

**Uncoordinated expression of the main contractile and relaxation
proteins in pathological skeletal muscles**

Summary of Ph.D. thesis

Andrea Zsófia Szabó

Supervisor: Dr. Ernő Zádor

Department of Biochemistry
Faculty of Medicine
University of Szeged

2012

INTRODUCTION

According to authoritative physiology textbooks skeletal muscle contributes about to 40% of body weight in human. Apparently this proportion shows a large individual variety, but in any case, skeletal muscle has a major part in balancing the functions of the whole organism. As a major ruler of general health conditions, skeletal muscle has been recognised ages ago, and beside this, nowadays it is rediscovered from endocrine and metabolic aspects. The considerable complexity of skeletal muscle is characterized by the type of its fibers. In mammals, four major fiber types are distinguished according to three main features; (1.) the dominantly expressed myosin heavy chain (MyHC), (2.) the twitch speed and (3.) the metabolic functions. These properties together characterise the muscle “phenotype”. The main contractile element MyHC isoforms are the most frequent markers, but they are not the only molecules that determine the muscle phenotype; the contraction speed is controlled by the MyHC isoforms, but the rate of relaxation depends on the SERCA isoforms.

The corresponding slow and fast MyHC and SERCA isoforms are usually expressed in coordination in muscle fibers suggesting that they are subject to common regulatory mechanism. This regulatory mechanism is mainly controlled by the electric component of innervation which is a major determinant of muscle type in normal use.

Based on a previous study from our laboratory on *regenerating* muscle, we hypothesized that the impact of nerve activity on the expression of SERCAs differs from that on the corresponding MyHC isoforms in the *normal* soleus muscle. In order to test this hypothesis, we denervated the hindlimb of the rat (HD). To further refine our study, we compared this intervention with selectively denervating the soleus (SD) thereby leaving the other hindlimb muscles intact and therefore render the innervated soleus to passive movement. The soleus muscle practically contains two fiber types, type I which has slow-twitch and oxidative metabolism and type IIA which has fast-twitch and oxidative-glycolytic metabolism. We assumed that the adaptation to passive movement might affect the levels of the MyHC and SERCA isoforms differently in SD than in HD soleus fibers.

The role of skeletal muscle is also significant in metabolism and as a major glucose user it is a main target of insulin. Insulin deficient diabetes (type I) can be induced in rodents by streptozotocin-treatment. Streptozotocin (STZ) is a fungal toxin which damages the insulin producing β -cells of the pancreatic island. Lack of insulin observed in type I diabetes restricts glucose uptake and severely affects metabolism, function and morphology of muscle. Stz-

rats develop peripheral neuropathy and a slow-to-fast transition of fiber types in the soleus. The conversion from slow to fast muscle phenotype declines the level of slow myosin heavy chain (MyHC1) and increases the levels of fast myosins (MyHC2a, MyHC2x and MyHC2b). The coordinated expression of the corresponding SERCA isoforms is functionally relevant and characteristic to this conversion. However, current research has shown that the level of calcineurin, a calcium-calmodulin dependent serine phosphatase is declined in stz-rats faster than the level of slow myosin, and the muscle specific overexpression of calcineurin improves insulin action, we suggested that regulators of the Ca²⁺ homeostasis like SERCAs might also respond relatively rapidly to blood glucose level in skeletal muscle.

AIMS

In this work we wanted to describe the changes of MyHC and SERCA levels in three pathological conditions of the rat soleus muscle: selective denervation (SD), hindlimb denervation (HD) and streptozotocin induced diabetes (stz-diabetes). We aimed to determine the mRNA, protein levels and fiber-expression of MyHC1, MyHC2a, SERCA2a and SERCA1 in these conditions. Putting together the results we wished to extend the present view about the regulation of MyHC and SERCA isoforms in skeletal muscle.

MATERIALS AND METHODS

Selective and hindlimb denervation. Male Wistar rats 3 months of age and weighing 280-350g were used for the experiments. Approximately 1 cm of the sciatic nerve high in thigh was cut out in the hindlimb-denervation (HD) group. In the selectively-denervated (SD) group, 0.3 cm of the soleus nerve was cut out. After denervation, the soleus muscles were dissected at 3, 7 or 14 days and frozen in isopentane cooled by boiling liquid nitrogen. Normal muscles were gained from untreated animals We also used the soleus of normal and HD 200-215g female Wistar rats to compare our results with those of Schulte et al. (1994).

