Gen. Physiol. Biophys. (2000), **19,** 195—205 

# Preventive Effect of Several Antioxidants after Oxidative Stress on Rat Brain Homogenates

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**Abstract.** Brain homogenate was used as a model system to study antioxidant properties of several natural and synthetic antioxidants under oxidative stress. Oxidative stress was induced by Fe/ascorbate system and lipid peroxidation as well as protein modification were studied. Thiobarbituric acid reactive substances (TBARS) were used as a marker of lipid peroxidation. The preventive effect concerning lipid peroxidation decreased in the order: buthylated hydroxytoluene (BHT) (3.5), stobadine (ST) (35), serotonin (54), trolox (98), U 74389G (160), melatonin (3100), (the numbers in the brackets represent IC50 in $\mu$mol/l). Methylprednisolone had no effect, and spin traps interfered with TBARS determination.

Concerning creatine kinase (CK) activity as a selected marker of oxidative modification of proteins, the preventive effect of antioxidants (30 $\mu$mol/l) decreased in the order: BHT (30), trolox (75), stobadine (ST) (77), $\alpha$-phenyl-N-tert-buthylnitrone (PBN) (87), sodium salt of N-tert-buthyl-C-(phenyl-2-sulfone) nitrone (SPBN) (90), (the numbers in the brackets represent the loss of CK activity in percentages, when 100 % was the loss of CK activity in the absence of any antioxidant). The nonglucocorticoid steroid U 74389G, methylprednisolone and serotonin had no preventive effects, while melatonin had antioxidant effect only in a higher concentration (1 mmol/l).

**Key words:** Thiobarbituric acid reactive substances (TBARS) — Creatine kinase modification — Antioxidants

## Introduction

Oxygen free radicals and their role in the modification of biological molecules, such as nucleic acids, lipids and proteins, have been suggested to be involved in the pathogenesis of a number of diseases as well as in certain processes, such as aging and ischemia reperfusion injury. The central nervous system (CNS) appears

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to provide an especially sensitive environment for free radical reactions (Clements and Panetta 1995).

The role of lipid peroxidation induced by oxygen free radicals has been well established in the pathophysiology of CNS injury and stroke (Braughler and Hall 1989; Hall and Braughler 1989; Kontos 1989; Halliwell 1991). Thus efforts have been directed towards the discovery of efficient lipid antioxidant compounds that can retard posttraumatic and postischemic neurodegeneration.

The biological significance of oxygen free radical damage to proteins is stressed by the demonstration that oxidative modification of proteins marks them for degradation by most common proteases. Oxidized proteins make up a substantial fraction of the catalytically inactive or less active, more thermolabile forms of enzymes that accumulate in cells during aging, oxidative stress, ischemia-reperfusion, as well as in a number of pathological states (Stadtman 1993).

The aim of this study was to compare the preventive effects of several antioxidants on rat brain homogenates after oxidative stress induced by reaction of Fenton type where the hydroxyl radical is generated. Biomolecules are injured by the hydroxyl radical when a metal is present in the environment of superoxide and hydrogen peroxide. Hydroxyl radicals can initiate the formation of other reactive oxygen species such as peroxyl radicals, singlet oxygen, etc. Some antioxidant enzymes or scavengers selectively decompose or scavenge individual reactive oxygen species. Superoxide dismutase degrades superoxide, catalase decomposes hydrogen peroxide. Ethylene diamine tetraacetic acid-$Na_2$ (EDTA) and histidine chelate iron, manitol is a selective scavenger of hydroxyl radicals. By using antioxidants with different affinities to individual oxygen radical species, we tried to assess which antioxidant property of the individual antioxidants studied was involved in its preventive effect on the enzyme CK, as a selected protein sensitive to oxidation.

