EXPERIMENTAL STUDY

In vitro effect of stobadine on Fe^{2+}-induced oxidative stress in rat liver mitochondria

Chavkova Z, Guzy J, Barnova E, Marekova M, Tomeckova V, Dubayova K, Kusnir J

Department of Medical Chemistry and Biochemistry, Faculty of Medicine Safarikiensis University, Kosice, Slovakia. **zchavko@central.medic.upjs.sk**

Abstract

The authors have studied the susceptibility of two key protective enzymes, glutathione peroxidase (GPX) and glutathione reductase (GR) to the reactive oxygen species (ROS) known to induce oxidative damage in vitro system containing Fe^{2+}/EDTA. The ability of ROS scavanger stobadine to prevent oxidative damage was also studied. Incubation of GPX with Fe^{2+}/EDTA resulted in the significant decrease in its enzyme activity while under the same condition the activity of GR was not changed. The presence of stobadine was effective in protecting GPX from the loss of its activity by in vitro oxidizing agents. The monitoring of the mitochondrial outer membrane dynamics by the method of synchronous fluorescence fingerprint showed that the membrane is involved in these processes. *(Fig. 3, Ref. 35.)*

Key words: glutathione peroxidase, glutathione reductase, antioxidant enzymes, oxidative stress, reactive oxygen species (ROS), stobadine, mitochondria, synchronous fluorescence fingerprint.

Oxidation stress is defined as an imbalance between production and elimination of reactive oxygen species (ROS) and excessive accumulation of ROS in tissue. ROS accumulation results in the induction of self-catalyzed reactions leading to the oxidative damage of biologically significant macromolecules and to the cell damage.

Oxidative stress is known to play a decisive role in pathogenesis of many diseases (Diplock, 1994) and ROS are also a key factor in the „mitochondrial theory of aging" (Miquel et al, 1980; Ozawa, 1997) and apopthotic cell death (France-Lanord et al, 1997; Susin et al, 1998; Petit et al, 1996).

The primary site of ROS interaction in the cells are macromolecules located at the near vicinity of ROS production, namely mitochondrial structures (Ernster, 1993). Mitochondrial respiratory chain is likely the main source of ROS in the cell (Chance et al, 1979). As much as 1—4 % of total oxygen consumed in the respiratory chain reactions is reduced to superoxide radical and to H_2O_2 (Richter et al, 1988). Possible targets for ROS in mitochondria include peroxidation of membrane lipids (Ernster et al, 1993), protein oxidation (Stadtman, 1993) and DNA damage (Richter et al, 1988 a, b; Dizdaroglu, 1991). Oxidation of macromolecules results in the breakdown of enzyme function, as well as in transport and receptor function and leading to the cell damage and death (Lenaz et al, 1994).

At physiological situation, ROS are eliminated by antioxidant defense system including both, enzyme and non-enzyme mechanisms. Some drugs possess an antioxidant activity and can produce some beneficial effects such as membrane stabilization and elimination of free radicals by ROS scavanging mechanisms. These compounds by ROS scavanging, binding metal ions and degrading peroxides can inhibit propagation of ROS reaction or repair already induced damage (Jakus and Lopuchova, 1999).

Stobadine (ST) is an effective, neuro- and cardioprotective drug with a potent antioxidative properties, a substance with a multicomponent effect affecting both, central and peripheral structures (Dubovicky et al, 1999). Because of its scavenging effect on ROS, it can be used as a drug in prevention and treatment of oxidative stress.

