

The slow sarco/endoplasmic reticulum Ca²⁺-ATPase declines independently of slow myosin in soleus muscle of diabetic rats

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The sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) isoforms are normally expressed in coordination with the corresponding myosin heavy chain (MyHC) isoforms in the fibers of skeletal muscle but this coordination is often disrupted in pathological conditions. In the streptozotocin-induced diabetes of rats (stz-rats), the soleus muscle showed peripheral neuropathy and the SERCA2a level decreased in type I (slow-oxidative) fibers compared to the control muscles, whereas the expression of the corresponding slow MyHC1 did not change. No difference was found at the mRNA and protein levels of SERCA and MyHC isoforms in the whole soleus, except that the level of the SERCA2a protein specifically declined in stz-rats compared to the controls. This shows that the coordinated expression of SERCA2a and MyHC1 is disrupted at the SERCA2a protein level in the diabetic soleus. The results are in line with previous observations that regulators of the Ca-homeostasis may adapt faster to type I diabetes than the contractile elements.

Keywords: SERCA, myosin, muscle fibers, diabetic rat

INTRODUCTION

Skeletal muscle is a major target of insulin. Decline of insulin level observed in type I diabetes restricts glucose uptake and severely affects metabolism, function and morphology of muscle (Klueber & Feczko, 1994; Aughstien *et al.*, 2006; reviewed by Sun *et al.*, 2008). In a rodent model of type I diabetes, hyperglycemia is induced by streptozotocin (streptozotocin), a fungal toxin damaging the insulin-producing pancreatic beta-cells (reviewed in Szkudelski, 2001). Rats with streptozotocin-induced diabetes (stz-rats) develop peripheral neuropathy (Klueber & Feczko, 1994; Snow *et al.*, 2005) and a slow-to-fast transition of fiber types in the soleus (Punkt *et al.*, 1999; Snow *et al.*, 2005). 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) (Pette & Staron, 2000). Myosin is responsible for muscle contraction and the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) for relaxation, therefore the coordinated expression of the corresponding MyHC and SERCA isoforms is functionally relevant and characteristic to the fiber types of normal muscle. The slow SERCA2a is coexpressed with the MyHC1 isoform in the type I (slow-oxidative) fibers. The fast type SERCA1a isoform is expressed together with MyHC2a, MyHC2x and MyHC2b in the type IIA, IIX/d and IIb (from fast-oxidative to fast-glycolytic) fibers, respectively (Talmadge *et al.*, 1996; Mendler *et al.*, 1998; Zador *et al.*, 2007). In spite of the coordinated expressions, current research has shown that factors controlling the levels of myosins and SERCAs

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Abbreviations: AGPC method, acid guanidinium thiocyanate/phenol/chloroform method; CSA, cross sectional area; DAB, diaminobenzidine; DM, diabetes mellitus; HRP, horseradish peroxidase; IRS, insulin receptor substrate; MEF-2, muscle enhancing factor-2; MyHC, myosin heavy chain; NFAT, nuclear factor of activated T-cells; PI3-K, phosphatidylinositol 3-kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SERCA, sarcoplasmic reticulum Ca²⁺-adenosine triphosphatase; stz-rats, streptozotocin-induced diabetic rats.

are different in both normal and pathological conditions (Zador & Wuytack, 2003; Zador *et al.*, 2005; Talmadge & Paalani, 2007; Szabo *et al.*, 2008).

Regulators of the Ca^{2+} homeostasis respond relatively rapidly to blood glucose level in skeletal muscle. The calcineurin-NFAT pathway is down-regulated, and the DNA binding of NFAT (nuclear factor of activated T-cells) and MEF-2 (muscle enhancing factor-2) is lower in rat skeletal muscle three weeks after streptozocin treatment (Costelli *et al.*, 2007). Insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) increases binding to SERCA1 and SERCA2 in normoglycemic rat after insulin stimulation and phosphorylation by insulin receptor kinase (Algenstaedt *et al.*, 1997). This suggests that SERCAs are targets of insulin action and hints at a direct link between the insulin-dependent and Ca^{2+} -signaling pathways. The MyHC1 level decreases in the rat soleus at 2–3 months after streptozotocin treatment (Punkt *et al.*, 1999; Snow *et al.*, 2005), but no parallel detection of MyHC and SERCA isoforms has been presented in the same diabetic muscle. Here we monitored SERCA together with MyHC at the mRNA and protein levels in the same soleus muscles of stz-rats four weeks after streptozocin treatment, in order to describe the extent of their coexpression.

