHC isoform separation, the gels were either silver-stained (Blum *et al*. 1987) or stained by Coomassie Brilliant Blue and Bismarck Brown R (Choi *et al.* 1996). The individual MyHC isoforms were densitometrically evaluated at two gels from each sample using the imaging system (Fujilab, Japan) and the AIDA 3.28 computer program (Advanced Image Data Analyser, Germany) (for further details see Říčný and Soukup 2011).

Quantitative morphological analysis

The numerical (N) proportions (%) of muscle fiber types were assessed by 2-D stereological methods using the principles of an unbiased counting frame and point counting (Zacharova and Kubinova 1995). The stereological measurements were performed using the C.A.S.T. Grid System (Olympus, Albertslund, Denmark). In order to achieve a realistic estimate of the measured parameters, the concrete arrangement of the stereological system (number of points, size of the counting frame, scanning interval) was selected according to muscle section size and fiber composition on the basis of efficacy analysis described in our previous papers (Zacharova and Kubinova 1995, Zacharova *et al.* 1997, 1999, 2005).

Statistical analysis

The data were expressed as mean ± SD. Differences between transcript and protein levels and fiber type composition were evaluated using the Kruskal-Wallis One Way Analysis of Variance on Ranks followed by all pairwise multiple comparison procedures (Dunn´s method), *mRNA* difference between EDL and SOL muscles (Fig. 3) by Mann-Whitney Rank Sum Test (SigmaStat program, Systat Software, Germany).

Results

MyHC mRNAs analysis by qRT-PCR

Our measurements revealed that the analyzed EDL and SOL muscles of adult Lewis rats expressed all four *MyHC-1, -2a, -2x/d* and *-2b* transcripts (Figs 1-3). The highest expression levels in the EDL were demonstrated for fast *MyHC-2b* followed by *MyHC-2x/d* isoform transcripts, which together formed more than 90 % of all *MyHC* transcripts and exceeded the level of the slower *MyHC-2a* isoform transcript almost ten times and that of *MyHC-1* nearly 30 times (Figs 1 and 3). On the other hand, in the SOL muscle, although all four *MyHC* RNAs were detected, the major *MyHC-1* transcript was two to three orders higher than the fast isoforms and it formed more than 99 % of all *MyHC* isoform RNAs (Figs 2 and 3). Differences in the level of individual transcripts between both muscles were highly significant (Fig. 3).

Fig. 1.

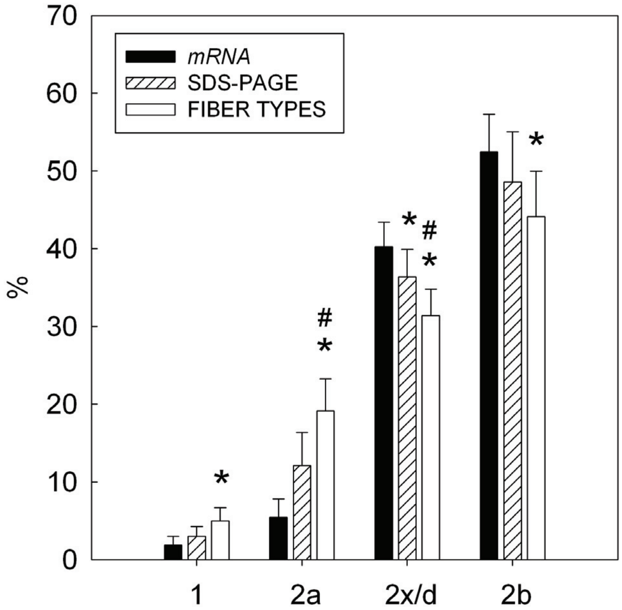
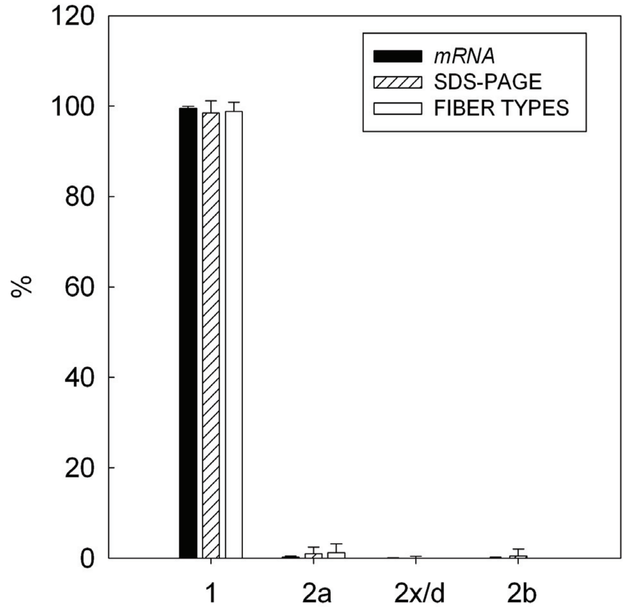


