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# Age-Associated Changes in $Ca^{2+}$-ATPase and Oxidative Damage in Sarcoplasmic Reticulum of Rat Heart

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**Summary**

Altered $Ca^{2+}$ handling may be responsible for the development of cardiac contractile dysfunctions with advanced age. In the present study, we investigated the roles of oxidative damage to sarcoplasmic reticulum (SR) and expression of $Ca^{2+}$-ATPase (SERCA 2a) and phospholamban in age-associated dysfunction of cardiac SR. SR vesicles were prepared from hearts of 2-, 6-, 15-, and 26-month-old *Wistar* rats. Although activity of $Ca^{2+}$-ATPase decreased with advancing age, no differences in relative amounts of SERCA 2a and phospholamban protein were observed. On the other hand, significant accumulation of protein oxidative damage occurred with aging. The results of this study suggest that age-related alteration in $Ca^{2+}$-ATPase activity in the rat heart is not a consequence of decreased protein levels of SERCA 2a and phospholamban, but could arise from oxidative modifications of SR proteins. Cellular oxidative damage caused by reactive oxygen species could contribute to age-related alternations in myocardial relaxation.

**Key words**

Aging • $Ca^{2+}$-ATPase • Protein damage • Heart • Oxidative stress

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## Introduction

Aging of the heart is connected to many molecular, structural, ionic, biophysical and biochemical changes, including the reduction of contractile performance, prolongation of the relaxing phase and moderation of β-adrenergic receptor-mediated contractile and inotropic responses (Lakatta 1993, Yang *et al.* 1996). The incidence of left ventricular hypertrophy, atrial fibrillation and heart failure grow dramatically during aging (Tribulová *et al.* 1999, Lakatta and Levy 2003). The main degeneration is prolonged calcium transient after depolarization, which results in prolonged contractile time and decelerated relaxation (Roffe 1998, Schmidt *et al.* 2000). Several studies have demonstrated relationship between age-related impairment of ventricular relaxation and decline in ability of sarcoplasmic reticulum (SR) to sequester $Ca^{2+}$ ions (Xu and Narayanan 1998, Schmidt *et al.* 2000, Knyushko *et al.* 2005, for review see Puzianowska-Kuznicka and Kuznicki 2009, Janczewski and Lakatta 2010). Some studies suggest that altered SR function is related to diminished level of SR $Ca^{2+}$-ATPase (SERCA 2a) protein or reduced SERCA 2a to phospholamban (PLN) ratio (Cain *et al.* 1998, Lim *et al.* 1999, Schmidt *et al.* 2000, 2005). SR dysfunction could also arise from posttranslational modifications of proteins. Altered phosphorylation and dysregulation of SR $Ca^{2+}$ cycling proteins was shown in aged rat myocardium (Xu and Narayanan 1998, Slack *et al.* 2001). Our previous studies suggest that aging is associated with increased protein oxidative damage and susceptibility to oxidative stress and loss in antioxidant capacity (Sivoňová *et al.* 2007, Babušíková *et al.* 2008). Changes in reactive oxygen species (ROS) induced cellular damage occur already during early postnatal development and are thought to accumulate with age (Ošťádalová *et al.* 2010). Protein oxidation includes numerous changes and all amino acids

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are sensitive to ROS, but sulfur-containing amino acids (cysteine and methionine) and aromatic amino acids (tyrosine, fenylalanine and tryptophan) are most prone to oxidative damage (Davies 1987). Aging was shown to increase reversible and irreversible oxidative modifications of protein sulfhydryl groups (Thomas and Mallis 2001), formation of protein carbonyls (Judge *et al.* 2005) and modifications of tyrosines to dityrosines (Leeuwenburgh *et al.* 1997) or 3-nitrotyrosines (Sharov *et al.* 2006a). Lipid peroxidation (LPO) is another ROS-induced degenerative process which interferes with cell membranes under conditions of oxidative stress (Girotti 1998). LPO can mediate protein dysfunction through alterations of membrane environment or through its products, reactive aldehydes, capable of binding to proteins (Petersen and Doorn 2004). Also LPO-mediated protein damage was shown to be accumulated with aging in various tissues and organs, including the heart (Yan and Sohal 1998, Judge *et al.* 2005). However, both LPO-mediated and direct protein oxidative damage was demonstrated either in whole tissue homogenates or isolated mitochondria, but data on age-related modifications of SR proteins are rare and/or are not correlated with SR function (Schöneich *et al.* 2006, Babušíková *et al.* 2008).

