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Hydrogen Peroxide Production by Alveolar Macrophages Is Increased and Its Concentration Is Elevated in the Breath of Rats Exposed to Hypoxia: Relationship to Lung Lipid Peroxidation

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Summary

Hypoxic exposure triggers a generation of reactive oxygen species that initiate free radical damage to the lung. Hydrogen peroxide is the product of alveolar macrophages detectable in the expired breath. We evaluated the significance of breath H_2O_2 concentration for the assessment of lung damage after hypoxic exposure and during posthypoxic period. Adult male rats were exposed to normobaric hypoxia (10 % O_2) for 3 hours or 5 days. Immediately after the hypoxic exposure and then after 7 days or 14 days of air breathing, H_2O_2 was determined in the breath condensate and in isolated lung macrophages. Lipid peroxidation was measured in lung homogenates. Three-hour hypoxia did not cause immediate increase in the breath H_2O_2; 5-day hypoxia increased breath H_2O_2 level to 458 %. After 7 days of subsequent air breathing H_2O_2 was elevated in both groups exposed to hypoxia. Increased production of H_2O_2 by macrophages was observed after 5 days of hypoxia and during the 7 days of subsequent air breathing. Lipid peroxidation increased in the periods of enhanced H_2O_2 generation by macrophages. As the major increase (1040 %) in the breath H_2O_2 concentration found 7 days after 3 hours of hypoxia was not accompanied by lipid peroxidation, it can be concluded that the breath H_2O_2 is not a reliable indicator of lung oxidative damage.

Key words
Hydrogen peroxide • Alveolar macrophages • Lipid peroxidation • Lung • Breath

Introduction

Several studies have shown that hypoxic exposure induces free radical oxidative damage mediated by membrane lipid peroxidation (Block *et al.* 1989, Nakanishi *et al.* 1995, Wilhelm and Herget 1999, Veselá and Wilhelm 2002). While the underlying mechanism may be multifactorial, various aspects indicate a key role for hydrogen peroxide (Russell and Jackson 1994, Kinnula *et al.* 1995).

Hydrogen peroxide participates in many biochemical and physiological processes. Its primary effect may be damaging. Its ability to cross membranes enables it to spread the damage to a relatively large distance, and by interaction with transition metal ions it can produce hydroxyl radicals, the ultimate biological

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oxidant (Vissers and Winterbourn 1991). On the other hand, there are multiple indications that H_2O_2 can participate in cellular signalling. It was shown that the major enzyme responsible for peroxide production, NADPH oxidase, is located in membranes of various non-phagocytic cells and the amount of peroxide formed is not sufficient to kill bacteria (for review see Bauerle *et al.* 1996).

The important source of H_2O_2 in the lung is represented by NADPH oxidase in alveolar macrophages. Hypoxia enhances H_2O_2 production. It was found that hypoxia primes alveolar macrophages for a large release of H_2O_2 under *in vitro* conditions (Tuohy *et al.* 1993). Alveolar macrophages isolated from rats exposed to hypoxia produced more hydrogen peroxide than macrophages from control animals (Wilhelm *et al.* 1995). Hypoxia also activated NADPH oxidase in smooth muscle cells of pulmonary arteries (Marshall *et al.* 1996).

Hydrogen peroxide can be detected in the breath (Wilson *et al.* 1993, Madden *et al.* 1997). Recently we have observed an increase in H_2O_2 amount in the expired breath of rats exposed to a short-term hypoxia (Wilhelm *et al.* 1999). Hypoxia thus increases the concentration of H_2O_2 in the breath, primes alveolar macrophages for higher H_2O_2 production *ex vivo*, and induces lung lipid peroxidation. All these parameters were investigated in separate studies. We have intended to compare the effect of hypoxia on these parameters in the same experimental animals in order to elucidate their mutual relationship. We have followed the effects of reoxygenation during the posthypoxic period of 14 days, which have not yet been studied in this respect.

Methods

Two month-old male Wistar rats weighing 265 ± 33 g (mean ± S.D.) were divided into six groups with 12 animals per group. The first group served as a control, two other groups were exposed to hypoxia for 3 hours, and three groups were exposed to hypoxia for 5 days in an isobaric hypoxic chamber (FiO2 = 0.1) (Hampl and Herget 1990). During the exposure to hypoxia the experimental animals had free access to food and water.

Immediately after the hypoxic exposure the individual awake animals were placed into a perspex body box, volume 4.5 l, flushed with air at a constant flow of 2.5 l/min and the vapor was collected for one hour as in our previous study (Wilhelm *et al.* 1999). The outlet of the box was connected to the glass chamber submerged in acetone cooled with dry ice. The frozen water vapor was weighed and analyzed for H_2O_2. The collection of breath samples was repeated after 7 days of recovery in air in animals exposed to hypoxia for 3 hours and 5 days, and after 14 days of recovery in air in animals exposed to hypoxia for 5 days. The control group was measured in the same manner.

The amount of hydrogen peroxide was assayed luminometrically, on the basis of the reaction of H_2O_2 with luminol, catalyzed by horse-radish peroxidase, as described in a previous study (Wilhelm *et al.* 1999).

After collecting the breath samples, alveolar macrophages were isolated according to the published protocol (Wilhelm *et al.* 1995). Shortly, the animals were anesthetized by halothane, and their lungs were excised and lavaged with Hank's buffered salt solution without Ca^{2+} and Mg^{2+} containing 0.6 mM EDTA. The cells were collected by centrifugation (400 x *g*, 10 min, 20 ºC), washed and sedimented under the same conditions. The number of cells was counted under a light microscope, the viability of the cells was measured using trypan blue exclusion test and cells were immediately used for chemiluminescence measurement. They were kept at room temperature and in atmospheric air.

