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Modulation of SERCA in the chronic phase of adjuvant arthritis as a possible adaptation mechanism of redox imbalance

MIRIAM STROSOVA[1], JANA KARLOVSKA[2], CORINNE M. SPICKETT[3], ZUZANA ORSZAGOVA[4], SILVESTER PONIST[1], KATARINA BAUEROVA[1], DANICA MIHALOVA[1], & LUBICA HORAKOVA[1]

[1]*Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia,* [2]*Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia,* [3]*Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, Scotland, UK, and* [4]*Department of Medical Chemistry and Biochemistry, Faculty of Medicine, Comenius University, Bratislava, Slovakia*

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Abstract

Adjuvant arthritis (AA) is a condition that involves systemic oxidative stress. Unexpectedly, it was found that sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity was elevated in muscles of rats with AA compared to controls, suggesting possible conformational changes in the enzyme. There was no alteration in the nucleotide binding site but rather in the transmembrane domain according to the tryptophan polar/non-polar fluorescence ratio. Higher relative expression of SERCA, higher content of nitrotyrosine but no increase in phospholipid oxidation in AA SR was found. *In vitro* treatments of SR with HOCl showed that in AA animals SERCA activity was more susceptible to oxidative stress, but SR phospholipids were more resistant and SERCA could also be activated by phosphatidic acid. It was concluded that increased SERCA activity in AA was due to increased levels of SERCA protein and structural changes to the protein, probably induced by direct and specific oxidation involving reactive nitrogen species.

Keywords: *Adjuvant arthritis, oxidative stress, Ca^{2+}-ATPase, sarcoplasmic reticulum, nitrotyrosine*

Abbreviations: *AA, adjuvant arthritis; a.u., arbitrary units; BSA, bovine serum albumin; Ca_i, intracellular calcium; Ca^{2+}_{free}, free calcium ions; Co, control; DNPH, dinitrophenylehydrazine; FITC, fluorescein-5-isothiocyanate; GSH, reduced glutathione; I, fluorescence intensity; IC_{50}, half maximal inhibitory concentration; LC-MS, liquid chromatography-mass spectrometry; NO, nitric oxide; pATP, negative logarithm of the concentration of ATP; PA, phosphatidic acid; PCs, phosphatidylcholines; PEs, phosphatidylethanolamines; PLs, phospholipids; 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; TBARS, thiobarbituric acid reactive substances*

Introduction

Oxidative stress and redox imbalance contribute to the pathogenesis of chronic inflammatory diseases, including rheumatoid arthritis (RA). The chronic vascular inflammation associated with RA results in accumulation of numerous cell types (activated macrophages, neutrophils, mast cells and lymphocytes) in the synovium and when activated these cell types can generate ROS [1–3]. In addition to the direct damaging effects of oxidative stress, numerous studies have indicated that oxidative stress exerts also more subtle effects on cellular function. The tightly

Correspondence: Lubica Horakova, Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dubravska cesta 9, 841 04 Bratislava, Slovakia. Tel: +421 2 5941 0656. Fax: +421 2 5477 5928. Email: lubica.horakova@savba.sk

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regulated homeostatic control of ROS/RNS influences the activity of diverse intracellular molecules and signalling pathways [4–7]. Therefore ROS/RNS are not only toxic agents, but also mediators of physiological function by serving as second messengers. Their role in regulating various inflammatory processes can contribute to the pathogenesis of inflammatory diseases [2,5,7].

Numerous animal studies using a variety of experimental models have documented increased levels of ROS/RNS in joints and plasma of animals with experimental arthritis [2] and markers of lipid and protein oxidation were increased in joint and plasma of arthritic animals [2,8,9]. Correspondingly, antioxidants such as reduced glutathione (GSH), vitamins E and C [10] and the antioxidant enzymes catalase, glutathione reductase and superoxide dismutase have been found to be decreased in joints and plasma of arthritic animals [11–13].

Oxidative stress is tightly connected with an imbalance of calcium homeostasis. Intracellular calcium levels play a role in many signalling functions and are modulated by calcium-regulating proteins, including the Ca^{2+}-ATPase from sarco/endoplasmic reticulum (SERCA) and calsequestrin [14–16]. SERCA pumps calcium from the cytosol into the sarcoplasmic reticulum against a large concentration gradient (i.e. 10 000-fold) [16], which requires a major energy expediture within excitable cells and is tightly coupled to the rate of oxidative phosphorylation and the generation of ROS in mitochondria. It has been found that Ca^{2+}-ATPase pumps, especially SERCA, are highly susceptible to damage by ROS, leading to a decrease of SERCA activity, which may in turn result in elevation of the intracellular calcium concentration, although it has also been reported that NO causes glutathionylation of SERCA, resulting in its activation [16]. Alterations in calcium balance affect many aspects of cellular regulation and may have deleterious effects.

It has been observed that loss of calcium homeostasis occurs during biological ageing and is correlated with oxidative modifications of SERCA and another calcium regulatory protein, calmodulin [16–19]. As oxidative stress also occurs in rheumatoid arthritis and animal models of AA [2], it is likely that SERCA activity and calcium homeostasis will be disrupted in these conditions, but as yet the relationship between redox imbalance in RA or AA and modulation of calcium homeostasis has not been studied. A better understanding of the molecular mechanisms underlying RA is essential to the introduction of new therapies, for example by determining whether therapies that restore the redox balance may have beneficial effects on the disease process [2].

To address these questions on the relationship between AA, oxidative stress and SERCA function, we analysed SR of skeletal muscles from rats with adjuvant arthritis and compared it with SR from control animals. We hypothesized that functional alterations of SERCA would correlate with post-translational and conformational changes of the SERCA protein and that SR lipid oxidation or changes in phospholipid composition during AA might also contribute to modulation of SERCA function.

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 (~160 g). SR vesicles were isolated from skeletal muscle of hind paws of rats according to the method of Warren et al. [20,21]. The skeletal muscle for SR isolation was withdrawn from the hind paws in the chronic phase of AA on day 28 after *Mycobacterium butyricum* injection. SR vesicles were prepared from control and AA muscle essentially as described by Warren et al. [20,21].

Oxidation of SR vesicles

SR vesicles (100 μg protein/ml) from control rats and rats suffering from AA were oxidized by HOCl (50–200 μM) at 25°C, pH 7.2 for 3 min. The oxidation was stopped by addition of cysteine (1 mM).