Of the natural antioxidants present in the living organism, we studied the watersoluble derivative of alpha tocopherol, trolox; melatonin a neurohormone secreted by the pineal gland, and its precursor serotonin. Of the synthetic antioxidants we tested the glucocorticoid steroid methylprednisolone and the nonglucocorticoid 21 aminosteroid U 74389G, as well as spin traps $\alpha$-phenyl-N-tert-buthylnitrone (PBN) and sodium salt of N-tert-buthyl-C-(phenyl-2-sulfone) nitrone (SPBN). Buthylated hydroxytoluene (BHT), another synthetic antioxidant, which is not available for in vivo treatment, was used as a very strong antioxidant only for comparison with the other compounds tested.

## Materials and Methods

*Animals*

Male normotensive rats of inbred Wistar strain (250-300 g) were used.

*Agents and chemicals*

Melatonin, serotonin and BHT were from Sigma, USA; U 74389G and methylprednisolone were from Upjohn, Belgium; PBN and SPBN were gifts from W.U.

Cao, Centaur Pharmaceuticals Ltd, USA Sunnyvale, CA. Stobadine, (-)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido(4,3b-indole) was prepared at the Institute of Experimental Pharmacology SAS, Bratislava (Štolc et al. 1983). Catalase, SOD, manitol were from Serva, Germany, histidine was from Reanal, Budapest. Protease inhibitors leupeptin, pepstatin aprotinin and phenylmethylsulfonyl fluoride (PMSF) were from Sigma, USA.

*Brain homogenate preparation*

Animals were sacrificed by decapitation, the brains were removed and 10 % homogenates were prepared by brain homogenization in icecold HEPES, pH 7.4, which contained (in mmol/l): NaCl 137; KCl 4.6; $KH_2PO_4$1.1; $MgSO_4$ 0.6; and protease inhibitors (in $\mu$g/ml): leupeptin 0.5; pepstatin 0.7; aprotinin 0.5; phenylmethylsulfonyl fluoride (PMSF) 40 to prevent proteins from degradation by most common proteases. Janke Kunkel Ultra Turrax T 25 was used for homogenization. The brain homogenates were divided into small volumes and stored at −20 ℃. The levels of TBARS remained without any significant changes during up to two months of storage.

*Oxidation of homogenates*

Brain homogenates diluted by potassium phosphate buffer pH 7.4 to the final concentration of 1 mg prot./ml were oxidized by $FeSO_4$ and ascorbic acid to the final concentration of 0.1 mmol/l and 0.5 mmol/l, respectively. The control sample contained only homogenate. Oxidation was started with ascorbic acid.

The antioxidants were dissolved in water or in ethanol (BHT, U-74389G, trolox, melatonin). Low concentrations of ethanol, not influencing TBARS determination and CK activity, were used. Antioxidants were added to the homogenates before the oxidative system, in the final concentrations of 10, 30, 100 $\mu$mol/l. Some of them were tested also in lower concentrations (BHT: 1, 3 $\mu$mol/l) or in higher concentrations (U-74389G: 300 $\mu$mol/l; melatonin: 1, 3, 5 mmol/l) to determine the extent of lipid peroxidation; or in the concentration of 30 $\mu$mol/l, if not indicated differently, to determine CK oxidative modification. The total volume of the reaction mixture was 0.5 ml.

All samples were incubated at 37 ℃ for 30 min. The oxidation was stopped by addition of TCA (20 %), with BHT prepared as indicated in *TBARS determination.* To study CK modification, oxidation was finished by addition of deferoxamine in the final concentration of 10 $\mu$mol/l, the samples were centrifuged at 10,000 × $g$ and 5 ℃ for 5 min, frozen and stored at −20 ℃ until the following day when CK activity was determined. Each sample was investigated in four parallels for TBARS as well as CK.

*TBARS determination*

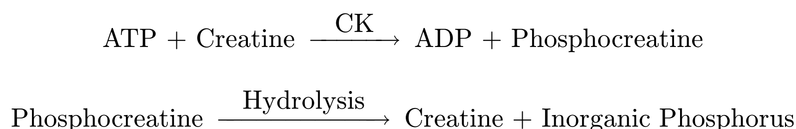
TBARS were determined according to Buege and Aust (1978). Briefly, 0.35 ml of 20 % TCA with BHT (20 mg BHT/ml ethanol per 200 ml of TCA) was added to 0.5 ml of each brain homogenate sample to stop the oxidation. The samples

were centrifuged for 5 min. The supernatants were quantitatively transferred to test tubes with 0.5 ml of thiobarbituric acid (TBA), (0.72 g TBA + 4 ml NaOH mol/l + 46 ml $H_2O$), heated to 80 ℃ for 15 min, and the reaction was stopped by cooling to 0 ℃.

