The effect of ascorbate supplementation on the activity of antioxidative enzymes in the rat hypothalamus and adrenals

Sinisa Djurasevic, Gordana Cvijic, Jelena Djordjevic, Iva Djordjevic, Nebojsa Jasnic and Predrag Vujovic

Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Republic of Serbia

Abstract. We investigated the effect of vitamin C on the oxidative status in the hypothalamus and adrenal glands of rats supplemented by its two doses over a four-week period. The results obtained have shown that vitamin C exerts effects which are tissue specific. In hypothalamus, it decreased the activity of copper zinc superoxide dismutase (CuZnSOD), the concentration of hydrogen peroxide (H_2O_2), as well as the activity of catalase and the level of lipid peroxidation, thus causing effects which are obviously antioxidative. On the other hand, the changes detected in adrenals indicate that vitamin C there performs some other, specific functions. They are followed by an increase in the activity of both CuZnSOD and MnSOD, as well as with the consequent rise of H_2O_2 content. However, these changes seem not to be of pro-oxidative nature since the level of lipid peroxidation in adrenals remains unchanged as compared to the controls.

Key words: Rats — Vitamin C — Hypothalamus — Adrenals — Oxidative status

Introduction

Vitamin C (ascorbic acid, ascorbate) is a vitamin which takes part in many biochemical processes in organism. Chemically capable of reacting with most of the physiologically important radicals and oxidants (Buettner 1993) it acts as a proven hydrosoluble antioxidant (Halliwell 1996). Another important biological function of ascorbate is to serve as a cosubstrate for several hydroxylase and oxygenase enzymes, maintaining their active center metal ions in a reduced state for optimal enzyme activity (Carr and Frei 1999). One of these enzymes is dopamine β-hydroxylase (DBH), which in different neuroendocrine tissues synthesizes noradrenaline through hydroxylation of dopamine (Diliberto et al. 1987).

It is known that the level of the DBH activity highly depends on the intracellular vitamin C content (Levine 1986), which, in turn, depends on the activity of members of the family of mammalian Na^+-dependent L-ascorbic acid plasma membrane transporters (Tsukaguchi et al. 1999). There are two types of these transporters, SVCT1 and SVCT2 (Daruwala et al. 1999), and the latter is widely expressed in the brain and adrenals (Liang et al. 2001). It has been shown that mutant mice lacking SVCT2 have severely reduced (99%) ascorbic acid levels in both brain and adrenals (Sotiriou et al. 2002). While this fall in vitamin C concentration shows no implication on the brain levels of dopamine and noradrenaline, it causes marked reduction in adrenal level of DBH activity; it seems that deranged catecholamine system function in SVCT2 null mice is largely restricted to the adrenal medulla (Bornstein et al. 2003).

According to previously mentioned, we assumed that the same tissue-specific response of hypothalamus and adrenals could be achieved even in normal rats, as a result of increased vitamin C supply. Since the activity of DBH involves the accumulation of an activated oxygen intermediate, with the properties of a copper-peroxo or copper-oxo species (Evans et al. 2003), we decided to study the oxidative status of rats supplemented by two different doses of ascorbic acid over a four-week period of time. Thus, the activities of copper zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD) and catalase, hydrogen peroxide (H_2O_2) concentration, the level of lipid peroxidation and

Correspondence to: Sinisa Djurasevic, Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, P.O.Box S2, Republic of Serbia
E-mail: sine@bio.bg.ac.rs

vitamin C content were determined in the hypothalamus and adrenal glands of the experimental animals, as well as their serum vitamin C level.

Materials and Methods

Design of experiment

Male rats of the Wistar strain (*Rattus norvegicus*) weighing 200 ± 45 g were used for the experiments. The animals were acclimated to 22 ± 1°C and maintained under conditions of 12-h periods of light and dark, with unlimited access to tap water and commercial rat food.

The rats were divided into three groups, each consisting of six animals. The control group consisted of rats which drank tap water. The second and the third group formed animals supplemented with a low or high dose of vitamin C dissolved in tap water.

Vitamin C supplementation

The vitamin C doses were chosen as 0.75 mg (low dose) and 25 mg (high dose) ascorbic acid/kg rat body weight per day. According to our previous experiment performed on a group of six animals during four weeks, the daily consumption of water in rats was linear in respect to their body weight, with average value of 240 ± 5 ml/kg. Therefore, both doses of vitamin C were dissolved in adequate volume of tap water every day (i.e. 3.125 mg ascorbic acid/l water and 104.167 mg ascorbic acid/l water, respectively) and administered to appropriate group of animals. This supplementation was permanent throughout four weeks.

