Cell Calcium 60 (2016) 245–255

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Reversible oxidation of vicinal-thiols motif in sarcoplasmic reticulum calcium regulatory proteins is involved in muscle fatigue mechanism

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ARTICLE INFO

Article history:
Received 17 February 2016
Received in revised form 1 June 2016
Accepted 3 June 2016
Available online 29 June 2016

Keywords:
Skeletal muscle
Vicinal thiols
Fatigue
SERCA
Ryanodine receptor
Redox

ABSTRACT

The mechanism underlying fatigue in skeletal muscle (SM) related to the redox-potential hypothesis, ranges from a direct effect of oxygen reactive species, to a number of other free radical intermediates targeting specific amino acids in the Ca^{2+}-regulatory proteins of the sarcoplasmic reticulum (SR). In the present study, we investigate the selective oxidation/reduction of the protein motif Cys-(Xn = 2–6)-Cys, known as a vicinal thiol group (VTG), present in the SR Ca^{2+}-ATPase (SERCA) and in the Ca^{2+}-channel ryanodine receptor (RyR) which are modified during muscle fatigue in SM. Selective oxidation of VTG with phenyl arsine oxide (PAO) increases fatigue in rat isolated SM and fatigue is prevented when muscle is previously incubated with a VTG selective reducing agent, 2,3-dimercaptopropanol (British anti-Lewisite (BAL)). In isolated SR membranes, PAO [<0.1 mM] modifies SERCA conformation and inhibits ATPase activity but does not affect Ca^{2+}-release. However, PAO at [>0.1 mM] inhibits SERCA and RyR activities in a reversible manner by selectively reducing them. Interestingly, as observed by differential scanning calorimetry, the conformation of SERCA from fatigued muscle changed in a similar manner as when SERCA VTG where oxidized. The addition of BAL to fatigued muscle restored the structural conformation and activity of SERCA with full recovery of muscle force production after fatigue. We conclude that VTG reversible oxidation of SR Ca^{2+} regulatory proteins are involved in muscle contraction/relaxation and are a molecular mechanism to be considered for muscle fatigue.

1. Introduction

Skeletal muscle cells are highly specialised for contraction/relaxation processes. In general, two types of muscle fibres result from muscle cell differentiation: slow fibres (type I) and fast fibres (type II), which have oxidative and glycolytic metabolism, respectively. Fast skeletal muscle experience a physiological phenomenon known as fatigue [1]. Muscle fatigue is defined as a failure to maintain the force of contraction resulting from repetitive stimulation, which renders the muscle incapable of subsequent contraction/relaxation cycles and refractory to further stimulation [2]. However, muscle force can be recovered after a period of rest. The fast skeletal muscle undergoes fatigue as a mechanism to prevent irreversible damage [3]. In contrast, slow skeletal muscle is less vulnerable to fatigue and as a result, a more intense stimulation protocol is needed to reach fatigue [4]. Under pathological conditions, such as muscular dystrophy, the irreversible loss of force for contraction after repetitive stimulation is an indicator of muscle damage, since muscle does not recover after a period of rest [5–7]. Membrane proteins such as the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase type 1 (SERCA1) and the Ca^{2+}-channel ryanodine receptor type 1 (RyR1) play a major role in the maintenance of the mechanical properties of muscle by regulating the intracellular calcium concentration ($[Ca^{2+}]_i$). Transverse Tubule membrane Ca^{2+}-ATPase (TT-PMCA) is also involved in Ca^{2+} regulation during prolonged muscle activity [8].

The mechanical activity of muscle is always accompanied by an increase in O_2 consumption and reactive oxygen species (ROS) production. Nitric oxide (NO) production by nitric oxide synthase (NOS-1) is stimulated by mechanical activity. The superoxide anion ($O_2^{\bullet -}$) is the first free radical to appear during oxidative

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http://dx.doi.org/10.1016/j.ceca.2016.06.001
0143-4160/

stress [9,10]. Nitrogen radical species (NRS) are found in low concentrations in the resting skeletal muscle but increase during periods of prolonged muscle activity [11,12]. NRS derivatives have modulatory effects on several skeletal muscle functions, such as excitation/contraction coupling (ECC), glucose metabolism, mitochondrial energy production, auto regulation of blood flow [13–15] and inhibitory effects on muscle force development [15]. High concentrations of exogenous ROS and NO have been used to diminish the force of contraction in experimental conditions [16,17]. The thiol oxidation of sarcoplasmic reticulum (SR) membrane proteins is a critical event for the activation of RyR1 [18,19] and the inhibition of SERCA1 during muscle mechanical activity [20]. We previously suggested that reversible oxidation of VTG in SERCA1 and RyR1 is a mechanism involved in the redox hypothesis for muscle fatigue [21].

