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Comparison of New ELISA Method With Established SDS-PAGE Method for Determination of Muscle Myosin Heavy Chain Isoforms

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Summary

We developed a new method for the quantitative determination of myosin heavy chain (MyHC) isoforms taking advantage of immunochemical differences and based on the ELISA principle. In the present paper we compare analysis of MyHC isoforms using the SDS-PAGE and the ELISA methods in the same samples of adult female inbred Lewis strain euthyroid, hyperthyroid and hypothyroid rats. In all thyroid states, the same composition and corresponding changes of MyHC isoforms were determined using both methodological approaches in the slow soleus and the fast extensor digitorum longus muscles. Our results showed that ELISA can be used for a "semi-quantitative" or "comparative" measurement of MyHC isoforms in multiple muscle samples, but that it is neither more exact nor faster compared to SDS-PAGE.

Key words

Muscle fiber types • Myosin heavy chains • SDS-PAGE • Immunoreactions-ELISA

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Introduction

Mammalian skeletal muscles contain 4 genes called MYH1, 2, 4 and 7 responsible for expression of three fast myosin heavy chain (MyHC) isoforms 2x/d, 2b, 2a and one slow 1 isoform, respectively (Rat Gene Database: http://rgd.mcw.edu/, Yoon *et al*. 1992, Schiaffino and Reggiani 1996, Weiss *et al*. 1999a, b). The fact that fast (and developmental) MyHC isoform genes cluster on a single chromosome (Remmers *et al*. 1992, Yoon *et al.* 1992) suggests that their protein isoforms share a great similarity of primary structure, which hampers their separation and quantification. At present, there are two frequently used ways for identification, separation and quantification of MyHC isoform content in muscle fiber types. The first method is the histochemical or immunocytochemical determination of fiber types with known content of MyHC isoform on muscle cross section. The percentage of each fiber type can be assessed e.g. by a 2-D stereological method using the principles of an unbiased counting frame and point counting (Zachařová and Kubínová 1995). This method provides information about the number of individual fibers expressing a given MyHC isoform or exhibiting a given mATPase activity (Zachařová *et al.* 1997, 1999, 2005, Soukup and Jirmanová 2000, Soukup *et al.* 2009, Novák *et al*. 2010, Novák and Soukup 2011). The second method is sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which on the other hand provides information about total MyHC isoform content in the whole muscle and individual MyHC isoforms can be determined by subsequent Western blotting (Zachařová *et al.* 2005, Smerdu and Soukup 2008, Soukup *et al.* 2009). However, the individual MyHC isoforms separated by SDS-PAGE, and especially separation of 2a and 2x/d bands, is often not complete and therefore the quantitative analysis may be affected by the subjective determination of borders among individual isoforms. We have therefore attempted to develop a new method based on the ELISA principle for quantitative determination of MyHC isoforms benefiting from

immunochemical differences among myosin isoforms. In the present paper, we compare SDS-PAGE and ELISA methods on the same muscle sample in rats with altered thyroid status.

Materials and Methods

Animals

Eighteen 4-week-old female inbred Lewis strain rats were obtained from the authorized laboratory rat-breeding unit of the Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Prague, (Accreditation No. 1020/491/A/00). 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. After experimental treatment, the adult 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. The soleus (SOL) and extensor digitorum longus (EDL) muscles were excised and frozen in liquid nitrogen. Frozen muscles were kept at –80 °C until further processing.

Alteration of thyroid status

The hyperthyroid status (TH) was induced in 4-week-old animals and maintained during the experiment by intraperitoneal injections of 3,3',5-triiodo-L-thyronine (sodium salt, T3, 0.15 μg/kg body weight) three times a week. The hypothyroid (HY) status was induced in 4-week-old animals and maintained during the experiment with a 0.05 % solution of methimazole (2-mercapto-1-methylimidazole) in drinking water. The euthyroid (EU) rats were age-matched littermates of the TH and HY animals.

SDS-PAGE

For SDS-PAGE the muscle samples (approximately 30 mg) were thoroughly cut by 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/12 000 x g. The pellet was resuspended in 3 volumes of extraction buffer (100 mM pyrophosphate pH 8.4, 5mM EGTA, 1 mM dithiothreitol), extracted for 30 min on ice by shaking and centrifuged 5 min/12 000 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 PROTEAN MINI GEL III, BIORAD, at a constant voltage (100 V) for 18-19 hrs at 4 °C. After the MyHC isoform separation, the gels were either silver-stained (Blum *et al.* 1987) or stained by Coomassie Brilliant Blue and Bismarck Brown R (CBB&BBR) (Choi *et al.* 1996). The individual MyHC isoforms were densitometrically evaluated using the LAS-1000 imaging system (Fujilab, Japan) and the AIDA 3.28 computer program (Advanced Image Data Analyser, Germany) at two gels from each sample.