The TBARS concentration was determined from the absorbance at 534 nm and expressed in nmol/mg protein. Malondialdehyde *bis* (dimethylacetal), (Merck, Germany) was used as a standard for TBARS determination.

*CK activity determination*

SIGMA diagnostics KIT, Procedure No. 661, was used for CK activity determination. Creatine kinase catalyses the reversible phosphorylation of adenosine-5′-diphosphate (ADP) by phosphocreatine to form adenosine-5′-triphosphate (ATP) and free creatine. The colorimetric method of the kit is based on the generation of phosphorus by the following reactions:



$$\text{ATP} + \text{Creatine} \xrightarrow{\text{CK}} \text{ADP} + \text{Phosphocreatine}$$

$$\text{Phosphocreatine} \xrightarrow{\text{Hydrolysis}} \text{Creatine} + \text{Inorganic Phosphorus}$$

The inorganic phosphorus produced was then measured colorimetrically and is proportional to CK activity. In our experiments the rat brain supernatant (10,000 × $g$ in the volume of 150 $\mu$l per sample) was analysed for CK activity.

*Protein determination*

The protein concentration was determined according to the method of Lowry et al. (1951). Bovine albumine, (Serva, Germany) was used as a standard for protein determination.

## Results

The inhibitory effects of several natural and synthetic antioxidants on lipid peroxidation in rat brain homogenates were tested after oxidation induced by $FeSO_4$ (0.1 mmol/l) and ascorbic acid (0.5 mmol/l). Control, oxidized samples, and samples oxidized and treated with antioxidants were compared with respect to their lipid peroxidation degree. Each sample was investigated in four parallels. In the individual experiments, the levels of TBARS were in the range of 0.034 ± 0.00043 – 0.07 ± 0.0026 nmol/mg prot. for control samples, in the oxidated samples they ranged between 0.345 ± 0.0044 – 0.511 ± 0.0187 nmol/mg prot.

IC50 was calculated from the dependence of the inhibitory effect versus antioxidant concentration (mol/l) using linear regression. Values of $-\log \text{IC50} \pm \text{SEM}$ were calculated by the program LIREKO.

Table 1 summarizes the inhibitory effects of the antioxidants tested on lipid peroxidation in rat brain homogenates, expressed in IC50 ($\mu$mol/l). The preventive

**Table 1.** Inhibition of lipid peroxidation induced by $Fe^{2+}$/ascorbic acid in rat brain homogenates.

| Antioxidant | IC50 ($\mu$mol/l) |
|---|---|
| BHT | 3.5 |
| ST | 35 |
| SEROTONIN | 54 |
| TROLOX | 98 |
| U 74389G | 160 |
| MELATONIN | 3100 |

effects of the antioxidants on lipid peroxidation decreased in the order: BHT (3.5), ST (35), serotonin (54), trolox (98), U 74389G (160), melatonin (3100), where the numbers in the brackets are IC50 in $\mu$mol/l. Methylprednisolone had no effect, and spin traps interfered with TBARS determination.