MATERIALS AND METHODS

Animals and treatments. Experiments were performed on 4-week-old male Wistar rats (weighing 170 ± 40 g). Rats were housed at a constant temperature (20°C), humidity, 12 h light-dark cycle, and were allowed free access to standard chow and water. All experimental protocols were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (TUKÉB 99/94). Rats were rendered diabetic with streptozotocin (65 mg/kg *i.v.*, Sigma Chemical Co.) dissolved in 0.1 M citrate buffer (pH 4.5). Only streptozocin-treated animals with plasma glucose concentrations above 15 mmol/l were considered diabetic and 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 (40 mg/kg pentobarbital sodium, Abbott Laboratories) and sacrificed by decapitation. Muscles, blood and post mortem urinary samples were collected for further investigations. The soleus muscles were dissected and frozen in isopentane cooled in liquid nitrogen and stored at -80°C . Non-fasting serum glucose concentration was measured using a kit from Roche Diagnostics, Ltd.

RT-PCR. Total RNA was extracted by AGPC-method and the specific mRNA levels were determined by RT-PCR as described (Zádor *et al.*, 1996; Fenyvesi *et al.*, 2004). The extracted RNA was measured at 260 nm and reverse transcription was made on 1 μg RNA using oligo (dT) primers. The proportion of SERCA1 and SERCA2 PCR fragments was measured by ratio RT-PCR according to the method detailed in (Zador *et al.*, 1996). Shortly, the same primers were used to amplify cDNA of both SERCA1 and SERCA2 in one PCR mix. The resulted amplification was divided into aliquots which were digested with either *StyI* (hydrolysing SERCA1) or *Trpu9I* (hydrolysing SERCA2) and a control digestion with both restriction enzymes was also applied. The digested aliquots were loaded onto 6% polyacrylamide gel. The ratio of the SERCA transcripts was estimated from the level of the undigested fragments of the different restriction enzyme reactions. The fragments were stained with ethidium bromide and quantified by Gel Doc 2000 (Bio-Rad Laboratories; Hercules, CA, USA) and Quantity One software. The level of other mRNAs was estimated by relative RT-PCR method described earlier (Fenyvesi *et al.*, 2004). All PCR amplifications were checked to be in the linear range.

Immunostaining, morphometry and immunoblotting. Frozen sections were taken from the central part of the muscles and immunostained as described in (Zádor *et al.*, 1998). Primary antibodies to MyHC1 (BA-D5, mouse, 1:50; Schiaffino *et al.*, 1989), MyHC2a (SC-71, mouse, 1:20; Schiaffino *et al.*, 1989), SERCA1 (A3, mouse, 1:20; Zubrzycka-Gaarn *et al.*, 1984) and SERCA2a (R-15; rabbit, 1:400; Wuytack *et al.*, 1989) were used and immunohybridizations were detected with HRP-conjugated anti-mouse or anti-rabbit antibodies and peroxidase histochemistry. The fiber size (cross sectional area, CSA) was measured by Cell software (Olympus Soft Imaging Solution GmbH, Münster) at least for 100 fibers from each muscle.