The SR membrane proteins undergo post-transcriptional changes, such as phosphorylation, nitrosylation and thiolation, to regulate the $[Ca^{2+}]_i$ during muscle contraction/relaxation [22–25]. SERCA1 and RyR1 are vicinal thiol proteins (VT-proteins), which are defined as proteins that contain one or more VTG [21,26]. In VT-proteins the thiol group of each cysteinyl side chain is susceptible to a number of oxidative modifications, such as the formation of inter- or intramolecular disulphide linkages between protein thiols (P-S-S-P) and low-molecular-weight thiols, such as glutathione (P-S-SG), oxidation to sulphenic (P-SOH), sulphinic ($P\text{-}SO_2H$) and sulphonic ($P\text{-}SO_3H$) acids, *S*-carbonylation (carbonyl groups introduced to proteins causing irreversible oxidation) [27], *S*-nitrosation and *S*-nitrosylation (nitroso groups introduced to proteins causing reversible oxidation) [25,28]. These changes alter numerous protein functions that contain structurally important cysteines. VTG located at the protein surface are able to interact with oxidants and disulphide-reducing enzymes regulated by redox potential [29,30]. Membrane proteins, such as SERCA1, have a VTG in close proximity to the enzyme nucleotide binding and phosphorylation sites located in the cytosolic domain. *Oxidised* VTG-SERCA1 (SERCA1-S_2), results in diminished ATPase and calcium transport activities, thereby inhibiting muscle relaxation [21]. We previously showed that the *reduced* VTG-SERCA1 (SERCA1-$(SH)_2$) is fully active.

In this study, we aimed to demonstrate that SERCA1 and RyR1 are targets for reversible VTG oxidation during muscle fatigue. Isolated fast skeletal muscle is resistant to fatigue when the VT-proteins are reduced and undergo fatigue more quickly when the VT-proteins are oxidised. The ability of VT-proteins to undergo reversible disulphide formation enables these proteins to function as nano-switches and control the $[Ca^{2+}]_i$ in a redox-dependent manner, thereby regulating muscle contraction/relaxation. Excess cytosolic Ca^{2+} in muscle cells after prolonged muscle activity is extruded through PMCA, delaying the effects of fatigue [8]. The TT membrane PMCA is not affected by thiol-oxidising or -reducing agents and is active under a muscle transient oxidative stress.

2. Materials and methods

2.1. Animals

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the United States and approved by the Ethics Committee of the School of Medicine of the National Autonomous University of Mexico (UNAM) (NOM-062-ZOO1999).

2.2. Materials

Phenylarsine oxide (PAO), 2,3-dimercaptopropanol (British anti-lewisite; BAL), *N*-ethylmaleimide (NEM) and dithiothreitol (DTT) were purchased from Sigma-Aldrich Co.

2.3. Muscle preparation

Male Wistar rats weighing 280–300 g were euthanized by cervical dislocation, and the *extensor digitorum longus* (EDL) was isolated at room temperature. The isolated muscle was placed in an acrylic chamber equipped with platinum electrodes along each side of the chamber wall, so that the electrodes contacted the entire length of the muscle. Krebs solution containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM dextrose, 1 mM $NaPO_4$ (dibasic) and 15 mM $NaHCO_3$ was bubbled with 95% O_2 and 5% CO_2 to reach pH 7.0. The EDL muscle was fastened by the distal tendon to forceps and by the proximal tendon to a force transducer. The platinum electrodes were connected in parallel to two stimulators (SD9; Grass).

2.4. Stimulation protocol

Single electrical pulses of 0.6 ms were used to reach the voltage for maximal tension. To obtain the optimal sarcomere length (2.4 μm), the muscles were stretched to the length at which the twitch force was maximal. Tetanic stimulation was accomplished by subjecting the muscles to 75 Hz at 60 V for 1 s followed by 3 min rest. This protocol was repeated several times with 3-min periods of rest to ensure that at least 3 control tetanic forces were achieved. This force was considered as the control maximal tetanic tension. At the end of the protocol, the muscle was rested for 10 min before beginning the fatigue protocol.

2.5. Single twitch stimulation training protocol for fatigue

A single electrical stimulation of 100 V for 0.6 ms was applied in succession every 0.3 s for 20 min. When the tension reached 30% of the original tension, the training was discontinued and the muscle was rested for 5 min before starting the tension recovery experiment. Previously described stimulation protocols [31,32] that used tetanic stimulation of 75 Hz at 60 V for 1 s followed by a single twitch for 1.5 s were used to restore muscle force.

2.6. Isolation of transverse tubule (TT) and sarcoplasmic reticulum membranes

TT and SR membranes were obtained from the fast skeletal muscle (forelimb, hindlimb and back muscles). The isolation was performed by differential centrifugation in a discontinuous sucrose gradient as previously described [31,32]. Membrane isolation was performed in the absence of any reducing agent in the buffer. The microsomal fraction was placed in a sucrose gradient of 25%, 27.5% and 35% (w/v). The 25/27.5% interface had the maximal signal for dihydropyridin receptor, as determined by immunoblotting, indicating the presence of TT membranes. When required, the isolated TT membranes were incubated in a Ca^{2+} loading solution in the presence of 5 mM potassium oxalate and centrifuged through a 25/45% discontinuous sucrose gradient to remove any Ca^{2+} oxalate-loaded vesicles [34]. Light SR (LSR) was isolated from the 35% band obtained from the first discontinuous sucrose gradient as previously described [33]. Measuring the maximum ATPase activity stimulated by Ca^{2+} identified the LSR. The protein concentration of each sample was determined using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) with BSA as the standard.

2.7. Immunoblotting

SR and TT membrane protein samples were subjected to 4–20% SDS-PAGE (Pierce) and transferred to nitrocellulose membranes (Millipore Corporation). Immunoblotting was performed using the