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Short Report

Reduced expression of sarcalumenin and related Ca^{2+}-regulatory proteins in aged rat skeletal muscle

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ABSTRACT

In skeletal muscle, Ca^{2+}-cycling through the sarcoplasm regulates the excitation–contraction–relaxation cycle. Since uncoupling between sarcolemmal excitation and fibre contraction may play a key role in the functional decline of aged muscle, this study has evaluated the expression levels of key Ca^{2+}-handling proteins in senescent preparations using immunoblotting and confocal microscopy. Sarcalumenin, a major luminal Ca^{2+}-binding protein that mediates ion shuttling in the longitudinal sarcoplasmic reticulum, was found to be greatly reduced in aged rat tibialis anterior, gastrocnemius and soleus muscle as compared to adult specimens. Minor sarcolemmal components of Ca^{2+}-extrusion, such as the surface Ca^{2+}-ATPase and the Na^{+}–Ca^{2+}-exchanger, were also diminished in senescent fibres. No major changes were observed for calsequestrin, sarcoplasmic reticulum Ca^{2+}-ATPase and the ryanodine receptor Ca^{2+}-release channel. In contrast, the age-dependent reduction in the α_{1S}-subunit of the dihydropryridine receptor was confirmed. Hence, this report has shown that downstream from the well-established defect in coupling between the t-tubular voltage sensor and the junctional Ca^{2+}-release channel complex, additional age-related alterations exist in the expression of essential Ca^{2+}-handling proteins. This may trigger abnormal luminal Ca^{2+}-buffering and/or decreased plasmalemmal Ca^{2+}-removal, which could exacerbate impaired signaling or disturbed intracellular ion balance in aged fibres, thereby causing contractile weakness.

1. Introduction

Alterations in the cytosolic Ca^{2+}-concentration play a key regulatory role in a variety of cellular processes. In skeletal muscle fibres, the precise spatial and temporal control of Ca^{2+}-uptake, Ca^{2+}-buffering and Ca^{2+}-release is maintained by highly organised interactions between cytoplasmic and luminal Ca^{2+}-binding proteins, voltage-dependent Ca^{2+}-channels, Ca^{2+}-exchangers, Ca^{2+}-release channels, Ca^{2+}-shuttle proteins and Ca^{2+}-ATPases. As a second messenger system, sarcoplasmic Ca^{2+}-ions are involved in a variety of physiological processes in muscle including the regulation of excitation–contraction coupling, fibre relaxation, and fibre type shifting (Berchtold et al., 2000). Pathophysiological modifications of Ca^{2+}-cycling are a major factor in neuromuscular pathologies, such as malignant hyperthermia and Brody's disease (MacLennan, 2000). Sarcopenia of old age, defined as the age-related decline in muscle mass and strength (Marzetti and Leeuwenburgh, 2006), has also been associated with impaired Ca^{2+}-homeostasis (Delbono et al., 1995). Excitation–contraction uncoupling due to age-induced alterations in the ratio of the voltage-sensing dihydropyridine (DHP) receptor and the ryanodine receptor (RyR) Ca^{2+}-release channel of the sarcoplasmic reticulum (SR) may cause a reduced Ca^{2+}-supply to the contractile apparatus, thereby triggering reduced contraction forces with aging (Renganathan et al., 1997; Ryan et al., 2001).

