Oxidative alternations in rat heart homogenate and mitochondria during ageing

E. Babusikova[1], M. Jesenak[2], P. Racay[1], D. Dobrota[1] and P. Kaplan[1]

[1] *Department of Medical Biochemistry, Centre of Excellence for Cardiovascular Research of Slovak Academy of Sciences, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Malá Hora 4, 036 01 Martin, Slovakia*
[2] *Department of Paediatrics, Martin Faculty Hospital, Kollárova 2, 036 59 Martin, Slovakia*

Abstract. Our understanding of the role played by reactive oxygen and nitrogen species in disease pathology and ageing is still insufficient. Reactive oxygen species and reactive nitrogen species can initiate protein and lipid oxidative damage that may be the most important contribution to ageing and age-related heart diseases. In the present study, we investigated the effect of ageing on oxidative damage of protein amino acid residues and lipids in heart homogenate and mitochondria of 4- and 26-month-old Wistar rats. Levels of dityrosine and levels of lysine conjugates increased in heart homogenate during ageing, although levels of conjugated dienes did not change. We observed significantly oxidative modification of tryptophan in heart mitochondria and increased levels of dityrosine with advancing age. However, levels of lysine conjugates, conjugated dienes as well as relative level of cytochrome c oxidase were unchanged in heart mitochondria during ageing. The results of this study suggest a different mechanism of oxidative modification in heart compartments during ageing and moreover, mitochondria and other cellular compartments are targets for oxidative modifications.

Key words*:* Ageing — Protein damage — Oxidative stress — Heart — Mitochondria

Introduction

Ageing is characterised by a progressive deterioration in physiological and metabolic processes. Ageing is an extremely complex, multifactorial process and numerous ageing theories have been proposed. During the past few years, the theories of Free Radical/Oxidative Stress theory of ageing and reactive oxygen and nitrogen species (ROS/RNS) have achieved increasing interest in experimental and clinical medicine.

The heart, an organ which has post-mitotic cells and one of the highest oxygen consumption rates in the body, has a slow turnover of antioxidant enzymes and is therefore highly susceptible to ROS/RNS. Ageing is associated with numerous molecular, ionic, biophysical and biochemical changes in the heart (Lakatta and Sollott 2002). Organisms are constantly exposed to many different forms of ROS/RNS that damage biomolecules, leading to the loss of biological function. For many years, the focus has been on lipid oxidation as a main result of ROS/RNS damage. Lipid peroxidation is a degenerative process that interferes with cell membranes under conditions of oxidative stress (Girotti 1998; Halliwell and Guteridge 1999). Lipid peroxidation products, such as unsaturated aldehydes, malondialdehyde, and 4-hydroxy-2-nonenal are cytotoxic and mutagenic (Dhalla et al. 2000) and can cause other oxidative damage to proteins. Presently, proteins are recognized as one of the most important targets of oxidative damage not just because they play elementary roles in organisms. Proteins can go through many covalent changes after exposing to oxidants (Halliwell and Guteridge 1999; Levine and Stadtman 2001). Protein modifications, elicited by the direct oxidative attack on Lys, Arg, Pro or Thr, by secondary reaction of Cys, His or Lys residues with reactive carbonyl compounds, can lead to the formation of protein carbonyl derivatives (Dalle-Donne et al. 2003) which cause numerous changes in protein structure and as a consequence, it is difficult to find easy-to-measure and representative parameters.

Correspondence to: Eva Babusikova, Department of Medical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Malá Hora 4, 036 01 Martin, Slovakia
E-mail: babusikova@jfmed.uniba.sk

Dysfunction in the mitochondrial respiratory chain can lead to initiation and worsening of cardiac failure. It assumes that mitochondria are considerably participating at oxidative damage during ageing as well as in the process of ageing because they are a place of free radical creation (Harman 1972; Lee and Wei 2001). The mitochondria releases H_2O_2 to the cytosol, and the general level of oxidative stress in the cell will depend on the balance between H_2O_2 generation and its elimination by cellular antioxidants (Davies et al. 2001). The ageing process in mitochondria can lead to the release of a higher amount of ROS, higher mitochondrial and cytosol damage (Lee and Wei 2001) and lower mitochondrial source of low-molecular antioxidants (Hernanz et al. 2000). Furthermore, previous studies in our laboratory performed on rat brain showed mitochondria protein oxidative damage (Babusikova et al. 2007). There are inconsistent results about the effect of ROS/RNS and ageing on mitochondria enzymatic activities (Miró et al. 2000; Choksi et al. 2004; Navarro and Boveris 2004, 2007) and therefore it is difficult to gauge the nature of the functional changes to mitochondria and the link between oxidative damage and metabolic dysfunction is still unclear.