Streptozotocin-induced diabetes. Rats with this treatment were prepared in the laboratory of Dr. Ágota Vér, (Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University). In case of hyperglycaemic model the experiments were performed on 4-week-old male Wistar rats (weighing 170 ± 40 g). Rats were rendered diabetic with streptozotocin (65 mg/kg i.v) and only with plasma glucose concentrations above 15 mmol /l were included in the study. Vehicle-treated rats served

as controls. Four weeks after the induction of DM (defined as day 0) the animals were anaesthetized and sacrificed by decapitation. The soleus muscles were dissected.

Ratio RT-PCR. RNA extraction and reverse transcription were carried out as described previously. The RNA was extracted with the shorter version of acid-guanidinium-phenol-chlorophorm method. Total RNA level was determined from its UV-absorption at 260nm and 280nm. The cDNA was transcribed from 1µg total RNA. For determination of SERCA1a/1b or SERCA1/SERCA2 ratios the same primers were used in one PCR mix. In case of SERCA1/SERCA2 the amplified fragments were identified by restriction digest. All PCR cycles were adjusted to the logarithmic/linear amplification phase and the products analyzed on a 6% (wt/wt) polyacrylamide gel.

Protein determination. Total protein levels were measured by the BCA method using the NanoDrop spectrophotometer. The crude homogenate made in the first step of the SERCA extraction was used to determine the total protein level.

Immunoblotting. SERCA and MyHC protein isoforms were measured in extracts of the same muscles. The muscles were homogenized in 2.5 ml of 20% sucrose, 5 mM HEPES (pH 7.5) and proteinase inhibitor cocktail in the cold room. To extract myosin the first centrifugation (1000g x 10 min) pellet was used. For SERCA determination the mitochondrial-microsomal (sarcoplasmic) pellet (200 000g x 30 min) was analysed. Amounts of extracts corresponding to equal parts of the muscles were loaded on each lane of the gel both for SERCA and myosin analysis. The primary antibodies were hybridized with HRP-conjugated 2nd antibodies and visualized by Ni-enhanced DAB staining or, in case of MyHC2a, by the ECL-method and quantified by densitometry.

Immunocytochemistry. Serial cryosections of 20 µm thickness from the central part of the muscles were cut and processed for immunostaining. Sections were incubated in 5% dried milk in PBS for 20 min to block nonspecific binding sites then incubated overnight with primary antibodies and then with peroxidase-conjugated secondary for 60 min. The immunocomplexes were visualized by DAB staining.

Fiber cross-sectional areas. The cryosections were made in the same way as in immunocytochemistry. The sections were stained by hematoxylin-eosin. We calculated the fiber cross-sectional areas (CSAs).

Statistics. We used unpaired t-tests to determine the significant differences. We considered $p < 0.05$ significant, $p < 0.01$ very significant and $p < 0.001$ highly significant. 2–4 animals were used for the mRNA studies, 3–4 animals for the immunoblots, 3 animals for the

immunohistochemistry and at least one hundred fibers were inspected for fiber types from each muscle.

RESULTS

Denervation models

Fresh weight, fiber cross-sectional area, total RNA and total protein levels.

In both conditions of denervation, the fresh weight of the muscles decreased from day 7, however this decrease was less pronounced in selectively-denervated (SD) than in hindlimb-denervated (HD) muscles on day 14. Also the fiber cross-sectional area was smaller compared to normal muscle at all stages in both types of denervation. Upon SD, this decrease was larger after day 3, whereas upon HD it was more pronounced after day 7 and 14.

The RNA content decreased in HD soleus at day 7 and 14 while in SD muscles it decreased only after two weeks. In SD muscles the RNA level increased first at day 3 and 7 compared to the level in HD muscle.

The total protein levels also decreased after 14 days in both types of denervation but more dramatically upon HD than SD.

All together, the fresh weight, the fiber cross-sectional area, the total RNA and protein contents were more reduced after two weeks in HD than SD.

The mRNA levels

The slow-twitch MyHC1 mRNA level declined in from day 7 in HD and on day 14 upon SD.

The level of the slow-twitch muscle-specific SERCA2a mRNA was significantly elevated above normal ($P < 0.05$) on day 7 of SD. The mRNA level of the fast oxidative MyHC2a changed differently in both types of denervation: in SD it increased on days 3 and 7 compared to HD on the same days.

