ORIGINAL ARTICLE

Oxidative impairment of plasma and skeletal muscle sarcoplasmic reticulum in rats with adjuvant arthritis – effects of pyridoindole antioxidants

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Submitted: 2008-06-30 *Accepted: 2008-09-04*

Key words: **adjuvant arthritis; plasma; sarcoplasmic reticulum; SERCA; pyridoindole antioxidants**

Neuroendocrinol Lett 2008; **29**(5):706–711 **PMID:** 18987589 NEL290508A29
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Abstract

OBJECTIVES: To study possible oxidation of proteins and lipids in plasma and sarcoplasmic reticulum (SR) from skeletal muscles and to assess the effects of pyridoindole antioxidants in rats with adjuvant arthritis (AA) and to analyze modulation of Ca-ATPase activity from SR (SERCA).
METHODS: SR was isolated by ultracentrifugation, protein carbonyls in plasma and SR were determined by ELISA. Lipid peroxidation was analyzed by TBARS determination and by mass spectrometry. ATPase activity of SERCA was measured by NADH-coupled enzyme assay. Tryptophan fluorescence was used to analyze conformational alterations.
RESULTS: Increase of protein carbonyls and lipid peroxidation was observed in plasma of rats with adjuvant arthritis. Pyridoindole antioxidant stobadine and its methylated derivative SMe1 decreased protein carbonyl formation in plasma, effect of stobadine was significant. Lipid peroxidation of plasma was without any effect of pyridoindole derivatives. Neither protein oxidation nor lipid peroxidation was identified in SR from AA rats. SERCA activity from AA rats increased significantly, stobadine and SMe1 diminished enzyme activity. Ratio of tryptophan fluorescence intensity in SR of AA rats increased and was not influenced by antioxidants.
CONCLUSION: Plasma proteins and lipids were oxidatively injured in rats with AA; antioxidants exerted protection only with respect to proteins. In SR, SERCA activity was altered, apparently induced by its conformational changes, as supported by study of tryptophan fluorescence. Stobadine and SMe1 induced a decrease of SERCA activity, elevated in AA rats, but they did not affect conformational changes associated with tryptophan fluorescence.

Abbreviations

AA	- adjuvant arthritis
Ca^{2+}	- calcium ions
Co	- control
ELISA	- enzyme linked immunosorbent assay
HOCl	- hypochlorous acid
MB	- Mycobacterium butyricum
LC-MS	- liquid chromatography-mass spectrometry
LDL	- low density lipoprotein
NADH	- β-nicotinamide adenine dinucleotide hydrate
QSAR	- quantitative structure-activity relationship
RA	- rheumatoid arthritis
ROS	- reactive oxygen species
SERCA	- sarco/endoplasmic reticulum Ca-ATPase
SH groups	- sulfhydril groups
SR	- sarcoplasmic reticulum
SMe1	- methylated racemic derivate of stobadine, 8-methoxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3b]indolinium dichloride
STB	- (–)-cis-2,8-dimetyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3b]indolinium dichloride
TBARS	- thiobarbituric acid reactive substances
Trp	- tryptophan

INTRODUCTION

Sarco/endoplasmic reticulum Ca-ATPase (SERCA) plays a key role in the relaxation of smooth, cardiac and skeletal muscle through the transport of cytosolic Ca^{2+} into the sarcoplasmic/endoplasmic reticulum (SR/ER) (East, 2000). Some physiological and pathological processes, such as cell proliferation and apoptosis (Sharov *et al*., 2006) are associated with abnormal activity or expression of SERCA. Modulation of SERCA activity may be a contributing factor in the development of some cardiovascular, neurodegenerative or skeletal muscle diseases. An important feature of SERCA is its high sensitivity towards modification by reactive oxygen species. Concerning investigations *in vivo,* biological aging was studied most intensively and resulted in oxidation and nitration of SERCA (Sharov *et al*., 2006).