In healthy adult skeletal muscle, charge movement in response to surface membrane depolarization activates the α_{1S}-subunit of the DHP receptor. This is followed by the direct physical coupling between the II and III loop domain of the voltage sensor and the cytosolic foot region of the RyR tetramer, which initiates the fast release of luminal Ca^{2+}-ions (Bannister, 2007). Skeletal muscle relaxation is mostly induced by the energy-dependent re-uptake of Ca^{2+}-ions via SR Ca^{2+}-pumps (SERCA) of the longitudinal tubules and terminal cisternae. In addition, Ca^{2+}-extrusion is mediated by the surface Na^{+}/Ca^{2+}-exchanger (NCX) and the plasmalemmal Ca^{2+}-ATPase (PMCA). A physiological mediator between Ca^{2+}-release and Ca^{2+}-uptake is represented by the luminal SR Ca^{2+}-reservoir system made up of calsequestrin (CSQ), sarcalumenin (SAR), junctate and related Ca^{2+}-binding proteins (Rossi and Dirksen, 2006). Both, CSQ and SAR belong to the class of high-capacity, medium-affinity Ca^{2+}-binding proteins. Recent studies suggest that luminal Ca^{2+}-binding proteins are probably multi-functional SR elements, which act as a Ca^{2+}-buffering complex, endogenous regulator of Ca^{2+}-release and as luminal chaperones. While CSQ and junctin are enriched in the junctional SR region in order to

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enhance Ca^{2+}-storage close to the site of the RyR Ca^{2+}-release complex, SAR was shown to function as the major non-junctional SR Ca^{2+}-binding protein (Leberer et al., 1990).

Previous aging studies suggest that no major changes occur in SR protein expression levels in skeletal muscle, as reviewed by Margreth et al., 1999. However, it is important to stress that subcellular fractionation methodology may introduce artifacts with respect to estimating Ca^{2+}-regulatory protein levels. Here, we have determined the density of key Ca^{2+}-handling elements in young adult versus senescent rat skeletal muscle preparations. In addition to the previously reported decrease in the α_{1S}-DHP receptor, three other protein species, SAR, PMCA and NCX were shown to be reduced in aged muscle. These abnormal muscle protein levels might play a pathophysiological role in impaired ion homeostasis and triad signaling observed in sarcopenia.

2. Materials and methods

2.1. Materials

Primary antibodies were purchased from Affinity Bioreagents, Golden, CO (MA3-913 to CSQ_f, PA1-913 to CSQ_s, MA3-912 to SERCA1, MA3-919 to SERCA2, MA3-914 to PMCA, MA3-920 to α_{1S}-DHPR, and MA3-925 to RyR1), and Sigma, Dorset, UK (N216 to NCX). Secondary antibodies used for immunoblotting and immunofluorescence microscopy were obtained from Chemicon International (Temecula, CA) and Molecular Probes (Eugene, OR), respectively. Protease inhibitors were from Roche Diagnostics (Mannheim, Germany). Acrylamide stock solutions were obtained from National Diagnostics (Atlanta, GA). Coomassie Blue R-350 was from Amersham Biosciences/GE Healthcare (Little Chalfont, Bucks, UK), and nitrocellulose membranes were from Millipore (Bedford, MA, USA). Chemiluminescence substrate was purchased from Pierce and Warriner (Chester, UK). Superfrost Plus positively-charged microscope slides were from Menzel Glässer (Braunschweig, Germany). All other chemicals used were of analytical grade and obtained from Sigma (Dorset, UK).

2.2. Preparation of aged muscle specimens

Freshly dissected muscle samples were obtained from the Animal Facility of the Department of Physiology, Trinity College Dublin. Wistar rats are an established model system for studying sarcopenia with a life expectancy of approximately 28–32 months. Animals were kept at a standard light–dark cycle and fed at libidum. Comparative studies were performed with young adult (3-month-old), adult (6-month-old) and senescent (30-month-old) tibialis anterior, gastrocnemius and soleus preparations. Muscle specimens (100 mg wet weight) were pulverized in liquid nitrogen using a mortar and pestle, and the resulting powder was placed into 1 ml of homogenisation buffer consisting of 0.5 M HEPES, pH 7.4, 200 mM EGTA, 10% (w/v) sucrose, 3 mM $MgCl_2$ solution and 0.1% (w/v) NaN_3, as well as a protease inhibitor cocktail (Doran et al., 2007). Tissue suspensions were sonicated for 10 s, subsequently incubated on ice with vortexing every 10 min for 10 s over a 4 h period, and then centrifuged for 20 min at 20,000*g*. Finally, protein samples were resuspended in an equal volume of reducing electrophoresis buffer, containing 0.05 M Tris, 3% (w/v) sodium dodecyl sulfate, 75 mM dithiothreitol, 20% (w/v) sucrose and 0.05% (w/v) bromophenol blue tracking dye.