Our aim was to investigate changes in the levels of dityrosine, tryptophan and the level of cytochrome c oxidase. We also studied 1-anilino-8-naphthalenesulfonate (ANS) fluorescence, levels of lysine conjugates with lipid peroxidation (LPO) products and conjugated dienes in the heart homogenate and mitochondria aged rats.

Materials and Methods

Animals

Male Wistar rats supplied by Institute of Experimental Pharmacology, Slovak Academy of Sciences (Dobrá Voda, Slovakia) were divided into two groups according to age, 4-month-old and 26-month-old. The animals were allowed free access to food and water, and were maintained in an air-conditioned room. Experiments were approved by the Ethics Committee of the Jessenius Faculty of Medicine in Martin (Slovakia), as well as by the rules issued by the State Veterinary and Alimentary Administration of the Slovakia.

Preparation of tissue samples

The rats were decapitated 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 7.0) with a Ultra-Turrax T 25 homogenizer (three times for 10 s; 20,500 rpm).

Mitochondria were prepared from tissue homogenate by differential centrifugation. The supernatant from homogenate centrifugation (3200 rpm for 10 min) was centrifuged at 15,100 rpm for 35 min. The pellet was suspended in 30 mmol/l imidazole, 60 mmol/l KCl, 2 mmol/l $MgCl_2$ (pH 7.0). All isolation steps were performed at 4°C.

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

Fluorescence measurements

Fluorescence measurements were performed in solution containing 50 μg proteins per milliliter, 10 mmol/l HEPES, 100 mmol/l KCl (pH 7.0) at 25°C using Shimadzu RF 540 spectroflourimeter. Fluorescence spectrums were measured as previously (Babusikova et al. 2004). For tryptophan, fluorescence were measured at emission spectra (from 300 to 450 nm) by excitation at 295 nm (2 nm slit width). Emission spectra of dityrosine were recorded in range 380 to 440 nm at excitation wavelength 325 nm. Emission spectra (from 425 to 480 nm) of lysine conjugates with LPO products were recovered at an excitation of 365 nm. Excitation spectra (from 325 to 380 nm) were measured at 440 nm. ANS fluorescence was measured following 15 min incubation of the probe with heart homogenate as well as mitochondria. The excitation and emission wavelengths were 365 and 480 nm, respectively.

Measurement of conjugated dienes and TBARS

Conjugated diene formation was determined from the absorbance ratio A_{233nm}/A_{215nm} of heart homogenate and mitochondria dispensed in concentration 20 μg/ml protein in solution with 10 mmol/l phosphate buffer containing 1% Lubrol (Braughler et al. 1986; 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.

Western blot analysis

The protein 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 and 15% gels. After electrophoresis, proteins were transferred to nitrocellulose membrane blots. Blots were blocked in 5% top nonfat milk in TBST buffer (tris buffered saline with addition of 0.05% of Tween 20) for

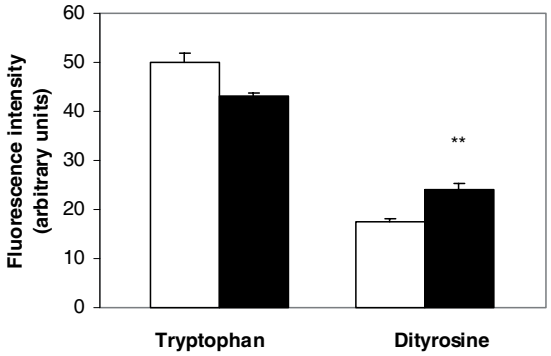

Figure 1. Effect of ageing on fluorescence intensity of tryptophan and dityrosine in rat heart homogenate. The results are expressed as means ± S.E.M. of 5 experiments. ** $p < 0.01$, significantly different as compared to 4 month-old animals; □ 4-month-old rats, ■ 26-month-old rats.