In the present study we determined activities of two enzymes participating in the cell antioxidative defense mechanism, glutathione peroxidase (GPX) and glutathione reductase (GR) in the liver mitochondria after incubation with ROS producing system containing Fe^{2+}/EDTA. We also determined the effect of sto-

Department of Medical Chemistry and Biochemistry, Faculty of Medicine Safarikiensis University, Kosice, Slovakia

Address for correspondence: Z. Chavkova, Ing, PhD, Dept of Medical Chemistry and Biochemistry, LF UPJS, Trieda SNP 1, SK-040 66 Košice, Slovakia.
Phone: +421.95.6423849, Fax: +421.95.64238

badine alone and in the combination with Fe^{2+}/EDTA on activities of both enzymes. The mitochondrial outer membrane dynamics was simultaneously monitored by the method of synchronous fluorescence fingerprint (Phelan, 1994; Kušnír et al, 1998). The purpose of our experiments was to find if presence of stobadine will protect against the loss of enzyme activities at given oxidizing system.

Material and methods

Glutathione reductase (E.C.1.6.4.2) Type III from baker yeast, ß-nicotinamide adenine dinucleotide phosphate, reduced (NADPH), reduced and oxidized glutathione were purchased from Sigma (St. Louis, MO).

(-)-cis-2,8-dimetyl-2,3,4,4a5,9b-hexahydro-1H-pyrido [4,3-b] indole (stobadine, ST) in the form of hydrochloride (m.w. 715,2) was obtained from Institut of Experimental Pharmacology SAV, Bratislava (Slovak Republic).

Female Wistar rats weighing 200—250 g fed on a standard laboratory diet and tap water were used in experiment. Animals were sacrificed by decapitation, their livers were quickly removed and mitochondria were isolated according to Johnson and Lardy (1967).

The effect of Fe^{2+}/EDTA, stobadine, and Fe^{2+}/EDTA and stobadine on mitochondrial enzyme activities was studied in three separate experimental groups (4—6 experiments in each group).

The effect of Fe^{2+}/EDTA on the enzymes was studied by incubating mixture containing mitochondrial proteins in phosphate buffer after addition of 100 µM $FeSO_4$/mg protein and 10 mmol EDTA for 60 min. The effect of stobadine or stobadine and Fe^{2+}/EDTA was studied under the same conditions, with 100 µM stobadine in the incubation mixture.

GPX activity (E.C.1.11.1.9) was measured by the consecutive glutathione reductase reaction. This reaction was monitored by oxidation of NADPH assayed at 366 nm (Flohe et al, 1984). The assay mixture contained 120 µl mitochondria suspension, 120 µl of 10 mM GSH, 120 µl glutathione reductase (0.24 U), 120 µl 1.5 mM NADPH in 1 % $NaHCO_3$ and 600 µl 0.1 M phosphate buffer (pH=7). The overal reaction was started by adding 120 µl of prewarmed 1.5 mM H_2O_2. The decrease in absorption at 365 nm was monitored for 5 min. Activity of enzyme was expressed as the decrease in NADPH concentration in units U/kg of protein.

Activity of glutathione reductase (E.C.1.6.4.2) was determined by the method of Calberg and Mannervick (1985). Reaction mixture contained in 1 ml 0.2 M potassium buffer and 2 mM EDTA, pH=7, 100 µl 2 mM NADPH in 10 mM Tris-HCl (pH=7) and 100 µl 20 mM GSSG. The reaction was initiated by the addition of 10 µl mitochondria suspension to the reaction mixture and the decrease in absorbance at 340 nm was monitored at 37 °C for 5 min.

Glutathione reductase activity was expressed as the amount of enzyme which catalyzes reduction of 1 mol of NADPH per second per kg of protein.

The natural synchronous fluorescence spectra were generated by constantly setting of the wave lengths differences $\Delta\lambda = 15$, 30, 50, 70, 90 between emission and excitation monochromators of fluorescence spectrometer Perkin-Elmer Model 3000. The countour line maps were made by the computer from the records of individual synchronous spectra settled in the space one after the other as vertical projection of their three dimensional form. Each countour line represents the value of relative fluorescence elevated by 100.