Redox imbalance contributes to the pathogenesis of rheumatoid arthritis (RA) and experimental animal models of adjuvant arthritis (AA), provoking also systemic damage (Kunsch *et al*., 2005). The inflammatory environment of the joint in RA probably contributes to the changes in intracellular Ca^{2+} (Carruthers *et al*., 2000). Relationship between the structural and functional alterations of SERCA and modification of SERCA by antioxidants has not yet been studied and may be important issues in the search for new therapeutic approaches to the treatment of RA.

MATERIAL AND METHODS

Experimental model

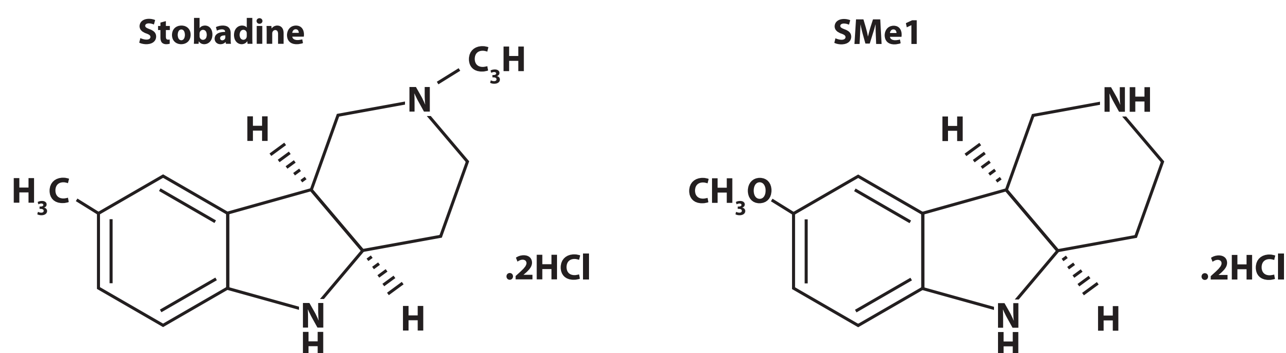
Male Lewis rats (Breeding Farm Dobrá Voda, Slovakia), weighing 150–170 g, 8 weeks of age, were used. AA was induced by heat inactivated *Mycobacterium butyricum* (MB) in incomplete Freund´s adjuvant (Difco Laboratories, Detroit, MI, USA), administered as single intradermal injection into the base of the rat`s tail. Changes of the hind paw volume were a parameter of arthritis progression (Bauerová *et al*., 2005, 2006). Oxidative injury of plasma and SR from skeletal muscles of hind paws was analyzed on day 28 after MB injection. Pyridoindole antioxidants were administered orally, daily dose 15 mg/kg b.w. with intragastic gavage, in solution of 0.5% methylcellulose in drinking water. First dose was approximately 2 h after MB injection. On day 28, the rats were sacrificed in anesthesia and plasma was prepared from heart blood. Solutions of anaesthetics, ketamine (100 mg/kg) and xylazine (16 mg/kg) b.w were mixed and administred by a single subcutaneous injection. SR vesicles were isolated from skeletal muscle of hind paws of rats according to Warren *et al*. (1974).

Ca-ATPase activity

SERCA activity from SR of rat hind paws was measured by NADH-coupled enzyme assay at 37 °C (Warren *et al*., 1974).

Protein carbonyls

Enzyme linked immunosorbent assay (ELISA) was used for quantitative determination of protein carbonyls in SR vesicles and plasma (Buss *et al*. 1997).



Pyridoindole antioxidants synthetized at the Institute of Experimental Pharmacology, Bratislava (Štolc *et al*., 1983; 2003).

Tryptophan (Trp) fluorescence

The fluorescence intensity (I) of Trp residues in water is low with the emission maximum at 358 nm and in dioxane (nonpolar environment) is high, with the emission maximum at 336 nm (Restall *et al.*, 1986). The intensities 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 Trp conformational changes. Trp fluorescence in membrane proteins of SR was measured on a Perkin Elmer LS45 spectrofluorometer 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 (Carney *et al.*, 2007).