2.3. Gel electrophoretic separation and immunoblot analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed by standard methodology with a Mini-Protean III gel system (BioRad Laboratories, Hemel-Hempstead, Herts., UK) using 10% resolving gels and 5% stacking gels. Fifty micrograms of protein was loaded per well and gels were electrophoresed for 1 h at 80 V, followed by a 130 V step until the bromphenol blue dye front had just ran off the gel. Gels were stained with Coomassie Blue or transferred to nitrocellulose membranes at 100 V for 70 min using a Transblot Cell from BioRad Laboratories (Hemel-Hempstead, Herts., UK). Blocking, washing, incubation with antibodies and enhanced chemiluminescence detection of immuno-decorated proteins was carried out by optimized methodology (Doran et al., 2007).

2.4. Confocal microscopy

Transverse cryosections with an approximate 10 μm thickness were prepared on a Thermo Shandon Cryotome (Life Science International Ltd., Cheshire, UK) and mounted on positively-charged microscope slides. Fixation, washing and immunolabelling with sufficiently diluted primary antibodies were carried out as previously described in detail (Doran et al., 2007). If Ca^{2+}-handling proteins of interest showed internal labelling patterns, fluorescent staining of laminin was employed to outline the fibre periphery. Alexa Fluor 488 dye or Alexa Fluor 594 dye conjugated secondary antibodies were used. Sections were viewed with a FluoView FV1000 confocal laser scanning microscope from Olympus (Hamburg, Germany), using the Olympus FluoView Version 1.3c software package.

3. Results

3.1. Immunoblot analysis of sarcalumenin in aged skeletal muscle

Following the gel electrophoretic separation of crude muscle extracts from 3-month, 6-month and 30-month old tibialis anterior, gastrocnemius and soleus muscle, the expression levels of key Ca^{2+}-handling proteins was determined by immunoblot analysis. While the overall protein band pattern was very comparable between young adult, adult and senescent fibre preparations from all three types of muscle (Fig. 1A), immunoblotting revealed drastic differences in the expression levels of important elements of excitation–contraction coupling (Fig. 1B and C). The analysis of SAR showed a severe reduction in its main 160 kDa band (Fig. 1D). In analogy to the reduced levels of this luminal Ca^{2+}-binding protein, the expression of two minor Ca^{2+}-extruding surface proteins, NCX and the PMCA-type of Ca^{2+}-ATPases, was found to be drastically reduced (Fig. 1E and F). Immunoblotting with antibodies to the fast and slow isoforms of the terminal cisternae Ca^{2+}-binding element calsequestrin and the SR Ca^{2+}-ATPase indicated a potential shift to a slower phenotype, but with the exception of increased levels of SERCA2 in aged soleus muscle these results were found not to be statistically significant (Fig. 1G, H, J and K). In contrast, as illustrated in the graph of Fig. 1B and C, the altered density of SAR, NCX and PMCA in aged fibres was clearly significant. The previously reported diminished levels of the voltage-sensing α_{1S}-DHP receptor were confirmed in this study (Delbono et al., 1995). In contrast to relatively comparable levels of the RyR1 Ca^{2+}-release channel (Fig. 1L), the principal ion channel-forming subunit of the transverse tubular Ca^{2+}-channel is greatly reduced in aged muscle (Fig. 1B and I).

