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Hypercapnia protects erythrocytes against free radical damage induced by hypoxia in exposed rats

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Several studies report that hypoxic exposure induces free radical oxidative damage in various tissues. The mechanism of this damage includes membrane lipid peroxidation which can be easily detected by measuring fluorescent end-products of the process, i.e. lipofuscin-like pigments. Four day exposure of rats to hypoxia (10% O_2) increased the level of lipofuscin-like pigments in erythrocytes up to 9 fold. This increase was completely prevented when the animals were exposed to hypercapnia (4.3% CO_2) in addition to hypoxia. We studied the possible mechanism of the hypercapnic protection on isolated erythrocyte membranes *in vitro*. Lipid peroxidation was initiated by incubation of the membranes with iron ions and ascorbate. Production of malonaldehyde, the precursor of lipofuscin-like pigments, was strongly inhibited in bicarbonate buffer. Similarly the production of lipofuscin-like products was damped. These experiments suggest that the protective effect of hypercapnia might consist in direct interaction of CO_2 with free radical processes. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS — Red blood cells; CO_2; lipid peroxidation; lipofuscin-like pigments

INTRODUCTION

Exposure of animals to hypoxia induces several mechanisms producing free radicals. Hypoxia accelerates the production of reactive oxygen species (ROS), namely superoxide and hydrogen peroxide.[1–3] Increased production of ROS under hypoxic conditions was observed in alveolar macrophages,[4] and smooth muscle cells isolated from pulmonary arteries.[5,6]

ROS produced during hypoxia preferentially attack membrane unsaturated fatty acids, initiating deteriorative process of membrane lipid peroxidation. For instance, hydrogen peroxide, formed in mitochondria of different lung cells or by activated phagocytes[7] can mediate oxidative lung damage by initiating membrane lipid peroxidation.[8] Exposure of rats to hypoxia increased the concentration of malonaldehyde, a lipid peroxidation decomposition product, in lung and other tissues.[9] The end-products of lipid peroxidation are fluorescent, and therefore the so-called lipofuscin-like pigments (LFP) are widely used as an indicator of oxidative damage in various biological systems.[10–15] Recently we have observed an induction of LFP formation in erythrocytes and the spleen of rats exposed to short-term, and chronic hypoxia.[16]

Carbon dioxide is an important component of biological acid-base reactions and it produces various physiological effects. Recently, a relatively new role of CO_2 was observed. It was found that CO_2 interacts with several free radical species and in dependence on many factors, it can either propagate or inhibit the free radical chain reactions. The protective role of hypercapnia against free radical damage induced by hypoxia in vivo was observed in some studies.[17–19]

A key role in the initiation and propagation of free radical reactions is played by iron ions.[20–22] Transferrin is the major iron binding protein in blood plasma

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and the capacity to bind iron enables transferrin to protect cells from oxidative damage. Oxidative stress destabilizes the iron-transferrin complex and increases the levels of catalytic metal ions. The protective effects of CO_2 seems to consist in stabilisation of the iron-transferrin binding due to a synergistic action of bicarbonate.[23] The fact that iron binding by transferrin requires bicarbonate anions offers an explanation for the different effects of lactic acid and hypercapnic acidosis on lipid peroxidation.[24]

In the present study we have investigated the effects of hypercapnia on the free radical damage induced in erythrocytes of animals exposed to hypoxia. As we observed a protective effect, we further studied the mechanism of erythrocyte lipid peroxidation *in vitro* in the presence of bicarbonate. The results indicate that bicarbonate could also inhibit lipid peroxidation in a direct manner, not involving transferrin.

MATERIALS AND METHODS

Treatment and sampling of animals

Two month old male Wistar rats weighing 220 ± 15 g were divided into 3 groups with 10 animals per group. The first group served as a control and was sacrificed at the same time as the experimental groups. The second group was exposed to hypoxia for 4 days in an isobaric hypoxic chamber ($FiO_2 = 0.1$).[25] During the exposure to hypoxia the experimental animals had free access to food and water. The third group was exposed to a hypoxic mixture containing 4.3% CO_2. The animals were sampled under pentobarbital anesthesia. Blood was taken from the abdominal aorta into a syringe with heparin, the blood sample was mixed with an equal volume of physiological saline and centrifuged (400*g*, 5 min.). The sedimented erythrocytes were washed under the same conditions and the final sediment was made to approximately 1.5 ml. A part (50 μl) was used for protein determination, 1 ml of suspension was pipetted into plastic vials and stored at -70°C until analysis.