**Table 2.** Effects of antioxidants (30 $\mu$mol/l) and other compounds on inactivation of CK by $Fe^{2+}$/ascorbate system in rat brain homogenates.

| Antioxidant | Loss of CPK activity in percentages |
|---|---|
| none | 100 |
| BHT | 30 |
| TROLOX | 75 |
| ST | 77 |
| PBN | 87 |
| SPBN | 90 |
| U 74389G | 100 |
| METHYLPREDNISOLONE | 121 |
| SEROTONIN (30 $\mu$mol/l) | 123 |
| SEROTONIN (100 $\mu$mol/l) | 119 |
| SEROTONIN (300 $\mu$mol/l) | 121 |
| MELATONIN (30 $\mu$mol/l) | 126 |
| MELATONIN (1 mmol/l) | 69 |
| Other compounds | |
| HISTIDINE (100 $\mu$mol/l) | 131 |
| EDTA (200 $\mu$mol/l) | 197 |
| MANITOL (30 $\mu$mol/l) | 97 |
| MANITOL (100 $\mu$mol/l) | 124 |
| CATALASE (49 000 U/ml) | 49 |
| SOD (630 U/ml) | 134 |

Protein injury and preventive effects of the antioxidants were also studied in the rat brain homogenates under the same oxidative conditions. We used CK activity as a marker of protein oxidative modification. The activities of CK in the control samples of the individual experiments were in the range of $129.25 \pm 1.65 - 147 \pm 4.24$ U/mg protein, and those in the oxidized samples were in the range of $57.25 \pm 1.03 - 88.63 \pm 2.27$ U/mg protein. Unless indicated differently, the antioxidants were tested in the concentration of 30 $\mu$mol/l. The results are summarized in Table 2. The differences in the activity of CK between the control and the oxidized samples were set as 100 percent loss of CK activity. The preventive effect of the antioxidants tested in the concentration of 30 $\mu$mol/l on CK activity decreased in the order: BHT (30), trolox (75), ST (77), PBN (87), SPBN (90), where the numbers in the brackets mean the loss of CK activity. U 74389G had no effect, methylprednisolone had a prooxidant effect, and serotonin showed a concentration-independent prooxidant effect. Melatonin had a prooxidant effect in the lower concentration tested (30 $\mu$mol/l) and an antioxidant effect in the higher concentration tested (1 mmol/l).

To explain the antioxidant effect of the antioxidants tested on CK activity, we studied also several other compounds with known specific effects: histidine, EDTA, manitol, catalase and superoxide dismutase (SOD). Histidine and EDTA stimulated the activity of CK, manitol in the lower concentration tested (30 $\mu$mol/l) was without any effect, and stimulated CK activity in the higher concentration. Catalase had a preventive effect on CK activity, SOD was without any preventive effect.

## Discussion

In *in vitro* experiments, the effect of stobadine, a pyridoindole compound on the brain homogenates was compared with those of natural and synthetic antioxidants which may be used (except BHT) in the treatment of brain pathological states connected with oxidative stress. The antioxidant which was the most potent in our experiments, BHT, is a typical chain breaking antioxidant, a very strong scavenger of peroxyl and hydroxyl radicals (Simic 1990). Stobadine is a peroxyl radical scavenger comparable with BHT (Kagan et al. 1993) and it is also a strong scavenger of hydroxyl radicals (Štefek and Beneš 1991). According to our results, BHT was 10 times more effective than stobadine. However, the above mentioned study was performed on liposomes, while we used brain homogenates, where stobadine due to its lipo-hydrophilic properties may be present both in hydrophilic and in hydrophobic phases of biological tissues (Kagan et al. 1993). We found melatonin to be a relatively weak antioxidant. Several recent reviews have supported claims that melatonin possesses a strong antioxidant activity (Reiter et al. 1994, 1995, 1997, Reiter 1996). A recent paper concluded that according to its structure, the "antioxidant activity" of melatonin is more likely due to its action as a preventive antioxidant than as a chain breaking antioxidant (Antunes et al. 1999).

In an experiment on mouse brain homogenates, which was very similar to our

study, the antioxidant effect of melatonin was compared with that of serotonin and with a liver metabolite of melatonin, 6-hydroxymelatonin (6-OH-MLT), as well as with BHT. According to the IC50 value, the antioxidant effect decreased in the order: BHT, 6-OH-MLT, serotonin, melatonin (Pierrefiche et al. 1993). This is in good correlation with our results, as we also found a significantly stronger antioxidant effect of serotonin in comparison with melatonin. The authors of the study mentioned above explained the strong antioxidant effect of melatonin *in vivo* in contrast with the weak effect *in vitro* by the fact that this effect may be combined with the effect of the liver metabolite 6-OH-MLT. This hypothesis is also supported by the short life time of melatonin in the organism after i.p. administration (20–30 min). In *in vivo* experiments, the strong antioxidant effect of the melatonin may be caused also by the ability of melatonin to activate enzymes with antioxidant capacity (Reiter et al. 1997).

