

The Effects of Hyperoxia, Hypoxia, and Ischemia/Reperfusion on the Activity of Cytochrome Oxidase from the Rat Retina

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Summary

Cytochrome oxidase activity from the retina can be enhanced or depressed by free radical-mediated reactions both in positive and negative aspect. The greatest effect was exerted by ischemia/reperfusion, which significantly increased the fluorescent products of lipid peroxidation (358 %, $P < 0.01$) and inhibited the enzyme activity (14 %, $P < 0.001$). After hyperoxia the fluorescent products slightly increased (192 %, $P < 0.05$) as well as the enzyme activity (133 %, $P < 0.05$). Hypoxia had no effect on any of these parameters. Specific changes in the composition of fluorophores after ischemia/reperfusion were revealed in the fluorescence spectra. The fact that increased lipid peroxidation after hyperoxia and after ischemia/reperfusion does not produce the same effect upon cytochrome oxidase activity might be explained by changes in the kinetic behavior of cytochrome oxidase. In the control enzyme preparation, two binding sites for cytochrome c were observed. One was of the low-affinity ($K_m = 60 \mu\text{M}$) and the other of the high-affinity ($K_m = 1.12 \mu\text{M}$). After *in vitro*-initiated lipid peroxidation, the low-affinity binding site was lost and the activity measured under "optimum" conditions at a single cytochrome concentration was higher than in the controls. This implies that oxidative damage to cytochrome oxidase *in vivo* can be site-specific and its extent should be estimated by performing detailed kinetic analysis as otherwise the results might be misleading.

Key words

Cytochrome oxidase • Retina • Hypoxia • Hyperoxia • Ischemia/reperfusion • Lipid peroxidation

Introduction

Retina is a tissue highly dependent on its oxygen supply. According to its specific consumption, the retina belongs to tissues with the highest metabolic rate. Most of the consumed oxygen is processed by cytochrome oxidase that is the terminal oxidase of cellular respiration catalyzing the transfer of electrons from reduced

cytochrome c to molecular oxygen. The free energy released in this reaction is used for pumping protons from the mitochondrial matrix to cytosol, generating a proton electrochemical gradient across the inner mitochondrial membrane that is used for ATP synthesis. Kinetic analysis of cytochrome oxidase activity shows concave Eadie-Hofstee plots which can be explained by two binding sites for the cytochrome c on the enzyme. One

site has a low affinity and high activity, while the other exhibits a high affinity and low activity (Sinjorgo *et al.* 1984). The steady-state oxidation of reduced cytochrome c by cytochrome oxidase revealed biphasic kinetics of the purified enzyme, while membrane-bound enzyme exhibited multiphasic kinetics with extended low affinity phase. The kinetics was modulated by phospholipid composition in the reconstituted enzyme (Speck *et al.* 1983). The lipids with which they are in contact affect the function of intrinsic membrane proteins. Alterations in membrane fatty acid composition bring about changes in enzymatic activities of membrane-bound enzymes (Vik *et al.* 1981). The phospholipid is required for binding of cytochrome c by cytochrome oxidase and the reaction of cytochrome c with cytochrome oxidase is proportional to the concentration of phospholipids in the enzyme (Tzagoloff and MacLennan 1965).

We have been interested in the effects of modulated oxygen concentrations as they are accompanied by free radical reactions that might modify the membrane lipid composition. At low concentrations of oxygen, the catalytic activity of cytochrome oxidase is inhibited through an allosteric effect of molecular oxygen on the enzyme (Chandel *et al.* 1996). Cytochrome oxidase was partially inhibited after exposure of isolated hepatocytes to hypoxia, and it was suggested that the oxidase functions as an oxygen sensor in intact hepatocytes (Chandel *et al.* 1997). Other experiments have shown that hypoxia induces lipid peroxidation in various tissues (Nakanishi *et al.* 1995, Wilhelm and Herget 1999). Reactive oxygen species (ROS) can also be generated in the retina during ischemia or during following reperfusion (Bonne *et al.* 1998). In a hyperoxic environment, ROS can be produced mainly by mitochondria, where large amounts of polyunsaturated fatty acids are also localized (Jamieson *et al.* 1986). Treatment of the rats with increased oxygen concentrations induced retinopathy in the affected animals (Ricci 1990).