Sample preparation

At the end of the vitamin C supplementation period, animals were killed by decapitation with Harvard guillotine without anesthesia, as recommended by the Local Ethical Committee. After this, hypothalamus and adrenal glands were removed and divided in two equal portions.

One sample of each tissue was homogenized in 9 volumes of 25 mmol/l phosphate buffered saline (PBS), pH 7.0, and centrifuged at 9000 g in a semi-preparative Sorvall Super T21 centrifuge for 20 min at 4°C. Supernatants were used for determination of catalase, CuZnSOD and MnSOD activities, as well as for the measurement of the concentration of H_2O_2e and total lipid hydroperoxides (the level of lipid peroxidation).

Another set of tissue samples, as well as blood serum, were used for determination of vitamin C content by using the similar procedure, except that 6% trichloroacetic acid was used instead of PBS.

Methods

The vitamin C concentration was determined in trichloroacetic acid samples by the method of Roe and Kuether (1943) and calculated against vitamin C standard curve absorption values.

The total superoxide dismutase activity was determined in the PBS samples by the adrenaline method of Misra and Fridovich (1972), using potassium cyanide as a CuZnSOD inhibitor for differential calculation of MnSOD activity (Weisiger and Fridovich 1973).

The catalase activity was measured in the PBS samples by the method of Beutler 1982), based on the rate of H_2O_2 degradation by the action of the catalase contained in the examined samples.

The measurement of the total lipid hydroperoxides and H_2O_2 content were both based on the ferrous ion oxidation by xylenol orange (FOX) assay (Wolf 1994). The two versions of FOX assays are described in the literature, FOX-1 and FOX-2 (Banerjee et al. 2002). The concentration of H_2O_2 was measured in PBS samples by the FOX-1 assay (Gay and Gebicki 2000) and calculated against H_2O_2 standard curve absorption values. The concentration of lipid hydroperoxides was measured in PBS samples by the FOX-2 assay (Jiang et al. 1991) with the level of lipid peroxidation in samples being expressed as a percent of lipid peroxidation level of control animal group.

Statistics

The data were expressed as means ± SEM. One-way ANOVA was undertaken for multiple range comparison, while significant differences among groups were determined by the Tukey posterior test. The probability of significance of differences was set at $p < 0.05$.

Results

In the vitamin C-supplemented rats its endogenous concentration in the serum was dose-dependently elevated, which results in its corresponding accumulation in the adrenal glands, but not in the hypothalamus of these animals (Fig. 1).

As can be seen from Fig. 2, the activity of CuZnSOD, but not the MnSOD, is depressed in the hypothalamus of rats fed with vitamin C. This probably results in decreased production of H_2O_2 and consequent decline of catalase activity in this tissue (Fig. 2). In accordance with this, feeding with vitamin C also decreases the level of lipid peroxidation in the hypothalamus (Fig. 2).

In the adrenal glands, activity of both forms of SOD was elevated as a result of vitamin C treatment (Fig. 3).