It is known that methylprednisolone is a significantly less effective inhibitor of lipid peroxidation dependent on Fe than 21 aminosteroids (Hall and McCall 1994). No preventive effect of methylprednisolone was observed in our experiments, in concentrations below 100 $\mu$mol/l, however 21 aminosteroid U 74389G was effective (IC50 = 160 $\mu$mol/l), though less effective than trolox (IC50 = 98 $\mu$mol/l). This result is in good correlation with the results of the above mentioned publication concluding that 21 aminosteroids are effective scavengers of peroxyl and phenoxyl radicals in the presence of lipid soluble azo-initiator of peroxyl radicals 2,2′-azo-bis (2,4-dimethylvaleronitrile) (AMVN) which induces lipid peroxidation; however, they have lower rate constants than vitamin E.

When we take into account the results of our study of lipid peroxidation of brain homogenates and the antioxidant capacity of the compouds tested against individual reactive oxygen species, we may conclude that the ability to scavenge peroxyl radicals and hydroxyl radicals probably plays the most important role in the inhibition of brain lipid peroxidation. The involvement of the peroxyl radical scavenging effect is supported by the fact that the most effective compounds inhibiting lipid peroxidation in our experiments, i.e. BHT, trolox and stobadine, are typical chain breaking antioxidants scavenging peroxy radicals. All of them are also strong OH radical scavengers. Serotonin, which appeared to be a strong inhibitor of lipid peroxidation, exerts its antioxidant effect mainly due to trapping OH radicals. We observed an extremely weak preventive effect of melatonin on lipid peroxidation, as it is much more likely to be a preventive antioxidant of the metal ion deactivating subclass than a chain breaking antioxidant.

The oxidative modification of the selected protein (CK) was studied in rat brain homogenates under the same oxidative conditions as lipid peroxidation. CK, an enzyme important for energy metabolism in cells of high and fluctuating energy requirements, catalyses the reversible transfer of a phosphoryl group from phospocreatine to ADP. CK belongs to the enzymes sensitive to metal catalyzed oxidative modification (Fucci et al. 1983; Levine 1983; Aksenov et al. 1997). Kinases belong to the most susceptible enzymes which require divalent cations for activity, and usually contain a histidine residue at or near the catalytic site (Fucci

et al. 1983). Single histidine residue is lost during the inactivation of glutamine synthetase, phosphoglycerate kinase or superoxide dismutase (Fucci et al. 1983). Recently it was shown that three histidine residues are located close to the active site of CK, and two of them are involved in the binding of creatine and ATP in the active site (Forstner et al. 1997). Previously, it was supposed that in a site-specific process binding of Fe(II) at the metal binding site on the enzyme might be followed by peroxidation of Fe(II) enzyme complex to generate an activated oxygen derivative (hydroxyl radical or singlet oxygen) that attacks a histidine or another oxidizable amino acid in the catalytic site and therefore might be shielded from attack by radical scavengers (Fucci et al. 1983; Stadtman 1993). According to recent knowledge, hydroxyl radical was excluded from the direct effect on protein residues and a new mechanism of oxidative impairment of protein was suggested with the main oxidant in the catalytic center being the ferryl complex (Meyerstein 1997).

The metal catalyzed modification of proteins is relatively insensitive to inhibition by free radical scavengers (Stadtman 1993). Catalase and chelators are usually able to inhibit the metal catalyzed oxidation of enzymes (Fucci et al. 1983; Levine 1983) The scavengers of OH radicals did not inhibit this reaction. Similarly, SOD, which decomposes the superoxide radical had no preventive effect (Fucci et al. 1983).