The level of the fast SERCA1 mRNA was significantly increased at day 3 in HD and at day 3 and 7 in SD. SERCA1 exists in two splice variants: a neonatal SERCA1b isoform (lacking a 40-bp alternative exon) and an adult SERCA1a form. Interestingly, whereas the level of the adult SERCA1a mRNA did not increase in any of the denervated muscles, the mRNA level of the neonatal SERCA1b was higher than normal in all stages of both treatments, except the 14th day of SD muscles. The increase of SERCA1b mRNA levels was higher in SD than in HD soleus muscles during the first week.

The MyHC2x mRNA levels showed a pronounced increase in both types of denervations, most remarkably on day 3 and 7 in SD, when it was significantly higher than in the HD muscles.

The levels of GAPDH mRNA did not change during the denervations so it could be used as an adequate reference control level for total mRNA.

Protein levels

The MyHC1 protein levels declined significantly only at day 14 of HD, but it decreased remarkably in all stages of SD. It seems that the loss of MyHC1 protein is slower in HD than in SD muscles.

The SERCA2a protein levels were not significantly altered in any of the types of denervations. The MyHC2a protein levels did not seem to change significantly compared to the normal level in any of the stages of both types of denervation. However it showed a significant decrease at day 14 in HD and at day 7 and 14 in SD compared to the previous stages of the same denervation.

The total SERCA1 (SERCA1a + SERCA1b) protein levels dropped significantly only at day 14 of HD, but it did not change in SD. We also analyzed female rats weighing 200g using our method. In this cases the SERCA1 protein level was significantly lower after 14 days in HD than in normal muscles.

Immunohistochemical results

MyHC fiber types: The percentage of slow MyHC1-positive type I fibers decreased in all stages of both types of denervation. The decrease was more pronounced in SD than in HD at day 3. In agreement with this, the decrease of MyHC1 protein level was larger in SD when compared to the HD muscles.

In HD muscles the ratio of MyHC2a-expressing type IIA fibers increased on day 7 and 14 in comparison with the normal soleus muscles. In SD muscles, the percentage of IIA fibers didn't change compared to normal muscles. On day 7, the ratio of type IIA fibers differed significantly between the hindlimb and selective denervation.

Hybrid fibers: There were more MyHC-hybrid fibers in both types of denervation than in the normal muscles. The numbers of MyHC-hybrid fibers were higher on day 7 in SD than in HD muscles.

SERCA fiber types: Parallel sections were also stained for SERCA1 and SERCA2a. The

percentage of pure SERCA2a fibers decreased at day 14 in HD, while in SD muscles it was already lower at day 3 and 7. The SERCA1-positive fibers increased only in SD at day 7 and 14. The number of SERCA-hybrid fibers increased in both types of denervation, but this was more pronounced in SD muscles.

Coexpression of MyHCs with the corresponding SERCAs: Next we explored to what extent the expression of the corresponding slow or fast isoforms of Ca²⁺ pumps and myosins remained correlated during denervation. First we looked at the expressions of the slow isoforms. The correlation between SERCA2a-positive fibers and MyHC1-positive fibers was tight in the normal muscles. In HD, the correlation loosened and it totally disappeared in SD at day 14. The fast isoforms, the SERCA1-positive and MyHC2a-positive fibers retained a relatively tight correlation during both types of denervation.

Diabetic model

Fresh weight, fiber cross-sectional area, total RNA and total protein levels

The weight and protein content of soleus of stz-rats was not significantly different but the cross sectional area was only 83% of that of the control muscles.

The mRNA levels

The level of *GAPDH* mRNA was not different in stz-soleus compared to the controls, therefore we used it as an RT PCR control. Normalized levels of *SERCA1a* and *SERCA2a* mRNAs were not significantly lower in the soleus of stz-rats than in the controls, neither were the relative levels of *SERCA1* and *SERCA2* transcripts. Similarly, there were no significant differences in the *MyHC1/MyHC2a* mRNA levels of stz-treated and control rats.

The protein levels

In accordance with the mRNA levels, the MyHC1, MyHC2a and SERCA1 protein levels were not different on immunoblots of the soleus of stz-treated and control rats. However, the SERCA2a level was about 50% lower in the soleus of stz-treated rats compared to that of the controls. This was in agreement with the observation made on transverse sections that the SERCA2a level was decreased in type I fibers of the soleus of stz-rats.

Immunohistochemical results

The percentage of type I fibers showed a tendency to decline in stz-rats but it was not statistically different from that of the controls. The ratio of type IIA fibers was also not significantly different in stz-soleus compared to the control. The number of hybrid fibers expressing both MyHC1 and MyHC2a was about the same in the soleus of diabetic and controls rats. The percentage of associated type IIA fibers (two or more fibers together) was higher in stz-rats than in the controls. This indicated peripheral neuropathy and a higher rate of reinnervation in the stz-treated soleus than in the control muscles.