We have exposed animals to hypoxia (10 % O₂ for 3 days) or hyperoxia (35 % O₂ for 3 days) or to ischemia/reperfusion (occlusion of the central retinal artery for 90 min, followed by reperfusion for 2 h). The activity of cytochrome oxidase was measured after these treatments in relation to membrane oxidative damage estimated by the production of fluorescent end-products of lipid peroxidation. The fluorescent products were characterized by tridimensional fluorescence spectral

arrays. The mechanism of action of lipid peroxidation upon cytochrome oxidase activity was further studied by kinetic analysis.

Methods

Treatment and sampling of animals

Two-month-old male Wistar rats weighing 235±16 g (mean ± S.D.) were divided into four groups with 9 animals per group. The first group served as a control for animals exposed to hypoxia and hyperoxia. The second group was exposed to hypoxia for 3 days in an isobaric hypoxic chamber (F_iO₂=0.1) as in our previous study (Wilhelm and Herget 1999, Wilhelm *et al.* 1999). The same equipment was used for exposing the animals to hyperoxia. The third group was exposed to 35 % O₂ for 3 days. The experimental animals had free access to food and water during the exposure to hypoxia or hyperoxia. The animals were killed by an overdose of halothane, and their retinas were separated after eye enucleation. The fourth group was deeply anesthetized by urethane. Under anesthesia, the central retinal artery of one eye was occluded in each animal with a suture while the other eye remained unoccluded and served as a control. The occlusion lasted 90 min, and then reperfusion was permitted for 2 h. Throughout the entire experiment the animals were kept under anesthesia. Then they were killed by decapitation, their eyes were enucleated and retinas separated.

Preparation of retinal homogenates

Eighteen retinas were homogenized in an all-glass homogenizer standing on ice in 5 ml of 60 mM ice-cold phosphate buffer (pH=7.4) containing 0.6 % lauryl maltoside. The homogenate was centrifuged (10 000 x g, 3 min) and filtered through a 25 µm membrane filter. The filtered homogenate was divided into two parts. In one part *in vitro* lipid peroxidation was initiated by incubation with 300 µM FeSO₄ plus 600 µM ascorbic acid. The samples were taken at 0 and after 120 min of incubation. The other part of the homogenate was analyzed directly, thus supplying data about the effects of individual treatments of animals. All the measured parameters were related to the protein concentration determined according to Lowry *et al.* (1951).

Assay of cytochrome oxidase activity

The activity of cytochrome c oxidase was measured by the spectrophotometric method following

the rate of oxidation of reduced cytochrome c at 550 nm (Yonetani 1967) with the use of HP8453 diode array spectrophotometer. The procedure was modified with respect to the optimum concentration of the potassium phosphate buffer that was found around 60 mM (see Results) and the detergent used – lauryl maltoside was used instead of Tween-80. Cytochrome c (type VI, Sigma) was reduced by sodium dithionite and desalted by gel filtration through a Sephadex G-25 column. The concentration of reduced cytochrome c was assayed spectrophotometrically. The cuvette contained 0.1 mg of protein in total volume of 3 ml and the final concentration of lauryl maltoside was 0.12 %, which corresponds to 36 mg/mg protein. The estimation of cytochrome oxidase in the groups after individual treatments was performed in hexaplicate of the homogenate prepared from pooled retinas using a constant 30 μ M concentration of reduced cytochrome c. Kinetic analysis of cytochrome oxidase activity after *in vitro* lipid peroxidation was performed on retinas from control animals using a range of cytochrome concentrations between 0.6 μ M and 30 μ M.

Measurement of fluorescence

The aliquots of the homogenate (0.6 ml) were extracted in 3 ml of a chloroform-methanol mixture (2:1, v/v) on a motor-driven shaker for 1 h. Water was then added to achieve phase separation and the sample was mixed and centrifuged. The lower chloroform layer was used for the measurements. At first, tridimensional spectral arrays were recorded using Perkin Elmer LS-5 spectrofluorometer coupled through an analog-digital converter to a computer where the data were stored. The division into 3D spectral arrays was made using software developed in our laboratory. The emission spectra were measured in the range of 390-460 nm for excitation adjusted between 280-375 nm in 10 nm steps. The quantitative estimation of LFP was based on the excitation and emission maxima found in 3D spectral arrays. The amount of specific fluorophores was expressed in relative fluorescence units related to the protein concentration (RFU/mg protein). The fluorometer was calibrated with standard No. 2 of the instrument manufacturer.