Fluorescence measurements

LFP in erythrocytes were determined using the method of Goldstein and McDonagh.[10] One ml of frozen erythrocyte suspension was added to 6 ml of chloroform!isopropanol mixture and extracted under nitrogen for 1 h on a motor!driven shaker. After extraction, 2 ml of distilled water was added, mixed, and the mixture was cetrifuged (400 g, 10 min.). After centrifugation the lower chloroform phase was separated and used for measurements. Fluorescence excitation spectra of chloroform extracts were measured on the Aminco-Bowman Series 2 spectrofluorometer coupled to a computer where the data were stored in the form of tridimensional spectral arrays. The excitation spectra were measured in the range of 250–400 nm for emission adjusted between 300–500 nm with a step of 10 nm. The quantitative estimation of LFP was based on the excitation and emission maxima found in 3 D spectral arrays. The fluorometer was calibrated with standard No. 2 of the instrument manufacturer and the LFP concentration was expressed in relative fluorescence units per mg of protein.

The same procedure was adopted for the membranes peroxidized *in vitro* where 0.5 ml of the incubation mixture was used.

Preparation of erythrocyte membranes

Blood was obtained from the hematology department. The samples remaining after examination of blood donors, anticoagulated with EDTA, were pooled and lysed in hypotonic solution containing 0.02 M NH_4Cl for 1 hour. The lysate was centrifuged at 45 000*g* for 50 min. The sediment was washed with physiological saline and centrifuged again under the same conditions. The washing step was repeated one more time, then the final sediment was made to approximately 5 ml and dialyzed overnight at 4°C. The dialyzed membranes were stored at -70°C.

In vitro membrane lipid peroxidation

The erythrocyte membrane suspension was divided into two parts. One part was buffered with 100 mM Tris-HCl, pH 7.8, the other was adjusted to the same pH with 100 mM bicarbonate buffer. Both solutions contained 300 μM EDTA, and lipid peroxidation was initiated by the addition of ascorbate (600 μM, final concentration) plus $FeSO_4$ (300 μM, final concentration). Both solutions contained 2 mg of protein/ml. This protein concentration was chosen after preliminary experiments which showed that higher protein concentrations inhibited lipid peroxidation reactions. The membranes were incubated for up to 330 min. and the process of lipid peroxidation was followed on the basis of production of malonaldehyde and LFP. The samples were taken at 0, 70, 150, 240, and 330 min.

Assay of malonaldehyde

For malonaldehyde determination the thiobarbituric acid methods of Asakawa and Matsushita[26] were

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adapted. Briefly, 200 μl of the incubated sample were pipetted into a glass test tube, 2 ml of 0.36% thiobarbituric acid was added together with 1 ml of 35% trichloroacetic acid. The test tube was placed in a boiling water bath for 15 min. Then it was cooled in a tap water, 1 ml of 100% acetic acid was added and the sample was mixed with 2 ml of chloroform. The mixture was centrifuged (400*g*, 10 min) and the absorbance of the pink layer was read at 532 nm. The concentration of MDA was calculated from the calibration curve constructed from MDA standard (Oxis International, USA).

The statistical evaluations were made using ANOVA with Scheffe post-hoc test, and the results are shown as means ± SEM.

Luminol chemiluminescence

We used the same concentrations of buffers, iron, and ascorbate as in *in vitro* lipid peroxidation system. The membranes were omitted and, instead, 0.25 mM luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione, Sigma) was added. Chemiluminescence was measured on a Luminometer 1250 (LKB-Wallac Oy, Finland), coupled through a custom made interface to a computer, where the data were collected at 1 s periods.

Detection of ferrous ions

We incubated the buffer systems with iron ions and ascorbic acid without membranes and in 30 min. intervals we withdrew 1 ml aliquots for the reaction of Fe^{2+} with 50 μl of 0.2% 2,2'-bipyridyl (Fluka) dissolved in 0.1 M HCl. The pink complex was measured at 525 nm using a HP 84453 diode array spectrophotometer (Hewlett Packard, Czech Republic).