The SERCA and MyHC isoforms were isolated from the same muscle and measured by immunoblotting as detailed in (Zádor & Wuytack, 2003; Szabó *et al.*, 2008). Shortly, the muscles were homogenized in 2.5 ml of 20% sucrose, 5 mM Hepes (pH 7.5) and proteinase inhibitor cocktail (Roche) in the cold room. The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was further centrifuged at $200000 \times g$ for 30 min to sediment the mitochondrial+microsomal fraction for SERCA isolation (Zádor *et al.*, 1998). The pellet of the $1000 \times g$ centrifugation was subjected to myosin extraction (Hämäläinen & Pette, 1997). The protein content of the crude homogenate and the end fractions of the isolations was measured by the BCA method. Gel loading was controlled after blotting onto PVDF membrane (Immobilon-P, Millipore) by Ponceau

staining. The membranes were hybridized with MyHC and SERCA antibodies, then with HRP-conjugated 2nd antibodies and developed with Ni-DAB (Wuytack *et al.*, 1994). The immunoblots were quantified by Gel Doc 2000 (Bio-Rad Laboratories; Hercules, CA, USA) and Quantity One software.

Statistics. Unpaired *t*-test was used to find significant differences, 2–4 animals were used for the mRNA studies, 3–4 animals for the immunoblots and 3 animals for the immunohistochemistry at least one hundred fibers were inspected for fiber types from each muscle.

RESULTS

Immunohistochemistry of streptozotocin-(streptozotocin) treated rat soleus muscles

The weight and protein content of soleus of stz-rats was not significantly different compared

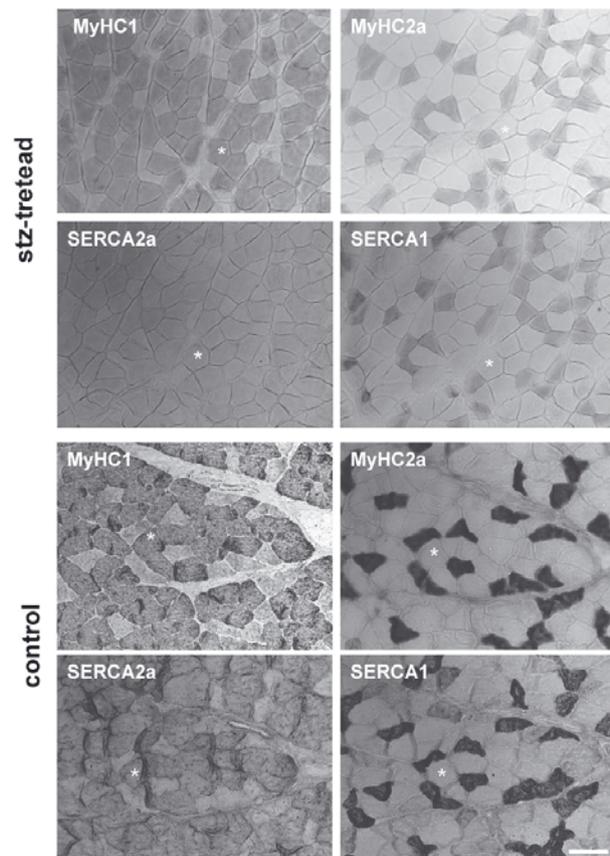


Figure 1. Myosin and SERCA isoforms in fibers of soleus muscle of stz-rats.

Immunostaining was made on parallel sections. Please note coexpression of MyHC1 with SERCA2a in type I fibers and coexpression of MyHC2a with SERCA1 in type IIA fibers of control and stz-rats. Expression of SERCA2a is practically not distinguishable in type I and type IIA fibers in stz-rats unlike in the control, suggesting lower level of the Ca^{2+} pump. *Indicate identical positions on sections. Bar is 100 μ m.

Table 1. Morphological parameters of diabetic and control rat soleus muscles.

¹Measured from the first homogenate; ²two or more adjacent fibers, **P*<0.05, ***P*<0.01, mean \pm S.E., *n*=4 for the weight and protein content and *n*=3 for the fiber parameters.