Statistics

Statistical evaluations were made using ANOVA with Scheffé *post-hoc* test, and the results are shown as means \pm S.E.M.

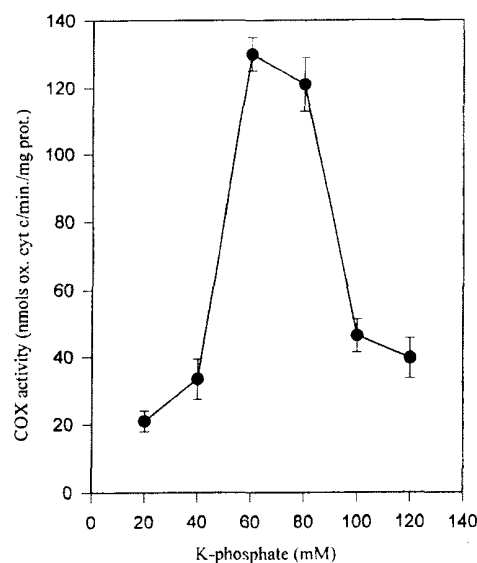


Fig. 1. Effect of potassium phosphate on the COX activity. Enzymatic activity was assayed with 30 μ M cytochrome c, each point represents a mean of six measurements \pm S.D.

Results

Optimal concentration of phosphate buffer in cytochrome oxidase assays

When cytochrome oxidase activity is being measured with a single cytochrome c concentration, it is recommended to run this assay under optimum conditions for obtaining maximum enzyme activity. One of the critical parameters is represented by the concentration of potassium phosphate, the optimum concentration of which varies in different tissues. Hence we started our study by seeking the concentration of potassium phosphate that would provide the maximum activity at a constant concentration of cytochrome c. The results, shown in Figure 1, indicate that the optimum concentration of potassium phosphate was found to be around 60 mM.

COX activity and the products of lipid peroxidation in animals exposed to change in oxygen concentration

We measured the activity of cytochrome oxidase in particular groups exposed to normoxia, hypoxia, hyperoxia, and ischemia/reperfusion. The results are summarized in Figure 2 together with the concentrations of fluorescent end-products of lipid peroxidation (LFP). Hypoxia did not influence COX activity significantly,

after hyperoxia it slightly increased (133 %, $P < 0.05$), and after ischemia/reperfusion it significantly decreased (14 %, $P < 0.001$). Apparently, COX activity was not related to the LFP concentration that significantly

increased after hyperoxia (192 %, $P < 0.05$), and after ischemia/reperfusion it increased to 358 % ($P < 0.01$). The change after hypoxia was not statistically significant.