Thus, it is not yet clear whether age-associated impairment in SR $Ca^{2+}$ handling is caused by altered expression of $Ca^{2+}$ cycling proteins or their posttranslational-oxidative modifications. Therefore, present study was performed to evaluate the roles of protein expression and oxidative damage to SR in age-related $Ca^{2+}$-ATPase dysfunction in rat hearts.

## Materials and Methods

*Animals*

Male *Wistar* rats (IEP SAS Dobra Voda, Slovakia) were grouped by age: young (2-month-old), adult (6-month-old), old (15-month-old) and senescent (26-month-old). The animals were maintained in an air-conditioned room (21±2 °C, 12 h light/dark cycle). Food and water were available *ad libitum*. Experiments were approved by The Ethics Committee of the Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava. All studies were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by The US National Institute of Health (NIH publication NO 85-23, revised 1996), as well as with the rules issued by the State Veterinary and Alimentary of the Slovak Republic.

*Preparation of sarcoplasmic reticulum vesicles*

Each group of animals consisted of 8 animals. The rats were decapitated after 5 minutes of halothane anesthesia (3 % halothane in oxygen/nitrous oxide, 1:2), and hearts were excised. Hearts were washed, minced and homogenized in 10 vol of ice-cold solution containing 30 mmol/l $KH_2PO_4$, 5 mmol/l EDTA, 0.3 mol/l sucrose, 0.5 mmol/l ditiothreitol, 0.3 mmol/l phenylmethyl-sulfonyl fluoride, 1 μmol/l leupeptine, 1 μmol/l pepstatine (pH 6.8) with a Ultra-Turrax T 25 homogenizer (three times for 10 sec, 20 500 rpm). The homogenate was centrifuged at 4 000×g for 15 minutes. The supernatant was poured through four layers of cheesecloth and centrifuged at 14 000×g for 15 minutes. The supernatant was centrifuged at 120 000×g for 70 minutes. The supernatant was discarded and the pellet was resuspended in 10 mmol/l imidazole, 0.65 mol/l KCl, pH 6.8, kept on ice for 30 minutes and centrifuged at 4 400×g for 10 minutes. The resulting supernatant was centrifuged at 120 000×g for 70 min. The final pellet was suspended in 400-600 μl of 0.29 mol/l sucrose, 100 mmol/l KCl, 10 mmol/l HEPES, pH 6.8. All isolation steps were performed at 4 °C.

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

*Determination of $Ca^{2+}$-ATPase activity*

The SR $Mg^{2+}$-dependent, $Ca^{2+}$-stimulated ATPase activity was determined as described by Kaplán *et al.* (2003). The $Ca^{2+}$-ATPase activity was calculated as a difference between $Ca^{2+}$, $Mg^{2+}$-ATPase activity and background $Mg^{2+}$-ATPase activity determined in the absence of $CaCl_2$ in medium containing 3 mmol/l EGTA. Kinetic parameters of $Ca^{2+}$-ATPase activity, maximum ATPase activity ($V_{max}$), half-maximally activating free $[Ca^{2+}]$ ($K_{Ca}$) and Hill coefficient ($n_{Hill}$) were calculated by non-linear least-squares fitting of the experimental data to the Hill equation

$$v = \frac{V_{max}}{1 + (K_{Ca}/[Ca^{2+}])^n}$$

where v is the rate of the ATPase reaction and $[Ca^{2+}]$ is the free $Ca^{2+}$ concentration.