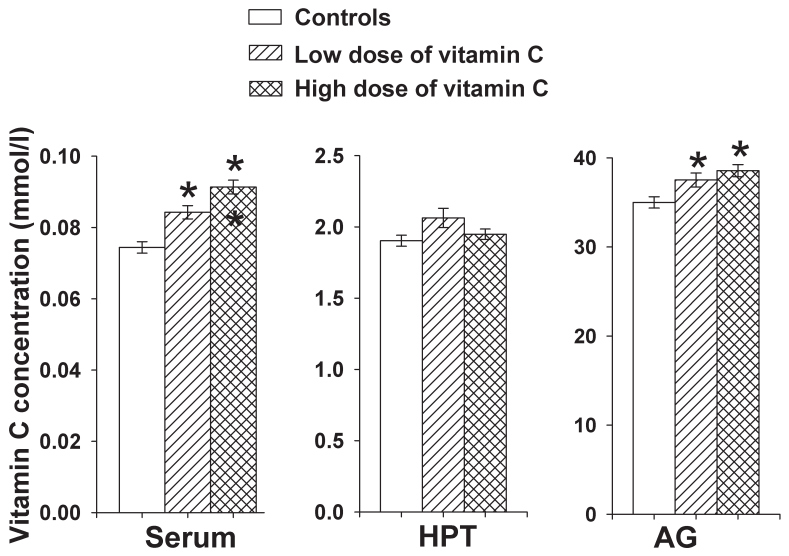

Figure 1. The effect of low and high dose of vitamin C supplementation on its concentration in rat serum, hypothalamus (HPT) and adrenal glands (AG). The data are expressed as means ± SEM. Statistically significant differences ($p < 0.05$) in relation to the control are marked with an asterisk above the columns, while statistically significant differences between two doses of the vitamin C are marked with an asterisk inside the columns.

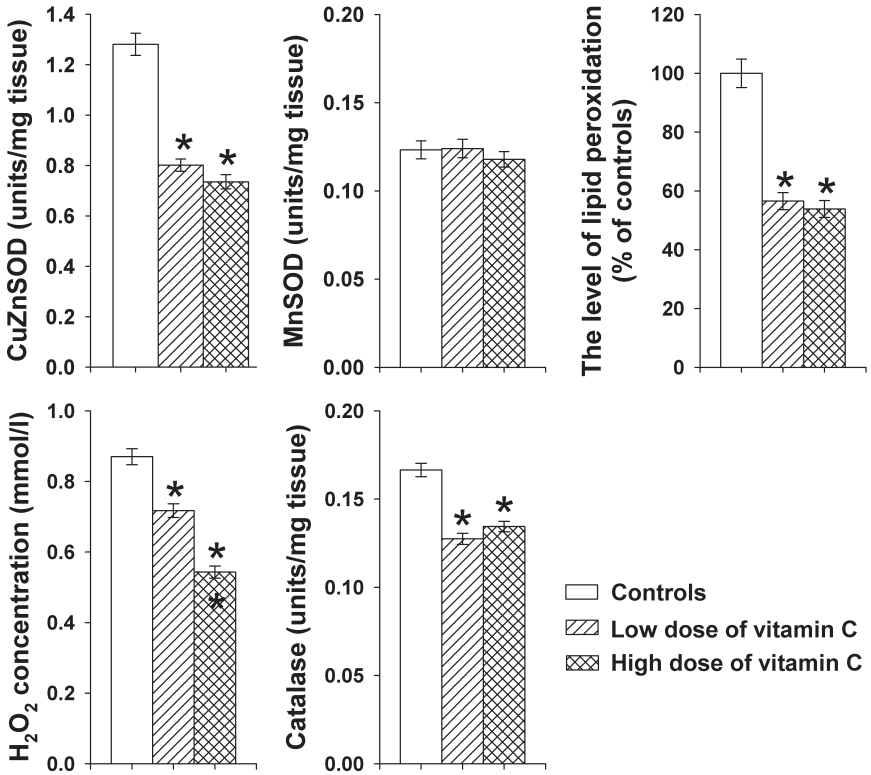

Figure 2. The effect of low and high dose of vitamin C supplementation on the oxidative status of rat hypothalamus. The data are expressed as means ± SEM. Statistically significant differences ($p < 0.05$) in relation to the control are marked with an asterisk above the columns, while statistically significant differences between two doses of the vitamin C are marked with an asterisk inside the columns.