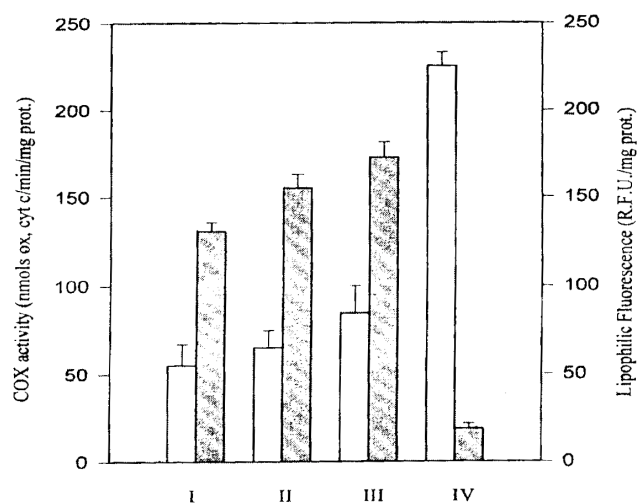
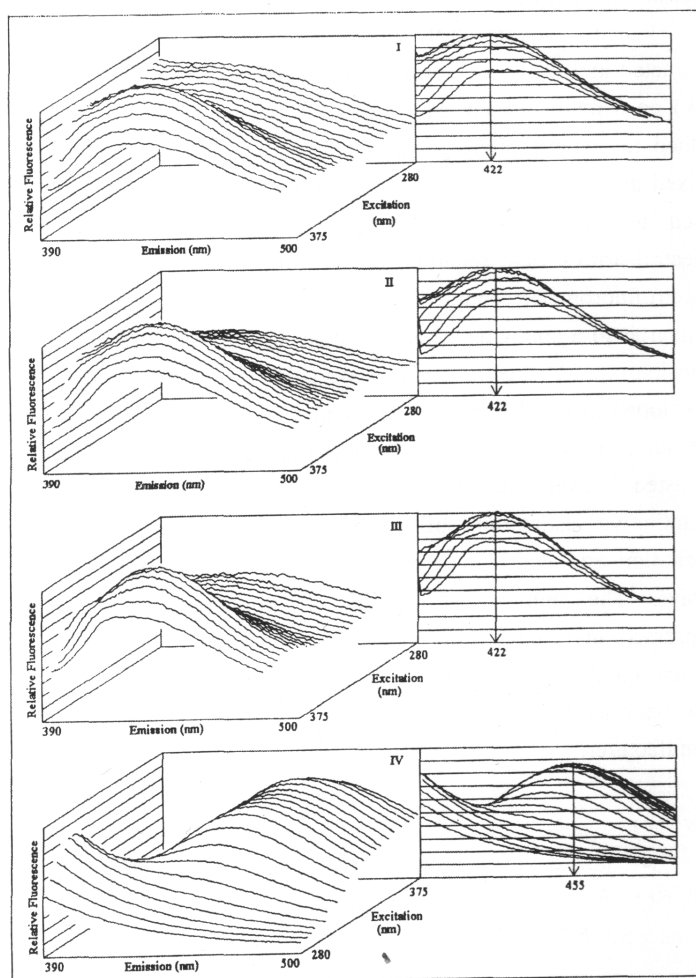


Fig. 2. COX activity and LFP levels in animals with manipulated oxygen concentration. I - control, II - hypoxia, III - hyperoxia, IV - ischemia/reperfusion. Empty columns represent the concentration of LFP, hatched columns indicate COX activity.

Fig. 3. Tridimensional fluorescence spectral arrays of chloroform extracts in individual groups. Roman numerals describe the groups as in Fig. 2.



Fluorescence spectra of the end-products of lipid peroxidation

The composition of lipophilic extracts from the retina in individual groups after manipulation with the oxygen concentration was investigated by measuring their fluorescence spectra. Figure 3 shows the 3D spectral arrays of emission spectra. In the control group, the emission maximum was found at 422 nm, as well as in the groups after hypoxia or hyperoxia. Furthermore, the position of excitation maximum was the same in all cases, namely around 325 nm. With regard to the fact that the 3D spectral arrays represent a "fingerprint" of a given composition, the overall shape of the spectral arrays after hypoxia and hyperoxia indicates changes in their composition relative to the controls. The greatest difference was observed in the spectra after ischemia/reperfusion. The emission maximum was shifted to 455 nm and excitation maximum to 335 nm.

Kinetic analysis of COX activity

We intended to elucidate the mechanism of the effect of lipid peroxidation on the activity of COX. The activation of COX after hyperoxia accompanied by increased lipid peroxidation is seemingly paradoxical when compared to the decreased activity after ischemia/reperfusion, when the products of lipid peroxidation are also increased. We therefore measured the kinetic properties of COX in control preparations and after *in vitro*-initiated lipid peroxidation. The level of fluorophores concentration increased to 160 % after 2 h of incubation. The summary of kinetic measurements is given in Figure 4. The Eadie-Hofstee plot of control preparations was concave and corresponded to two binding sites for cytochrome c. The high-affinity binding site had an apparent $K_m=1.12 \mu\text{M}$ and $V_{max}=178$ nmols ox.cyt c/min/mg protein. The low-affinity binding site had an apparent $K_m=60 \mu\text{M}$ and $V_{max}=1200$ nmols ox.cyt c/min/mg protein. Measurements performed after *in vitro* lipid peroxidation revealed that the low-affinity binding site was absent and that both V_{max} and K_m increased in comparison with the high-affinity kinetic branch.