Results

The mitochondrial GPX activity significantly decreased upon exposure to Fe^{2+}/EDTA (Fig. 1). Incubation of mitochondria with stobadine alone for 1 hour had no effect on the activity of GPX. Addition of stobadine to Fe^{2+}/EDTA containing incubation mixture prevented decrease of GPX activity caused by the presence of Fe^{2+}/EDTA.

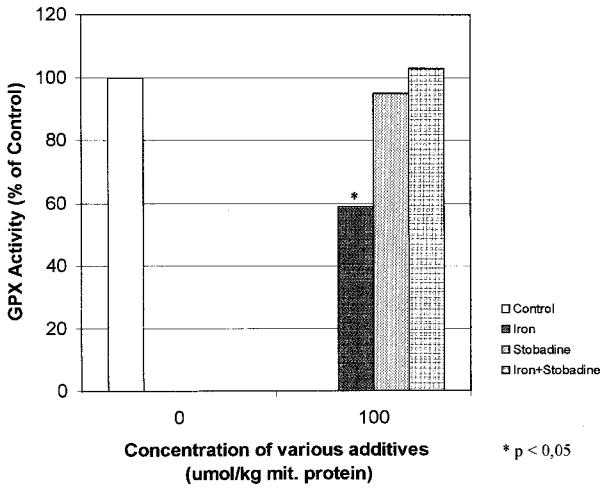

Fig. 1. Changes in glutathione peroxidase activity (GPX) in rat liver mitochondria (%) in dependence on concentration of various additives (iron, stobadine, and their combination). Absolute values of activity GPX determined in U/kg mitochondrial protein were statistically evaluated by Student t-test on the level of significance p<0.05 (in the figures marked by asterisk). From these results relative percentage have been calculated for comparison with control values (100 %). The control (without additives) and experimental groups contained 4 samples.

Incubation of mitochondria with Fe^{2+}/EDTA did not markedly influence GR activity (Fig. 2). Neither, stobadine itself or in combination with Fe^{2+}/EDTA had any effect on the GR activity as illustrated in Fig. 2.

A figure 3A is a grafical (spectral) definition of control rat liver mitochondria suspension in respiration medium. It graphically defines the state of the mitochondrial outer membrane. The area $\Delta\lambda = 15$—30 nm is an area of dispersion fenomena (dispersion of light at the surface of mitochondria). The density of countour lines in this area is high what represents a high scattering intensity. The mitochondria are not changed and behave accord-

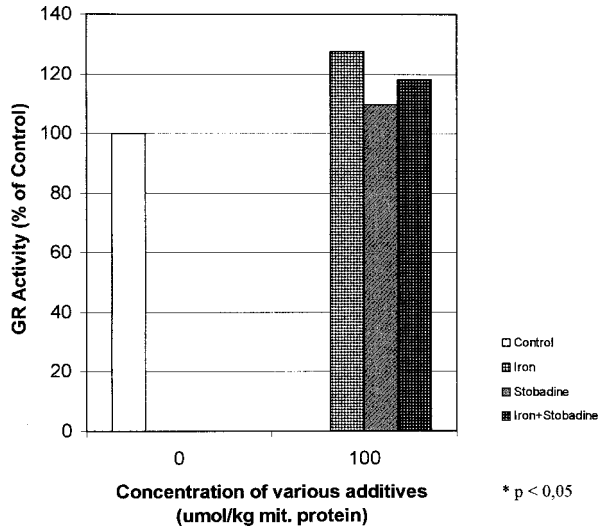

Fig. 2. Changes in glutathione reductase activity (GR) in rat liver mitochondria (%) in dependence on concentration of various additives (iron, stobadine, and their combination). Absolute values of activity GR determined in mol/sec/kg mitochondrial protein were statistically evaluated by Student t-test on the level of significance p<0.05 (in the figures marked by asterisk). From these results relative percentage have been calculated for comparison with control values (100 %). The control (without additives) and experimental groups contained 4 samples.

ing to their fluorescence spectrum (Fig. 3A) as large particles. The countour lines in the area >30 nm represents the area of a native fluorescence of the mitochondrial surface components. The countour lines are dense. This fluorescence is very characteristic for vital mitochondria after isolation. This record (Fig. 3A) was considered as a standard (control), for comparison other countour maps of shape changed mitochondria after addition of solution $FeSO_4$ — EDTA stobadine and combination $FeSO_4$ — EDTA with stobadine (Fig. 3B, 3C, 3D).