All antioxidants tested in this work, which inhibited the oxidative modification of CK, were strong scavengers of OH radicals (Simic 1990; Štefek and Beneš 1991; Sen and Phillis 1993). Nevertheless, also the compounds which did not exert any preventive effect on CK activity or even had prooxidative effects were previously found to be scavengers of OH radicals: 21 aminosteroids, melatonin and serotonin (Hall and McCall 1994; Daniels et al. 1996; Longoni et al. 1998). This may indicate that scavenging of OH radicals is not involved in the preventive effect on CK activity. We confirmed this fact by the study of manitol on CK activity in brain homogenates, where no preventive effect of this scavenger of OH radicals was observed. However the recent theory of the site-specific radical mechanism of oxidative inactivation of enzymes excluded direct OH radical involvement (Meyerstein 1997). The singlet oxygen quenching ability is expected to be involved in the oxidative modification of CK in the case of antioxidants able to quench this reactive oxygen species: BHT (Kohno et al. 1995), trolox and stobadine. Stobadine is approximately as effective a singlet oxygen quencher as are alpha tocopherols (Horáková et al. 1994). It is known that photooxidative inactivation of certain enzymes by singlet oxygen involves destruction of histidine residues (Wasserman 1970). Alpha tocopherols are scavengers of superoxide radicals (Serbinova and Packer 1994). But the involvement of superoxide in our experiments is also ruled out by the failure of SOD to inhibit the inactivation reaction.

Spin traps of PBN type are able to scavenge not only OH radical but also $H_2O_2$ (Sen and Phillis 1993), which could contribute to the protection of CK as catalase diminished the loss of its activity in our experiments.

Pure CK was found to be inactivated by the ascorbate system by mechanisms

different from the metal catalyzed inactivation of other pure enzymes having histidine in the metal-binding site, like pyruvate kinase or glutamine synthetase (Levine 1983). In contrast to pyruvate kinase and glutamine synthetase histidine, EDTA and other chelating agents stimulated the inactivation of CK by ascorbate indicating complete protection (Levine 1983). The protection exerted by histidine in this work was explained by its ability to chelate the required metal cation. These results of inactivation of pure CPK are in agreement with our results on CPK in brain homogenates, where histidine and EDTA stimulated the inactivation of CPK. According to a review by Stadtman (1993), metal catalyzed oxidation of a number of enzymes is completely inhibited by EDTA at concentrations equal to or greater than the amount of the metal ion present. Such enzymes posses tight metal-binding sites. Oxidation of other proteins is stimulated by EDTA and it is supposed that these proteins lack a tight metal-binding site but have the ability to bind the EDTA-metal chelate complex. EDTA would thus serve as a vehicle to carry the metal to the EDTA- binding site and thereby promote site-specific modification of amino acid residues at the EDTA-binding site. As in the case of direct metal-binding, the site-specific oxidations catalyzed at EDTA-binding sites on proteins might also be resistant to inhibition by free radical scavengers. Manitol, a scavenger of hydroxyl radicals, did not prevent oxidative modification of CK in our experiments on rat brain homogenates. The resistance of these oxidative modifications to hydroxyl radical scavengers may indicate a site-specific mechanism of protein oxidation (Stadtman 1993).

Our experiments support the idea that oxidative modification of CK in rat brain homogenates may be a site-specific process, and CK probably possesses the ability to bind the EDTA-metal chelate complex. Individual reactive oxygen species, like superoxide radical and OH radical, are probably not directly involved in oxidative inactivation of the enzyme, but hydrogen peroxide is included in the oxidative injury of CK. The resistance of CK to reactive oxygen species is only relative as known antioxidants, such as BHT, trolox, stobadine and spin traps, prevented CK from being oxidatively modified. We suppose that a singlet oxygen quenching ability and scavenging of $H_2O_2$were involved in the CK-protecting ability of the compounds tested.

**Acknowledgements.** This study was supported by Slovak Grant Agency for Science VEGA No. 2/6023/99, and by research grant awarded by Centaur Pharmaceuticals Ltd., Sunnywale CA, USA.

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Final version accepted March 13, 2000