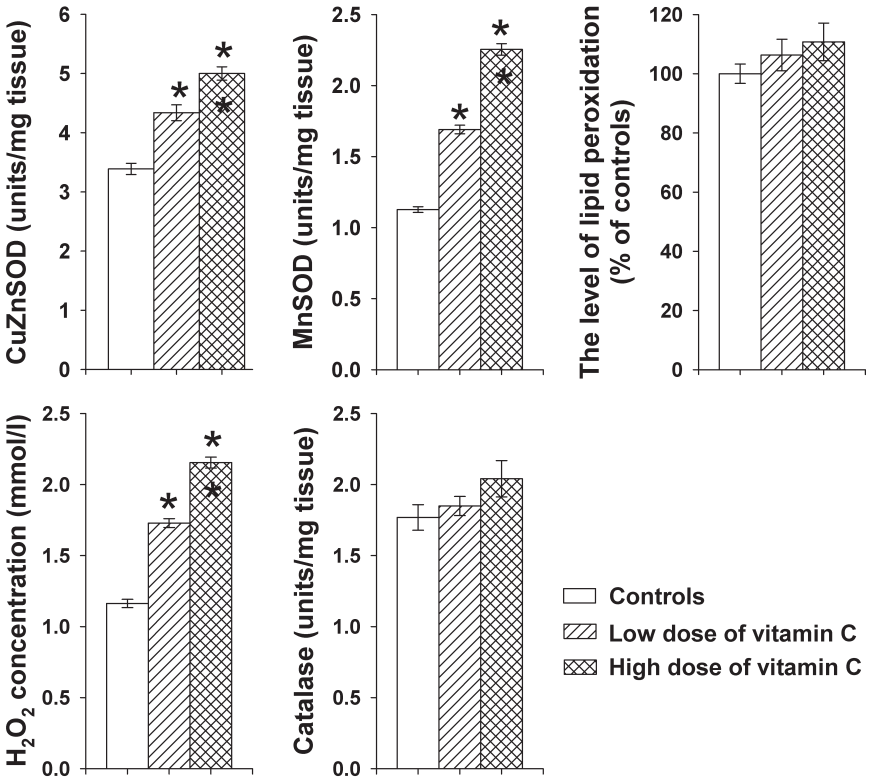

Figure 3. The effect of low and high dose of vitamin C supplementation on the oxidative status of rat adrenal glands. The data are expressed as means ± SEM. Statistically significant differences ($p < 0.05$) in relation to the control are marked with an asterisk above the columns, while statistically significant differences between two doses of the vitamin C are marked with an asterisk inside the columns.

This was followed with accompanying increase in the concentration of H_2O_2, but not with the increase in the activity of catalase, which remains unchanged under the influence of vitamin C, as well as the level of lipid peroxidation (Fig. 3).

Discussion

As can be seen from our results, vitamin C decreases the activity of CuZnSOD in hypothalamus. The mechanism of this action probably involves a removal of superoxide radical as its respective substrate (Buettner 1993), with the similar effect already described in cultured rat brain astrocytes after incubation with ascorbate (Kao et al. 2003). In accordance to this, the concentration of H_2O_2, a product of superoxide dismutases activity, is also lowered in the hypothalamus, followed with the consequent catalase activity decline. Proved with the further level of lipid peroxidation decrease, these results implicate the antioxidant effect of ascorbate in hypothalamus. This vitamin C outcome is of the exceptional importance for the neural tissue, being particularly sensitive to oxidative damage due to the high concentrations of unsaturated lipids (Sevanian and McLeod 1997). Indeed, the brain ascorbate level in anoxia-tolerant reptiles is known to be several times higher than in mammals, in order to prevent oxidative damage during reoxygenation after a hypoxic dive (Rice et al. 1995).

The ascorbic acid concentration in hypothalamus is of the order of several millimoles, which is in complete agreement with the literature data (Oke et al. 1987). Conversely, the vitamin C itself does not alter this concentration, thus making difficult to interpret its antioxidative effects in hypothalamus. Being already among the highest in the organism (Kratzing et al. 1982), the concentration of ascorbic acid in the brain is known to be under strong homeostatic regulation (Rice 2000). Realized through rapid ascorbate turnover among neural cells and blood (Spector 1977), this regulation probably combines fast neuronal SVCT2 mediate ascorbic acid uptake and simultaneously dehydroascorbic acid upset (mediated by some of the members of glucose transporters family). Thus maintained stable intracellular ascorbic acid level most likely causes the ability of dietary vitamin C to increase its brain content controversially (Hediger 2002).

In sharp contrast to hypothalamus, vitamin C supplementation in adrenals exerts completely different pattern of changes. It is not just that the activities of both forms of SODs are elevated under the influence of ascorbate, but also the concentration of H_2O_2, as a consequent product of their activity. The source of increased superoxide radical production, as a respective substrate of superoxide dismutases activity, remains to stay unclear. However, there are indications that the DBH activity may be followed by increased oxidative stress since the mechanism of dopamine hydroxylation involves the accumulation of an activated oxygen intermediate with the properties of a copper-peroxo or copper-oxo species (Evans et al. 2003). In accordance with the previous statement, the increased formation of H_2O_2 could be involved in modulation of the DBH activity. To be more specific, it is known that the enzyme activity leads to increased generation of H_2O_2 (Levin and Kaufman 1961), which in turn causes further DBH inactivation (Slama et al. 2001). Here we propose that both H_2O_2 formations during dopamine hydroxylation, as well as its inhibitory action on DBH, represent segments of the same mechanism of negative auto-regulation of the enzyme activity. In this sense, the function of appropriate peroxidase activity would be the maintenance of H_2O_2 at the optimal concentration, in agreement with our results showing that the adrenal catalase activity remains unchanged under the influence of vitamin C. Nevertheless, this mechanism seems not to be pro-oxidative, since the level of lipid peroxidation remains unchanged as compared to the controls.