Ca^{2+}-ATPase activity

Enzyme activity of SR Ca^{2+}-ATPase (SERCA) from AA samples was measured by the NADH-coupled enzyme assay outlined by Warren et al. [20,21]. 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 15 min. The calcium ionophore A-23187, 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. The reaction was started by the 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.

We studied the ATPase activity also as a function of ATP and free Ca^{2+} concentrations. Concentrations of free Ca^{2+} were calculated by the computer program Maxchelator [22] using the binding affinities described by Gould et al. [23]. The dependence of ATPase activity on Ca^{2+}_{free} concentration was fitted to the Hill equation:

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

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 the $V_{\max}$ 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 [24,25]:

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

where $V^i_{\max}$ is the activity at saturating concentrations of the substrate and $K_m{}^i$ is the Michaelis constant. The indices $I = \mathrm{I}$ 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. Unilamelar liposomes were obtained by sonication of this PA lipid suspension for 5–10 min followed by 30 extrusions through a polycarbonate filter (Nuclepore) with pores of diameter 200 nm, using the LiposoFast Basic extruder (Avestin) fitted with two gas tight Hamilton syringes (Hamilton) as described by MacDonald et al. [26]. 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. Afterwards, 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 denaturizing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [27] 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% bromphenole 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% stacking-gel). The separation was performed for 0.5 h at 50 V and afterwards for ~2 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 Colour 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 semi-dry immunoblotting (Hoefer Scientific Instuments) 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 PVDF membrane with the primary antibodies was performed overnight at 4°C. After washing, a secondary antibody was used. The secondary antibody for all three antibodies SERCA1, calsequestrin1 and nitrotyrosine was monoclonal anti-rabbit-IgG-antibody peroxidase conjugate (Sigma). The bands were visualized by the ECL Immobilon™ Western detection kit (Milli pore) according to the manufacturer's instructions and the images were captured on a Kodak X-ray film using a Kodak-developer/fixer kit (Fisher).

ELISA

A sensitive enzyme linked immunosorbent assay (ELISA) as described by Buss et al. [28] was used for the quantitative determination of protein carbonyls in SR vesicles and plasma. Protein samples were derivatized with dinitrophenyl-hydrazine (DNPH) and adsorbed in multiwell-plates (Nunc Immunosorp plates, Roskilde, Denmark). A biotin-conjugated anti-dinitrophenyl-rabbit-IgG-antiserum (Sigma, USA) was used as primary antibody and a monoclonal anti-rabbit-IgG-antibody peroxidase conjugate (Sigma, USA) as secondary antibody. The development was performed with o-phenylendiamine. Absorbance was determined at 492 nm. The method was calibrated using oxidized bovine serum albumin (BSA). Oxidized and reduced BSA were prepared according to the method of Buss et al. [28].

Sulphydryl group determination

The content of reduced SH groups in the SR was determined spectrophotometrically by measuring the absorbance at 412 nm after derivatization with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to Favero et al. [29]. SR (100 μg) was diluted into the assay medium containing 50 mM tris(hydroxymethyl) aminomethane (pH 7.0), 1% sodium dodecyl sulphate (SDS), 1 mM EDTA and 1 mM DTNB. Absorbance changes were monitored for 8 min, after which no additional absorbance changes could be observed. GSH standard dilutions were used for the calibration. Free SH groups were

calculated according to the Beers-Lambert relationship by using a molar extinction coefficient of 5300.

Tryptophan (Trp) fluorescence

The fluorescence intensity (I) of Trp residues is lower in water with the emission maximum at 358 nm and higher in dioxane (non-polar environment) with the emission maximum at 336 nm [30]. The intensity ratio I_{358nm}/I_{336nm} corresponds to fluorescence of Trp residues in polar (cytosol) and non-polar (membrane) environment and can be a marker of SERCA Trp conformational changes. Trp fluorescence in membrane proteins of SR was measured on a Perkin Elmer LS45 spectrofluorometer (Waltham) at 25°C. Trp emission spectra were collected by exciting at 290 nm and the emission spectra were recorded in the range of 300–450 nm [31]. The SR vesicles (final concentration 15 μg protein/cuvette) were added to the Hepes buffer (pH 7.2) containing 20 mM Hepes, 0.1 M KCl, 5.1 mM $MgSO_4$, 25 μM EGTA.

Labelling of Ca^{2+}-ATPase by FITC

ATPase from SR was labelled with fluorescein-5-isothiocyanate (FITC) according to the method of Fround and Lee [32]. FITC 'Isomer I' was obtained from Invitrogen™. When FITC:ATPase labelling ratios are below 1:1, at pH 8, reaction of binding FITC to ATPase is complete, so that separation of bound and free FITC becomes unnecessary [32].

We used FITC:ATPase labelling ratio of ~0.5:1. SR (0.6 mg) in 1 M KCl, 0.25 M sucrose and 50 mM Hepes, pH 8, in the volume 35 μl. The reaction mixture was incubated with 2.5 nmol of FITC. Stock solution of FITC (6 mM) was prepared in dry dimethylformamide. The reaction mixture was left to stand at room temperature in the dark for 1 h and then diluted with 250 μl of 0.2 M sucrose and 50 mM Tris-HCl pH 7. Labelled samples were stabilized for 30 min at room temperature and stored on ice until use.

Fluorescence spectra were measured on a Perkin Elmer LS45 spectrofluorometer (Waltham) at 25°C and relative fluorescence units (RFUs) were recorded. Labelled protein (15 μg) was added to 1 ml of 5 mM $MgSO_4$, 100 mM KCl and 50 mM Tris-HCl buffer, pH 7, at 25°C. EGTA and $CaCl_2$ were added from stock solutions to give total concentrations of 25 μM and 0.8 mM, respectively. Fluorescence spectra were collected by exciting at 485 nm and the emission spectra were recorded in the range of 500–600 nm.

Determination of lipid peroxidation

The concentration of thiobarbituric acid reactive substances (TBARS) was used as the index of lipid peroxidation as described previously [33]. Briefly, 1.5 ml of the reaction mixture consisting of 750 μl of 0.67% thiobarbituric acid and 750 μl of 20% trichloroacetic acid were added to the plasma (150 μl) diluted with 350 μl of PBS. The samples were incubated in a water bath (90°C) for 30 min and after being cooled on ice, the samples were centrifuged (10 min, 2000 g). The absorbance of the supernatant was measured at 532 nm.