Fig. 2.



Figs 1. and 2. Relative values (expressed as percentage) of slow *MyHC-1* and fast *MyHC-2a, -2x/d* and *-2b* mRNAs as determined by qRT-PCR (dark columns), of MyHC-1, -2a, -2x/d and -2b isoform content as determined by SDS-PAGE (hatched columns) and fiber type proportions of type 1, 2A, 2X/D and 2B as determined by immunocytochemistry and quantitative fiber type analysis (blank columns) in the extensor digitorum longus (**Fig. 1.**) and soleus (**Fig. 2.**) muscles of adult female Lewis strain rats. Note the similarity of proportions between mRNA level, MyHC isoform content and fiber type percentage in case of each isoform. Statistics was performed using the Kruskal-Wallis One Way Analysis of Variance on Ranks. * indicates significant difference ($p \leq 0.05$) against *MyHC* transcript; # indicates significant difference ($p \leq 0.05$) against SDS-PAGE.

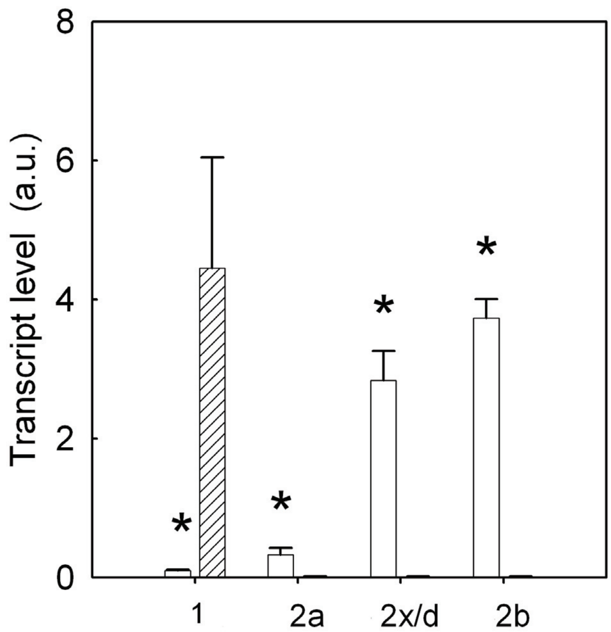


Fig. 3. Expression of slow *MyHC-1* and fast *MyHC-2a, -2x/d* and *-2b* mRNAs in the extensor digitorum longus (EDL, blank columns) and the soleus (SOL, hatched columns) muscles of adult female inbred Lewis strain rats. Results are expressed in arbitrary units (a.u.). Note the great difference between the transcript levels in the SOL compared to the differences present in the EDL muscle. Statistics was performed using Mann-Whitney Rank Sum Test. Significant difference between same *MyHC* transcripts in the EDL against SOL: * $p \leq 0.01$.

MyHC protein analysis by SDS-PAGE

The SDS-PAGE technique also confirmed the presence of all four MyHC-1, -2a, -2x/d and -2b protein isoforms, but consistently only in the EDL muscles. A major proportion consisted of the fastest MyHC-2b and -2x/d isoforms forming about 85 % of all MyHC protein, followed by the -2a isoform. The three fast isoforms thus formed 97 % of MyHC content, the rest consisting of the slow MyHC-1 isoform (Fig. 1). In contrast to EDL, SOL muscle contained a dominant 98.5 % proportion of MyHC-1 protein isoform supplemented by MyHC-2a (Fig. 2). MyHC-2x/d and -2b isoforms were absent in all but one analyzed muscle.

