# **Physiological Research Pre-Press Article**

## Age-dependent effect of oxidative stress on cardiac sarcoplasmic reticulum vesicles

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Short title: Effect of oxidative stress on sarcoplasmic reticulum

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## **Summary**

The oxidative stress hypothesis of ageing suggests that accumulation of oxidative damage is a key factor of the alternations in physiological function during ageing. We studied age-related sensitivity to oxidative modifications of proteins and lipids of cardiac sarcoplasmic reticulum (SR) isolated from 6-, 15- and 26-month-old rats. Oxidative stress was generated *in vitro* by exposing SR vesicles to 0.1 mmol/l FeSO<sub>4</sub>/EDTA + 1 mmol/l H<sub>2</sub>O<sub>2</sub> at 37°C for 60 minutes. In all groups, oxidative stress was associated with decreased membrane surface hydrophobicity, as detected by probe 1-anilino-8-naphthalenesulfonate. Structural changes in SR membranes were accompanied by degradation of tryptophan and significant accumulation of protein dityrosines, protein conjugates with lipid peroxidation products, conjugated dienes and thiobarbituric acid reactive substances. The sensitivity to oxidative damage was most pronounced in SR of 26-month-old rat. Our results indicate that ageing and oxidative stress are associated with accumulation of oxidative damaged proteins and lipids and these changes could contribute to cardiovascular injury.

# Key words

Oxidative stress, reactive oxygen species, protein damage, heart

## Introduction

All organisms are permanently exposed to exogenously and endogenously generated reactive oxygen and nitrogen species (ROS/RNS). ROS/RNS serve as signal molecules at low concentration, but evoke harmful, pernicious effects if they are produced in oversize amounts (Dhalla *et al.* 2000). Although cells has a complex net of antioxidant defence (Bergendi *et al.* 1999; Pollack and Leeuwenburgh 1999, Sivoňová *et al.* 2007) this defence is not quite effective, and therefore molecular damage may occur. Unbalance between pro-oxidants and antioxidants in profit of pro-oxidants causes elevated oxidative stress. Oxidative damage of biomolecules can accumulate (Babušíková *et al.* 2007; Catacay *et al.* 2003, Krajčovičová-Kudláčková *et al.* 2006), and gene expression can be altered (Kaplán *et al.* 2007) with advancing age. Some of the oxidative damages may be a base of a functional decay and failure associated with old age and diseases (Ames *et al.* 1993).

There is evidence that the most important mechanism of oxidative damage to proteins is metal-catalysed oxidation (Berlett and Stadtman 1997). Hydrogen peroxide is a precursor of hydroxyl radical (the most reactive ROS). Each biological system which produces superoxide also produces hydrogen peroxide by dismutation reaction. Besides superoxid dismutase there are another enzymes producing  $H_2O_2$ : amino acid oxidase, glycolate oxidase, urate oxidase, monoamino oxidase xanthine oxidase, NADPH oxidase (Reiter 1998; Dupuy *et al.* 1991). Hydrogen peroxide is reduced to hydroxyl radical in the presence of transient metal (Fe<sup>2+</sup>, Cu<sup>2+</sup>) by Haber-Weiss or Fenton reaction (Halliwell and Gutteridge 1990). Transient metal are able to split organic hydroperoxides to radicals which initiate chain reaction and initiate lipid peroxidation (Spiteller 2001; Dean *et al.* 1997). Age-related reduction of ability to degrade oxidative modified proteins can contribute to accumulation of damaged dysfunctional molecules in cell (Shringarpure and Davies 2002). All amino acids are sensitive to hydroxyl damage (Fu *et al.* 1998; Davies 1987). In the presence of Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> protein modification occurs at amino acid side chains with metal-binding sites (Stadman and Berlett 1997). Metal catalysed protein oxidation is probably included in many physiological processes and pathological damages. Ageing has a powerful effect on increased susceptibility to cardiovascular diseases even in optimal healthy individuals. Increasing evidence indicates that ROS production in cells increase with the age in mammals (Smith *et* al. 1991). However cause relationship between protein damage and etiology and development of age-related diseases is not exactly established. The aim of the present study was to study changes and sensitivity of heart sarcoplasmic reticulum vesicles with advancing age to *in vitro* induced oxidative stress.