Myosin preparation and ELISA procedure

Crude myosin extract from SOL and EDL muscles was prepared by Ultra-Turrax homogenization of muscle in 50 vol (w/v) of homogenization buffer (25 mM K-phosphate pH 7.6, 100 mM KCl, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 10 mM ATP) followed by 30 min centrifugation at 2 000 x g. The supernatant was discarded and the pellet was extracted for 30 min in 10 vol (orig. tissue w/v) of extraction buffer (150 mM K-phosphate pH 6.8, 300 mM KCl, 2 mM EDTA, 2 mM DTT, 10 mM ATP). After centrifugation (15 min at 10 000 x g) 1 volume of 50 % glycerol,150 mM K-phosphate pH 6.8, 300 mM KCl was added to the 3 volumes of supernatant and the crude myosin extract was stored frozen at –80 °C. The crude myosin extract was found to be adequate for direct coating of microplates. We compared this with purified myosin preparations (approx. 95 % purity) and found no appreciable differences.

Polystyrene microtitration plates (96 wells, flat-bottom, NUNC MaxiSorp) were coated with appropriate muscle crude myosin extracts (10 μg protein/ml; 100 mM Na-carbonate pH 9.5; 0.1 ml/well) for 3 h/RT; the remaining binding was quenched by incubation with 0.3 ml/well of 10 mg BSA/100 mM Na-carbonate pH 9.5 for 2 h/RT. Three rows of wells were usually coated with the extract from selected muscles. After washing (3 x 0.3 ml/well of wash buffer: 10 mM K-phosphate pH 7.2, 100 mM KCl, 0.05 % Tween 20), 0.1 ml/well of an appropriately diluted selected primary antibody was

added and incubated for 1 h/RT and at 4-6 °C overnight. The amount of total adsorbed fast myosin was measured by anti fast MyHC antibody for the EDL and the amount of total adsorbed slow myosin by anti slow MyHC antibody for the SOL muscle (both provided by Biotrend or Medac/Novocastra, Germany). The content of specific isoforms was measured with isoform-specific monoclonal antibodies BA-D5 (MyHC-1), SC-71 (MyHC-2a) and BF-F3 (MyHC-2b) (Schiaffino *et al.* 1989, Smerdu and Soukup 2008). Triplicates were routinely used with a given antibody. Antibodies were used at the following dilutions: SC-71 1:10; BF-F3 1:20; BA-D5 1:60-600; aF MyHC-fast 1:10 000-25 000; aS MyHC-slow 1:10 000-25 000, giving the absorbance signal between 0.5-1.5 absorbance units (AU). The plate was washed again (4x wash buffer) and incubated with HRP-labeled secondary antibody (0.3 ml/well 12 000x diluted donkey AffiniPure antimouse IgG-HRP, Jackson ImmunoResearch Lab.) for 2 h/RT. After washing (5x washing buffer), 0.2 ml/well of substrate solution (0.1 M Na-acetate pH 5.5, 0.1 mg/ml tetramethylbenzidine and 0.003 % H_2O_2) was added and the reaction was stopped after the appropriate incubation time (15-45 min/RT) by pipeting 0.05 ml of 0.5 M H_2SO_4. The absorbance signal generated by labeled secondary antibody was measured using a microplate reader (Victor 1420 Multilabel Counter, Wallac) at 450 nm. Because we could not utilize individual protein MyHC isoforms as standards, our data from control and experimental animals are expressed as relative values. The signals obtained by subtype-specific antibodies were corrected accordingly and effects of TH and HY states were compared to control EU animals. The relationship between the amount of adsorbed protein and the obtained signal is not strictly linear, but as the differences between our samples were relatively small, we believe that such correction is valid.

Results

In the great majority of the analyzed SOL muscles in all three thyroid states, SDS-PAGE revealed only MyHC-1 and -2a isoforms (Fig. 1 three left lines), although in few gels it also revealed traces (1 to 6 %) of MyHC-2x/d and/or of -2b isoforms as well. In the EDL muscles, four MyHC isoforms MyHC-1, -2a, -2x/d and -2b were demonstrated by the SDS-PAGE technique in all three thyroid statuses and in all muscle samples with typical quantitative changes in their proportions in different thyroid statuses (Fig. 1 three right lines).