Determination of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) (Brown *et al.*, 1994) were used as the index of lipid peroxidation in plasma. Lipids of SR were analyzed by LC-MS.

Liquid chromatography-mass spectrometry (LC-MS)

Reverse phase LC-MS was carried out essentially (Spickett *et al.*, 2001), using the Thermo Finnigan Surveyor system and a Luna C8 column (5 µm RP-Sct, 1 mm inner diameter × 150 mm, Phenomenex, UK). The MS spectra were acquired also in positive ion mode on a 2000 Q TRAP mass spectrometer.

Statistical analysis

Unpaired one-way ANOVA test with Tukey comparison was used for statistical analysis. 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

Protein carbonyls were used as a marker of oxidative injury of plasma and SR from skeletal muscles of hind rat paws. In spite of a significant increase of protein carbonyls found in plasma of AA rats (Figure 1a), no alterations were observed in SR either in untreated AA rats or in those treated with antioxidants (Figure 1a). Stobadine (STB) and its methylated derivative SMe1 decreased protein carbonyls in plasma, and the STB effect proved significant (Figure 1a). TBARS and LC-MS were used to analyze lipid peroxidation in plasma and SR, respectively. Significant increase of TBARS was observed in plasma of AA rats, yet no protection by pyridoindole antioxidants was found (Figure 1b). The native SR membranes from rat muscles consisted according to LC-MS analysis mainly of phosphatidylcholines and phosphatidylethanolamines 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. No significant changes in lipid composition or increase of lipid oxidative products were identified between SR of control, AA rats or rats treated with antioxidants (Figure 2). The phosphatidylethanolamine content was also similar, although the adjuvant arthritis sample used to obtain the precursor of 141 *m/z* data contained less sodium, hence the peaks are shifted by –22 *m/z* compared to the other samples.

Compared to controls, SERCA activity significantly increased on day 28 after inducing AA by MB injection. In STB and SMe1 treated rats SERCA activity was significantly decreased (Figure 3a). Ratio of Trp fluorescence intensity I_{358nm}/I_{336nm} (polar/nonpolar environment) increased in SR from rats with AA. Antioxidants had no effect on I_{358nm}/I_{336nm} elevation (Figure 3b).