Unlike hypothalamus, feeding with ascorbic acid significantly increases its adrenal concentration, confirming the glands ability to accumulate vitamin C under normal physiological conditions. This is very important for the function of adrenals as a main organism depot of vitamin C, capable of releasing it during stress response (Lahiri and Lloyd 1962; Padayatty et al. 2007) with pattern which has been shown to be stressor specific (Djordjević et al. 2006).

In conclusion, our results have shown that vitamin C exerts effects which are tissue-specific and not necessarily dose-dependent. Indeed, the fact that SVCTs are fully saturated at low extracellular ascorbic acid concentrations (Washko et al. 1989) probably explains the relative inefficiency of high vitamin C dose in animals, as it is already shown in humans (Levine et al. 1996). Also, it has been demonstrated that the use of high doses of ascorbic acid causes strong decrease in SVCT1 expression in the apical surface of the intestinal membrane (MacDonald et al. 2002), thus confirming the old thesis that the main limiting factor of the vitamin C bioavailability is its intestinal absorption (Hodges et al. 1969).

Acknowledgement. This work was supported by the Serbian Ministry of Science and Technology, grant No. 143050.

References

Banerjee D., Kumar A., Kumar B., Madhusoodanan U. K., Nayak S., Jacob J. (2002): Determination of absolute hydrogen peroxide concentration by spectrophotometric method. Curr. Sci. **83**, 1193–1194

Beutler E. (1982): Catalase. In: Red Cell Metabolism, a Manual of Biochemical Methods. pp. 105–106, Grune & Stratton, New York

Bornstein S. R., Yoshida-Hiroi M., Sotiriou S., Levine M., Hartwig H. G., Nussbaum R. L., Eisenhofer G. (2003): Impaired adrenal catecholamine system function in mice with deficiency of the ascorbic acid transporter (SVCT2). FASEB J. **17**, 1928–1930

Buettner G. R. (1993): The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. Arch. Biochem. Biophys. **300**, 535–543

Carr A., Frei B. (1999): Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J. **13**, 1007–1024

Daruwala R., Song J., Koh W. S., Rumsey S. C., Levine M. (1999): Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. FEBS Lett. **460**, 480–484

Diliberto E. J. Jr., Menniti F. S., Knoth J., Daniels A. J., Kizer J. S., Viveros O. H. (1987): Adrenomedullary chromaffin cells as a model to study the neurobiology of ascorbic acid: from monooxygenation to neuromodulation. Ann. N. Y. Acad. Sci. **498**, 28–53

Djordjević J., Djurašević S., Vučković T., Jasnić N., Cvijić G. (2006): Effect of cold and heat stress on the rat adrenal, serum and liver ascorbic acid concentration. Arch. Biol. Sci. **58**, 161–164

Evans J. P., Ahn K., Klinman J. P. (2003): Evidence that dioxygen and substrate activation are tightly coupled in dopamine β-monooxygenase. Implications for the reactive oxygen species. J. Biol. Chem. **278**, 49691–49698

Gay C., Gebicki J. M. (2000): A critical evaluation of the effect of sorbitol on the ferric-xylenol orange hydroperoxide assay. Anal. Biochem. **284**, 217–220

Halliwell B. (1996): Vitamin C: antioxidant or pro-oxidant *in vivo*? Free Radic Res. **25**, 439–454

Hediger M. A. (2002): New view at C. Nat. Med. **8**, 445–446

Hodges R. E., Baker E. M., Hood J., Sauberlich H. E., March S. C. (1969): Experimental scurvy in man. Am. J. Clin. Nutr. **22**, 535–548

Jiang Z. Y., Woollard A. C., Wolff S. P. (1991): Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipids **26**, 853–856