3.2. Confocal microscopy of aged muscle fibres

As illustrated in Fig. 2A and B, a fast-to-slow transition process appears to occur during muscle aging. Although the above-outlined immunoblot analysis did not show major differences in the total

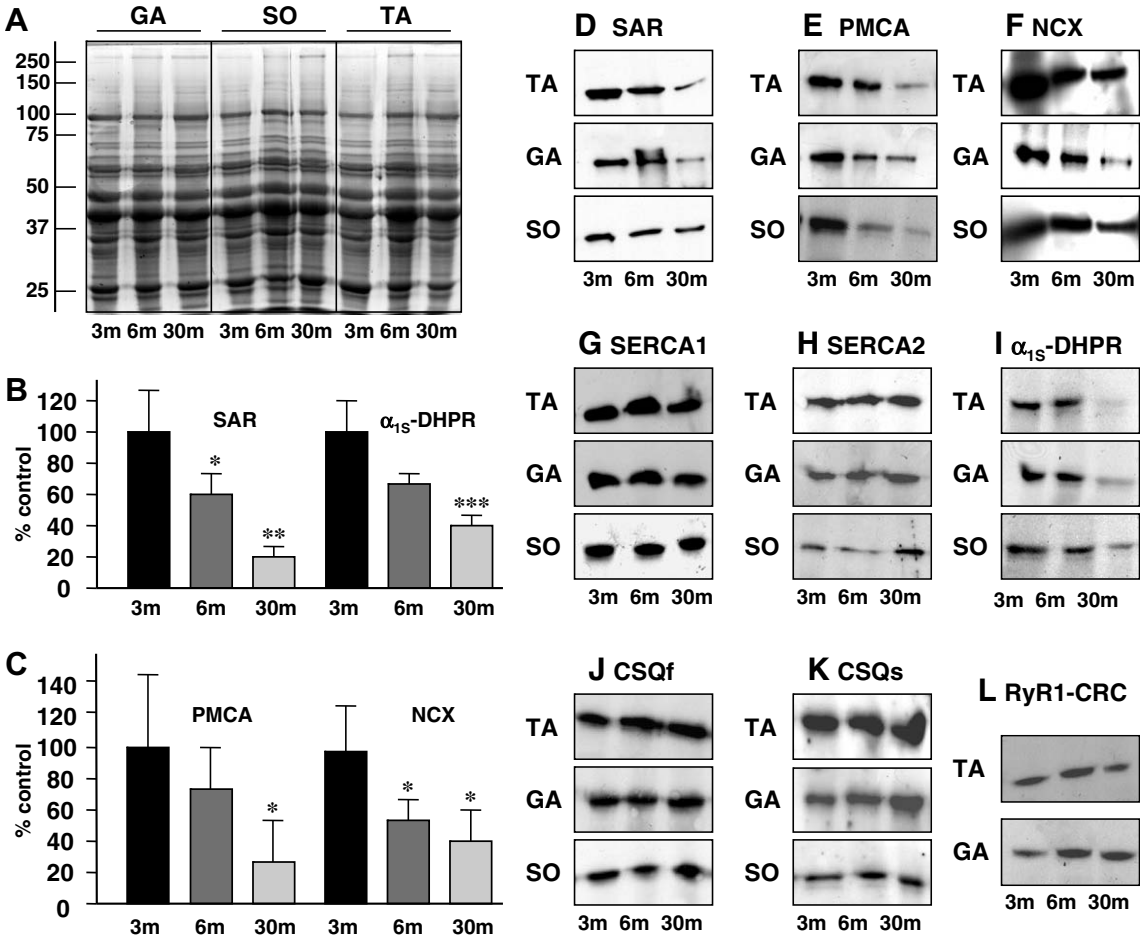

Fig. 1. Comparative immunoblot analysis of key Ca^{2+}-handling proteins in young adult, adult and senescent rat skeletal muscle. Shown is a Coomassie-stained gel (A) and immunoblots labeled with antibodies to sarcalumenin (D, SAR), the plasmalemmal Ca^{2+}-ATPase (E, PMCA), the Na^{+}–Ca^{2+}-exchanger (F, NCX), fast and slow sarcoplasmic reticulum Ca^{2+}-ATPase (G, SERCA1; H, SERCA2), the α_{1S}-subunit of the dihydropryridine receptor (I, α_{1S}-DHPR), fast and slow calsequestrin (J, CSQf; K, CSQs), and the ryanodine receptor Ca^{2+}-release channel (L, RyR1-CRC). 3-month (3 m), 6-month (6 m) and 30-month (30 m) old samples were isolated from tibialis anterior (TA), gastrocnemius (GA) and soleus (SO) muscle. Molecular mass standards (in kDa) are indicated on the left of the Coomassie-stained gel panel. The comparative blotting of gastrocnemius preparations was statistically evaluated using an unpaired Student's *t*-test ($n = 6$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; panels B and C).