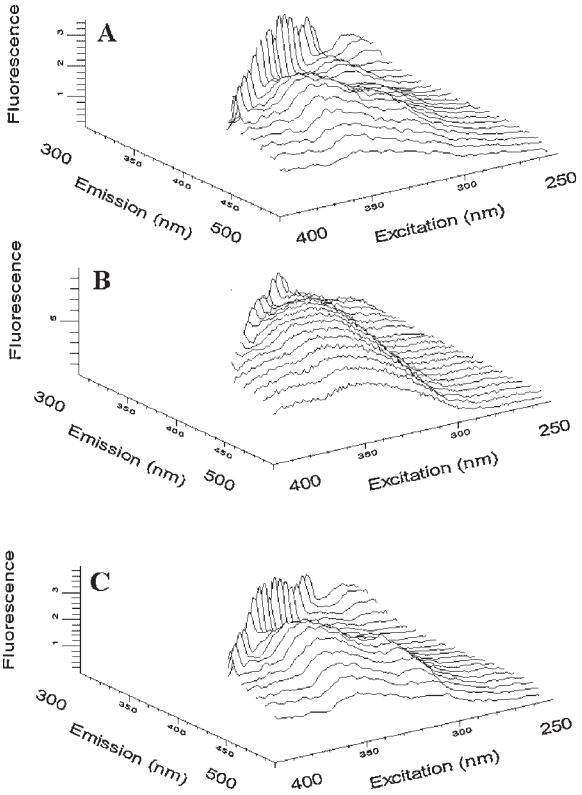

Figure 1. Fluorescence excitation spectral arrays of erythrocyte LFP. A: Control group, B: hypoxic group, C: hypoxic/hypercapnic group. Fluorescence intensity is given in arbitrary units.

RESULTS

In accordance with our previous studies we have observed increased levels of LFP in erythrocytes of animals exposed to 4-day hypoxia. The fluorescence spectra of erythrocyte extracts are summarized in Figure 1. A typical spectral array of hypoxic erythrocytes is shown in Figure 1B. It contains similar fluorophores to the control group (Figure 1A), but some fluorophores are specifically increased. Particularly it was F260/410 and F355/410 (excitation/emission, nm). When comparing the hypoxic/hypercapnic group (Figure 1C) to controls, it is apparent that the formation of the specific fluorophores was prevented. We measured these effects quantitatively and the results are summarized in Figure 2. Hypoxia induced significant increase in both fluorophores. F260/410 increased more then twofold and F355/410 more than ninefold, when compared to controls. The fluorophores of the hypoxic/hypercapnic group did not differ significantly from the controls.

Further we intended to investigate the mechanism of the protective action of hypercapnia on the peroxidative damage to erythrocytes. As LFP originate from the reactions of aldehydic products of membrane lipid peroxidation, we studied the lipid peroxidation of erythrocyte membranes *in vitro* in the presence and the absence of bicarbonate. Figure 3 documents the inhibitory effect of bicarbonate on MDA production in relation to Tris buffer of the same pH. While in the Tris buffer MDA increases during the incubation about 16-fold, the increase in the presence of bicarbonate is no higher than twofold.

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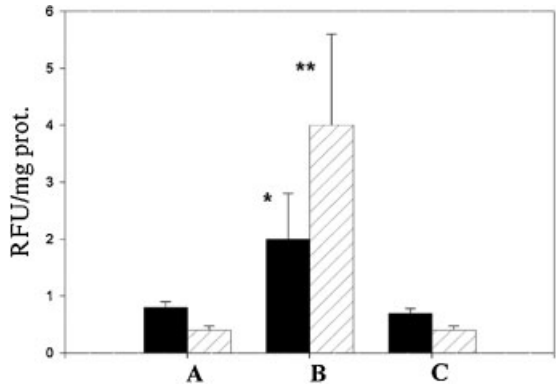

Figure 2. Quantitative expression of LFP fluorophores. Black columns: F260/410, hatched columns: F365/410. A: Control group, B: hypoxic group, C: hypoxic/hypercapnic group. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, related to controls.