The countour map after addition $FeSO_4$ — EDTA to the mixture of mitochondria (Fig. 3B) shows slightly increased fluorescence and density of countour lines as compared with control mitochondria (Fig. 3A). Probable swelling process releases hidden natural fluorophores on the surface of mitochondrial outer membrane due to supposed membrane refolding, as a result of an intensive activity of mitochondria defence system against oxidative stress.

The countour line map after addition of stobadine to mitochondria (Fig. 3C) showes no change at the light scattering area, but decrease of fluorescence at fluorescence area caused probably by soft surface changes of mitochondria (Fig. 3E). The countour line maps after addition of combination $FeSO_4$ — EDTA with stobadine to mitochondria (Fig. 3D) has no effect, on mitochondria surface in both fluorescence and light scattering area. Moreover combination $FeSO_4$ — EDTA with stobadine causes no backround fluorescence.

Discussion

Stress is a decisive factor in the development of many pathological processes such as atherosclerosis, myocardial infarction, stroke and many neurodegenerative diseases.

In many experimental studies has been demonstrated that antioxidants, chemical compounds that eliminate ROS can also protect against these serious and frequently occurring diseases (Jakuš and Lopuchová, 1999; Horáková and Štolc, 1998).

In our *in vitro* experiments, the oxidizing system Fe^{2+}/EDTA had the same effect as an oxidative stress factor to mitochondria (Morimoto et al, 1997). Iron and copper ions are powerful promoters of free-radical damage, causing formation of hydroxyl radicals and accelerating lipid peroxidation, so their effect is thought to be free radical-mediated (Anderson and Philips, 1999).

GPX and GR are two important enzymes involved in the cellular defense against oxidative stress. It has been shown that the controlled decrease of the level of antioxidant enzymes catalase, SOD, GPX, and GR in human fibroplasts results in lowering the cell viability under normal and hyperoxic conditions. The cell viability is more drastically affected when GSH-related enzymes, particularly GPX, are inhibited (Michiels et al, 1988). Similarly, a systematic increase in the level of these enzymes, GPX in particular, through micro injection results in higher resistance of the cells toward oxidative stress (Remacle et al, 1992).

In our study we observed a significant decrease in the GPX activity along with no change in the GR activity after incubation of mitochondria in oxidative conditions. Our results are in agreement with the studies of Tabatabaie and Floyd (1994). They investigated the effect of Fe(II) or Fe(III)/ascorbate system on the activities of both enzymes by incubating the enzymes with different concentration of $FeSO_4$ or $FeCl_3$ and other oxidants and determined the formation of protein carbonyls as an index of oxidative modification in relation to the activity of both enzymes. The loss of enzymatic activity is considered to be the consequence of oxidative modification of amino acid residues at or in the vinicity of the active site resulting from metal catalyzed oxidation of proteins (Stadtman et al, 1991). The treatment of GR with hydroxyl radical-generating system resulted in the formation of carbonyl groups and inactivation of enzyme activity, while the incubation of enzyme in the presence of iron and increase in the carbonyl formation was not followed by a significant loss in enzyme activity. GPX was inactivated by various oxidizing agents including iron, but no increase in carbonyls was detected.

In the case of GR, no change in enzyme activity accompanied by a large increase in the carbonyl content could be due to Fe binding at protein molecule in the site far away from the active site of the enzyme, so that ensuing modification of amino acid is not occuring at a position critical to the activity. Therefore, the enzymatic activity is spared even though the enzyme is oxidatively modified (Tabatabaie and Floyd, 1994).