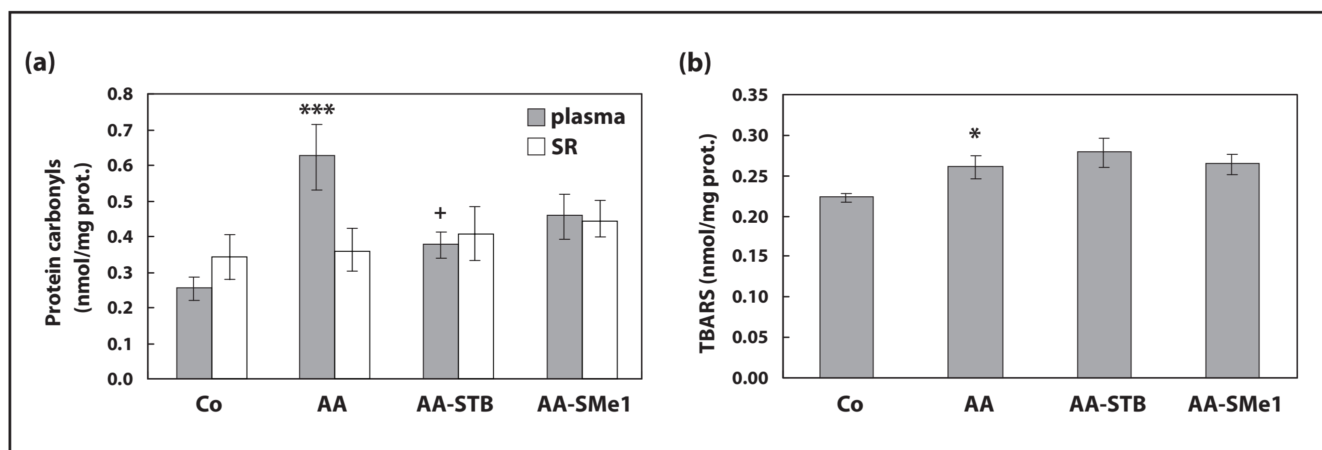


Figure 1. Protein carbonyls (a) in plasma and SR and TBARS (b) in plasma.
Co - control, AA - adjuvant arthritis, AA-STB, AA-SMe1 - treated with antioxidants. Values are mean ± SEM, the number of animals 6-8. Statistical significance with respect to control $^{*}p<0.05$, $^{***}p<0.001$ and to AA $^{+}p<0.05$.

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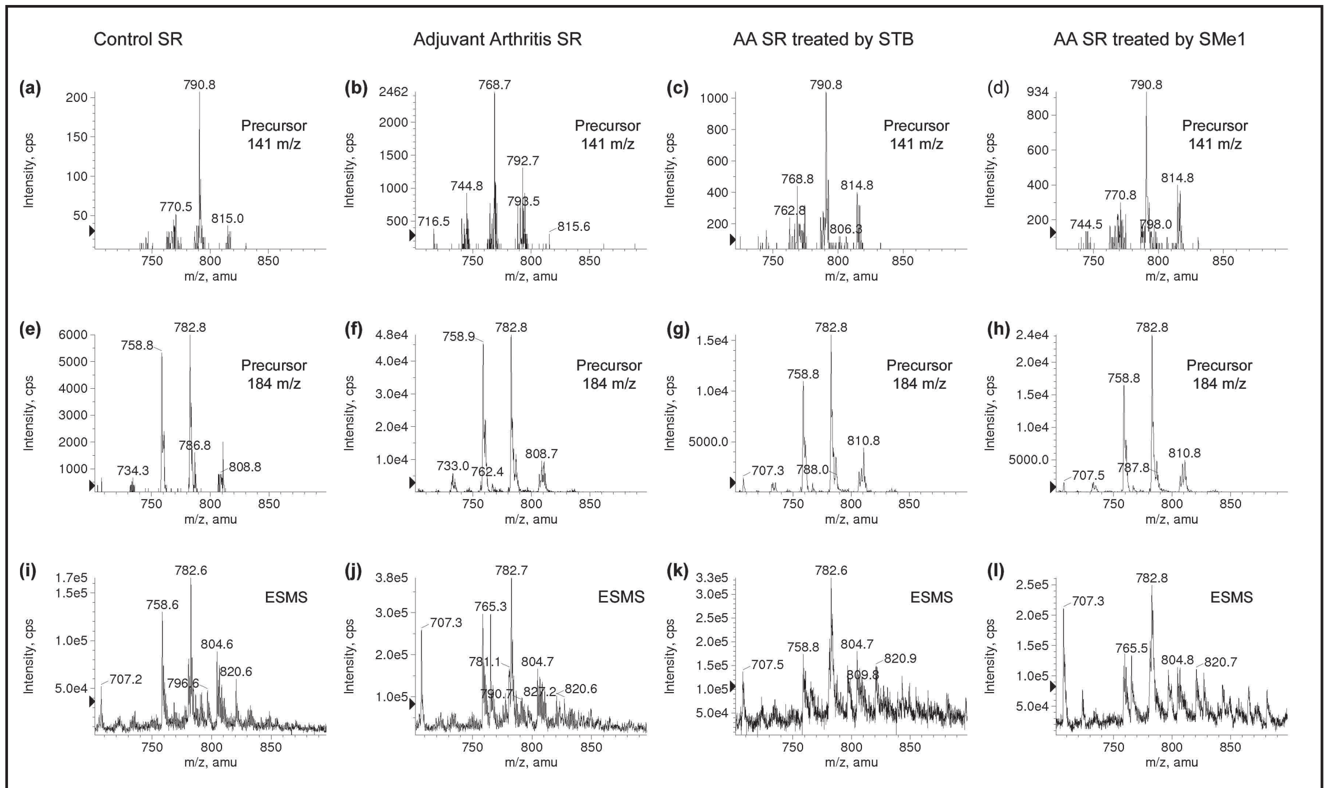