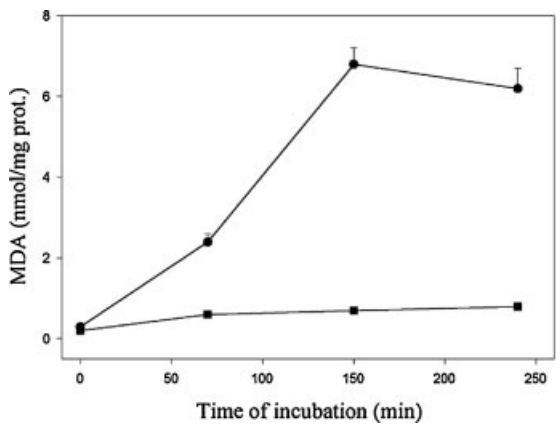

Figure 3. Time course of MDA production during *in vitro* peroxidation of erythrocyte membranes. Circles: Tris buffer; squares: bicarbonate buffer. SD bars for the bicarbonate buffer are smaller than the square symbols.

The spectra of LFP formed during *in vitro* lipid peroxidation are shown in Figure 4. Non-peroxidized membranes are presented in Figure 4A, the end-point of peroxidation in Tris buffer is shown in Figure 4B and incubation in bicarbonate buffer in Figure 4C. The fluorophore accumulated in the Tris buffer with maximum intensity F355/430 was used for the quantitative evaluation of LFP production during *in vitro* lipid peroxidation. The summary of the incubation results shown in Figure 5 documents inhibition of LFP production in the presence of bicarbonate in comparison with the Tris buffer.

In order to elucidate the mechanism of bicarbonate inhibition of free radical production more deeply, we tested its effect on chemiluminescence of luminol.

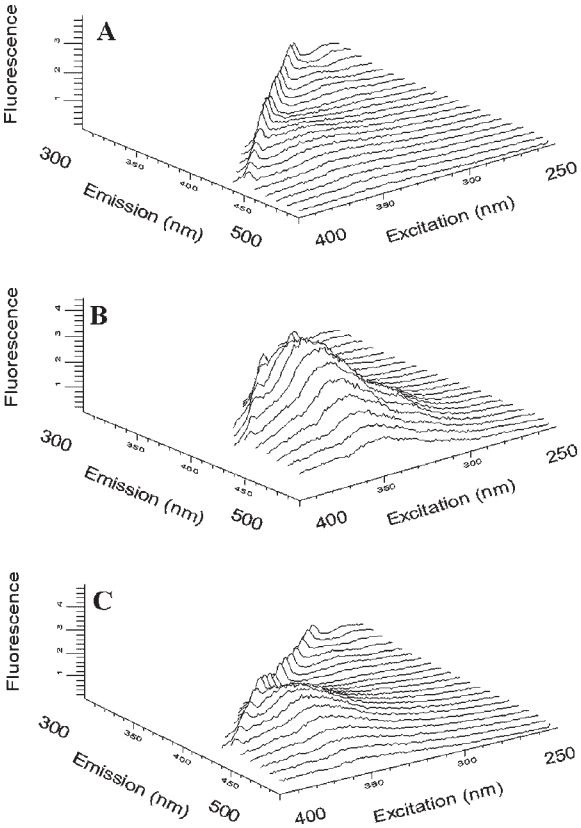

Figure 4. Fluorescence excitation spectral arrays of LFP fluorophores produced during *in vitro* peroxidation of erythrocyte membranes. A: Non-peroxidized membranes, B: membranes peroxidized in Tris buffer, C: membranes peroxidized in bicarbonate buffer. Fluorescence intensity is given in arbitrary units.