#### **Materials and Methods**

#### Animals

Male *Wistar* rats (supplied by IEP SAS Dobra Voda, Slovakia) were divided into three groups according to age, as adult (6-month-old), old (15-month-old) and senescent (26-month-old). The animals were maintained in air-conditioned room ( $21 \pm 2^{\circ}$ C, 12 h light/dark cycle). The animals were allowed free access to food and water. Experiments were approved by Ethics Committee of the Jessenius Faculty of Medicine in Martin, as well as with the rules issued by the State Veterinary and Alimentary Administration of the Slovak republic.

## Preparation of tissue samples

Animals were divided into three groups according to age. Each group consisted of 5 animals. The rats were decapitated after 5 min halothane anaesthesia and hearts were excised. Hearts were washed, minced and homogenized in 10 vol of 30 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 5 mmol/l EDTA, 0.3 mol/l sucrose, 0.5 mmol/l ditiothreitol, 0.3 mmol/l phenylmethylsulfonyl fluoride, 1µmol/l leupeptine, 1 µmol/l pepstatine (pH 6.8) with a Ultra-Turrax T 25 homogenizer (three times for 10 sec, 20500 rpm). Homogenates were stored at -80°C or were used for

sarcoplasmic reticulum preparation. The sarcoplasmic reticulum (SR) vesicles were prepared as described (Kaplán *et al.* 2003).

Protein assay was performed by method of Lowry et al. (1951), using bovine serum albumin as a standard.

Oxidation of cardiac sarcoplasmic reticulum vesicles

Oxidative stress was induced by incubation of sarcoplasmic reticulum vesicles (3 mg/ml) with 1 mmol/l  $H_2O_2$ , 0.1 mmol/l FeSO<sub>4</sub>/EDTA at 37°C for 60 min. Controls in each age group were incubated in the same manner without the treatment with free radical generating system. After the appropriate time intervals, the aliquots of SR vesicles were taken for fluorescence measurements.

#### Fluorescence measurements

Fluorescence measurements were performed in solution containing 50 µg of proteins per ml, 10 mmol/l HEPES, 100 mmol/l KCl (pH 7.0) at 25°C using Shimadzu RF 540 spectroflourimeter. Fluorescence spectra were measured as previously (Babušíková *et al.* 2004).

#### Measurement of lipid peroxidation

Heart homogenate and sarcoplasmic reticulum vesicles were dispensed in concentration of 20  $\mu$ g/ml protein in solution with 10 mmol/l phosphate buffer containing 1% Lubrol (Braughler *et al.* 1986). The absorption spectrum was then recorded using Pharmacia LKB Ultrospec III spectrophotometer. The rate of conjugated diene formation was estimated according to the lipid oxidation index, A<sub>233nm</sub>/A<sub>215nm</sub>, which provides a sensitive method for determination of lipid peroxidation (Klein 1970). Determination of thiobarbituric acid-reactive substances (TBARS) formation was performed according to Das (1994). TBARS concentration was determined from the absorbance at 532 nm.

#### Data analysis

The results are presented as mean  $\pm$  S.E.M. One-way analysis of variance was first carried out to test for differences between groups. Differences between the means of the individual groups were assessed by Newman-Keuls test. A value of p < 0.05 was considered to be statistically significant.

#### Results

#### Protein oxidative modification

Effects of oxidative stress on protein structure were studied by measuring of tryptophan and dityrosine fluorescence. Amount of tryptophan was significantly decreased in consequence of *in vitro* induced oxidative stress in each age group (Tab. 1). The intensity of intrinsic tryptophan fluorescence was reduced by  $17.4 \pm 1.8\%$  in SR vesicles from adult animals compared to the control sample without treatment. Similar effect of oxidative stress we observed in group of old and senescent animals (-  $17.8 \pm 1.4\%$ ; -  $25.0 \pm 1.3\%$ ).

The intensity of dityrosine fluorescence was elevated in the samples after free radical treatment (Tab. 1). Significant changes were observed in SR vesicles from of adult, old and senescent rats  $(18.2 \pm 5.1\%; 9.2 \pm 1.6\%; 15.0 \pm 2.6\%)$ .

#### Oxidative modification of lysine

The fluorescence intensity (excitation and emission spectra) of lysine conjugates with products of lipid peroxidation increased after induction of oxidative stress. Exposure of SR vesicles from adult rats caused  $38.8 \pm 3.8\%$  and  $31.9 \pm 4.6\%$  (Tab. 2) increase of excitation and emission fluorescence, respectively after H<sub>2</sub>O<sub>2</sub> + FeSO<sub>4</sub>/EDTA treatment. Exposure of SR vesicles from old rats caused an increase by  $20.6 \pm 3.8\%$  and  $18.9 \pm 3.6\%$ . Table 2 also shows that oxidative modification in cardiac SR vesicles form senescent rats enhanced to  $135.1 \pm 3\%$  and to  $133.2 \pm 3.3\%$ , respectively compared to SR vesicles without treatment.