*Fluorescence measurements*

Fluorescence measurements were performed in solution containing 50 µg protein per ml, 10 mmol/l HEPES, 100 mmol/l KCl (pH 7.0) at 25 °C using Shimadzu RF 540 spectroflourimeter (Kaplán *et al.* 2003, Babušíková *et al.* 2004).

Emission spectra of dityrosine, a product of tyrosine oxidation, were recorded in the range of 380 to 440 nm (5 nm slit width) at excitation wavelength of 325 nm (5 nm slit width) (Giulivi and Davies 1994).

Emission spectra (from 425 to 480 nm, 5 nm slit width) of adducts of lipid peroxidation end-products with proteins were recorded after excitation at 365 nm (5 nm slit width).

*Free sulfhydryl group measurement*

The contents of free sulfhydryl (–SH) groups were measured by a DTNB (dithiobisnitrobenzoic acid) assay (Hu 1994) in a Tris buffer, (pH 8.2). Absorbance was read at 412 nm and the –SH group content was calculated using a molar absorption coefficient of 13 600 $M^{-1}cm^{-1}$ after the subtraction of the blank absorbance from the absorbance of the sample.

*Western blot analysis*

The homogenates were solubilized in 0.5 mol/l Tris-HCl, pH 6.8, 10 % glycerol, 5 % SDS, 5 % 2-β-mercaptoethanol, and 0.5 % bromphenol blue. Equal amounts of protein from each sample (20 µg protein/lane) were subjected to SDS-PAGE with the use of 8 % gel for detection of SERCA2a and 15 % gel for detection of phospholamban. After electrophoresis, proteins were transferred to nitrocellulose membrane blots. Blots were blocked in 5 % top nonfat milk in a TBST buffer (TBS with addition of 0.05 % of Tween 20) for 1 hour at room temperature. The blots were incubated at 4 °C overnight with primary monoclonal antibodies to SERCA2a or PLN (Upstate Biotechnology) diluted in an antibody buffer (5 % nonfat milk diluted in TBST). PLN antibody recognizes phosphorylated and nonphosphorylated forms of phospholamban as well as the breakdown protein derivatives. The blots were then washed in TBST and incubated in a secondary antibody solution for 1 h or 1.5 h at room temperature. The blots were washed in TBST, incubated in chemiluminescent substrate (Pierce) for 3 min and exposed to film. The bands corresponding to a particular protein were visualized and quantified by the Molecular Imager (Bio-Rad). Monoclonal anti-phospholamban antibody, clone A1 recognizes phosphorylated and nonphosphorylated PLN.

*Statistics*

The results are presented as a 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. P-values less than or equal to 0.05 were considered statistically significant.

## Results

*Body weight and heart weight*

The weights of young, adult, old and senescent Wistar rats were 212±2.8, 341±7.4, 494±6.2 and 460±5.8 g, respectively. The old and senescent rats were significantly heavier than the adult rats ($p<0.001$) and the young rats were significantly lighter than the adult rats ($p<0.001$). The heart weights of young, adult, old and senescent rats were 0.838±0.024 g, 1.026±0.036 g, 1.103±0.022 g and 1.400±0.031 g, respectively. Only the heart weights of senescent rats were significantly different from those of adult rats ($p<0.001$), but the heart weight-to-body weight ratio, an indicator of cardiac hypertrophy, was not changed (6-month old 3.09±0.19 mg/g, 15-month old 2.70±0.13 mg/g and 26-month old 3.30±0.16 mg/g). The heart weight-to-body weight ratio of young rats was significantly higher (3.89±0.12 mg/g, $p<0.01$) when compared to adult rats. We did not observe any pathological changes in aged hearts.