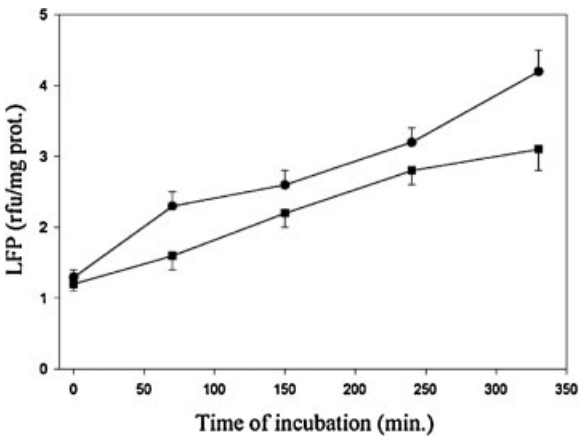

Figure 5. Time course of LFP production during *in vitro* peroxidation of erythrocyte membranes. Circles: Tris buffer, squares: bicarbonate buffer. Concentration of fluorophore F355/430 is shown.

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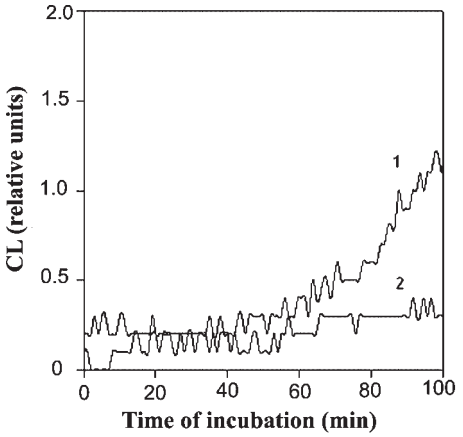

Figure 6. Production of luminol chemiluminescence during incubation in the absence of membranes. Curve 1: Tris buffer, curve 2: bicarbonate buffer. Concentrations of buffers, iron ions, and ascorbate are the same as in Fig. 3.

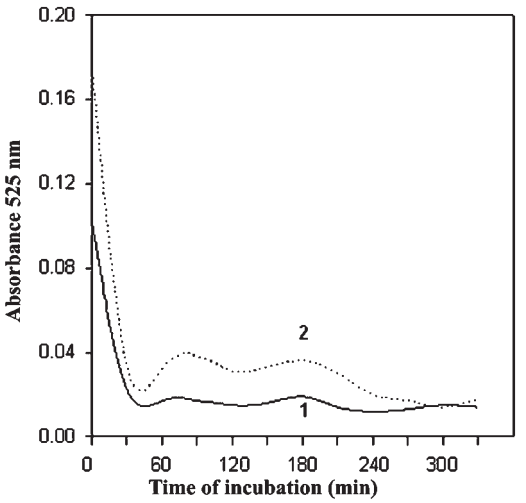

Figure 7. Time course of the concentration of ferrous ions during incubation in the absence of membranes. Curve 1: Tris buffer, curve 2: bicarbonate buffer.

Figure 6 shows luminol chemiluminescence generated in the reaction mixtures used for initiation of lipid peroxidation *in vitro.* Curve 1 was obtained in Tris buffer. It is apparent that after 40 min of incubation the light production started to rise. On the other hand, curve 2, measured in bicarbonate buffer indicates only slight increase in light production.

As the free radical generation in our system depended on autooxidation of ferrous ions, we tested the ability of ascorbic acid to generate ferrous ions throughout the whole incubation time by detecting the pink complex of Fe^{2+} with bipyridyl. In Figure 7 it can be seen that more Fe^{2+} is generated in bicarbonate buffer (curve 2) than in the Tris buffer (curve 1). Thus bicarbonate does not inhibit ascorbate reduction of Fe^{3+}.

DISCUSSION

In our previous study we found that short-term hypoxia induces peroxidative damage to rat erythrocytes which is revealed in increased formation of the fluorescent end-products of lipid peroxidation – LFP.[16] LFP have distinct fluorescence properties that enable their sensitive detection. Here we demonstrate that hypercapnia inhibits the peroxidation induced by hypoxia.

Several studies reported the inhibitory effect of CO_2 on free radicals derived both from oxygen or nitrogen.[17–19] The protective effect on erythrocytes has not been studied before. Many mechanisms were suggested to explain the protective effect of CO_2 *in vivo*.