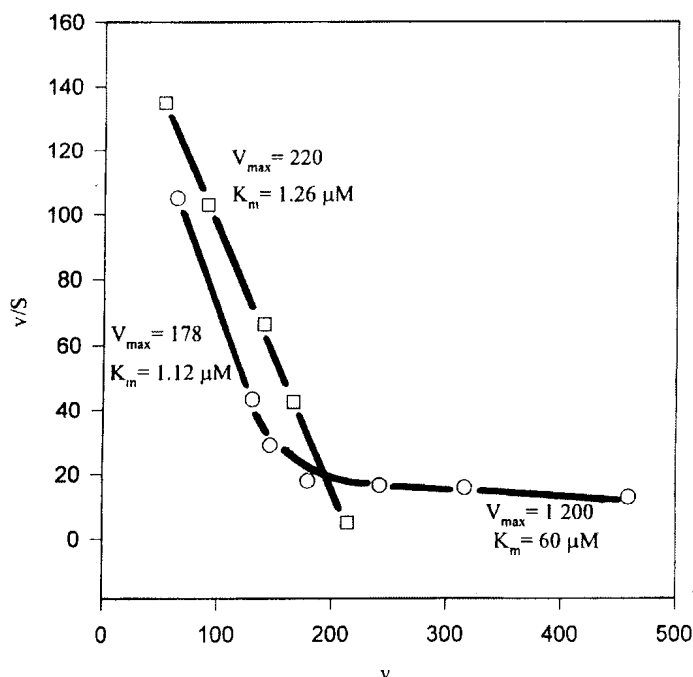


Fig. 4. Eadie-Hofstee plot of COX activity after *in vitro*-initiated lipid peroxidation. Dots represent non-peroxidized control, squares indicate the sample exposed to lipid peroxidation for 2 h.

Discussion

When measuring the cytochrome oxidase activity at a single cytochrome c concentration it is important to find the optimum concentration of salts in the assay buffer. Both K_m and V_{max} of the high-affinity

reaction increase rapidly with increasing ionic strength. Although the K_m of the low-affinity reaction increases rapidly with increasing ionic strength, the V_{max} increases slowly. These effects give an apparent maximum in plots at a single cytochrome c concentration (Cooper 1990). When measuring the cytochrome oxidase activity in

detergent extracts, the quality of the detergent plays an important role. Cholate, for instance, is a known inhibitor of cytochrome oxidase. Lauryl maltoside, on the other hand, is known for its stimulation of cytochrome oxidase activity. It increases the turnover number of both reactions without affecting the K_m values (Sinjorgo *et al.* 1984). Therefore, the potential substitution of some phospholipids interacting with the enzyme with the molecules of detergent should not induce inhibition or a change in the ratio of low-affinity and high-affinity reactions. However, it has been observed that removal of diphosphatidylglycerol from the preparation of beef heart cytochrome oxidase resulted in the loss of the low-affinity phase of electron transfer (Vik *et al.* 1981). We observed the same kinetic effect after initiation of lipid peroxidation in our experiments. It is thus possible that the critical lipid moieties have been affected by this treatment.

In another study, free radical production initiated by whole-body gamma irradiation inhibited cytochrome oxidase activity not only in the exposed brain, but also in shielded liver. It was suggested that aldehydic products of lipid peroxidation (Wilhelm *et al.* 1984) could exert the inhibiting effect on the liver. In the pig heart, submitochondrial particles of cytochrome oxidase were inhibited after redox cycling of doxorubicin. This anticancer drug produces reactive oxygen species and initiates lipid peroxidation. The low-affinity binding site of cytochrome oxidase was inhibited preferentially (Demant, 1991).

In the present study, both hyperoxia and ischemia/reperfusion induced lipid peroxidation in the retina of exposed animals. However, the activity of COX increased after hyperoxia while after ischemia reperfusion the enzymatic activity decreased. On the basis of an analogy with *in vitro* lipid peroxidation, we can deduce that hyperoxia might have produced a similar effect by inhibiting the low-affinity binding site which resulted in an increase in total activity. The extent of the oxidative damage induced by hyperoxia (192 %) was similar to that produced by *in vitro* peroxidation (160 %). Under conditions of more extensive oxidative damage

induced by ischemia reperfusion (increase to 358 %), the inhibition probably also involves the other active site and a decrease in total enzymatic activity is observed. It is important to realize that the differences in COX activity after manipulating the ambient oxygen concentration do not stem from the different availability of oxygen but from the peroxidation of membrane lipids. The response of a tissue to particular treatment is dependent on the lipid composition and the antioxidant capacity of the tissue. Because of its specific composition (high concentration of polyunsaturated fatty acids and high amount of mitochondria) the retina might respond to hypoxia differently from other tissues. Though both hypoxia and ischemia decrease oxygen concentration, they do not generate the equal amounts of ROS, and probably produce individual ROS in different ratios. Therefore the damage caused by hypoxia or ischemia will differ in extent and quality. However, further studies are needed to elucidate this problem.