Figure 2. ES-MS spectra of sarcoplasmic reticulum from rats. The spectra were acquired in positive ion mode on a 2000 Q TRAP mass spectrometer. (a), (b), (c) and (d) show the precursors of 141 *m/z*, corresponding to phosphatidylethanolamines; (e), (f), (g) and (h) show the precursors 184 *m/z*, corresponding to phosphatidylcholines; and (i), (j), (k) and (l) show the complete spectra.

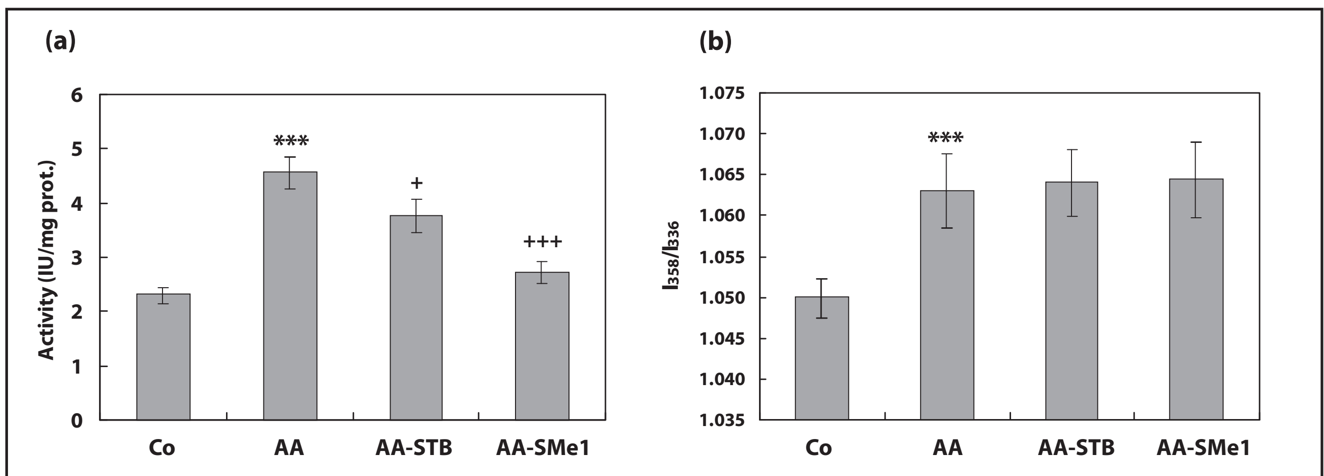

Figure 3. Ca-ATPase activity (a) and intensity of tryptophan fluorescence (b) in SR.
Co - control, AA - adjuvant arthritis, AA-STB, AA-SMe1 - treated with antioxidants. I_{358}nm/I_{336}nm, means fluorescence intensity of tryptophan residues in polar (emission maximum at 358 nm) and nonpolar (emission maximum at 336 nm) environment. Values are mean ± SEM, the number of animals 5-8. Statistical significance with respect to control ****p*<0.001 and to AA ^{+}p<0.05, ^{+++}p<0.001.

DISCUSSION

In contrast to human RA, experimental AA is partially reversible. Animals slowly recover from adjuvant arthritis after experimental day 28. Although the active inflammatory responses gradually subside, the swelling and apparent anatomical deformities may last for a longer period (Wauben *et al*., 1994) The systemic inflammatory process and systemic oxidative stress will disappear in AA, in contrast to human RA, which is a lifelong chronic autoimmune disease.