### ANS fluorescence intensity

Fluorescence intensity of 1-anilino-8-naphthalenesulfonate probe was decreased after induction of oxidative stress (Tab. 3). Exposure of old and senescent heart SR vesicles caused  $6.1 \pm 3.5\%$  and  $10.6 \pm 1.4\%$ , respectively, decrease in ANS fluorescence.

## Levels of conjugated dienes and TBARS

Modifications in lipid structure were assayed by measurement of changes in levels of conjugated dienes and determination of thiobarbituric acid-reactive substances (TBARS). Oxidative stress caused accumulation of conjugated dienes in cardiac sarcoplasmic reticulum vesicles (Fig. 1). In adult animals the level of conjugated dienes was elevated to  $118.8 \pm 0.2\%$ . Levels of conjugated dienes increased to  $115.1 \pm 0.4\%$  in sarcoplasmic reticulum vesicles from old animals, and to  $117.2 \pm 0.5\%$  in sarcoplasmic reticulum of senescent animals.

Levels of TBARS elevated dramatically in each age group after induction of oxidative stress (Fig. 2). Levels of TBARS increased to  $327.5 \pm 5.8\%$ ,  $378.5 \pm 0.06\%$ , and to  $427.4 \pm 2.3\%$  with advancing age.

## Discussion

There is growing evidence that protein oxidative damage is involved in ageing process and disease. It is assumed that levels of ROS/RNS increase in ageing process (Smith *et* al. 1991). In addition, with advancing age antioxidant defence of organism deteriorates and susceptibility of biomolecules to oxidative stress may be elevated. In this study we have shown that oxidative damage to proteins and lipids of cardiac SR increases during *in vitro* generated oxidative stress. Oxidative damage increased in all age groups, but was the most pronounced in senescent rats.

Our previous studies demonstrated damaging effect of hydroxyl radicals on protein structure in cardiac mitochondria, myofibrils (Babušíková et al. 2004) and sarcoplasmic reticulum (Kaplán et al. 2003) in adult rats. It is assumed, that rise in the levels of iron and ROS with subsequent disturbation in  $Ca^{2+}$  homeostasis could be associated with cardiovascular diseases (Reddy and Clarck 2004; Chen et al. 2002; Ermak and Davies, 2002). SR plays an essential role in the regulation of intracellular  $Ca^{2+}$  concentration and cardiac contraction and relaxation. Although free-radical mediated dysfunction of SR was observed in numerous of studies (Morris and Sulakhe 1997; Xu et al. 1997; Kukreja et al. 1988) a mechanism of this damage is not yet completely understood. Levels of amino acid oxidative damage increased with advancing age in the control groups. Age-associated damage was observed in the skeletal muscle and the heart of mice (Catacay et al. 2003; Leeuwenburgh et al. 1997) and in rat brain (Babušíková et al. 2007). We observed significant protein and lipid oxidative damage after exposure of sarcoplamic reticulum vesicles to hydroxyl radicals. Levels of tryptophan decreased and levels of dityrosine and lysine conjugates increased in all age groups, however the most pronounced changes and the highest levels of investigated protein and lipid oxidative markers were observed in 26-month-old animals. Amino acid residues such as Trp, Tyr and Lys have a crucial role in the function of  $Ca^{2+}-ATPase$ (Andersen and Vilsen 1995; Yamagata et al. 1993) and their modifications can contribute to observed loss in function of  $Ca^{2+}$ -ATPase during ageing. Another important mechanism of SR dysfunction caused by ROS/RNS is related to alternation in membrane lipid bilayer. As shown fluorescence studies with ANS probe, SR membrane hydrophobicity was significantly altered by hydroxyl radicals. Consequently, changes in SR permeability to Ca<sup>2+</sup> may contribute to disturbation of Ca<sup>2+</sup> homeostasis. Moreover, lipid radicals or aldehydes formed during lipid peroxidation can react with Cys and Lys and inhibit membrane transport and enzymatic activities (Refsgaard et al. 2000). Changes in capacity to preserve a normal

calcium homeostasis could be a base of reduced cell function in ageing process and they can cause that senescent organisms are more sensitive to different diseases. Post-translation modifications as well as fundamental protein structural changes may result in age-related decline.