ES-MS and LC-MS

ES-MS was performed in positive-ion mode on a LCQ Duo ion trap mass spectrometer (Thermo-Finnigan). For direct infusion of samples the solvent system was 9:1 methanol/water (v/v), with a flow rate of 5 μl/min. The capillary temperature was set to 200°C, with a nebulizing gas flow of 20 l/h and a drying gas flow of 400 l/h. Data were collected between 400–1000 m/z. Rat SR vesicles were prepared in 9:1 methanol/water (v/v) (usually 1 μl in 100 μl) and diluted 10-fold in this solvent.

Reverse phase liquid chromatography-mass spectrometry (LC-MS) was carried out essentially as decribed previously [34], using the Thermo Finnigan Surveyor system and a Luna C8 column (5 μm RP-Sct, 1 mm inner diameter × 150 mm, Phenomenex). This column was operated at a flow rate of 100 μl/min with an isocratic solvent system of 71:4:8 (by vol.) methanol/hexane/0.1 M aq. ammonium acetate. Rat SR vesicles were reconstituted in 9:1 methanol/water as above and diluted 5-fold in running solvent; 20 μl of sample were injected per run.

Reconstructed ion chromatograms showing individual molecular species were generated using Excalibur software (Thermo-Finnigan) and were mean-smoothed. In all the spectra and chromatograms shown the percentage scale on the vertical axes corresponds to intensity related to that of the largest peak in the region analysed, unless otherwise stated.

Analysis of phospholipids was also carried out on a QTrap 2000 mass spectrometer (Applied Biosystems) running Analyst software version 1.4.1. SR samples were diluted at least 1 in 1000 in 9:1 methanol/water and infused by nanospray using Proxeon nanospray tips mounted on a Proxeon nanospray source. The spray voltage was set to 800 V and the curtain gas to the maximum flow that did not reduce signal intensity. Data were collected to give sufficient time for a good signal-to-noise ratio in the summed spectra (usually 2–5 min). Standard spectra were acquired in positive ion mode in the range 400–1000 Da. Precursor ion of m/z 185 experiments were performed in positive ion mode, using a collision energy of 40 V scanning 500–900 Da with a step size of 0.1 Da and a scan time of 4 s and Q1 and Q3 set to unit resolution. Neutral loss of m/z 141 experiments

was run under identical conditions to the precursor ion except using collision energy of 50 V.

Statistical data analysis

The statistical analyses of the obtained data was performed using unpaired two-tailed *t*-test. The values were expressed as mean ± SEM. Statistical significance stages were set at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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, Slovak Academy of Sciences and by the State Veterinary and Food Administration of the Slovak Republic.

Results

Oxidative stress in plasma

To confirm that AA resulted in a systemic oxidative stress, lipid and protein oxidation markers were measured in plasma of control and AA animals (Figure 1). Protein carbonyls were determined by ELISA and a substantial (~3-fold) increase in protein carbonyls was found in the plasma of AA rats compared to control animals (Figure 1A). Lipid peroxidation in plasma was measured by TBARS and a small but significant elevation of these products was found in plasma of rats suffering from AA (Figure 1B).

SERCA activity

The activity of Ca^{2+}-ATPase is dependent on the presence of the two substrates, free calcium ions (Ca^{2+}_{free}) and ATP. The ATPase activity of SERCA in the presence of optimal $[Ca^{2+}_{free}] = 1.7\ \mu M$ and $[ATP] = 2.1$ mM concentrations is depicted in Figure 2A and is in good correlation with other authors [35,36]. The animals suffering from AA had approximately twice the enzyme activity, compared with controls. The presence of the Ca^{2+} ionophore A-23187 increased the Ca^{2+}-ATPase activity by 47% in control and 53% in adjuvant SR samples, but approximately the same increase between control and AA samples was observed in presence or absence of calcium ionophore (Figure 2A).

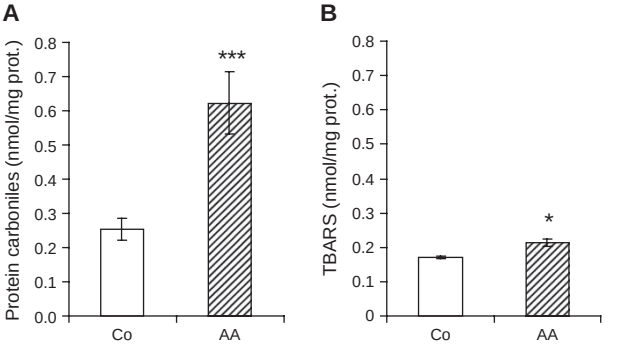

Figure 1. Protein carbonyls and TBARS formation in plasma. Protein carbonyls in plasma of control and AA rats were determined by ELISA. Carbonyls of protein samples were derivatized with dinitrophenyl-hydrazine (DNPH). A biotin-conjugated anti-dinitrophenyl-rabbit-IgG antiserum was used as primary antibody and a monoclonal anti-rabbit-IgG antibody peroxidase conjugate as secondary antibody (A). Plasma (150 µl) of control and AA rats diluted with 350 µl of PBS was used to determine TBARS (B). Values are mean ± SEM. The number of animals in each group was 6–8. Significance compared to control, $^{*}p < 0.05$, $^{***}p < 0.001$.

The dependence of SERCA activity in control and AA samples on the substrate concentrations was analysed in more detail (Figures 2B and C). With respect to increasing Ca^{2+}_{free} concentration, the maximal rate of SERCA reaction from AA ($V_{max} = 6.38 \pm 2.66$ IU/mg) was elevated compared with control animals ($V_{max} = 3.83 \pm 0.85$ IU/mg), ~1.7-fold. The kinetic constant K (the concentration of substrate at half maximal enzyme velocity of reaction) was not significantly changed, indicating that the affinity of SERCA for Ca^{2+} binding was unchanged in SR from AA animals.

According to Coll and Murphy [37], data of ATPase activity suggest the existence of two ATP-binding sites, a high-affinity (catalytic) site and a low-affinity (regulatory) one. However, with the structural resolution of the purified SR Ca^{2+}-ATPase, only a single ATP binding site was likely to exist [38]. It may thus be that the ATP binding site changes its affinity for ATP subsequent to phosphoryl transfer. Following phosphorylation of the pump, ATP may bind (at a lower affinity) and increase the Ca^{2+}-ATPase activity [35]. The increase of ATP concentrations caused a 1.4-fold increase with respect to the catalytical binding site: for control samples $V_{max} = 1.10 \pm 0.19$ IU/mg and for samples from AA animals $V_{max} = 1.58 \pm 0.26$ IU/mg.