1 h at room temperature. The blots were incubated 90 min in primary antibodies to cytochrome c oxidase (Molecular Probes, 1 : 1000) diluted in antibody buffer (5% nonfat milk diluted in TBST). The blots were then washed in TBST and incubated in secondary antibody solution for 90 min 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 particular protein were visualized and quantified by the Molecular Imager (Bio-Rad).

Data analysis

The results are presented as mean ± S.E.M. One-way analysis of variance was first carried out to test for the differences between groups. Between individual groups comparisons were made using an unpaired Student's *t*-test. A value of $p < 0.05$, $p < 0.01$, $p < 0.001$ were considered to be statistically significant.

Results

Weights of 4-month-old and 26-month-old Wistar rats were 348.75 ± 10.28 g and 407.5 ± 20.54 g ($p < 0.05$).

Oxidative modification in heart homogenate

During ageing we observed oxidative damage in rat heart homogenate. Fig. 1 shows changes in intensity of intrinsic tryptophan and dityrosine fluorescence. Tryptophan fluorescence of 26-month-old rats was not significantly changed compared with the 4-month-old rats ($p > 0.05$). Levels of dityrosine increased significantly with ageing

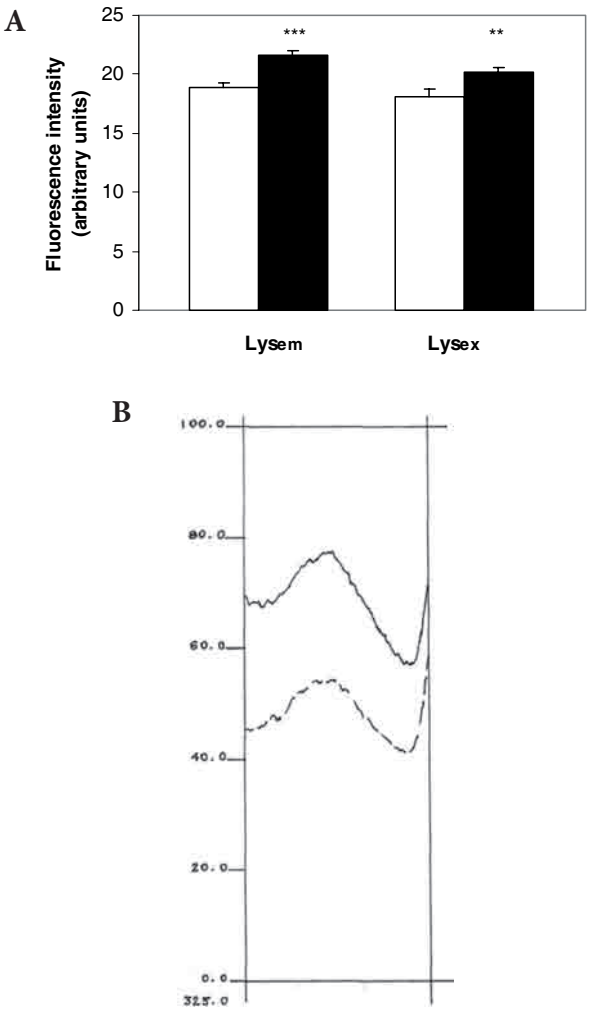

Figure 2. A. Effect of ageing on fluorescence emission and excitation of conjugates of lysine with lipid peroxidation (LPO) products in rat heart homogenate. **B.** Excitation fluorescence spectrum of conjugates of lysine with LPO products in rat heart homogenate. The results are expressed as means ± S.E.M. of 5 experiments. Lys_{em}, emission of conjugates of lysine with lipid peroxidation products; Lys_{ex}, excitation of conjugates of lysine with lipid peroxidation products; ** $p < 0.01$, *** $p < 0.001$, significantly different as compared to 4 month-old animals; □ 4-month-old rats, ■ 26-month-old rats.