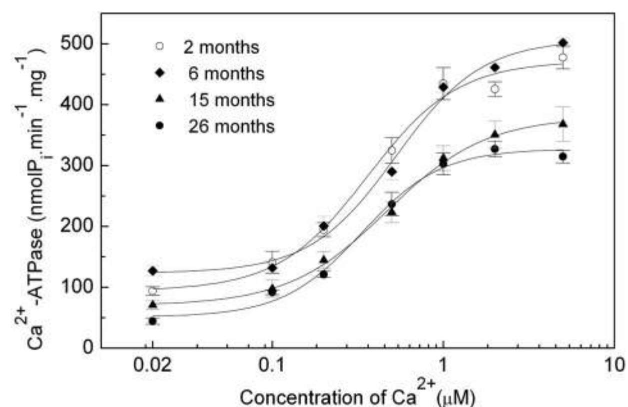


**Fig. 1.** Calcium dependence of $Ca^{2+}$-ATPase activity in cardiac sarcoplasmic reticulum vesicles. The results are expressed as means ± S.E.M. of 8 experiments.

*Changes in heart sarcoplasmic reticulum calcium ATPase*

The activity of $Ca^{2+}$-ATPase was determined in the cardiac SR vesicles. While the difference in pump activity between young and adult rats was not significant aging was associated with significant loss of activity (Fig. 1). Maximum velocity, $V_{max}$, decreased to 75.2±5.1 % (p<0.01) in old animals and to 64.5±2.5 % (p<0.001) in senescent animals as compared to the values of adult animals. The half-maximally activating free $[Ca^{2+}]$, $K_{Ca}$, and the Hill coefficient, $n_{Hill}$, were unchanged (Table 1). Although the activity of SR $Ca^{2+}$-ATPase dramatically declined, the protein levels of $Ca^{2+}$-ATPase and phospholamban were not changed with aging as demonstrated by Western blot analysis (Fig. 2).

**Table 1.** Effect of age on kinetic parameters of SR $Ca^{2+}$-ATPase.

| Age | $V_{max}$ (nmol $P_i$/min/mg) | $K_{Ca}$ (µmol/l) | $n_{Hill}$ |
|---|---|---|---|
| *2 months* | 472.2 ± 17.6 | 0.46 ± 0.05 | 1.65 ± 0.31 |
| *6 months* | 507.4 ± 13.1 | 0.62 ± 0.07 | 1.73 ± 0.22 |
| *15 months* | 381.7 ± 26.1 ** | 0.63 ± 0.08 | 1.66 ± 0.46 |
| *26 months* | 327.3 ± 12.8 *** | 0.50 ± 0.04 | 1.87 ± 0.37 |

$V_{max}$ – maximum $Ca^{2+}$-ATPase activity; $K_{Ca}$ – half-maximally activating free $[Ca^{2+}]$; $n_{Hill}$ – the Hill coefficient. Values are expressed as means ± S.E.M. of 8 experiments. ** $p$<0.01; *** $p$<0.001; significantly different as compared to 6 months animals.