The K_m for ATP for the catalytic binding site in SR samples from AA animals ($3.12 \pm 1.49\ \mu M$) was, within error margins, equal to that of control samples ($3.60 \pm 1.84\ \mu M$). Analogously, the K_m for ATP regulatory binding site related to AA samples $K_m = 0.41 \pm 0.17$ mM was, within error margins, equal to that of control samples ($K_m = 0.50 \pm 0.18$ mM).

Analysis of SERCA and calsequestrin expression and oxidation

To investigate whether the increase in SERCA activity was due to a change in protein level in the SR, where in rabbit 70–90% and in rat 40% of the protein is Ca^{2+}-ATPase [20,39,40], the protein composition of SR from AA and CO animals was analysed by electrophoresis (Figure 3). The band at ~110 kDa corresponding to SERCA in AA SR was not changed (210.2 a.u.) compared with control sample (213.1 a.u.) in the coomassie-stained gel

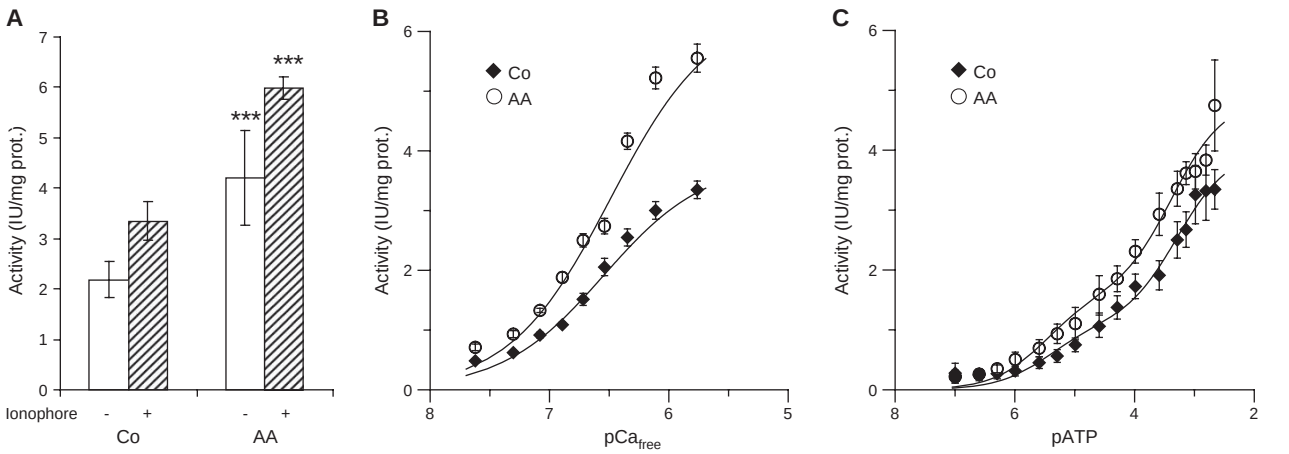

Figure 2. Ca^{2+}-ATPase activity of SR from control (Co) rats and rats suffering from AA in the presence or absence of Ca^{2+} ionophore A-23187 (A) and as a function of free Ca^{2+} (B) and ATP (C). Activities of the Ca^{2+}-ATPase in SR from control rats and rats on day 28 after MB injection were measured at 37°C using coupled enzyme assay, at pH 7.2. In panel (A) results are expressed as mean ± SEM of measurements from 6–8 animals, each of them measured in three parallels. ***$p < 0.001$ means significant increase of enzyme activity in rats with AA compared to controls. In panels (B) and (C) results are means ± SEM of two independent experiments, where in each experiment three parallel measurements were performed. pCa_{free} and pATP means a negative logarithm of concentration of free Ca^{2+} ions or ATP and were calculated from concentrations expressed in Molar.

(Figure 3A). However, more exact immunoblotting using a monoclonal antibody against SERCA1 showed a small increase in SERCA1 band density (~110 kDa) in sarcoplasmic reticulum samples from AA rats (168.2 a.u.) compared to controls (147.9 a.u.) (Figure 3B). In contrast, immunoblotting for the calcium-regulating protein calsequestrin at ~47 kDa (Figure 3C) showed this protein was significantly decreased in AA samples (143.6 a.u.) in comparison to controls (191.0 a.u.). The ratio of band density of SERCA vs calsequestrin indicated higher SERCA expression in AA sarcoplasmic reticulum of skeletal muscle (1.17 vs 0.77, respectively).

In terms of oxidative damage to the proteins in SR, there was no evidence for either new bands or aggregation of SERCA in samples from AA rats, compared to control samples, indicating that there was no major damage to the protein in AA. No significant changes in protein carbonyls were observed in AA animals, 53.2 ± 9.50 pmol/mg protein compared to CO animals 54.5 ± 7.22 pmol/mg protein. Moreover, analysis of SH group content showed no significant changes in SR of rats with AA. The levels of SH free groups were 330.66 ± 17.62 and 297.61 ± 17.69 nmol/mg protein in control rats and rats with AA, respectively. However, western blotting with an anti-nitrotyrosine polyclonal antibody demonstrated an increase in nitrotyrosine in sarcoplasmic reticulum samples from AA rats (205.6 a.u.) to compared with controls (149.9 a.u.) (Figure 3D).