The apparent activation of COX activity after oxidative damage could be explained by a change in kinetic properties of the enzyme subjected to lipid peroxidation. Our preliminary data have shown that the changes of COX kinetics during *in vitro*-initiated lipid peroxidation are complex and depend on the extent of peroxidative damage. It is possible to obtain a whole array of possible responses, from activation of the "total" activity due to inhibition of the high affinity binding site to the complete inhibition of the enzyme activity. However, a specifically designed study will be needed for the detailed analysis of these effects.

In conclusion, it is important to stress that when evaluating the effects of free radical-initiated reactions upon cytochrome oxidase it seems necessary to perform a detailed kinetic analysis, as otherwise the results might be misleading.

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References

- BOONE C, MULLER A, VILLAIN M: Free radicals in retinal eschemia. *Gen Pharmacol* **30**: 275-280, 1998.
- COOPER CE: The steady-state kinetics of cytochrome c oxidation by cytochrome oxidase. *Biochim Biophys Acta* **1017**: 187-203, 1990.

- CHANDEL NS, BUDINGER GRS, CHOE SH, SCHUMACKER PT: Molecular oxygen modulates cytochrome c oxidase function. *J Biol Chem* **271**: 18672-18677, 1996.
- CHANDEL NS, BUDINGER GRS, CHOE SH, SCHUMACKER PT: Cellular respiration during hypoxia. *J Biol Chem* **272**: 18808-18816, 1997.
- DEMANT EJF: Inactivation of cytochrome c oxidase activity in mitochondrial membranes during redox cycling of doxorubicin. *Biochem Pharmacol* **41**: 543-552, 1991.
- JAMIESON D, CHANCE B, CADENAS E, BOVERIS A: The relation of free radical production to hyperoxia. *Annu Rev Physiol* **48**: 703-719, 1986.
- LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275, 1951.
- NAKANISHI K, TAJIMA F, NAKAMURA A, YAGURA S, OOKAWARA T, YAMASHITA H, SUZUKI K, TANIGUCHI N, OHNO H: Effects of hypobaric hypoxia on antioxidant enzymes in rats. *J Physiol Lond* **489**: 869-876, 1995.
- RICCI B: Oxygen-induced retinopathy in the rat model. *Doc Ophthalmol* **74**: 171-177, 1990.
- SINJORGO KMC, MEIJLING JH, MUIJSERS AO: The concept of high- and low-affinity reactions in bovine cytochrome c oxidase steady-state kinetics. *Biochim Biophys Acta* **767**: 48-56, 1984.
- SPECK SH, NEU CA, SWANSON MS, MARGOLIASH E: Role of phospholipid in the low affinity reactions between cytochrome c and cytochrome oxidase. *FEBS Lett* **164**: 379-382, 1983.
- TZAGOLOFF A, MACLENNAN DH: Studies of the electron-transfer system. LXIV. Role of phospholipid in cytochrome oxidase. *Biochim Biophys Acta* **99**: 476-485, 1965.
- VIK SB, GEORGEVICH G, CAPALDI RA: Diphosphatidylglycerol is required for optimal activity of beef heart cytochrome c oxidase. *Proc Natl Acad Sci USA* **78**: 1456-1460, 1981.
- WILHELM J, HERGET J: Hypoxia induces free radical damage to rat erythrocytes and spleen: analysis of the fluorescent end-products of lipid peroxidation. *Int J Biochem Cell Biol* **31**: 671-681, 1999.
- WILHELM J, FRYDRYCHOVÁ M, VÍZEK M: Hydrogen peroxide in the breath of rats: The effect of hypoxia and paraquat. *Physiol Res* **48**: 445-449, 1999.
- WILHELM J, KRATOCHVIL O, SONKA J: The changes in activities of some mitochondrial oxidases in the brain and liver of rats with gamma-irradiated heads. *Agressologie* **25**: 13-15, 1984.
- YONETANI T: Cytochrome oxidase: beef heart. *Methods Enzymol* **10**: 332-335, 1967.

Reprint requests

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