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Modulation of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase activity and oxidative modification during the development of adjuvant arthritis

Miriam K. Strosova [a], Janka Karlovska [b], Petronela Zizkova [a], Magdalena Kwolek-Mirek [c], Silvester Ponist [a], Corinne M. Spickett [d,e], Lubica Horakova [a,*]

[a] Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovakia
[b] Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia
[c] Department of Molecular Biophysics, University of Lodz, Poland
[d] Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, Scotland, UK
[e] School of Life & Health Sciences, Aston University, Birmingham, UK

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ABSTRACT

Adjuvant arthritis (AA) was induced by intradermal administration of *Mycobacterium butyricum* to the tail of Lewis rats. In sarcoplasmic reticulum (SR) of skeletal muscles, we investigated the development of AA.

SR Ca^{2+}-ATPase (SERCA) activity decreased on day 21, suggesting possible conformational changes in the transmembrane part of the enzyme, especially at the site of the calcium binding transmembrane part. These events were associated with an increased level of protein carbonyls, a decrease in cysteine SH groups, and alterations in SR membrane fluidity. There was no alteration in the nucleotide binding site at any time point of AA, as detected by a FITC fluorescence marker. Some changes observed on day 21 appeared to be reversible, as indicated by SERCA activity, cysteine SH groups, SR membrane fluidity, protein carbonyl content and fluorescence of an NCD-4 marker specific for the calcium binding site.

The reversibility may represent adaptive mechanisms of AA, induced by higher relative expression of SERCA, oxidation of cysteine, nitration of tyrosine and presence of acidic phospholipids such as phosphatidic acid. Nitric oxide may regulate cytoplasmic Ca^{2+} level through conformational alterations of SERCA, and decreasing levels of calsequestrin in SR may also play regulatory role in SERCA activity and expression.

Introduction

There is increasing evidence that oxidative/nitrative stress, involving increased levels of reactive oxygen and nitrogen species (ROS[1] and RNS), are involved both in rheumatoid arthritis, muscle dysfunction, and in a variety of animal models of experimental arthritis [1–4]. ROS/RNS can cause specific, reversible and/or irreversible oxidative modifications on sensitive proteins that may lead to a change in the activity or function of the oxidized protein. Under conditions of ROS/RNS stress, the thiols in cysteine residues are among the most susceptible oxidant-sensitive targets and can undergo various reversible and irreversible redox alterations [5]. The formation of nitrotyrosine is a major peroxynitrite-mediated protein modification [6]; increased levels of peroxynitrite are found in the joints of RA patients [7] and in an animal model of RA [8]. Accumulation of nitrotyrosine has been associated with reduced production of skeletal muscle force in pathological conditions [9].

Oxidative/nitrative stress may induce calcium homeostasis imbalance and in particular dysregulation of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA; EC 3.6.3.8). SERCA plays a key role in relaxation of smooth, cardiac and skeletal muscle through the transport of cytosolic Ca^{2+} into sarcoplasmic (SR) or endoplasmic reticulum (ER) [10]. Abnormal activity and/or expression of SERCA are associated with other physiological or pathological processes like cell proliferation, apoptosis, muscle diseases and cancer. An important feature of SERCA is its high susceptibility to modification by ROS, and its complex regulation by oxidative modification. Opposing effects may be induced by NO, according to its concentration. Low levels can cause site-specific glutathiolation of cysteine

* Corresponding author. Address: Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dubravska Cesta 9, 841 04 Bratislava, Slovakia. Fax: +421 2 5477 5928.
E-mail address: lubica.horakova@savba.sk (L. Horakova).

[1] *Abbreviations used:* AA, adjuvant arthritis; a.u., arbitrary units; Ca^{2+}_{free}, free calcium ions; Co, control; DTT, dithiotreitol; FITC, fluorescein-5-isothiocyanate; iNOS, inducible nitric oxide synthase; *M. butyricum*, *Mycobacterium butyricum*; NCD-4, N-cyclohexyl-N′-(4-dimethylamino-l-naphthyl) carbodiimide; NO, nitric oxide; pATP, negative logarithm of the concentration of ATP; PA, phosphatidic acid; pCa_{free}, negative logarithm of the concentration of free Ca^{2+} ions; RA, rheumatoid arthritis; RFUs, relative fluorescence units; RNS, reactive nitrogen species; ROS, reactive oxygen species; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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residues, associated with an increase in activity and subsequent NO-dependent cardiac, skeletal and vascular relaxation [10–13]. In contrast, higher, practically pathological levels of NO or peroxinitrite cause inactivation of SERCA, possibly associated with tyrosine nitration [14,10]. Nevertheless, it has been suggested that nitration can be considered as a regulatory feature, rather than one reflecting protein damage [12].

An opposing modulation of SERCA activity by ROS and RNS has been found *in vitro,* in aging, some pathological conditions and in muscle contraction/relaxation. Although it is clear that ROS and RNS play a role in AA, and can modulate SERCA activity, the role and mechanisms of SERCA modulation by ROS and RNS in the course of AA are unknown. In contrast to RA, systemic inflammation in the AA model falls spontaneously after day 21; adaptive and repairing mechanisms in tissues start to occur but the morphological and functional damage to the hind paw joint remains. SERCA activity and calcium status in the muscle may play a role in this adaptation.

We hypothesized that changes in SERCA activity induced by oxidative and nitrative stress may contribute to and reflect the disease state in AA, over the timecourse of the disease. In the present study, we investigated whether functional alterations of SERCA correlate with post-translational and conformational changes of the SERCA protein; the changed composition of SR lipids in AA (increase of acidic lipids) also contributes to the modulation of SERCA function; and finally, if nitric oxide (nitration of tyrosine) is involved in alterations of cytoplasmic Ca^{2+} level due to conformational alterations of SERCA.