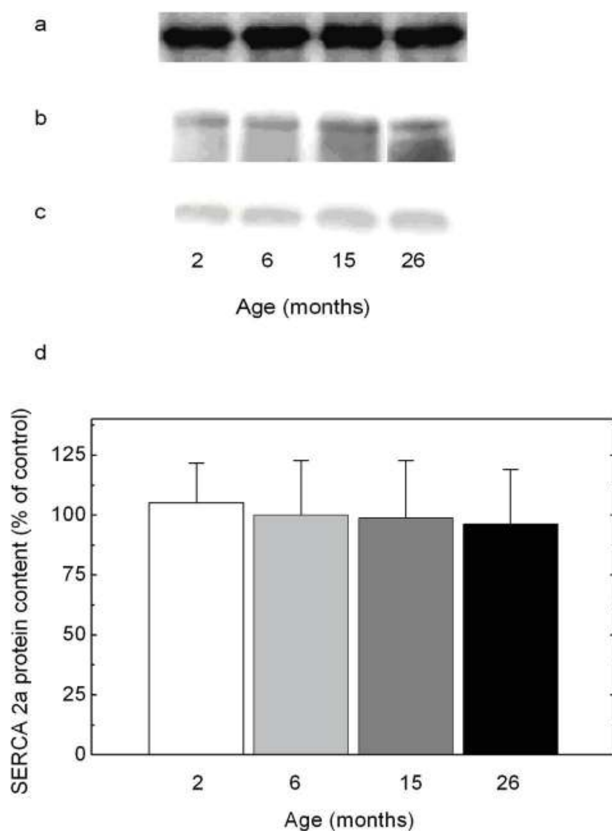


**Fig. 2.** Effect of aging on cardiac proteins. Representative western blot of SERCA2a (**a**), phospholamban (**b**), actin (**c**), and SERCA2a protein content expressed as percentage of average value in 6-months old rats (**d**). The results are expressed as means ± S.E.M. of 8 experiments.

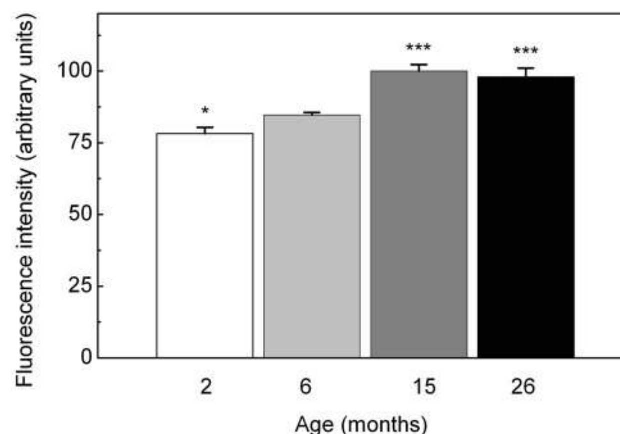


**Fig. 3.** Effect of aging on fluorescence intensity of dityrosines in cardiac sarcoplasmic reticulum. The results are expressed as means ± S.E.M. of 8 experiments. * $p$<0.05, *** $p$<0.001; significantly different as compared to 6 month-old animals.

*Oxidative modifications of SR*

During aging, accumulation of protein oxidative damage in the rat heart was observed. Fig. 3 shows the changes in intensity of dityrosine fluorescence in SR vesicles. There were no differences in dityrosine levels between young and adult rats. The intensity of dityrosine fluorescence was higher in old rats to 118±3 % (p<0.001) and in senescent rats to 116±4 % (p<0.001) of the adult value (Fig. 3). Level of dityrosine was lower in young rats (by 9.2±2.6 %, p<0.05) compared to adult rats.

In contrast, the free sulfhydryl group content was highest in young rats and it decreased progressively with age (Fig. 4).

To evaluate whether LPO contributes to protein modification we measured fluorescence spectra corresponding to adduct of LPO-end products with free amino groups of proteins. As Fig. 5 shows there were no significant changes in fluorescence intensity during aging.

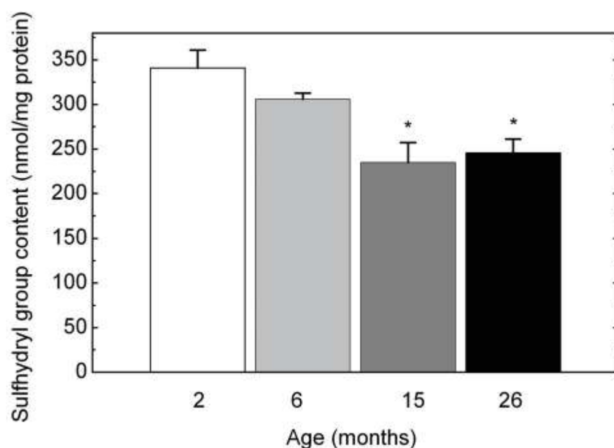


**Fig. 4.** Effect of aging on sulfhydryl group content in sarcoplasmic reticulum of rat heart. The results are expressed as means ± S.E.M. of 8 experiments. * $p<0.05$; significantly different as compared to 6 month-old animals.