In the diabetic muscles, SERCA1a was stained in type IIA fibers with similar intensity as in the control, but SERCA2a expression in type I fibers was practically not different from the background level found in the type IIA fibers.

DISCUSSION

Denervation models

The denervations highlight the difference in nerve control of expressions of the MyHC and SERCA isoform. In SD the soleus was passively moved by the other hindlimb muscles therefore the effect of passivity, which is an attribute of HD was eliminated. However, SD had another attribute, the passive movement. In spite of the different additional effects we could observe changes that were common in both types of denervation. One of these was the switch from slow to fast myosin heavy chains, the other was the less apparent switch from slow to fast SERCA isoforms. This extended the earlier observation of our group that the expression of SERCA isoforms in regenerating muscle are less or not nerve dependent compared to the myosin heavy chain isoforms. Similarly, the lack of decline in SERCA2a expression was described in spinal cord isolation, in spinal cord trans-section and in overloaded soleus muscles. The largest discrepancy in coordinated protein expression was found when upon selective denervation the MyHC1 protein level dramatically decreased whereas the SERCA2a did not change. This drop in the level of MyHC1 protein indicated that a selective degradation of MyHC1 protein accompanied the slow-to-fast transformation in the muscle adapting to passive movement. A parallel drop in the level of SERCA2a was not observed, again showing that myosin and Ca²⁺ pumps respond differently to passive movement. Upon denervation, the loss of coordination was more pronounced in selective denervation for the slow MyHC1 and SERCA2a than for the fast isoforms. The loss of coordinated expression resulted in the appearance of hybrid fibers. More SERCA-hybrid

fibers than MyHC-hybrid fibers were found in SD than in HD muscles and SERCA-hybrids appeared earlier. This also shows that the expressions of MyHC and of SERCA are differentially regulated.

In conclusion, the expression of SERCA and myosin isoforms seems to be separately controlled each by a unique set of regulatory factors in the soleus muscle. The slow innervation is a major controller for the slow type myosin, while the fast myosin (MyHC2a) is more expressed when the slow myosin is declining, however not balanced by an equimolar replacement of the fast isoform. The switch of SERCA2a to SERCA1a isoform does not coincide, but is rather delayed compared to the switch of the slow-to-fast myosin isoforms. As a consequence, the slow type SERCA is not expressed in coordination with the slow type myosin in many of the fibers.

Diabetic model

The soleus muscle of stz-rats was similar by weight and protein content but the fiber size was smaller than in the control. This suggests that the fiber size reflects muscle atrophy earlier than the fresh weight or protein content. An increased association of type IIA fibers - an indication of peripheral neuropathy - also appears to be an early marker of diabetes in stz-soleus. Parallel with fiber type association a slow-to-fast fiber transition occurs at 2–3 months after stz-treatment. In contrast, we did not detect a significant transition of slow-to-fast fibers at 4 weeks. Since the range of streptozotocin doses used for inducing type I diabetes is relative narrow in rats, the variance in fiber type shift is more likely due to the duration of hyperglycemia than to the applied amount of the toxin. According to the unchanged ratio of myosin and SERCA1a in fiber types, the stz-soleus showed similar MyHC1, MyHC2a and SERCA1a mRNA and protein levels compared to those of the control. However, the expression level of SERCA2a was lower in diabetic soleus type I fibers compared to the controls and this was supported by the immunoblot but not the RT PCR results. This shows that different mechanisms regulate the levels of the SERCA2a pump and the MyHC1 in the stz-soleus. The SERCA2a protein level may decrease in several ways; one of these is the increased level of glycolysation. Advanced glycation end products accumulate in the skeletal muscle and heart of diabetic animals. Although the levels of both SERCA2a and its inhibitor, the phospholamban decrease in diabetes, the phospholamban/SERCA2a ratio becomes higher. The levels of reactive oxygen and nitrogen species (ROS and RNS) are elevated and decrease the levels of specific proteins like SERCA2a. The expression of SERCA2 isoforms is largely

regulated at the level of RNA splicing in the normal heart and slow skeletal muscle, however no such mechanism was implicated in the diabetic soleus. The calcineurin-NFAT pathway is a key factor of muscle remodelling and it has been reported to decline in stz-induced diabetic rats parallel with muscle atrophy. Since SERCA2a is the major pump regulating the level of Ca^{2+} in the sarcoplasm, it is an interesting question how the decrease of SERCA2a level reported here is related to the decline of the calcineurin-NFAT pathway in diabetic muscle. Our results suggest that the SERCA2a protein level is an earlier marker than that of MyHC1 for muscle diabetes. This implicates that the Ca^{2+} metabolism reacts more readily to diabetic stress than the contractile elements do.