Parameter	Diabetic	Control
Weight (mg)	157 \pm 9	162 \pm 7
Protein content (mg) ¹	34.4 \pm 3	31.9 \pm 2
Fiber CSA (μ m ²)	2912 \pm 89**	3521 \pm 91
Type I fibers (%)	73.5 \pm 3.1	80.9 \pm 1.6
Type IIA fibers (%)	26.9 \pm 3	19.5 \pm 1.6
Hybrid fibers (%)	0.5 \pm 0.2	0.4 \pm 0.1
% of IIA fibers in association ²	57 \pm 4.3*	28.6 \pm 5

to the controls (Table 1). However, the fiber size (cross sectional area, CSA) was 83% of that of the control muscles (2912 \pm 89 *vs.* 3521 \pm 91 μ m², *P*<0.01, *n*=3). The percentage of type I fibers expressing myosin heavy chain (MyHC1) showed a tendency to decline but it was not statistically different from that of the controls (73.5 \pm 3.1 *vs.* 80.9 \pm 1.6%, *P*=0.128, *n*=3). The ratio of type IIA fibers expressing myosin heavy chain 2a (MyHC2a) was also not significantly different in streptozotocin-soleus compared to the control (26.9 \pm 3.0% *vs.* 19.5 \pm 1.6%, *P*=0.095, *n*=3).

The number of hybrid fibers expressing both MyHC1 and MyHC2a was about the same in the soleus of diabetic and controls rats (Table 1). However, in stz-rats the percentage of associated type IIA fibers (two or more fibers together) was higher than in the controls (57 \pm 4.3 *vs.* 28.6 \pm 5, *P*<0.05). This indicated peripheral neuropathy and a higher rate of reinnervation in the streptozotocin-treated soleus than in the control muscles (Karpati & Engel, 1968; Jaweed *et al.*, 1975).

The MyHC isoforms are the most frequently used but not the only markers of muscle fiber types (Sciaffino & Reggiani, 1996). The sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) isoforms are expressed in coordination with the corresponding MyHCs and can also be used for fiber typing (Talmadge *et al.*, 1996). We immunostained the muscle fibers on consecutive sections for SERCA2a and SERCA1 in addition to the MyHC1 and MyHC2a isoforms (Fig. 1). In adult soleus, SERCA1a practically accounted for the total amount of SERCA1 (Zádor *et al.*, 2007) and it was specifically expressed in the type IIA fibers, whereas SERCA2a was present exclusively in type I fibers. 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. Therefore we aimed to measure the difference at the mRNA and protein levels of the SERCA and MyHC isoforms in the whole soleus muscle.

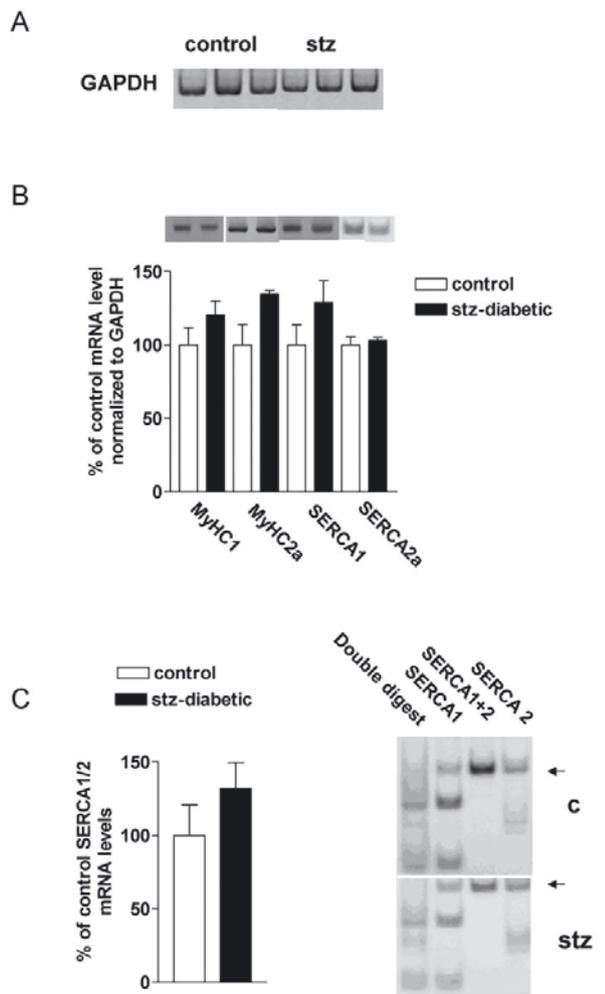


Figure 2. Myosin and SERCA mRNA levels in soleus of stz-diabetic rats.