amounts of SERCA2 present in crude extracts from young adult versus senescent gastrocnemius fibres, confocal microscopy indicates increased numbers of aged fibres expressing this slow SR marker protein. Labeling of the RyR1 Ca^{2+}-release channel seems to be slightly elevated in aged fibres (Fig. 2G and H). In analogy to the diminished levels of the α_{1S}-DHP receptor in 30-month-old gastrocnemius muscle (Fig. 2C and D), immunofluorescence microscopy confirmed the reduced levels of the luminal Ca^{2+}-shuttle protein SAR (Fig. 2E and F), the ion-exchanger NCX (Fig. 2I and J) and the sarcolemmal Ca^{2+}-ATPase PMCA (Fig. 1K and L).

4. Discussion

Ca^{2+}-cycling through the sarcoplasm regulates the contractile status of skeletal muscle fibres, making Ca^{2+}-ions key second messenger molecules of the excitation-contraction-relaxation cycle. Here, we could show that besides the previously established reduction in an L-type Ca^{2+}-channel of the transverse tubules, the voltage-sensing α_{1S}-DHP receptor (Ryan et al., 2001; Delbono et al., 1995), other essential components of Ca^{2+}-homeostasis are also affected during fibre aging. This includes the calmodulin-dependent PMCA of 140 kDa, which directly utilizes energy from ATP hydrolysis for lowering cytoplasmic Ca^{2+}-levels (Di Leva et al., 2008) and the electrogenic counter-transporter NCX, which indirectly uses the electrochemical transmembrane Na^{+}-gradient for moving Ca^{2+}-ions to the outside of the muscle fibre (Levitsky, 2007). However, since NCX and PMCA seem to play a less crucial role in Ca^{2+}-extrusion during fibre relaxation in skeletal muscle as compared to the heart, the most important finding of this study is the drastic reduction in the Ca^{2+}-shuttle element of the longitudinal SR. SAR of apparent 160 kDa contains in its amino-terminal region an acidic CSQ-like Ca^{2+}-binding domain and co-localizes with the SERCA-type Ca^{2+}-pumps (Leberer et al., 1990), which suggests that SAR plays a role in both Ca^{2+}-sequestration and Ca^{2+}-uptake mechanisms. Despite the potential role of SAR in enhancing SERCA stability, its reduction does not appear to affect the integrity of the overall SERCA population. Possibly reduced SAR levels do not directly alter SERCA expression in aged fibres, but diminish the luminal ion transport capacity from SERCA uptake domains towards CSQ-rich terminal cisternae regions. A reduced capacity of the longitudinal SR shuttle system would consequently also negatively influence the number of available ions for fast Ca^{2+}-release mechanisms, which could result in reduced muscle force. Impaired Ca^{2+}-cycling might trigger an abnormal excitation–contraction–relaxation cycle and thereby diminished contractile efficiency.

As reviewed by Rossi and Dirksen (2006), developmental studies have shown that SAR exhibits a gradual increase during fibre maturation in both skeletal and smooth muscle. The biochemical