(+37.21 ± 7.4%; $p < 0.001$). In order to assess modification of homogenate proteins by LPO-end products, the fluorescence excitation (350–360 nm) and emission (440–450 nm) spectra were measured. Fluorescence of lysine conjugates with LPO products were increased with age (+14.98%, $p < 0.001$; +12.0%, $p < 0.01$) (Fig. 2). Significant differences were observed in ANS fluorescence (+15.23%, $p < 0.001$) (Fig. 3). Modifications in lipid structure were also assayed by measurement of changes in

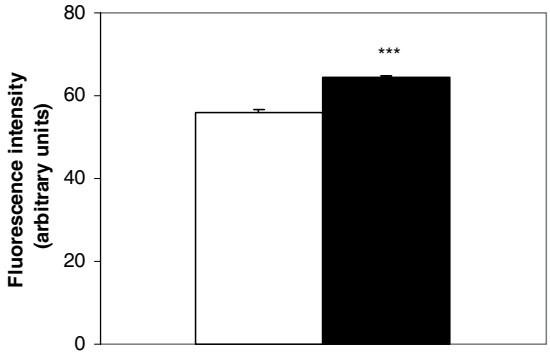

Figure 3. Effect of ageing on fluorescence intensity of ANS probe in rat heart homogenate. The results are expressed as means ± S.E.M. of 5 experiments. *** $p < 0.001$, significantly different as compared to 4 month-old animals; □ 4-month-old rats, ■ 26-month-old rats.

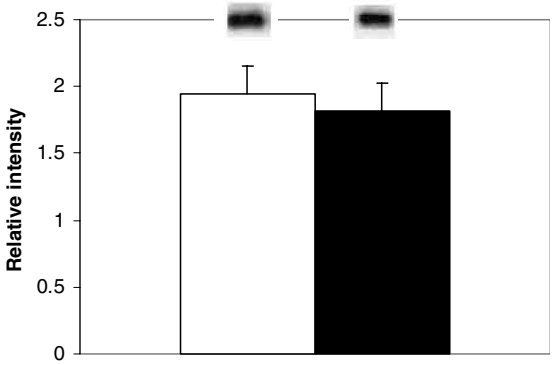

Figure 4. Representative western blot of cytochrome c oxidase and effect of ageing on protein level of cytochrome c oxidase. The results are expressed as means ± S.E.M. of 5 experiments. □ 4-month-old rats, ■ 26-month-old rats.

Table 1. Effect of ageing on level of conjugated dienes in heart homogenate

Age	CD A233/A215	TBARS (mmol/mg)
4 months	0.35 ± 0.0035	3.93 ± 0.389
26 months	0.40 ± 0.0026***	3.39 ± 0.078

CD, conjugated dienes, TBARS, thiobarbituric acid-reactive substances. The results are expressed as means ± S.E.M. of 5 experiments. *** $p < 0.001$, significantly different as compared to 4 month-old animals.

levels of conjugated dienes and TBARS (Tab. 1). Levels of conjugated dienes elevated significantly to 114.29 ± 0.7% and levels of TBARS were unchanged with advancing age. A relative amount of cytochrome c oxidase did not change during ageing (Fig. 4).

Oxidative damage in heart mitochondria

In rat brain mitochondria we observed significant changes only in the levels of intensity of intrinsic tryptophan (–10%) and dityrosine (+12%) fluorescence (Tab. 2). Fluorescence of lysine conjugates with LPO-end products did not change with advancing age and protein surface hydrophobicity, measured by ANS fluorescent probe, did not change as well (Tab. 2).

Discussion

Ageing has a powerful effect on increased susceptibility to cardiovascular diseases even in optimally healthy individuals. In this report we investigated the potential effect of the ageing process on protein and lipid modifications in rat heart homogenates and mitochondria. We observed protein and lipid oxidative damage in heart homogenate and mitochondria with advanced age; however, heart homogenate was more damaged than heart mitochondria (Tab. 3).