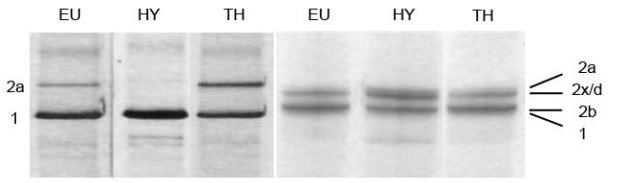

Fig. 1. Example of the silver stained SDS-PAGE gel of myosin heavy chain (MyHC) isoforms in slow-twitch soleus (three left lines) and fast-twitch extensor digitorum longus (three right lines) muscles from euthyroid (EU), hypothyroid (HY) and hyperthyroid (TH) adult female rats. The MyHC-1, -2a, -2x/d and -2b isoform bands are labeled. Note that in the SOL muscle, only MyHC-1 and -2a isoforms are demonstrated, while in the EDL muscle all four isoforms could be recognized.

Using ELISA, we confirmed the dominant content of the MyHC-1 isoform supplemented by a low and variable amount of MyHC-2a for the SOL muscle. Furthermore, comparison of rats with altered thyroid status showed that the MyHC-2a content in the SOL muscle was substantially increased in TH, while it was further decreased in HY status (Fig. 2). In the EDL muscle, the MyHC-1 isoform was slightly decreased in TH and substantially increased in HY status, the MyHC-2a isoform was increased in both experimental statuses, while the MyHC-2b isoform was enhanced in TH status and decreased in HY status (Fig. 3).

Discussion

We developed a new method for the quantitative determination of MyHC isoforms based on the ELISA principle. We found that the SDS-PAGE and ELISA methods performed on the same samples of SOL and EDL muscles of adult female inbred Lewis strain euthyroid, hyperthyroid and hypothyroid rats reveal the same composition and corresponding changes of MyHC isoforms. Our results thus show that ELISA can be used for a “semi-quantitative” or “comparative” measurement of MyHC isoforms in multiple muscle samples.

Quantitative information about MyHC content changes is necessary in many experimental situations. We have successfully used both a 2-D stereological method that analyzes fiber type composition in serial cross sections, as well as SDS-PAGE that reveals proportions of MyHC isoforms in muscle homogenates or extracts (Zachařová *et al.* 1997, 1999, 2005, Smerdu and Soukup 2008, Soukup *et al.* 2009, Novák *et al*. 2010, Novák and Soukup 2011). However, the existence of hybrid fibers with mixed reactions often hampered the exactness of

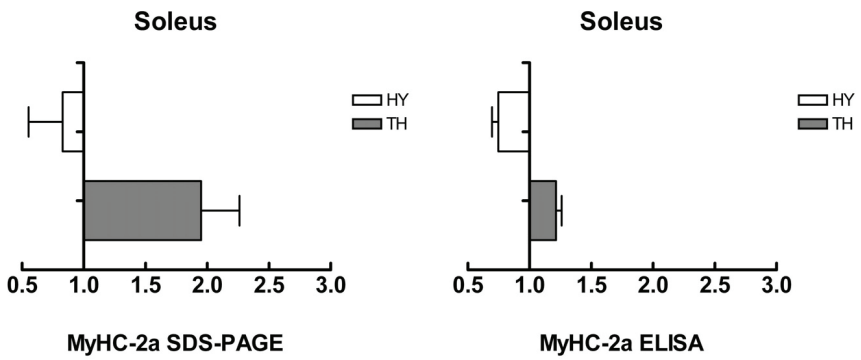

Fig. 2. Relative changes of MyHC-2a isoform in the soleus muscle as measured by SDS-PAGE (left; densitometry) or ELISA (right; reaction with SC-71 antibody). Levels of isoforms in hypothyroid (HY) and hyperthyroid (TH) rats are presented as their relation to EU levels, which was set as 1.