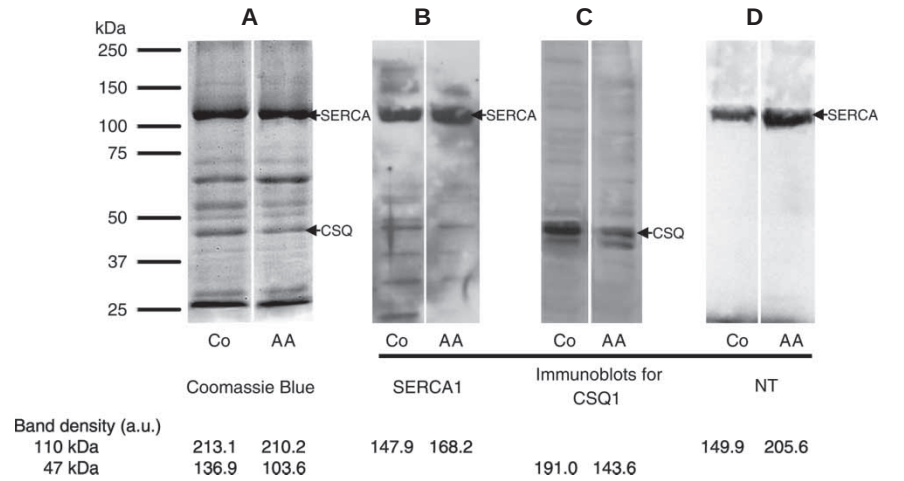

Figure 3. SDS-PAGE and immunoblots of SR proteins from control and AA rats. (A) Coomassie blue-stained gel of SR proteins electrophoretically separated on a 7.5% SDS gel. (B–D) Immunoblots using by antibodies specific to SERCA1 (B), calsequestrin1 (CSQ1; C) and nitrotyrosine (NT; D). In all gels the position of SR proteins are indicated and the data shown is representative of three independent experiments. The numbers underneath the gels indicate the density of the bands of interest, determined using adobe Photoshop 7.0 software.

Analysis of conformational changes

To investigate further the factors underlying the increase in SERCA activity, the fluorescent probe FITC was used to assess whether a structural alteration of the ATP (nucleotide) binding site of the SERCA protein had occurred, because FITC binding is localized at a specific lysine residue (Lys^{515}) in this binding site. No significant changes of total FITC fluorescence intensity were identified in AA samples compared to control (Figure 4). However, the ratio of fluorescence intensities I_{358nm}/I_{336nm} of Trp residues in polar (cytosol) and non-polar (membrane) environments significantly increased in SR from rats with AA (Figure 4), suggesting that a conformational change SERCA had occurred.

Analysis of SR phospholipid composition

As it is known that phospholipid membrane composition can affect the activity of membrane enzymes, including SERCA [40], the phospholipids present in SR from AA and CO animals were analysed by positive ion mass spectrometry. The native SR membranes from rat muscles consist mainly of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) containing saturated (palmitoyl 16:0, stearoyl 18:0) and unsaturated (oleoyl 18:1, linoleoyl 18:2, arachidonoyl 20:4 and docosahexenoyl C22:6) fatty acyl chains. Figure 5 shows the positive ion electrospray spectra in the region 700–900 m/z for SR membranes from control rats and rats with adjuvant arthritis, as well as the precursors of a fragment with m/z 184 (indicative of phosphocholine-containing lipids) and the precursors of a fragment with m/z 141 (indicative of phosphoethanolamine-containing lipids). The phosphatidylcholine profile of both types of SR appeared to be very similar. The same was true of the phosphatidylethanolamine profile, although the control sample used to obtain the precursor of 141 m/z data contained more sodium, hence the peaks are shifted by +22 m/z compared to the adjuvant arthritis sample. Whereas palmitoyl-containing PCs dominated (C16:0/18:2 at 758 m/z and C16:0/20:4 at 782 m/z), the PEs tended to contain stearate, such as C18:0/18:2 at 744 m/z, C18:0/20:4 at 768 m/z and C18:0/22:6 at 792 m/z. There were also fewer mono-unsaturated fatty acyl chains in the PEs, compared to PCs. Very low levels of TBARS were determined in SR (data not shown) and there was no evidence of increased phospholipid hydroperoxides or chlorohydrins in adjuvant arthritis samples according to the MS analysis (Figure 5). LCMS analyses also confirmed the absence of significant differences in the native phospholipid composition (data not shown).

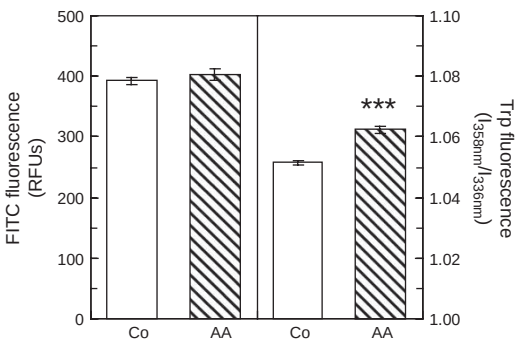

Figure 4. Maximum FITC fluorescence intensity and ratio of Trp fluorescence intensity I_{358nm}/I_{336nm} in SR vesicles. Fluorescence intensity was measured on a Perkin Elmer LS45 spectrofluorometer at 25°C and relative fluorescence units (RFUs) were recorded. I_{358nm}/I_{336nm} is the ratio of fluorescence intensity of tryptophan residues in polar (emission maximum at 358 nm) and non-polar (emission maximum at 336 nm) environments. Values are mean ± SEM; the number of animals was 6–8, measured in duplicate. Statistical significance with respect to control, ***$p < 0.001$.

Analysis of phospholipid oxidation in SR oxidized in vitro

In order to investigate further the possible mechanisms of SERCA modulation in AA and the susceptibility of SR to oxidative stress induced *in vitro* with HOCl, LCMS analysis of SR phospholipids was carried out. The SR phospholipids were only found to undergo oxidative damage at relatively high levels of HOCl and SR from control animals was found to be more susceptible to oxidation than SR from AA animals (Figure 6). Mono-peroxides of palmitoyl-linoleoyl PC (790 m/z) and palmitoyl-arachidonoyl PC (814 m/z; data not shown) could be observed strongly in the reconstructed ion chromatograms (RICs) of control samples, but only weakly in AA samples. A bis-peroxide of palmitoyl-arachidonoyl PC (846 m/z; data not shown) and its dehydration product were similarly apparent in control samples but less intense in AA samples. A chlorinated product, monochlorohydrin of palmitoyl-oleoyl PC at 812 m/z, was also detected more intensely in HOCl-oxidized control samples compared to AA SR extracts (Figure 6). The RICs of unoxidized SR samples showed no evidence of significant phospholipid oxidation products, based on lack of signals at the relevant elution times. There were no significant differences in the intensities of the native peaks (DPPC at 734 m/z, PAPC at 782 m/z and POPC at 760 m/z) in oxidized AA and control samples (data not shown), indicating that the concentration of lipid was comparable within each set of samples.