Aromatic amino acid residues have been considered the main target of oxidants. We measured dityrosine fluorescence as a sensitive marker of protein oxidation (Davies et al. 1999). Levels of dityrosine were increased in cardiac homogenate and in mitochondria; there was a significant decreased level of tryptophan. The cross-linking of protein tyrosyl residues can be a result of a normal physiological process as a result of a pathological response to oxidative

Table 2. Effect of ageing on fluorescence intensity in rat heart mitochondria

Age	Fluorescence intensity (arbitrary units)					CD
	Tryptophan	Dityrosine	Lys_{em}-LPO	Lys_{ex}-LPO	ANS	A233/A215
4 months	54.55 ± 0.3	72.86 ± 1.5	31.09 ± 0.3	30.59 ± 0.4	48.34 ± 0.9	0.2594 ± 0.0042
26 months	49.54 ± 0.8***	81.85 ± 1.8**	32.10 ± 0.8	31.90 ± 1.3	50.06 ± 1.7	0.2611 ± 0.0108

CD, conjugated dienes ; Lys_{em}-LPO, emission of conjugates of lysine with lipid peroxidation products; Lys_{ex}-LPO, excitation of conjugates of lysine with LPO products; ANS, 1-anilino-8-napthalenesulfonate. Values are expressed as means ± S.E.M. of 5 experiments. ** $p < 0.01$, *** $p < 0.001$, significantly different as compared to 4 month-old animals.

Table 3. Compare of oxidative changes in cardiac homogenate *versus* mitochondria with advancing age

Oxidative marker	H/Mit
Tryptophan	8.31/9.18
diTyrosine	37.20/12.34
Lysem	14.98/3.20
Lysex	12.0/4.28
ANS	15.23/3.56
CD	1.82/0.66

Lys_{em}, emission spectrum of conjugates of lysine; Lys_{ex}, excitation spectrum of conjugates of lysine; ANS, fluorescent probe 1-anilino-8-naphthalenesulfonate; CD, conjugated dienes; H, comparison of changes in homogenate between 4- and 26-month-old animals; Mit, comparison of changes in mitochondria between 4- and 26-month-old animals.

stress and disease. Age-related increase in oxidative damage of tyrosine was observed in mouse skeletal muscle and heart (Leeuwenburgh et al. 1997) and in rat brain (Babusikova et al. 2007). Similar amino acid residues oxidative modifications were observed in the heart after *in vitro*-induced oxidative stress (Kaplan et al. 2003; Babusikova et al. 2004) which suggest an indirect effect of reactive oxygen during ageing. In contrast, in the Davies et al. (2001) study, there were no observed age-related alternations (o-, m-tyrosine content) in brain, heart or liver. Several factors may account for the different results of these studies, including differences in animal species and the methods used for detection of protein oxidation. We observed also changes in the surface hydrophobicity in cardiac homogenate with advanced age by using ANS, an anionic probe. Changes in surface hydrophobicity are often associated with changes in protein conformation which can cause alternation in enzyme activities. Age-related changes in the surface hydrophobicity could be due to oxidation by ROS not only in rat liver (Chao et al. 1997) and brain (Babusikova et al. 2007) but also in the heart. We did not detect changes in mitochondrial ANS fluorescence and probably conformational changes occurred in other cardiac cellular compartments. We determined oxidative damage in heart sarcoplasmic reticulum during ageing (result does not show). Statistically significant oxidative lipid damage was observed only in heart homogenate, levels of conjugates of lysine with products of LPO and conjugated dienes increased in 26-month-old rats. There was higher sensitivity to protein oxidative damage than to lipid oxidative damage observed by Tian et al. (1998) as well. However, there were increased levels of proteins carbonyl and TBARS in the submitochondrial membranes of brain, heart, liver and kidney in ageing mice (Navarro et al. 2004). In contrast, increased accumulation of degraded lipid products such as malondialdehyd and 4-hydroxynonenal in mitochondria membranes were observed with advanced age (Esterbauer et al. 1991; Chen and Yu 1994). This indicates the possibility that the decline of enzymatic activities is rather connected with the accumulation of protein damage during ageing. The results of our study indicate age-related accumulation of fluorescent products within the heart which supports the idea that protein and oxidative modification increases with advanced age.