The mechanisms of reversibility may be based on modification of SERCA ATPase activity. Selective reversible oxidation at critical sites of SERCA results

in regulation of its function, including free radical formation. To date, modulation of SERCA activity has not been studied in animals with AA. Some agents with antioxidant properties may modulate SERCA activity in both ways, either decreasing or increasing it (Fusi *et al.*, 2001).

We found an increase of protein carbonyls and lipid peroxidation in rats with AA induced by MB, similarly as in several other models of AA (Kunsch *et al.*, 2005). In spite of this finding, the only significant alteration in SR was an unexpected increase of Ca-ATPase activity, which may indicate possible structural changes of SERCA observed by many other authors *in vitro* and *in vivo,* as reviewed previously (Squier and Bigelov, 2000). The SR Ca-ATPase contens 13 tryptophan residues, 11 of them located in the transmembrane, two in the cytoplasmic domain, and alteration in Trp fluorescence may be a marker of conformational changes of SERCA (Restall *et al.*, 1986). Elevation of SERCA activity in SR from AA may be associated with ratio of Trp fluorescence intensity I_{358nm}/I_{336nm} increase and may support the notion of conformational changes of SERCA in our experiments. Increased SERCA activity correlated with recently reported stimulated function of mitochondria under the same experimental conditions (Bauerová, *et al.*, 2005), probably indicative of adaptive mechanisms.

STB and its methylated racemic derivative SMe1 influenced only proteins, exhibiting no effect on lipids either in plasma or in SR. Missing of protection by pyridoidole derivatives against lipid peroxidation in plasma may be explained by following facts: i) plasma contains only low level of lipids ii) we found relatively slight increase of lipid peroxidation in plasma, to compare with protein carbonyls, iii) STB with redox potential 0.58V is able directly protect aminoacids (redox potential approximately about 1.00 V) against oxidation (Steenken *et al.*, 1992, Horáková *et al.*, 1996), nevertheless its activity against lipid peroxidation *in vivo* is dependent on the presence of vitamin E or ascorbate (Kagan *et al.*, 1993, Račková *et al.*, 2002).

STB was previously shown to be able to scavenge hydroxyl, peroxyl and alkoxyl radicals, to quench singlet oxygen, to repair oxidized amino acids, and to preserve oxidation of SH groups by one-electron donation (Horáková and Štolc, 1998). These effects originate from its ability to form a stable nitrogen-centred radical on indole nitrogen (Stenkeen *et al.*, 1992). STB decreased protein oxidation *in vitro* and *in vivo* (Kyseľová et. al., 2003; Štefek *et al.*, 2005) and protected LDL against Trp destruction (Horáková *et al.*, 1996). Stobadine prevented protein carbonyl formation *in vitro* after HOCl oxidation, probably due HOCl scavenging. Nevertheless, STB was found to have no protective effect against the decreased Ca-ATPase activity induced by HOCl (Štrosová *et al.*, 2005; Štrosová *et al.*, 2006) A higher antioxidant activity of SMe1 was supposed according to quantitative analysis between structure and biological effects of pyridoindole derivatives (QSAR) (Račková *et al.*, 2006). In our experiments SMe1 was more effective in protecting SERCA activity in SR from AA rats pretreated with SMe1 to compare with STB.

We conclude that the pyridoindole antioxidants studied exerted a protective effect on plasma proteins of AA rats. By its increasing activity, SERCA may participate in adaptative mechanisms of ROS formation in AA. Conformational changes associated with Trp fluorescence intensity ratio I_{358nm}/I_{336nm} increase may be involved in modulation of SERCA activity in AA rats. STB and SMe1 diminished adaptive increase of SERCA activity probably by other mechanisms as are conformational changes associated with Trp fluorescence.

ACNOWLEDGEMENTS.

This study was supported by VEGA 2/0090/08, APVV-51-017905 and by COST action B35.

Authors are thankful to Prof Balgavý for critical comments and advices, to Ing Danica Mihalová and Denisa Komendová for experimental help.

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