The important role of CO_2 in protection against free radical damage consists in stabilization of the iron-transferrin complex[23] which decreases the availability of catalytic iron ions for the initiation of free radical reactions. Besides this indirect action, CO_2 can directly interact with free radical species and thus either augment or inhibit the free radical process dependent on other conditions.[27]

To test the dirrect effect of CO_2 we studied lipid peroxidation of isolated erythrocyte membranes in the presence or absence of bicarbonate *in vitro*. This *in vitro* system eliminated most of the factors responsible for the inhibitory effect of CO_2 *in vivo*. We measured the effects of bicarbonate on aldehydes and LFP production. Both types of products were inhibited, although MDA was inhibited in the presence of bicarbonate to a greater extent than LFP. This suggests that the inhibitory effect of bicarbonate is aimed against free radical reactions generating MDA. LFP originate from non-radical reactions with aldehydic products of lipid peroxidation, and besides MDA, other aldehydes might be involved. *In the case when the production of the other aldehydes is not inhibited by bicarbonate to the same extent as MDA, they can still support formation of LFP at lower rate, as was observed.*

The major LFP fluorophore formed during *in vitro* lipid peroxidation was F355/430. A similar fluorophore (F355/F440) was found in erythrocytes of

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animals after hypoxic exposure, but it did not represent the major species. It appears that hypoxia might induce specific fluorophores. *The ultimate identification of these fluorophores is highly complicated, because the spectroscopically defined species can be resolved by HPLC to several distinct substances.*[16] *This can be achieved in future studies.*

We interpret our *in vitro* experiments that hypercapnia, and correspondingly increased bicarbonate concentration, inhibits free radical reactions directly by interferring with the process of lipid peroxidation. The *in vivo* inhibition of LFP formation by hypercapnia could be at least partially caused by this mechanism.

When we tested the effects of bicarbonate in purely chemical system by measuring luminol chemiluminescence, we found that bicarbonate inhibited the free radicals oxidizing luminol. Thus this experiment supports the idea of direct antiradical effect of bicarbonate. The possible explanation could be the formation of carbonate radical unable of luminol oxidation. However, we were not able to detect carbonate radical by measuring its absorbance at 600 nm.[28] The source of radicals in our *in vitro* system was autooxidation of ferrous ions. We found higher levels of ferrous ions in the bicarbonate buffer system than in Tris buffer throughout the whole incubation period. This indicates that the reducing power of ascorbic acid was not damped in the presence of bicarbonate. On the other hand, the increased levels of Fe^{2+} in the bicarbonate buffer might document its slower reoxidation and also decreased production of free radicals.

The finding that hypercapnia prevents hypoxia induced lipid peroxidation is of clinical importance. The effects of lung hypoxia and hypercapnia in patients with chronic respiratory failure may be different from the effects of hypoxia and hypocapnia in high altitude residents. In patients the moderate level of hypercapnia may antagonize the hypoxia induced lung oxidative damage.