(A) GAPDH mRNA levels used for normalization. (B) mRNA levels in percent of control (mean \pm S.E., $n=3-4$). Above columns representative bands of RT-PCR are shown. (C) The ratio of SERCA1/2 mRNA levels (mean \pm S.E., $n=3$ for the control and $n=2$ for the stz-diabetic rats) Left. Representative gels of ratio-RT-PCR analysis of SERCA1 and SERCA2 (right). Undigested SERCA isoforms are indicated above lanes. Only the upper undigested bands (indicated by small arrows) were used for quantitation; c, control; stz, streptozotocin-treated.

mRNA levels of MyHC and SERCA isoforms

The level of *GAPDH* mRNA was not different in streptozocin-soleus compared to the controls (Fig. 2A), therefore we used it as an RT-PCR control, reference mRNA. Normalized levels of *SERCA1a* and *SERCA2a* mRNAs were not significantly lower in the soleus of stz-rats than in the controls (Fig. 2B). Neither were the relative levels of *SERCA1* and *SERCA2* transcripts, measured by ratio-RT-PCR, different in the diabetic and control soleus (see right-hand columns in Fig. 2B and representative gel in Fig. 2C). Similarly, there were no significant

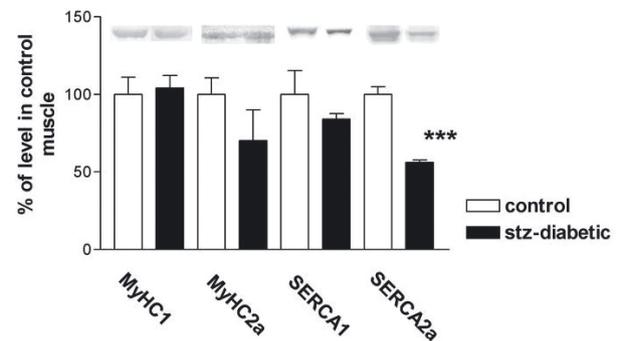


Figure 3. MyHC and SERCA protein levels in soleus of stz-rats.

Above columns representative bands of immunoblots are shown. *** $P<0.001$, $n=3-4$. Other symbols as in Fig. 2.

differences in the *MyHC1* and *MyHC2a* mRNA levels of soleus muscles of the streptozocin-treated and control rats.

Protein levels of MyHC and SERCA isoforms

In accordance with the mRNA levels, the *MyHC1*, *MyHC2a* and *SERCA1* protein levels were not different on immunoblots of the soleus of streptozocin-treated and control rats (Fig. 3). However, the *SERCA2a* level was about 50% lower ($P=0.0002$) in the soleus of streptozocin-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.

DISCUSSION

The soleus muscle of stz-rats showed similar weight and protein content but a smaller fiber size than the control. In another model, the selectively denervated soleus, a similar decrease in fiber size but not in fresh weight or protein content has been reported after 3 days, while both fresh weight and protein content decreased with fibers size after 7 days (Szabó *et al.*, 2008). This shows that fiber size reflects muscle atrophy earlier than the fresh weight or protein content.

An increased association of type IIA (fast-oxidative) fibers is an indication of peripheral neuropathy (Karpati & Engel, 1968; Jaweed *et al.*, 1975), and it also appears to be an early marker of diabetes in streptozocin-soleus (Snow *et al.*, 2005). Parallel with fiber type association, we detected a tendency but not a significant transition of slow to fast fiber types at four weeks after streptozocin treatment. We used only three animals for this experiment, but increasing the sample size above $n=14$ in both groups should result in a significant dif-