Fiber type immunohistochemical analysis

In the EDL muscles, we confirmed using quantitative fiber type analysis the presence of all four fiber types, namely slow type 1, fast 2A, 2X/D and 2B. The fastest 2B and 2X/D fibers formed more than 75 % and together with 2A fibers, they represented up to 95 % of all fibers (Fig. 1). In the SOL muscle, quantitative fiber type composition analysis determined by immunocytochemistry demonstrated a dominant presence of slow type 1 fibers stained with BA-D5 mAb specifically recognizing slow MyHC-1 isoform supplemented by about 1 % of fast 2A fibers stained by SC-71 mAb specifically recognizing fast MyHC-2a isoform. All fibers in the SOL muscle were stained by BF-35 mAb, recognizing all MyHC isoforms except MyHC-2x/d, which showed the absence of pure fast 2X/D fibers. Similarly, there were no 2B fibers stained by BF-F3 mAb specifically recognizing this fiber type. In both muscles transitional hybrid type 2C (1C) fibers were found, which stained to a variable extent by anti-slow and anti-fast mAbs. Figures of histochemical and immuno-cytochemical reactions were presented earlier in our papers (e.g. Soukup *et al.* 2002, 2009, 2012, Novák *et al*. 2010).

Discussion

Comparison of MyHC mRNA, protein level and fiber type

Our results confirmed the presence of similar proportions of *MyHC* mRNA transcripts, protein isoforms and fiber types of all four MyHC-1, -2a, -2x/d and -2b isoforms in the EDL and SOL muscles. Furthermore, in the SOL muscle, both fastest MyHC isoforms at the transcript and protein levels were markedly less represented suggesting an explanation for the absence of 2X/D and 2B fibers. In the EDL muscle, the highest levels of *MyHC-2b* and *-2x/d* mRNA were about one order higher than the level of the minor *MyHC-1* and even less than one order when compared to *MyHC-2a* mRNA. The lowest amount of the *MyHC-1* transcript corresponding to a minor representation of the slow MyHC-1 protein isoform and of the slow type 1 fibers in the EDL, as well as the dominant presence of the slow type 1 transcript, isoform and fiber type in the SOL muscle, are in agreement with the results of previous studies in Lewis rats (Soukup *et al.* 2002, 2009, Zacharova *et al.* 2005, Smerdu and Soukup 2008, Novák *et al.* 2010, Novák and Soukup 2011).

In the EDL, the amount of *MyHC* mRNAs increased in the same order as did the content of MyHC protein isoforms and the percentage of fiber types, i.e. type 1 < 2a < 2x/d < 2b (cf. Fig. 1). Interestingly, the 2A fibers in the EDL were present in a higher percentage than would correspond to the *MyHC-2a* transcript level and MyHC-2a isoform content. Our stereological evaluation suggested that about 5 to 10 % of fibers considered to be 2A fibers according to the reaction with SC-71 mAb were in fact 2C/1C fibers. The same can be true for the type 1 fibers, as some of these could be detected by BA-D5 mAb, although they would rather belong to the 1C/2C fibers. If so, then in the EDL, the percentage of 2A and type 1 fibers would be lower and that of 2X/D and 2B higher than shown in Figure1. In such case, the significant difference found between fiber type and MyHC isoform or transcript percentage might be lower or disappear. This would further support our suggestion that there is close correlation among transcript, protein and fiber type levels. The question of correct determination of fiber type percentage can be further raised by the difference in the avidity of mAbs used for the detection of MyHC isoforms. Both mAbs BA-D5 (anti slow MyHC-1) and SC-71 (anti fast MyHC-2a) can be used highly diluted (1:200), while BF-35 (anti MyHC-2x/d) and BF-F3 (anti MyHC-2b) usually have to be used undiluted. This could especially effect the determination of 2X/D fibers, because BF-35 mAb is a negative marker of 2X/D fibers. This means that even a small amount of MyHC-1 or -2a recognized by high affinity BAD-5 and SC-71 mAbs can mask the possible existence of 2X/D fibers. This problem can be partially overcome by using the new 6H1 mAb specifically recognizing the MyHC-2x/d isoform.