On the other hand, GPX undergoes considerable loss of activity upon incubation with Fe^{2+}/EDTA. It clearly indicates that GPX is more susceptible to oxidative modification and damage under the employed conditions then GR. That is in agreement with results of

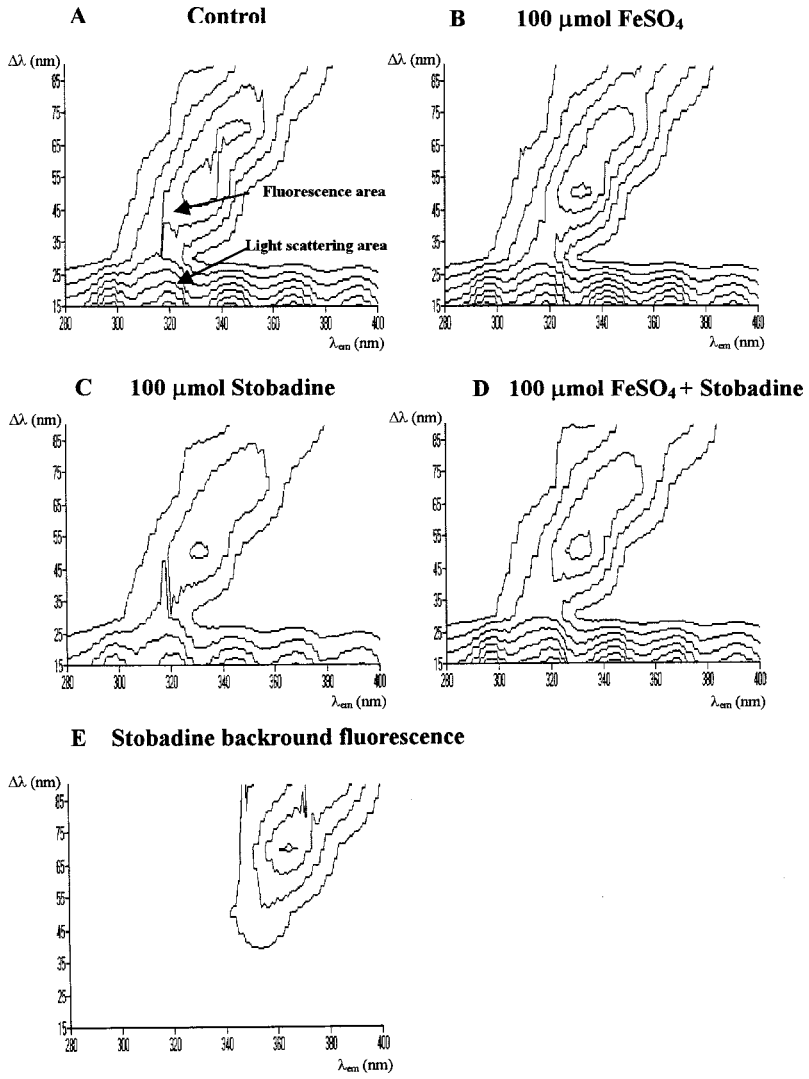

Fig. 3. The 3-D synchronous fluorescence countour maps of isolated rat liver mitochondria (10 μl/10 ml in respiration medium). Spectral changes indicate the participation of outer mitochondrial membrane in the effect of $FeSO_4$/EDTA ions stobadine and their combination. A — control sample of mitochondria, B — after addition 100 μmol $FeSO_4$/EDTA, C — after addition 100 μmol stobadine, D — after addition 100 μmol $FeSO_4$/EDTA with stobadine, E — background fluorescence of stobadine.

others (Trumper et al, 1989; Pigeolet et al, 1990). According to Epp et al (1983) GPX has a tryptophan residue in its active site. This tryptophan is probably involved in the binding of the hydrophobic peroxides and is particularly sensitive to hydroxyl radicals.