Kao P. F., Lee W. S., Liu J. C., Chan P., Tsai J. C., Hsu Y. H., Chang W. Y., Cheng T. H., Liao S. S. (2003): Downregulation of superoxide dismutase activity and gene expression in cultured rat brain astrocytes after incubation with vitamin C. Pharmacology **69**, 1–6

Kratzing C. C., Kelly J. D., Oelrichs B. A. (1982): Ascorbic acid in neural tissues. J. Neurochem. **39**, 625–627

Lahiri S., Lloyd B. B. (1962): The form of vitamin C released by the rat adrenal. Biochem. J. **84**, 474–477

Levin E. Y., Kaufman S. (1961): Studies on the enzyme catalyzing the conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. J. Biol. Chem. **236**, 2043–2049

Levine, M. (1986): Ascorbic acid specifically enhances dopamine β-monooxygenase activity in resting and stimulated chromaffin cells. J. Biol. Chem. **261**, 7347–7356

Levine M., Conry-Cantilena C., Wang Y., Welch R. W., Washko P. W., Dhariwal K. R., Park J. B., Lazarev A., Graumlich J. F., King J., Cantilena L. R. (1996): Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc. Natl. Acad. Sci. U.S.A. **93**, 3704–3709

Liang W. J., Johnson D., Jarvis S. M. (2001): Vitamin C transport systems of mammalian cells. Mol. Membr. Biol. **18**, 87–95

MacDonald L., Thumser A. E., Sharp P. (2002): Decreased expression of the vitamin C transporter SVCT1 by ascorbic acid in a human intestinal epithelial cell line. Br. J. Nutr. **87**, 97–100

Misra H. P., Fridovich I. (1972): The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. **247**, 3170–3175

Oke A. F., May L., Adams R. N. (1987): Ascorbic acid distribution patterns in human brain. A comparison with nonhuman mammalian species. Ann. N. Y. Acad. Sci. **498**, 1–12

Padayatty S. J., Doppman J. L., Chang R., Wang Y., Gill J., Papanicolaou D. A., Levine M. (2007): Human adrenal glands secrete vitamin C in response to adrenocorticotrophic hormone. Am. J. Clin. Nutr. **86**, 145–149

Rice M. E. (2000): Ascorbate regulation and its neuroprotective role in the brain. Trends Neurosci. **23**, 209–216

Rice M. E., Lee E. J., Choy Y. (1995): High levels of ascorbic acid, not glutathione, in the CNS of anoxia-tolerant reptiles contrasted with levels in anoxia-intolerant species. J. Neurochem. **64**, 1790–1799

Roe J. H., Kuether C. A. (1943): The determination of ascorbic acid in whole blood and irine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem. **147**, 399–407

Sevanian A., McLeod L. (1997): Formation and biological reactivity of lipid peroxidation products. In: Free Radical Toxicology. (Ed. K. B. Wallace), pp. 47–70, Taylor and Francis, Washington

Slama P., Jabre F., Tron T., Reglier M. (2001): Dopamine β-hydroxylase inactivation generates a protein-bound quinone derivative. FEBS Lett. **491**, 55–58

Sotiriou S., Gispert S., Cheng J., Wang Y., Chen A., Hoogstraten-Miller S., Miller G. F., Kwon O., Levine M., Guttentag S. H., Nussbaum R. L. (2002): Ascorbic-acid transporter Slc23a1 is essential for vitamin C transport into the brain and for perinatal survival. Nat. Med. **8**, 514–517

Spector R. (1977): Vitamin homeostasis in the central nervous system. N. Engl. J. Med. **296**, 1393–1398

Tsukaguchi H., Tokui T., Mackenzie B., Berger U. V., Chen X. Z., Wang Y., Brubaker R. F., Hediger M. A. (1999): A family of mammalian Na^+-dependent L-ascorbic acid transporters. Nature **399**, 70–75

Washko P., Rotrosen D., Levine M. (1989): Ascorbic acid transport and accumulation in human neutrophils. J. Biol. Chem. **264**, 18996–19002

Weisiger R. A., Fridovich I. (1973): Mitochondrial superoxide simutase. Site of synthesis and intramitochondrial localization. J. Biol. Chem. **248**, 4793–4796

Wolf S. P. (1994): Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. Methods Enzymol. **233**, 182–189