In summary, presented study suggested increased oxidative modification of amino acids and lipid oxidation in heart sarcoplasmic reticulum vesicles with advancing age as well as with induced oxidative stress. Combination of amino acid and lipid modifications can cause cardiac contractile dysfunction and could be included in ageing process and contribute to cardiovascular disease.

### **Conflict of Interest**

There is no conflict of interest.

#### Acknowledgements

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# Legends

**Figure 1.** Effect of H<sub>2</sub>O<sub>2</sub>, FeSO<sub>4</sub>/EDTA on conjugated dienes in heart sarcoplasmic reticulum vesicles. C-control (vesicles without treatment), OS-oxidative stress. The results are expressed as means  $\pm$  S.E.M. of 5 experiments. \*\*\*p < 0.001; significantly different as compared to appropriate control.

**Figure 2**: Effect of H<sub>2</sub>O<sub>2</sub>, FeSO<sub>4</sub>/EDTA on TBARS conjugated dienes in heart sarcoplasmic reticulum vesicles. C-control (vesicles without treatment), OS-oxidative stress. The results are expressed as means  $\pm$  S.E.M. of 5 experiments. \*\*\*p < 0.001; significantly different as compared to appropriate control.

| Sample        | Fluorescence intensity (arbitrary units) of |                  |
|---------------|---|------------------|
|               | Tryptophan                                  | Dityrosine       |
| 6 mo control  | $65.4 \pm 0.62$                             | $30.84 \pm 0.94$ |
| 6 mo OS       | 53.99 ± 1.23***                             | 36.45 ± 1.58*    |
| 15 mo control | $76.68 \pm 1.89$                            | $71.03 \pm 1.69$ |
| 15 mo OS      | 64.65 ± 1.12***                             | 77.53 ± 1.15**   |
| 26 mo control | $80.88 \pm 0.72$                            | $61.85 \pm 1.28$ |
| 26 mo OS      | 60.62 ± 1.06***                             | 71.11 ± 1.59***  |

**Table 1**: Effect of oxidative stress on cardiac sarcoplasmic reticulum vesicles.

OS-oxidative stress; Values are expressed as means  $\pm$  S.E.M. of 5 experiments. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; significantly different as compared to control.

**Table 2.** Effect of  $H_2O_2$ , FeSO<sub>4</sub>/EDTA on fluorescence emission (Lym) and excitation (Lyx) of conjugates of lysine with lipid peroxidation products in rat heart sarcoplasmic reticulum vesicles.

| Sample        | Fluorescence intensity (arbitrary units) of |                  |
|---------------|---|------------------|
|               | Lym   | Lyx              |
| 6 mo control  | $42.49 \pm 1.49$                            | 43.12 ± 1.49     |
| 6 mo OS       | 56.06 ± 1.96***                             | 59.86 ± 1.64***  |
| 15 mo control | $48.16 \pm 0.79$                            | $51.52 \pm 0.83$ |
| 15 mo OS      | 57.27 ± 1.73**                              | 62.14 ± 1.97**   |
| 26 mo control | $46.1 \pm 1$                                | $44.09 \pm 0.87$ |
| 26 mo OS      | 59.41 ± 1.38***                             | 59.56 ± 1.33***  |

OS-oxidative stress. The results are expressed as means  $\pm$  S.E.M. of 5 experiments. \*\*p < 0.01; \*\*\*p < 0.001; significantly different as compared to control.

| Sample        | Fluorescence intensity (arbitrary |  |
|---------------|-----------------------------------|--|
|               | units) of 1,8-ANS probe           |  |
| 6 mo control  | $76.95 \pm 1.44$                  |  |
| 6 mo OS       | $72.23 \pm 2.68$                  |  |
| 15 mo control | 94.24 ± 1.54                      |  |
| 15 mo OS      | 89.76 ± 1.11*                     |  |
| 26 mo control | 96.66 ± 1.84                      |  |
| 26 mo OS      | $86.44 \pm 1.40$ **               |  |

Table 3: Effect of H<sub>2</sub>O<sub>2</sub>, FeSO<sub>4</sub>/EDTA on fluorescence intensity of ANS.

OS-oxidative stress. The results are expressed as means  $\pm$  S.E.M. of 5 experiments. \*p < 0.05; \*\*p < 0.01; significantly different as compared to control (cardiac SR vesicles without treatment).





Fig.2