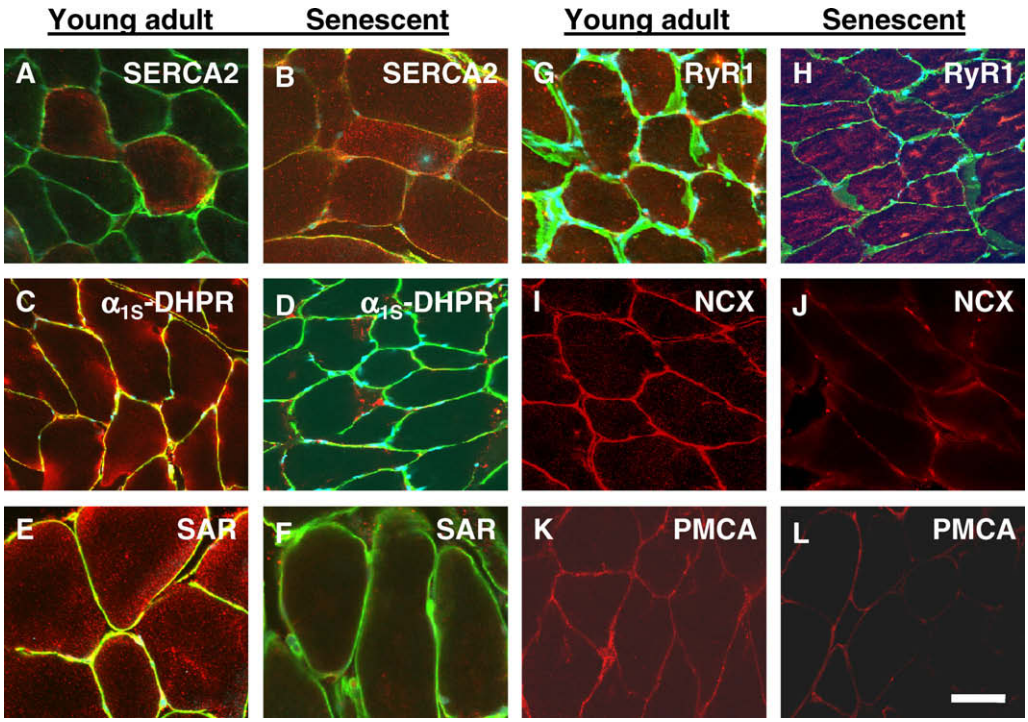

Fig. 2. Immunofluorescence survey of key Ca^{2+}-handling proteins in young adult versus senescent rat skeletal muscle. Indirect immunofluorescence microscopy was employed for the localization of the slow sarcoplasmic reticulum Ca^{2+}-ATPase (A and B; SERCA2), the α_{1S}-subunit of the dihydropyridine receptor (C and D; α_{1S}-DHPR), the Ca^{2+}-binding protein sarcalumenin (E and F; SAR), the ryanodine receptor Ca^{2+}-release channel (G and H; RyR1), the Na^{+}–Ca^{2+}-exchanger (I and J; NCX) and the plasmalemmal Ca^{2+}-ATPase (K and L; PMCA) in young adult (A, C, E, G, I, K) versus senescent (B, D, F, H, J, L) rat gastrocnemius muscle. Proteins of interest were labeled with red fluorescence, whereby in panels A–H green fluorescent staining of laminin was used to outline the cellular periphery. Bar = 40 μm.

analysis of nerve crushing and fast-to-slow muscle transformation has revealed a sharp decrease in SAR abundance following both denervation or chronic low-frequency simulation (Donoghue et al., 2004). SAR knockout mice exhibit enhanced resistance to fatigue and altered Ca^{2+}-handling properties (Zhao et al., 2005). SAR-deficient fibres show diminished Ca^{2+}-uptake into the SR lumen and enhanced store-operated Ca^{2+}-entry appears to be a result of chronic adaptation from SAR ablation (Yoshida et al., 2005). Taking into account the previously reported plasticity of SAR expression and the findings presented here, it can be concluded that aging-induced changes in SAR levels are due to secondary mechanisms of muscle degeneration and/or fibre transformation. This report has clearly shown that downstream from the impaired triadic receptor coupling process, age-dependent alterations also affect the density of other crucial Ca^{2+}-handling proteins. A decreased expression of SAR, NCX and PMCA may cause a lower capacity of luminal Ca^{2+}-shuttling from SERCA sites to CSQ-mediated storage domains and a decreased extrusion of Ca^{2+}-ions through the sarcolemma. Hence, abnormal Ca^{2+}-handling seems to play a pathophysiological role in the multi-factorial etiology of sarcopenia and might be directly involved in contractile weakness.

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