As a consequence to oxidative damage there is a decrease in bioenergetical function of mitochondria in tissues with age (Wei et al. 2001). The link between mitochondrial oxidative damage and metabolic dysfunction is still unclear. Activities of complexes I, II, III, IV and V have been variously shown to decline or remain unchanged with age (Sugiyama et al. 1993; Berrientos et al. 1996; Lenaz et al. 1997; Davies et al. 2001; Kumaran et al. 2004). Western blot analysis showed that ageing is associated with a selectively decreased content of complexes I, II and IV in human skeletal muscle, whereas complex III was not altered (Boffoli et al. 1994). We did not detect changes in amount of cytochrome c oxidase. Due to inconsistent results, it is difficult to gauge the nature of functional changes to mitochondria. In many enzymatic activities there are observed changes during ageing but the mechanism of how it occurs is still unclear. Our established oxidative damage and accumulation of damaged amino acids with age could contribute to the observed changes in enzymatic activities. Post-translate mechanism of protein oxidative damage can contribute to the increase in oxidative stress and to the physiological damage accumulation during ageing.

In conclusion, results of this study indicate that the susceptibility of tissues to undergo protein oxidative damage increases with age. This shows that protein oxidative modification is in a reciprocal relation to the ageing of an organism and its cells which may be sensitive to various degrees to increasing oxidative stress.

Acknowledgements. This work was partially supported by grant VEGA 1/0027/08.

References

Babušíková E., Kaplán P., Lehotský J., Jeseňák M., Dobrota D. (2004): Oxidative modification of rat cardiac mitochondrial membranes and myofibrils by hydroxyl radicals. Gen. Physiol. Biophys. **23**, 327–335

Babusikova E., Hatok J., Dobrota D., Kaplan P. (2007): Age-related oxidative modifications of proteins and lipids in rat brain. Neurochem. Res. **32,** 1351–1356

Barrientos A., Casademont J., Rötig A., Miró Ó., Urbano-Márquez Á., Rustin P., Cardellach F. (1996): Absence of relationship between the level of electron transport chain activities and ageing in human skeletal muscle. Biochem. Biophys. Res. Commun. **229**, 536–539

Boffoli D., Scacco S. C., Vergari R., Solarino G., Santacroce G., Papa S. (1994): Decline with age of the respiratory chain activity in human skeletal muscle. Biochim. Biophys. Acta **12**, 73–82

Braughler J. M., Duncan L. A., Chase R. L. (1986): The involvement of iron in lipid peroxidation. J. Biol. Chem. **261**, 10282–10289

Chao C. C., Ma Y. S., Stadtman E. R. (1997): Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. Proc. Natl. Acad. Sci. U.S.A. **94**, 2969–2974

Chen J. J., Yu B. P. (1994): Alterations in mitochondrial membrane fluidity by lipid peroxidation products. Free Radic. Biol. Med. **17**, 411–418

Choksi K. B., Boylston W. H., Rabek J. P., Widger W. R., Papaconstantinou J. (2004): Oxidatively damaged proteins of heart mitochondrial electron transport complexes. Biochim. Biophys. Acta **1688**, 95–101

Dalle-Donne I., Giustarini D., Colombo R., Rossi R., Milzani A. (2003): Protein carbonylation in human diseases. Trends Mol. Med. **9**, 169–176

Das D. K. (1994): Cellular, biochemical, and molecular aspects of reperfusion injury. Ann. N. Y. Acad. Sci. **723**, 118–124

Davies M. J., Fu S., Wang H., Dean R. T. (1999): Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Radic. Biol. Med. **27**, 1151–1163

Davies S. M. K., Poljak A., Duncan M. W., Smythe G. A., Murphy M. P. (2001): Measurements of protein carbonyls, ortho- and meta-tyrosine and oxidative phosphorylation complex activity in mitochondria from young and old rats. Free Radic. Biol. Med. **31**, 181–190

Dhalla N. S., Temsah R. M., Netticadan T. (2000): Role of oxidative stress in cardiovascular diseases. J. Hypertens. **18**, 655–673