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REFERENCES

1. Cadenas E, Boveris A, Ragan CI, Stoppani AOM. Production of superoxide radical and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef heart mitochondria. *Arch Biochem Biophys* 1977; **180**: 248–257.
2. Herget J, Wilhelm J, Novotna J, *et al*. A possible role of the oxidant tissue injury in the development of hypoxic pulmonary hypertension. *Physiol Res* 2000; **49**: 493–501.
3. Hoshikawa Y, Ono S, Suzuki S, *et al*. Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia. *J. Appl Physiol* 2001; **90**: 1299–1306.
4. Wilhelm J, Sojkova J, Herget J. Production of hydrogen peroxide by alveolar macrophages from rats exposed to subacute and chronic hypoxia. *Physiol Res* 1995; **45**: 185–191.
5. Marshall C, Mamary AJ, Verhoeven AJ, Marshall BE. Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. *Am J Respir Cell Mol Biol* 1996; **15**: 633–644.
6. Killilea DW, Hester R, Balczon R, Babal P, Gillespie MN. Free radical production in hypoxic pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**: L408–L412.
7. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* 1990; **141**: 471–501.
8. Block ER, Patel JM, Edwards D. Mechanism of hypoxic injury to pulmonary artery endothelial cell plasma membranes. *Am J Physiol* 1989; **257**: C223–C231.
9. Nakanishi K, Tajima F, Nakamura A, *et al*. Effects of hypobaric hypoxia on antioxidant enzymes in rats. *J. Physiol* 1995; **489**: 869–876.
10. Goldstein BD, McDonagh EM. Spectrofluorescent detection of in vivo red cell lipid peroxidation in patients treated with diaminodiphenylsulfone. *J. Clin Invest* 1976; **57**: 1302–1307.
11. Wilhelm J, Sonka J. Time-course of changes in lipofuscin-like pigments in rat liver homogenate and mitochondria after whole body gamma irradiation. *Experientia* 1981; **37**: 573–574.
12. Wilhelm J, Brzak P, Rejholcova M. Changes in lipofuscin-like pigments in erythrocytes and spleen after whole-body gamma irradiation of rats. *Radiat Res* 1989; **120**: 227–233.
13. Shimasaki H, Maeba R, Tachibana R, Ueta N. Lipid peroxidation and ceroid accumulation in macrophage cultured with oxidized low density lipoprotein. *Gerontology* 1995; **41** (Suppl. 2): 39–48.
14. Vasankari T, Kujala U, Heinonen O, Kapanen J, Ahotupa M. Measurement of serum lipid peroxidation during exercise using three different methods: diene conjugation, thiobarbituric acid reactive material and fluorescent chromolipids. *Clin Chim Acta* 1995; **234**: 63–69.
15. Skoumalova A, Rofina J, Schwippelova Z, Gruys E, Wilhelm J. The role of free radicals in canine counterpart of senile dementia of the Alzheimer type. *Exp Gerontol* 2003; **38**: 711–719.
16. 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* 1999; **31**: 671–681.
17. Vannucci RC, Brucklacher RM, Vannucci SJ. Effect of carbon dioxide on cerebral metabolism during hypoxia-ischemia in the immature rat. *Pediat Res* 1997; **42**: 24–29.
18. Barth A, Bauer R, Gedrange T, Walter B, Klinger W, Zwienger U. Influence of hypoxia and hypoxia/hypercapnia upon brain and blood peroxidative and glutathione status in normal weight and growth-restricted newborn piglets. *Exp Toxicol Pathol* 1998; **50**: 402–410.
19. Takeshita K, Suzuki Y, Nishio K, *et al*. Hypercapnic acidosis attenuates endotoxin-induced nuclear factor-B activation. *Am J Respir Cell Mol Biol* 2003; **29**: 124–132.
20. Rice-Evans C, Baysal E. Iron-mediated oxidative stress in erythrocytes. *Biochem J* 1987; **244**: 191–196.

DOI: 10.1002/cbf

21. Monteiro HP, Winterbourn CC. Releas of iron from ferritin by divicine, isouramil, acid-hydrolyzed vicine, and dialuric acid and initiation of lipid peroxidation. *Arch Biochem Biophys* 1989; **271**: 536–545.
22. Peuchant E, Carbonneau MA, Dubourg L, *et al*. Lipoperoxidation in plasma and red blood cells of patients undrgoing haemodialysis: vitamins A, E, and iron status. *Free Radic Biol Med* 1994; **16**: 339–346.
23. Edeker BL, Rasmussen GT, Britigan BE. Bicarbonate and phosphate ions protect transferrin from myeloperoxidase-mediated damage. *Leukocyte Biol* 1995; **58**: 59–64.
24. Rehncrona S, Hauge HN, Siesjo BK. Enhancement of iron-catalyzed free radical formation by acidosis in brain homogenates: Difference in effect by lactic acid and CO_2. *J. Cereb Blood Flow Metabol* 1989; **9**: 65–70.
25. Hampl V, Herget J. Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from chronic exposure to hypoxia. *Am Rev Respir Dis* 1990; **141**: 2830–2842.
26. Asakawa T, Matsushita S. Thiobarbituric acid test for detecting lipid peroxides. *Lipids* 1979; **14**: 401–406.
27. Vesela A, Wilhelm J. The role of carbon dioxide in free radical reactions of the organism. *Physiol Res* 2002; **51**: 335–339.
28. Shafirovich V, Douradin A, Huang W, Geacintov NE. The carbonate radical is a site-selective oxidizing agent of guanine in double-stranded oligonucleotides. *J. Biol Chem* 2001; **276**: 24621–24626.

DOI: 10.1002/cbf