SERCA inactivation in SR oxidized in vitro

Figure 7A shows the activity of SERCA in SR from control and AA animals after additional *in vitro* oxidation of SR by HOCl. The slope of SERCA activity decrease in SR from AA samples was significantly steeper with $IC_{50} = 52.8$ μM HOCl, $r = 0.993$, compared to controls $IC_{50} = 81.5$ μM HOCl,

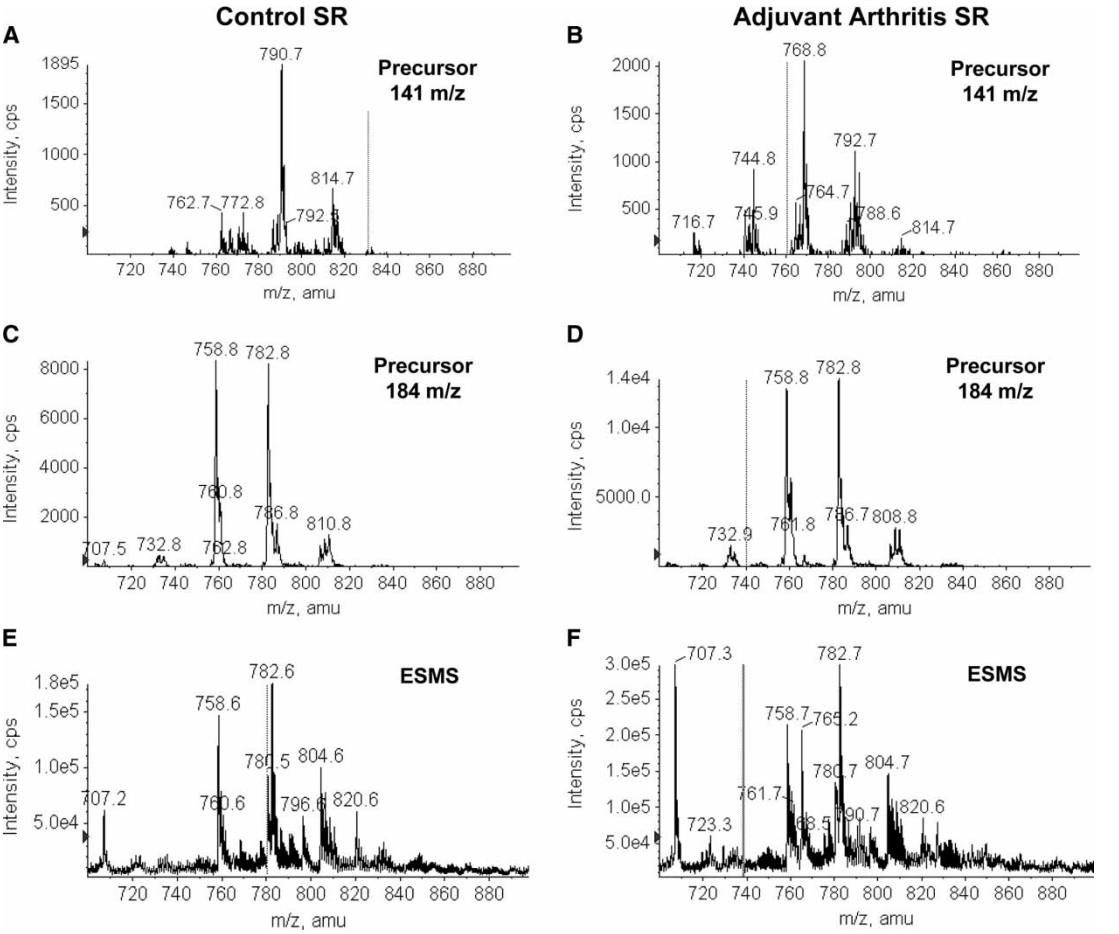

Figure 5. ESMS spectra of sarcoplasmic reticulum (unoxidized) from control rats and rats with adjuvant arthritis. (A, C, E) show SR from control animals; (B, D, F) show SR from AA animals. The spectra were acquired in positive ion mode on a 2000-QTrap mass spectrometer. (A) and (B) show the precursors of 141 m/z, corresponding to phosphatidylethanolamines; (C) and (D) show the precursors 184 m/z, corresponding to phosphatidylcholines; and (E) and (F) show the complete spectra.

$r = 0.966$. This suggests that SERCA from AA animals was more susceptible to oxidation by HOCl.

Effect of PA on SERCA activity

As SERCA activity has been reported to be dependent on acidic phospholipids, the potential activation of SERCA by increasing PA concentration was investigated (Figure 7B). At low concentrations, PA caused a sharp increase in the SERCA activity, but at concentrations above 5 μM it caused decrease of ATPase activity, although the SERCA activity remained higher than in the control sample in the absence of PA.

Discussion

The objective of this study was to investigate the relationship between AA, oxidative stress and SERCA function, based on the concept that the inflammatory environment of the joint in adjuvant arthritis (AA) may contribute to changes in intracellular Ca^{2+} and result in the development of the chronic phase of the disease [41]. We focused on the hypothesis that AA-induced oxidative stress causes inactivation of SERCA, either due to direct oxidation of the protein or indirectly by changes in lipid composition or oxidation. In previous work using the *Mycobacterium butyricum* injection model of AA we found evidence of systemic oxidative stress, specifically: increased protein carbonyls [42], increased lipid peroxidation [43] and a significant reduction of total antioxidant status [44] in plasma. Measurements of plasma protein carbonyls and TBARS in the present study confirmed that AA induces a systemic oxidative damage.

However, instead of the expected ROS-induced decrease of SERCA activity in rats with AA, an increase in activity with respect to both substrates (Ca^{2+} and ATP) was observed. The kinetic data suggested that an increase in V_{max} was responsible, as the affinity of the enzyme (K_m) to Ca^{2+} ions and ATP was not significantly affected. This increase in SERCA activity induced by AA is a novel and exciting finding and several potential contributing factors for this were pursued to investigate the underlying molecular processes.

