Age-Dependent Effect of Oxidative Stress on Cardiac Sarcoplasmic Reticulum Vesicles

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Summary

The oxidative stress hypothesis of aging suggests that accumulation of oxidative damage is a key factor of the alterations in physiological function during aging. 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 $FeSO_4$/EDTA + 1 mmol/l H_2O_2 at 37 °C for 60 min. In all groups, oxidative stress was associated with decreased membrane surface hydrophobicity, as detected by 1-anilino-8-naphthalenesulfonate as a probe. 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 aging and oxidative stress are associated with accumulation of oxidatively damaged proteins and lipids and these changes could contribute to cardiovascular injury.

Key words

Oxidative stress • Reactive oxygen species • Protein damage • Heart

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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 defense (Bergendi *et al.* 1999, Pollack and Leeuwenburgh 1999, Sivoňová *et al.* 2007) this defense 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, Cakatay *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-catalyzed 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 superoxide 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

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hydroxyl radical in the presence of transient metal (Fe^{2+}, Cu^{2+}) 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^{2+} and H_2O_2 protein modification occurs at amino acid side chains with metal-binding sites (Stadman and Berlett 1997). Metal catalyzed protein oxidation is probably included in many physiological processes and pathological damages. Aging 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.

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±2 °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_2PO_4, 5 mmol/l EDTA, 0.3 mol/l sucrose, 0.5 mmol/l ditiothreitol, 0.3 mmol/l phenylmethylsulfonyl fluoride, 1 μmol/l leupeptine, 1 μmol/l pepstatine (pH 6.8) with a Ultra-Turrax T 25 homogenizer (three times for 10 s, 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 $FeSO_4$/EDTA at 37ºC 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 spectrofluorimeter. 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 μg/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 Ultraspec III spectrophotometer. The rate of conjugated diene formation was estimated according to the lipid oxidation index, A_{233nm}/A_{215nm}, 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 ± 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.

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

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

OS – oxidative stress. Values are expressed as means ± 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, $FeSO_4$/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 ± 1.49	43.12 ± 1.49
6 mo OS	56.06 ± 1.96***	59.86 ± 1.64***
15 mo control	48.16 ± 0.79	51.52 ± 0.83
15 mo OS	57.27 ± 1.73**	62.14 ± 1.97**
26 mo control	46.1 ± 1	44.09 ± 0.87
26 mo OS	59.41 ± 1.38***	59.56 ± 1.33***

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

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 (Table 1). The intensity of intrinsic tryptophan fluorescence was reduced by 17.4±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±1.4 and –25.0±1.3 %).

The intensity of dityrosine fluorescence was elevated in the samples after free radical treatment (Table 1). Significant changes were observed in SR vesicles from of adult, old and senescent rats (18.2±5.1, 9.2±1.6 and 15.0±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±3.8 % and 31.9±4.6 % (Table 2) increase of excitation and emission fluorescence, respectively after H_2O_2 + $FeSO_4$/EDTA treatment. Exposure of SR vesicles from old rats caused an increase by 20.6±3.8 % and 18.9±3.6 %. Table 2 also shows that oxidative modification in cardiac SR vesicles form senescent rats enhanced to 135.1±3 % and to 133.2±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 (Table 3). Exposure of old and senescent heart SR vesicles caused 6.1±3.5 and 10.6±1.4 % 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±0.2 %. Levels of conjugated dienes increased to 115.1±0.4 % in sarcoplasmic reticulum vesicles from old animals, and to 117.2±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±5.8, 378.5±0.06 and 427.4 ± 2.3 % with advancing age.

Discussion

There is growing evidence that protein oxidative damage is involved in aging process and disease. It is assumed that levels of ROS/RNS increase in aging process (Smith *et al.* 1991). In addition, with advancing

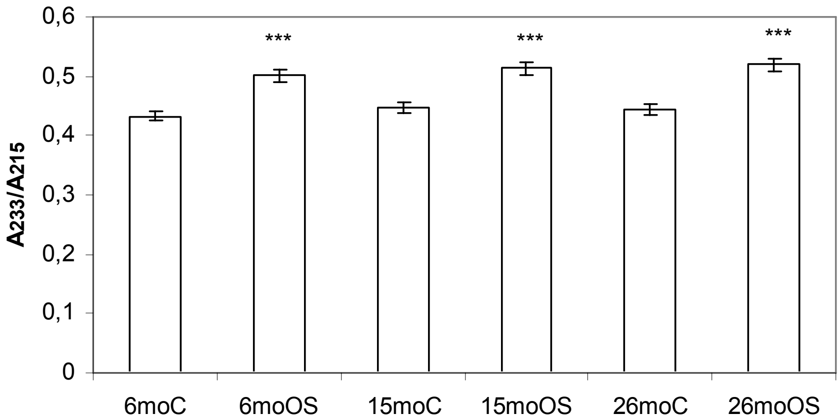

Fig. 1. Effect of H_2O_2, $FeSO_4$/EDTA on conjugated dienes in heart sarcoplasmic reticulum vesicles. C – control (vesicles without treatment), OS – oxidative stress. Values are expressed as means ± S.E.M. of 5 experiments. *** $p<0.001$; significantly different as compared to appropriate control.

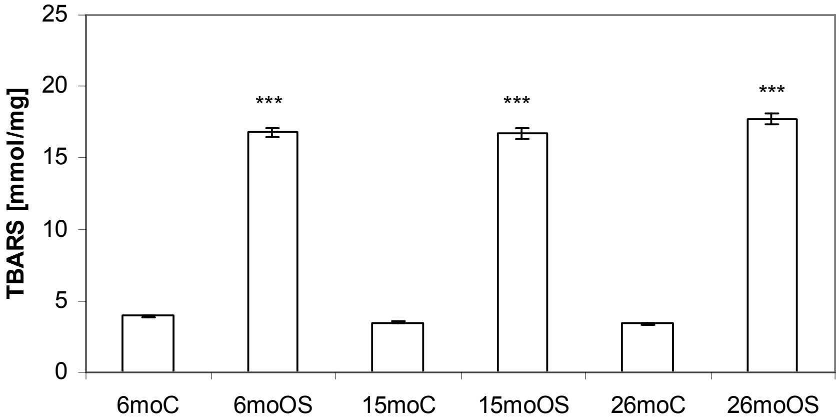

Fig. 2. Effect of H_2O_2, $FeSO_4$/EDTA on TBARS conjugated dienes in heart sarcoplasmic reticulum vesicles. C – control (vesicles without treatment), OS – oxidative stress. Values are expressed as means ± S.E.M. of 5 experiments. *** $p<0.001$; significantly different as compared to appropriate control.

Table 3. Effect of H_2O_2, $FeSO_4$/EDTA on fluorescence intensity of ANS.

Sample	**Fluorescence intensity (arbitrary units) of 1,8-ANS probe**
6 mo control	76.95 ± 1.44
6 mo OS	72.23 ± 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 ± 1.40**

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

age antioxidant defense 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 disturbance 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 (Cakatay *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 aging. 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^{2+} may contribute to disturbance of Ca^{2+} 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 aging 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 conclusion, 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 aging process and contribute to cardiovascular disease.

Conflict of Interest

There is no conflict of interest.

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