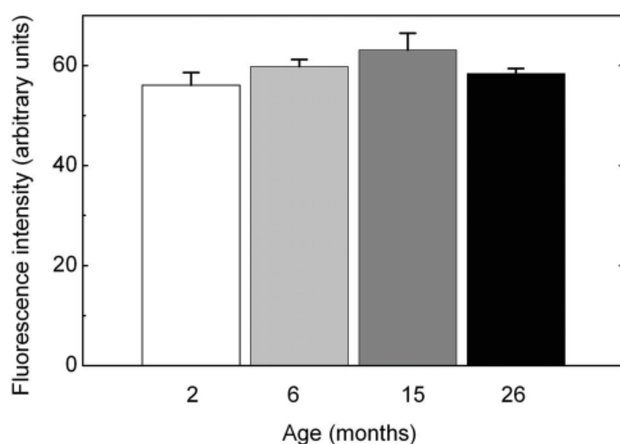


**Fig. 5.** Effect of aging on formation of adducts of LPO-end products with proteins in cardiac sarcoplasmic reticulum. The results are expressed as means ± S.E.M. of 8 experiments.

## Discussion

The present study shows age-related decrease in SR $Ca^{2+}$-ATPase activity in the rat heart. The loss in activity was not associated with alterations in protein contents of SERCA 2a and phospholamban, but was accompanied by significant accumulation of protein oxidative damage.

Age-dependent decline in cardiac SR $Ca^{2+}$-uptake or $Ca^{2+}$-ATPase activity was demonstrated in several studies (Xu and Narayanan 1998, Schmidt *et al.* 2000, 2005, Kaplán *et al.* 2007). Decreased SERCA2a content (Cain *et al.* 1998) or elevated concentration of $Ca^{2+}$-ATPase inhibitor PLN (Lim *et al.* 1999) were suggested as possible causes of this change. Moreover, adenoviral gene transfer of SERCA2a was shown to restore $Ca^{2+}$-ATPase activity and diastolic function in aged rat heart (Schmidt *et al.* 2000). However, other studies, including ours, have failed to show altered mRNA or protein levels of $Ca^{2+}$ handling proteins in SR of aged hearts (Xu and Narayanan 1998, Slack *et al.* 2001, Kaplán *et al.* 2007). The results presented here support the view that altered expression of proteins responsible for SR $Ca^{2+}$-uptake is not involved in age-dependent SR dysfunction. Xu and Narayanan (1998) were first to report that posttranslational modification of SR proteins occurs in aged rat hearts. Their study suggest that reduced phosphorylation of $Ca^{2+}$-ATPase and PLN by $Ca^{2+}$/calmodulin dependent protein kinase is responsible for loss in activity. $Ca^{2+}$-ATPase activity is regulated by PLN. Dephosphorylated PLN inhibits SERCA by lowering its apparent affinity to $Ca^{2}$, $K_{Ca}$, without altering its maximum activity, $V_{max}$, (Vangheluwe *et al.* 2005). PLN phosphorylation withdraws this inhibitory effect and stimulates $Ca^{2+}$ transport. Our study suggests that PLN phosphorylation is unchanged during aging since $K_{Ca}$ value, which is in inverse proportion to affinity, was similar in all aging groups. However, we cannot exclude the possibility that age-related changes in PLN phosphorylation are superimposed by phosphorylations caused by β-adrenergic stimulation triggered by decapitation of animals. Despite this limitation, our study showed gradual decrease of maximum activity, $V_{max}$, indicating other mechanisms of $Ca^{2+}$-ATPase inhibition, such as protein oxidation, nitration or nitrosylation. Knyushko *et al.* (2005) have demonstrated that decrease in $Ca^{2+}$-ATPase activity can be partly attributed to reduced SERCA 2a content, but the mechanism involves also increased protein nitration, suggesting the role of SR oxidative/nitrative stress. Evidence from a number of studies indicates that oxidative/nitrative stress plays a major role in the mechanism of aging (for review see Lakatta and Sollott 2002). Age-related accumulation of