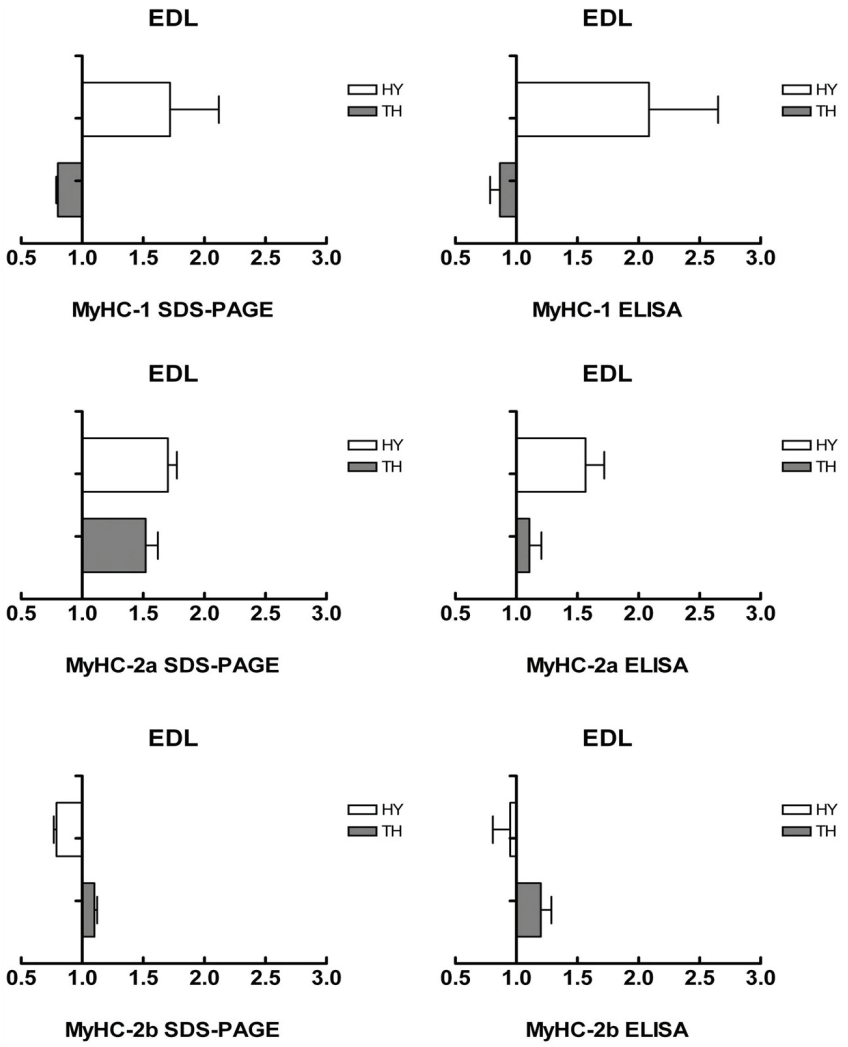

Fig. 3. Relative changes of MyHC isoforms in the extensor digitorum longus (EDL) muscle as measured by SDS-PAGE (left; densito-metry) or ELISA (right; reaction with appropriate antibodies: BA-D5 for MyHC-1; SC-71 for MyHC-2a or BF-F3 for MyHC-2b). Levels of isoforms in hypothyroid (HY) and hyperthyroid (TH) rats are presented as their relation to EU levels, which was set as 1.

fiber type evaluation and imperfect isoform separation deteriorated quantitative evaluation using SDS-PAGE. We have therefore tried to adopt an immunochemical approach similar to ELISA.

Myosin is by far the most prevailing protein in muscle tissue. Therefore, we expected that it would be possible to use muscle extracts as coating solutions for ELISA without extensive prepurification and, after allowing them to react with a battery of myosin-subtype specific antibodies, to obtain quantitative data representing proportions of individual MyHC isoforms. Our results at this stage must be taken as semi-quantitative and further refinement is necessary to reach a higher degree of accuracy. Specificity of the antibodies used in these experiments probably plays a crucial role. Experience with immunohistochemically stained muscle

fresh frozen sections has shown a high degree of discrimination between various myosin (MyHC) subtypes, but the "binding behavior" of these antibodies in ELISA-type assays may differ similarly to the different specificity and avidity observed between immunohistochemical staining of fresh frozen muscle sections and immuno-blots in the rat and human muscles (Smerdu and Soukup 2008). Unfortunately, individual protein MyHC isoforms, which would be crucial for resolving the issue of possible cross-reactivity, are not as yet available. The existence of a cell-line expressing a single MyHC subtype would provide enormous help, not only for this type of study.

We can conclude that ELISA can be used for measurement of MyHC isoforms in multiple (several tens) samples and the procedure, after initial optimalization, is quite simple. However, it is not superior to SDS-PAGE yielding only relative "semi-quantitative" data. It is neither faster nor cheaper compared to SDS-PAGE, and its reliability is strongly dependent on the specificity of the antibodies used. Still, we hope that its efficacy can be improved e.g. by utilizing individual protein MyHC isoforms as standards. In such case, the presented method could provide in the future additional "quantitative" information about MyHC isoform content alongside SDS-PAGE.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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