Absence of MyHC-2x/d and -2b isoforms and 2X/D and 2B fibers in the SOL muscle can be explained by the extremely low *MyHC-2x/d* and *-2b* transcript levels. The absence of 2X/D and 2B fibers in the SOL is supported by stereological analysis of mean fiber diameters (fiber areas), which revealed that all fibers in the SOL muscles have similar diameters in contrast to the EDL, where type 2B and 2X/D fibers always exhibit significantly greater diameters compared to 2A and type 1 fibers (unpublished data from Cast Grid analysis).

Mechanisms of regulations of MyHC genes transcription

It is generally supposed that the *MyHC* mRNA level defines the amount of subsequently synthesized protein (Pette and Staron 1990, Schiaffino and Reggiani 1996, Pette *et al.* 1999, Pette 2002, Caiozzo *et al.* 2003, Schiaffino 2010). Many of the dynamically regulated cell processes (e. g. mRNA stability, formation of splicing variants, regulation of translation or protein stability) may result in a lower correlation between mRNA expression and protein level. Evidently, fiber type composition can flexibly react to physiological demands within the given genetic range (Eržen *et al.* 1996, Snoj-Cvetko *et al.* 1996a,b) and, as already concluded, the extent of each MyHC isoform expression is determined by the level of their mRNA. The molecular control of muscle diversity and plasticity have been studied for a long time and it seems that the neural input generated *via* the nerve impulse pattern plays an important, if not decisive, role (for review see e.g. Pette and Staron 1993, Schiaffino and Reggiani 1994, 1996, Buonanno and Rosenthal 1996, Pette *et al.* 1999, Soukup and Jirmanová 2000, Pette 2002, Asmussen *et al.* 2003, Schiaffino 2010). However, the way of neural input conversion into gene expression is still a matter of speculation (for review see Buonanno and Fields 1999, Pette 2002, Schiaffino 2010). One of the most promising suggestions attempting to explain the effect of neural input on gene expression relies on the serine/threonine phosphatase called calcineurin. If the Ca^{2+} level in the sarcoplasm remains high due to tonic excitation (characteristic e. g. of the anti-gravity function of the SOL muscle), calcineurin is activated and can dephosphorylate the nuclear factor of activated thymocytes (NFAT) in the case of “slow muscle genes”, which afterwards enters the nucleus and associates with MEF2 (belonging to the family of myocyte enhancing factors). These can then bind together to the slow *MyHC-1* isoform promotor and thus induce expression of *MyHC-1* mRNA. In fast muscles, like EDL, the neural impulse pattern is characterized by irregular short bursts of activity resulting in fluctuation of Ca^{2+} concentration that is not high enough to activate calcineurin and start the whole cascade of events described above. On the other hand, it was suggested that calcineurin has only a modulatory role, rather than being a primary regulator of slow *MyHC* gene expression (Pandorf *et al.* 2009). It remains to be also established whether maturation of fast fibers is accompanied by epigenetic modifications at the *MyHC-1* gene locus that make this gene essentially inaccessible for transcription or whether gene expression is also controlled at the post-transcriptional level (Schiaffino 2010).

Conclusions

Using three different methods, we demonstrate the existence of corresponding proportions of MyHC at the level of mRNA transcripts (*MyHC-1, -2a, -2x/d, -2b*), protein isoforms (MyHC-1, -2a, -2x/d, -2b) and fiber types (type 1, 2A, 2X/D, 2B). Markedly low expression of *MyHC-2x/d* and *MyHC-2b* mRNAs in comparison with *MyHC-1* mRNA in the SOL muscle clearly shows why MyHC-2x/d and MyHC-2b protein isoforms are usually not present at amounts detectable by silver staining of electrophoretic gels and why there are no 2X/D and 2B fibers detected on muscle sections by immunocytochemical staining. As already suggested long time ago for RT-PCR (Wright *et al.* 1997), we conclude that Real Time RT-PCR can be used as a routine method wherever analysis of MyHC and/or muscle composition changes is needed. If the same correlation is proven in humans, Real Time RT-PCR could be used in the case of muscle biopsies, as it requires much smaller samples compared to the other two methods.

Conflict of Interest

There is no conflict of interest.

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