ference of fiber types according to a power analysis of our data (S.D. and mean difference). This was in agreement with Rutschmann *et al.* (1984) who also reported no decline of slow myosin level after the same duration (4 weeks) in stz-rats, in contrast to reports of lower slow myosin levels and significant slow-to-fast fiber type transition at 2–3 months after streptozocin treatment (Punkt *et al.*, 1999; Snow *et al.*, 2005). The above studies all used $n \leq 8$ rats per group. The range of streptozocin doses used for inducing type I diabetes in rat is relatively narrow (reviewed by Szkudelski, 2001), therefore the variance in the 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 streptozocin-soleus showed similar MyHC1, MyHC2a and SERCA1a mRNA and protein levels compared to those of the control. The lower expression level of SERCA2a in diabetic soleus type I fibers was supported by the immunoblotting data but not the RT-PCR results. This shows that different mechanisms dysregulate the levels of the SERCA2a pump and the slow myosin in the streptozocin-soleus than in the denervated soleus, because, in the latter one, the dysregulation occurred both at the mRNA and protein levels (Szabó *et al.*, 2008).

The SERCA2a protein level may decrease in several ways (reviewed by Vangheluwe *et al.* 2005). In diabetic rat heart, it declines because of an increased level of glycosylation (Bidasee *et al.*, 2004). Advanced glycation end products also accumulate in the skeletal muscle of diabetic animals (Snow *et al.*, 2006). Although the levels of both SERCA2a and its inhibitor phospholamban decrease in diabetes, the phospholamban/SERCA2a ratio becomes higher (Rupp *et al.*, 1989; Belke *et al.*, 2004; Bidasee *et al.*, 2004; Vasani *et al.*, 2004). The levels of reactive oxygen and nitrogen species (ROS and RNS) are elevated (Aragno *et al.*, 2004; Mastrocola *et al.*, 2008) and they decrease the levels of specific proteins like SERCA2a (Aragno *et al.*, 2004; Mastrocola *et al.*, 2008; reviewed by Vangheluwe *et al.*, 2005).

Both SERCA1 and SERCA2 isoforms interact with phosphorylated forms of insulin receptor substrates, IRS-1 and IRS-2 (Algenstaedt *et al.*, 1997). This interaction can be stimulated by insulin in the control but not in stz-rats (Algenstaedt *et al.*, 1997). It is an interesting question whether the decreased interaction of IRS and SERCA in streptozocin-diabetic rats (Algenstaedt *et al.*, 1997) is related to the decline of SERCA2a level reported here.

The pathological mechanism of diabetes involves inhibition of phosphatidylinositol 3-kinase (PI3-K) (Lee *et al.*, 2004). Low activity of the PI3-K/Akt pathway in the endothelium and/or skeletal

muscle may contribute to defective signals and subsequent catabolism (reviewed by Kobayashi *et al.*, 2005). However, a direct role of this pathway in the phosphorylation of SERCA2a or its regulator, phospholamban, has not been documented yet.

The expression of SERCA2 isoforms is largely regulated at the level of RNA splicing in the normal heart and slow skeletal muscle, i.e. the structure of the 3' end confers higher stability on SERCA2a than on SERCA2b mRNA (reviewed by Misquita *et al.*, 2006). However, no such mechanism was implicated in the diabetic soleus (in our experiments) or in the diabetic heart (Bidasee *et al.*, 2004), since the SERCA2a mRNA level was not different from that of the control. The splicing of SERCA1, SERCA2 and the muscle-specific insulin receptor are each regulated by muscleblind protein 1 (MLNB1) (Savkur *et al.*, 2001; Ho *et al.*, 2004; Hino *et al.*, 2007) but no such regulation was implicated in the streptozocin-soleus.

The calcineurin-NFAT pathway is a key factor of muscle remodeling (reviewed by Bassel-Duby & Olson, 2006) and it has been reported to decline in streptozocin-induced diabetic rats parallel with muscle atrophy (Costelli *et al.*, 2007). Conclusively, muscle specific overexpression of calcineurin improves insulin action and glucose metabolism in the soleus of transgenic mice (Ryder *et al.*, 2003; Long *et al.*, 2007). 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.

In conclusion, we 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 do the contractile elements. The separate regulation of SERCA2a and MyHC1 is a further support of this conjecture.

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