GPX is known to contain a selenocysteine group at the active site (Chaudiere et al, 1984). This rare amino acid is essential for GPX activity (Fujii and Tanaguchi, 1999). Oxidation of the selenium to a selenoic acid derivative is thougth to be responsible for inactivation of the enzyme by hydroperoxides and other oxidizing agents. On the other hand the results of Tabatabaie and Floyd (1996) indicated that selenocystamine, the compound known to possess GPX-like activity, preserves its activity after treatment with benzaldehyde, the compound implicated in ROS formation. The possibility of a direct interaction between selenol group of the GPX and benzaldehyde, cannot be completely ruled out, but inactivation of the enzyme may be due to the reactions between benzaldehyde and different amino acids (at critical sites of the enzyme), which may result in the disruption of enzymatic activity.

The addition of spin trap agent stobadine protected GPX against the loss of enzymatic activity in Fe^{2+} oxidation system. It is supposed that the effect of transition metal ions is mediated by free radicals and stobadine which is free radical scavanger protects by its ability to scavange the reactive primary radicals and prevent the propagation of free radical chain reactions. Another possibility is that some specific interaction exists between stobadine and the enzyme. Stobadine may bind to the amino acid residues around the catalytic site in the enzyme molecule and shields it from ROS attack.

Simultaneous monitoring of outer mitochondrial membrane dynamics was performed by the method of synchronous fluorescence fingerprint. The countour line maps of synchronous spectra are grafical definition of biological mixture (suspension of mitochondria) considered as one entity (Phelan, 1994; Kušnír et al, 1998). The comparison of the countour line maps of the mitochondria suspension in both scattering and fluorescence area help us to reveal changes in outer mitochondrial membrane dynamics of control and affected mitochondria. The rearangement of the mitochondrial shape is probably involved in response to ROS effect induced by Fe^{2+} and stobadine protection respectively.

Our study shows that the protection by stobadine against cell damage observed in some pathological situations such as ischemia (Štolc et al, 1997) may be related to the stobadine ability to protect against the loss of antioxidative defense mechanism.

References

Anderson D, Philips BJ: Comparative in vitro and in vivo effects of antioxidants. Food Chem Toxicol 1999; 37: 1015—1025.

Calberg I, Mannervik B: Glutathione reductase. Methods Enzymol 1985; 113: 484—485.

Dubovický M, Ujházy E, Kovačovský P, Rychlík I, Janšák J: Evaluation of long-term administration of the antioxidant stobadine on explorary behaviour in rats of both genders. J Appl Toxicol 1999; 19: 431—436.

Diplock AT: Antioxidants and disease prevention. Mol Aspects Med 1994; 15: 293—376.

Dizdaroglu M: Chemical determination of free radical-induced damage to DNA. Free Radical Biol Med 1991; 10: 225—242.

Ernster L: Lipid peroxidation in biological membranes: Mechanism and implications. In: Yagi K (Ed): Active oxygen, lipid peroxides and antioxidants. Boca Raton, CRC Press 1993, 1—38.

Flohé L, Gunzler WA: Assay of glutathione peroxidase. Methods Enzymol 1984; 105: 114—121.

France-Lanord V, Brugg B, Michel PP, Agid Y, Ruberg M: Mitochondrial free radical signal in ceramide-dependent apoptosis: A putative mechanism for neuronal death in Parkinson's disease. J Neurochem 1997; 69: 1612—1622.

Fujii J, Taniguchi N: Down regulation of superoxide dismutases and glutathione peroxidase by reactive oxygen and nitrogen species. Free Rad Res 1999; 31: 301—303.

Horáková Ľ, Štolc S: Antioxidant and pharmacodynamic effects of pyridoindole stobadine. Gen Pharmac 1998; 30: 627—638.

Chance B, Sies H, Boveris A: Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979; 59: 527—605.