Material and methods

Experimental model

AA was induced by intradermal injection of heat-inactivated *Mycobacterium butyricum* into the base of the tail of Lewis rats (approximately 160 g). SR vesicles were isolated from skeletal muscle of hind paws of control and AA rats according to the method of Warren et al. [15]. The skeletal muscle for SR isolation was taken from the hind paws at different times of AA: on days 7, 14, 21, 28 after *M. butyricum* injection.

All procedures involving animals were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, and by the State Veterinary and Food Administration of the Slovak Republic.

Ca^{2+}-ATPase activity

The activity of SR Ca^{2+}-ATPase (SERCA) from AA samples was measured by the NADH-coupled enzyme assay outlined by Warren et al. [15]. The SR vesicles (final concentration 12.5 μg protein/cuvette) were added to the assay mixture (40 mM Hepes pH 7.2, 0.1 M KCl, 5.1 mM $MgSO_4$, 2.1 mM ATP, 0.52 mM phosphoenolpyruvate, 1 mM EGTA, 0.15 mM NADH, 7.5 IU of pyruvate kinase, 18 IU of lactate dehydrogenase) and incubated at 37 °C for 10 min. The calcium ionophore A-23187 (SIGMA), at a final concentration of 0.2 μg/ml, was added to some key samples to demonstrate that alterations in membrane Ca^{2+} permeability were not an important factor with respect to Ca^{2+}-ATPase activity. The reaction was started by addition of $CaCl_2$ (final concentration 1 mM). The reaction rate was determined by measuring the decrease of NADH absorbance at 340 nm, at 37 °C.

ATPase activity was studied also as a function of ATP and free Ca^{2+} concentrations. Concentrations of free Ca^{2+} were calculated by the computer program Maxchelator [16] using the binding affinities described by Gould et al. [17].

The dependence of ATPase activity on Ca^{2+}_{free} concentration was fitted to the Hill equation:

$$A = \frac{V_{max}\left[Ca^{2+}_{free}\right]^h}{K^h + \left[Ca^{2+}_{free}\right]^h}$$

where V_{max} is the activity of Ca^{2+}-ATPase at saturating concentration of the substrate, K is the concentration of Ca^{2+}_{free} corresponding to one-half of V_{max}, and h is the Hill coefficient, an indicator of steepness of the curve. The dependence of ATPase activity on ATP concentration was fitted to the bi-Michaelis–Menten equation [18,19].

$$[EP] = \frac{V'_{max}\cdot[Mg\cdot ATP]}{K'_m + [Mg\cdot ATP]} + \frac{V''_{max}\cdot[Mg\cdot ATP]}{K''_m + [Mg\cdot ATP]}$$

where V^i_{max} is the activity at saturating concentrations of the substrate and K^i_m is the Michaelis constant. The indices i = I and II mean high- and low-affinity binding sites, respectively.

Effect of phosphatidic acid on SERCA activity

Phosphatidic acid (PA) was prepared in 40 mM Hepes (pH 7.2) with 0.1 M KCl. Unilamellar liposomes were obtained by sonication of the PA lipid suspension for 5–10 min followed by 51 extrusions through a polycarbonate filter (Nuclepore) with pores of 200 nm diameter using the LiposoFast Basic extruder (Avestin) fitted with two gas tight Hamilton syringes (Hamilton), as described by MacDonald et al. [20]. SR vesicles (12.5 μg protein/cuvette) were incubated for 20 min at 37 °C with suspensions of unilamellar liposomes with increasing concentrations of PA in the reaction buffer for determination of Ca^{2+}-ATPase activity. Afterward, the measurement of ATPase activity was started by addition of $MgSO_4$ (5.1 mM) and $CaCl_2$ (1 mM), as mentioned above.

SDS–PAGE

SR proteins (1 mg/ml) were separated by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli et al. [21] by using a mini-PROTEAN II electrophoresis cell (Bio-Rad). Aliquots of the samples in Laemmli-buffer (pH 6.8) containing 25 mM Tris, 1% SDS, 0.192 M glycine, 1% bromphenol blue and 5% mercaptoethanol added freshly to the buffer each time, were incubated for 10 min at 95 °C and then loaded onto an SDS–polyacrylamide gel (7.5% separating- and 4% sticking-gel). The separation was performed for 0.5 h at 50 V and afterward for approximately 1.5 h at 150 V, until the first marker reached the end of the gel. Visualization was performed by Coomassie Brilliant Blue staining. For size determination of the protein bands, the "Precision Plus Protein™ Dual Color Standard marker" (Bio-Rad) was used. The densities of the bands on the gels were determined using Adobe Photoshop 7.0 software.

Western blotting

After SDS–PAGE, the proteins were transferred to Immobilon-P PVDF membrane (Millipore) by semidry immunoblotting (Hoefer Scientific Instruments) at 250 A for 25 min. After blocking with 3% BSA, the membrane was exposed to the primary monoclonal antibody specific for rat SERCA1 (IIH11, Santa Cruz), which was diluted 1:200 in 1.5% BSA, or monoclonal antibody against calsequestrin1 (6D201, Santa Cruz), which was diluted 1:500 in 1.5% BSA. In addition, a polyclonal antibody against nitrotyrosine (PNK, Santa Cruz) diluted 1:200 in 1.5% BSA was used. Incubation of the