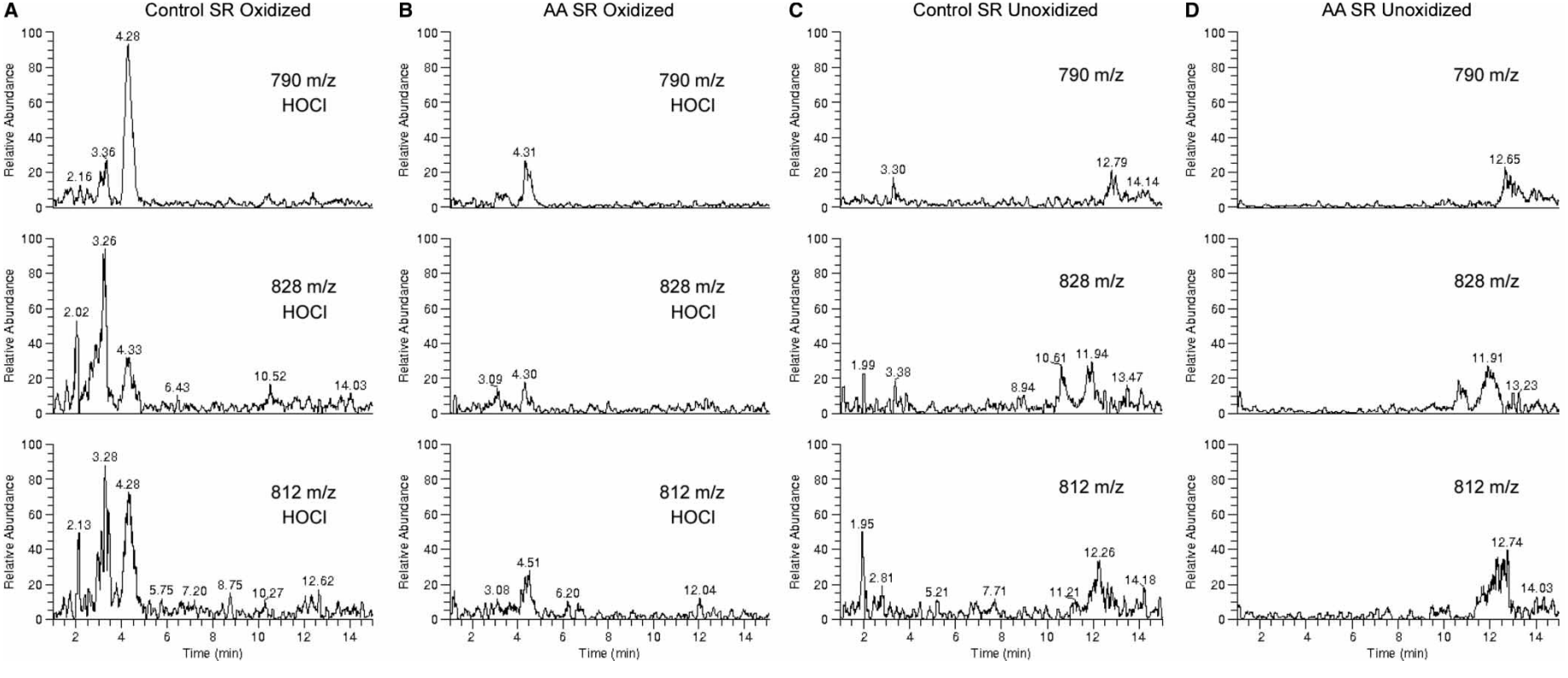

Figure 6. Reconstructed ion chromatograms of oxidized phosphatidylcholines in SR vesicles from control rats and rats with adjuvant arthritis. Spectra were recorded on a Thermo Finnigan LCQ. Panel (A) shows data from SR (1 mg prot/ml) from control rats oxidized with 10 mM HOCl. Panel (B) shows data from SR from AA rats oxidized as indicated. Panel (C) shows data from unoxidized control rats and panel (D) shows data from unoxidized SR from AA rats, as indicated. 790 m/z corresponds to the mono-peroxide of PLPC (100% = ion intensity of 7.5×10^5 counts in all panels); 828 m/z corresponds to the dehydration product of the bis-peroxide of PAPC at 846 m/z (100% = ion intensity of 3.7×10^5 counts in all panels); and 812 m/z corresponds to the mono-chlorohydrin of POPC (100% = ion intensity of 3×10^5 counts in all panels). The oxidized lipids elute early in the spectra (earlier than 6 min); the components eluting after 10 min in the unoxidized samples are unsaturated native lipids that have the same m/z as the oxidized lipid. The chromatograms shown are representative of all those obtained for each sample type (2–3 measurements were performed).

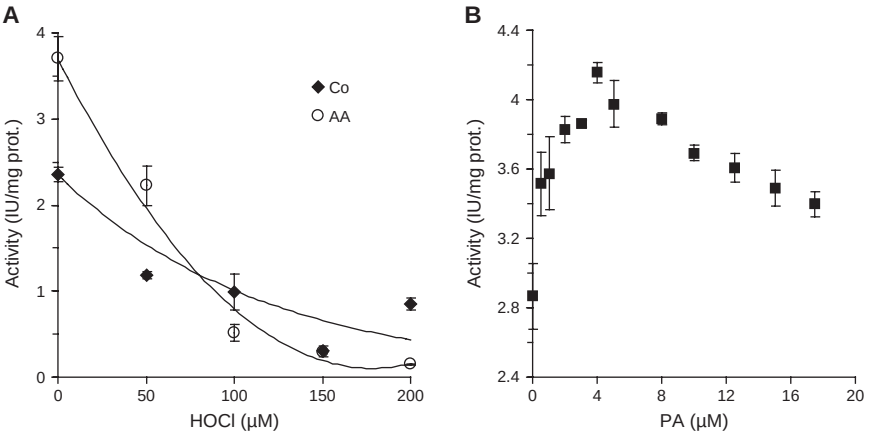

Figure 7. Effects of *in vitro* treatments on SERCA activity of control (Co) and AA rats. (A) Samples of SR from control rats and rats with AA were oxidized *in vitro* by HOCl (50–200 μM) at 25°C, pH 7.2 for 3 min. The oxidation was stopped by the addition of cysteine (1 mM). Results represent means ± SEM from two independent experiments measured in three parallels. Significant (***$p < 0.001$) decrease of enzyme activity was observed from 50 μM HOCl concerning control as well as AA samples. (B) SR vesicles (12.5 μg prot) were incubated with increasing concentrations of phoshatidic acid at 37°C for 20 min and then SERCA activity was measured at 37°C using a coupled enzyme assay, at pH 7.2. Results represent means ± SEM from two independent experiments measured in three parallels.