oxidative damage to cellular constituents was demonstrated in various tissues and organs, including the heart, and mitochondria were identified as a main source as well target of ROS (Tatarková *et al.* 2011, for review see Sastre *et al.* 2003, Navarro and Boveris 2007). However, little is known about age-related oxidative damage to cardiac SR, despite the fact that SERCA2a was shown to be inhibited by various ROS (Morris and Sulakhe 1997, Xu and Zweier 1997, Kaplán *et al.* 2003) and is known to be tightly modulated by post-translational modifications, such as glutathionylation, nitrosylation or sumoylation (Vangheluwe *et al.* 2005, Kho *et al.* 2011). To test the hypothesis of age-related oxidative stress to SR we measured protein oxidative damage. The lack of significant changes in adducts of proteins with LPO-end products suggests that LPO is not involved in the mechanism of SR dysfunction. On the other hand, loss of sulfhydryl groups and an increase in dityrosines suggest that aging is associated with significant oxidation of SR proteins. It is well known that cysteine residues play an important role in the transporting mechanism of $Ca^{2+}$ pump and modification of sulfhydryl groups results in the inhibition of activity (Saito-Nakatsuka *et al.* 1987). Sharov *et al.* (2006b) have demonstrated that oxidation of cysteine residues is involved in age-dependent loss in $Ca^{2+}$-ATPase activity in rat skeletal muscle. Since cardiac $Ca^{2+}$-ATPase is reversibly regulated through S-glutathionylation of cysteine residues; their irreversible oxidation may contribute to pump inactivation and impairment of contractile function. Also tyrosine modification of $Ca^{2+}$-ATPase was demonstrated in the aging heart. Knyushko *et al.* (2005) have demonstrated 3-nitrotyrosine modification of SERCA, which correlated with inhibition of $Ca^{2+}$ transport function. We have not identified which proteins were modified by ROS, but our study suggests that tyrosines in SR proteins can also undergo oxidation resulting in dityrosine formation. Since protein oxidative damage was accompanied by decrease in maximum velocity and not the calcium affinity ($K_{Ca}$) it could be possible that ROS inactivate fraction of $Ca^{2+}$-ATPase molecules without affecting properties of others. However, a limitation of this study is that it does not provide direct evidence for causal relation between loss of $Ca^{2+}$-ATPase activity and increased oxidative damage. Therefore, further studies will be necessary to directly assess oxidative and nitrative SERCA2a damage during aging. Alternative mechanisms, such as SERCA2a protein modification by advanced glycation end-products (AGE), N-glycosylation, glutathionylation and $Ca^{2+}$/calmodulin kinase-dependent phosphorylation and the role sarcolipin and other regulatory proteins (Vangheluwe *et al.* 2005) should also be investigated. Finally, calcium cycling is also determined by SR $Ca^{2+}$ release channel, ryanodine receptor (RyR). The role of RyR in age-related SR dysfunction was not investigated in the present study, however, several studies showed reduced expression, phosphorylation or function of RyR in the aging heart (for review see Puzianowska-Kuznicka and Kuznicki 2009, Janczewski and Lakatta 2010).

In summary, the present study demonstrates that aging is associated with progressive decline in $Ca^{2+}$-ATPase activity in cardiac sarcoplasmic reticulum. The loss in activity is not caused by altered SERCA2a protein expression, but may be due, at least in part, to protein oxidative modifications.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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