Esterbauer H., Schaur R. J., Zollner H. (1991): Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. Free Radic. Biol. Med. **11**, 81–128

Girotti A. W. (1998): Lipid hydroperoxide generation, turnover, and effector action in biological systems. J. Lipid Res. **39**, 1529–1542

Halliwell B., Gutteridge J. M. C. (1999): Free Radicals in Biology and Medicine (3rd ed.). Oxford, University Press, UK

Harman D. (1972): The biologic clock: the mitochondria? J. Am. Geriatr. Soc. **20**, 145–147

Hernanz A., Fernandez-Vivancos E., Montiel C., Vazquez J. J., Arnalich F. (2000): Changes in the intracellular homocysteine and glutathione content associated with aging. Life Sci. **67**, 1317–1324

Kaplan P., Babusikova E., Lehotsky J., Dobrota D. (2003): Free radical-induced protein modification and inhibition Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum. Mol. Cell. Biochem. **248,** 41–47

Klein R. A. (1970): The detection of oxidation in liposome preparations. Biochim. Biophys. Acta **210**, 483–486

Kumaran S., Subathra M., Balu M., Panneerselvam C. (2004): Age-associated decreased activities of mitochondrial electron transport chain complexes in heart and skeletal muscle: role of L-carnitine. Chem. Biol. Interact. **148**, 11–18

Lakatta E. G., Sollott S. J. (2002): Perspectives on mammalian cardiovascular ageing: humans to molecules. Comp. Biochem. Physiol., A **132**, 699–721

Lee H. C., Wei Y. H. (2001): Mitochondrial alterations, cellular response to oxidative stress and defective degradation of proteins in aging. Biogerontology **2**, 231–244

Leeuwenburgh C., Wagner P., Holloszy J. O., Sohal R. S., Heinecke J. W. (1997): Caloric restriction attenuates dityrosine cross-linking of cardiac and skeletal muscle proteins in aging mice. Arch. Biochem. Biophys. **346**, 74–80

Lenaz G., Bovina C., Castelluccio C., Fato R., Formiggini G., Genova M. L., Marchetti M., Pich M. M., Pallotti F., Parenti Castelli G., Biagini G. (1997): Mitochondrial complex I defects in aging. Mol. Cell. Biochem. **174**, 329–333

Levine R. L., Stadtman E. R. (2001): Oxidative modification of proteins during aging. Exp. Gerontol. **36**, 1495–1502

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275

Miro O., Casademont J., Casals E., Perea M., Urbano-Marquez A., Rustin P., Cardellach F. (2000): Aging is associated with increased lipid peroxidation in human hearts, but not with mitochondrial respiratory chain enzyme defects. Cardiovasc. Res. **47**, 624–631

Navarro A., Boveris A. (2004): Rat brain and liver mitochondria develop oxidative stress and loose enzymatic activities upon ageing. Am. J. Physiol., Regul. Integr. Comp. Physiol. **287**, R1244–1249

Navarro A., Boveris A. (2007): The mitochondrial energy transduction system and the aging process. Am. J. Physiol., Cell. Physiol. **292**, C670–686

Navarro A., Gomez C., López-Cepero J. M., Boveris A. (2004): Beneficial effects of moderate exercise on mice aging: survival, behavoir, oxidative stress, and mitochondrial electron transfer. Am. J. Physiol., Regul. Integr. Comp. Physiol. **286**, R505–511

Sugiyama S., Takasawa M., Hayakawa M., Ozawa T. (1993): Changes in skeletal muscle, heart and liver mitochondrial electron transport activities in rats and dogs of various ages. Biochem. Mol. Biol. Int. **30**, 937–944

Tian L., Cai Q., Wei H. (1998): Alterations of antioxidant enzymes and oxidative damage to macromolecules in different organs of rats during aging. Free Radic. Biol. Med. **24**, 1477–1484

Wei Y.-H., Ma Y.-S., Lee H.-C., Lee C.-F., Lu C.-Y. (2001): Mitochondrial theory of aging matures – roles of mtDNA mutation and oxidative stress in human aging. Chin. Med. J. (Taipei) **64**, 259–270

Final version accepted: April 29, 2008