One possible explanation of the increase in SERCA activity could involve conformational changes in the catalytic or substrate binding sites; it has been reported previously that conformational changes in the nucleotide binding site of the ATPase protein are induced *in vitro* through oxidation of skeletal muscle SR Ca^{2+}-ATPase by HOCl [29] or by Fenton reaction [39]. Biological ageing leads to oxidation and nitration of SERCA at cysteine and tyrosine [30] and to a conformationally altered nucleotide binding site [17]. However, in our experiments, the increase in SERCA activity in SR from AA rats was not associated with conformational changes in nucleotide binding site, as indicated by using the fluorescence label FITC, which agrees with the observation that the K_m for ATP was unchanged. On the other hand, an increase in the Trp fluorescence intensity ratio (I_{358nm}/I_{336nm}) suggested that in SERCA from AA animals there was a shift of tryptophan residues from a non-polar (membrane) to polar (cytosolic) environment. This suggests a conformational change in the transmembrane domain of SERCA, which contains 11 out of the 13 tryptophan residues present in SR Ca^{2+}-ATPase.

An obvious mechanism for an alteration of SERCA activity is the oxidative post-translational modification of the protein. SR analysis by PAGE showed that there were no major structural changes in the protein such as aggregation or fragmentation and no formation of protein carbonyls. As the redox state of specific Cys residues of SERCA is known to be important for enzymatic function (modification of selected Cys may result in either inhibition and activation of protein [19,45,46]), we analysed the thiol content of SR to test whether cys oxidation had occurred. However, there was no significant increase in thiol oxidation in SR of AA animals and as more than 90% of the free protein thiols in SR are located on SERCA [19,47], it is unlikely that the SERCA thiols were significantly oxidized. On the other hand, an increase in nitrotyrosine specifically in SERCA was observed in AA, suggesting that RNS are formed in this condition and cause damage to the protein. Macrophages, chondrocytes and synovial fibroblasts are all known sources of nitric oxide [2] and NO synthase has also been found to be abundant in fast-twich fibres [48]. Although NO-induced glutathionylation of Cys_{669} or Cys_{674} can activate SERCA, higher levels of peroxynitrite that cause nitrotyrosine formation have previously been reported to inhibit the enzyme; however, this loss of individual Cys coincided with the loss of SERCA activity only for the residues at positions 674, 675 and 938 [40,45]. SERCA1 is known to be less susceptible than SERCA2 to irreversible cysteine oxidation and inactivation. The lack of change in thiol status in AA suggested that glutathionylation of cys residues in SERCA is not the mechanism responsible for increased activity; however, nitrotyrosine is a more stable modification than glutathionylation and it is possible that thiol modification was reversed either *in vivo* or during preparation of the tissue.

Although the Ca^{2+}-ATPase activity was normalized to total SR protein, of which it represents a major fraction, we considered the possibility that increased levels of SERCA occurred in SR from AA animals and contributed to the elevated activity. To investigate this, the levels were visualized by western blotting with a monoclonal antibody against SERCA1 and the level compared with calsequestrin, which was used as a marker of SR protein. Calsequestrin functions as a Ca^{2+} storage reservoir in the SR and is also involved in negative regulation of the

RyR2 channel (responsible for release of calcium from SR) in a process involving polymerization and interaction with triadin and junctin [49]. It was found there was a clear increase in the ratio of SERCA1: calsequestrin proteins in AA SR, with both an increase in SERCA1 levels and a decrease in calsequestrin relative to control SR. Although the level of SERCA1 was increased in AA, it is not possible to determine from these experiments whether this was due to increased synthesis or decreased degradation of the SERCA. However, an attractive possibility is that tyrosine nitration causes some inactivation the enzyme, which then leads to increased synthesis as a compensatory mechanism.

A further mechanism for alteration of SERCA activity in AA relates to the phospholipid composition of the SR. The transmembrane domain of the Ca^{2+}-ATPase of skeletal muscle SR makes contact with about 30 lipid molecules in the membrane [50,51] and its activity depends on the phospholipid composition and structural state of the SR membrane lipid phase [52,53]. Furthermore, lipid oxidation resulting from inflammation and oxidative stress can induce structural changes in the lipid bilayer, which could indirectly inhibit the activity of the pump [54] and membrane proteins may be secondarily modified by molecules generated during lipid oxidation. HNE, an aldehydic product of lipid peroxidation, is able to form covalent cross-links with proteins and thus interact with key enzymes and ion transporters [55]. Analysis of SR by TBARS assay did not show any evidence of lipid oxidation in this compartment, so mass spectrometry was used as an additional approach to confirm this finding and also to investigate whether any alterations in profile of the major SR phospholipids, PC and PE, occurred in AA. However, the mass spectrometry results supported the conclusion that there was no significant oxidative damage to SR lipids in AA and there was no evidence of significant changes in profile of the positively charged phospholipids. However, it is possible that very minor changes in phospholipid components, beyond the resolution of this method, could affect the activity of SERCA and also that phospholipids other than those determined here are responsible for the increased SERCA activity in AA. Our results showing that phosphatidic acid can activate SERCA *in vitro* support this possibility, as it has been reported that levels of acidic phospholipids are increased in heart sarcolemmal and mitochondrial fractions [56].

Our studies on SR from control and AA animals suggest that, despite the systemic oxidative stress indicated by changes in plasma, there was little measureable oxidative damage to either proteins or lipids of SR and thus it appears that these components are relatively resistant to oxidation. To test this and investigate further the potential mechanisms of oxidative stress-induced changes, SR samples were subjected to oxidative stress *in vitro* using HOCl. This provided very interesting results, as it showed that SERCA and lipids had differential sensitivity between AA and control samples, with SERCA from AA samples having increased sensitivity to HOCl oxidation but more resistance to phospholipid oxidation, compared to control SR. This indicates that there were fundamental differences in SERCA structure and function in AA and supports the suggestion of a conformational change, as indicated by the Trp fluorescence data. It also suggests that HOCl-mediated oxidation is not a major contributor to changes in AA, as HOCl inhibited SERCA activity, rather than elevating it.

To summarize, we report the novel finding that in AA induced by *Mycobacterium butyricum* injection, SERCA1 activity was increased $\sim$2-fold. We conclude that a number of factors may contribute to this effect, including increased levels of SERCA protein relative to calsequestrin in AA SR, conformational changes in SERCA, increased levels of phosphatidic acid that can activate SERCA and possibly limited oxidative post-translational modifications. The increased SERCA activity may reflect an adaptive response during AA-induced inflammation or could contribute to the pathology of the disease by disrupting calcium balance.

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