Chaudiere J, Wilhelmsen, EC, Tappel AL: Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans. J Biol Chem 1984; 259: 1043—1050.

Jakuš V, Lopuchová M: Role of free radicals, oxidative stress and antioxidants systems in liver diseases. Bratisl Lek Listy 1999; 100: 548—559.

Johnson D, Lardy H: Isolation of liver or kidney mitochondria. Method Enzymol 1967; 10: 94—96.

Kušnír J, Dubayová K, Makuchová V: The reference library of synchronous fluorescence spectra — medicine and industrial application. Chem Papers 1998; 52: 291.

Lenaz G, Bovinna C, Formiggini G, Castelli GP: Mitochondria, oxidative stress, and antioxidant defences. Acta Biochim Polonica 1999; 46: 1—21.

Morimito RI, Kline MP, Bimston DM, Cotto JJ: The heat shock response: regulation and function of heat-shock proteins and molecular chaperones. Assays Biochem 1997; 32: 17—19.

Michiels C, Raes M, Zachary MD, Delaive E, Remacle J: Exp Cell Res 1989; 179: 589—591.

Michiels C, Remacle J: Use of the inhibition of enzymatic antioxidant systems in order to evaluate their physiological importance. Europ J Biochem 1988; 177 (2): 435—441.

Miquel J, Economos AC, Fleming J, Johnson JE, Jr: Mitochondrial role in cell aging. Exp Gerontol 1980; 15: 575—585.

Ozawa T: Genetic and functional changes in mitochondria associated with aging. Physiol Rev 1997; 77: 425—464.

Petit PX, Susin SA, Zamzami N, Mignotte B, Kroemer G: Mitochondria and programmed cell death: Back to the future. FEBS Lett 396; 396: 7—13.

Phelan V: Adding a new dimension to fluorescence analysis. Int Lab News 1994; 12: 7.

Pigeolet E, Corbisier P, Houbrion A, Lambert D, Michiels C, Raes M, Zachary MD, Remacle J: Glutathione peroxidase, superoxid dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. Mech Ageing Dev 1990; 51: 283—297.

Raes M, Michiels C, Remacle J: Comparative study of the enzymatic defense systems against oxygen derived free radicals: the key role of glutathione reductase. Free Rad Biol Med 1987; 3: 3—7.

Remacle J, Lambert D, Raes M, Pigeolet E, Michiels C: Importance of various antioxidant enzymes for cell stability. Confrontation between theoretical and experimental data. Biochem J 1992; 286 (1): 41—46.

Richter C, Park JW, Ames BN: Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci USA 1988 a; 85: 6465—6467.

Richter C: Do mitochondrial DNA fragments promote cancer and aging? FEBS Lett 1988 b; 241: 1—5.

Stadtman ER, Oliver CNJ: Metal-catalyzed oxidation of proteins. Physiological consequences. Biol Chem 1991; 266 (4): 2005—2008.

Stadtman ER: Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Ann Rev Biochem 1993; 62: 797—821.

Štolc S, Vlkolinský R, Pavlásek J: Neuroprotection by the pyridoindole stobadine: a minireview. Brain Res Bull 1997; 42 (5): 335—340.

Susin SA, Zamzami N, Kroemer G: Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta 1998; 1366: 151—165.

Tabatabaie T, Floyd RA: Susceptibility of glutathione peroxidase and glutathione reductase to oxidative damage and protective effect of spin trapping agents. Arch Biochem Biophys 1994; 314: 112—119.

Tabatabaie T, Floyd RA: Inactivation of glutathione peroxidase by benzaldehyde. Toxicol Appl Pharmacol 1996; 141: 389—393.

Trumper L, Noack H, Augustin W: The role of mitochondrial glutathione in the defence against Fe^{2+}/ ascorbate induced peroxidation of rat liver mitochondria. Biomed Biochem Acta 1989; 48 (10): 743—750.

Received September 4, 2001.
Accepted November 12, 2001.