Comparison of levels of SERCA and MyHC in denervation and diabetic models

The two denervation models are examples of the effects of acute lack of innervation while the diabetic model represents the effects of a chronic metabolic malfunction. These different pathological statuses explain the differences between the patterns of changes in the two models. In these experimental models fibers size adapts earlier than fresh weight and this represents an example of the partial overlap between the effects of acute and chronic conditions.

During denervation the decline of MyHC1 level was not followed by the slow-type SERCA2a, showing that this protein is not as strongly dependent on innervation as MyHC1.

In contrast with denervation, in the diabetic model the SERCA2a protein was the first to decrease and followed only much later by the decrease of MyHC1. These results show that the different mechanisms that regulate the levels of SERCA2a and slow myosin act also differently in denervated and diabetic soleus, especially because, the dysregulation occurred both at the mRNA and protein levels

Although the detailed patterns of changes were different in the two models, the directions of changes point to slow-to-fast transformation in both. Overall, these elements are underlining the different layers of regulation of corresponding MyHC and SERCA isoforms, showing that the MyHC1 is more dependent on innervation while SERCA2a is more dependent on metabolic changes.

Novel results in the thesis:

- The regulation of the corresponding MyHC and SERCA isoforms dyscoordinated in denervation, among these the MyHC1 is dependent, the SERCA2a is not dependent on

innervation.

- The selective denervation is introduced as a new experimental model to study gene expression.
- In the stz-induced diabetes the slow-type SERCA2a is more dependent on metabolic status than the slow-type MyHC1.
- The decline of fibers size (CSA) reflects muscle atrophy earlier than that of the fresh weight.

ACKNOWLEDGEMENTS

I would like to thank to my supervisor Dr. Ernő Zádor for his guidance, to Prof. Frank Wuytack (KULeuven, Belgium) whose laboratory I had the opportunity to work for one month and to my colleagues Dr. Gábor Zoltán Rácz and Dr. Noémi Tóth who helped my work and to Prof. László Dux for providing the opportunity to work in the Department of Biochemistry. This research was supported by Tét B20/04, ETT 168/2003 and TÁMOP-4.2.2/B-10.

List of publications

Articles related to the thesis

I. Szabó A, Wuytack F, Zádor E. (2008) The Effect of Passive Movement on Denervated Soleus Highlights a Differential Nerve Control on SERCA and MyHC Isoforms. *J Histochem Cytochem* **56(11)**: 1013-1022.

II. Rácz G, Szabó A, Vér A, Zádor E. (2009) The slow sarco/endoplasmic reticulum Ca²⁺-ATPase declines independently of slow myosin in soleus muscle of diabetic rats. *Acta Biochim Pol* **56(3)**: 487-493.

Article not related to the Thesis

I. Tóth N, Szabó A, Kacsala P, Héger J, Zádor E. (2008) 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. *Phytomedicine* **15(9)**: 691-698

Presentations as lectures and posters

1. **Szabó, A.** and E. Zádor (2005) Comparison of the effect of ischiadic and selective denervation on gene expression in rat soleus muscles. (XXXIV. European Muscle Conference, Hungary, Hortobágy) *J Muscle Res Cell Motil* 26: p63 Poster
2. Keresztes Sz, **Szabó A.** és Zádor E. (2006) Muscleblind mRNS-ek és a SERCA1 splicingja regenerálódó vázizomban. MÉT LXX. Vándorgyűlése (Szeged) P24 Poster
3. **Szabó A.** and E. Zádor (2006) Génkifejeződés összehasonlítása szelektív- és végtag denervált patkány soleus izomban. MÉT LXX. Vándorgyűlése (Szeged) P28 Poster
4. **Szabó A** (2007) Uncoordinated changes of the main contraction and relaxation protein in pathological skeletal muscle. TÉT BILAT Workshop KULeuven (Belgium)
Lecture
5. Tóth N., Báthori M., Ramirez G. G., **Szabó A.**, Kacsala P., Héger J., Zádor E. (2008) Effect of 20E on different rat muscle models. Ecdysone-Workshop, (Ulm, Germany)
Lecture