Hydrogen peroxide production by alveolar macrophages was measured using luminol-dependent chemiluminescence originating from the reaction between H_2O_2 and luminol catalyzed by horse-radish peroxidase (Wilhelm *et al.* 1995). For one measurement $1x10^6$ cells was used. They were stimulated either with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), final concentration 5 μM, or with protein kinase C activator phorbol-myristate-acetate (PMA), final concentration 1.6 μM. The measurements were carried out in plastic cuvette at 37 ºC for approximately 15 min with periodic agitations to prevent cell adherence. The maximum chemiluminescence was used as a parameter for characterization of H_2O_2 production. Its expresion related to the number of viable cells in the cuvette was given in relative units to compare the H_2O_2 production in individual rats of different experimental groups. A cell suspension obtained from one rat was used for triplicate assays of H_2O_2 production after stimulation with FMLP and also after stimulation with PMA. The basic production of H_2O_2 (without any stimulant) was measured at the beginning of each measurement before the addition of particular stimulant. A value characterizing particular experimental group was calculated as a mean value ± SD obtained from twelve animals. The maximum amount of H_2O_2 produced by activated cells was estimated by comparing the peak

chemiluminescence to that produced by the addition of known amount of H_2O_2. This was found to be in the hundred-nanomolar range.

After washing out the macrophages, the lungs were frozen between two alluminium blocks, precooled in liquid nitrogen and stored for other analyses at –75 ºC. About 0.5 g of frozen tissue was used to prepare 10 % homogenate in 10 mM phosphate buffer, pH 7.4. The aldehydic products of lipid peroxidation were assayed using the LPO-586 kit (Bioxytech S.A.). We employed the reaction in methanesulfonic acid, which is according to the manufacturer more sensitive to 4-hydroxy-alkenals, but also detects malonaldehyde. The concentration of aldehydes was read from the calibration curve constructed with 4-hydroxy-2-nonenal. It was expressed in nmols of aldehydes per mg of protein assayed according to Lowry *et al.* (1951).

Statistical evaluation was performed using ANOVA and the Scheffé *post-hoc* test.

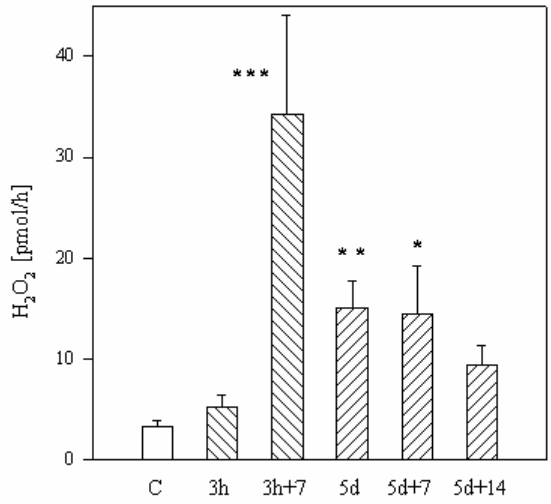

Fig. 1. *The amount of H_2O_2 in the breath samples of the experimental groups. C – controls; 3h – group exposed to hypoxia for 3 hours; 3h+7 – group exposed to hypoxia for 3 hours, measured after 7 days of air breathing; 5d – group exposed to 5 days of hypoxia; 5d+7 – group exposed to 5 days of hypoxia, measured after 7 days of air breathing; 5d+14 – group exposed to 5 days of hypoxia, measured after 14 days of air breathing. Significantly different from controls: *P<0.05, **P<0.01, *** P<0.001*

Results

We started our study by measuring exhaled H_2O_2 in the breath. With regard to the production of reactive oxygen species, the critical periods are observed during the transition from normoxia to hypoxia and from hypoxia to normoxia. We therefore investigated the effects of hypoxia immediately after hypoxic exposure and then during the subsequent period of air breathing. The time scale of the samplings was based on our previous study (Wilhelm *et al.* 1999).

The results are summarized in Figure 1, where the measured units are shown. In the following text, for better comparison of the time-course of individual parameters the results are expressed in percentages and related to 100 % of control value.

There was no significant change in H_2O_2 output immediately after 3 hours of hypoxic exposure. However, after subsequent 7 days of air breathing the production of H_2O_2 sharply rose to 1040 % ($P<0.001$). After 5 days of hypoxia the H_2O_2 output rose significantly to 458 % ($P<0.01$), and remained at this elevated level (436 %, $P<0.05$) during 7 days of subsequent air breathing. After 14 days of air breathing the difference relative to the control was not significant.

The production of H_2O_2 by isolated alveolar macrophages was measured after stimulation with either FMLP or PMA which activate different parts of the H_2O_2 biosynthetic pathway. The results are summarized in Figure 2. After stimulation with FMLP, the only significant increase in H_2O_2 production was observed after 7 days of recovery in air following the 5 days of hypoxic exposure (175 %, $P<0.01$). Macrophages stimulated by PMA produced increased amounts of H_2O_2 after 5 days of hypoxic exposure (142 %, $P<0.05$) and also after 7 days of recovery (145 %, $P<0.05$). After 14 days of air breathing the PMA-stimulated H_2O_2 production returned to the control level. Hypoxic exposure for 3 hours did not cause any changes in macrophage H_2O_2 production.

After washing out the macrophages, the lungs of the experimental animals were used for the assay of aldehydic products of lipid peroxidation. The results are shown in Figure 3. A significant increase was observed after 5 days of hypoxia (230 %, $P<0.01$). The increased level of aldehydes lasted for 7 days of air breathing (212 %, $P<0.01$), but it returned to control values after 14 days of air breathing. Exposure to hypoxia for 3 hours did not produce any changes.

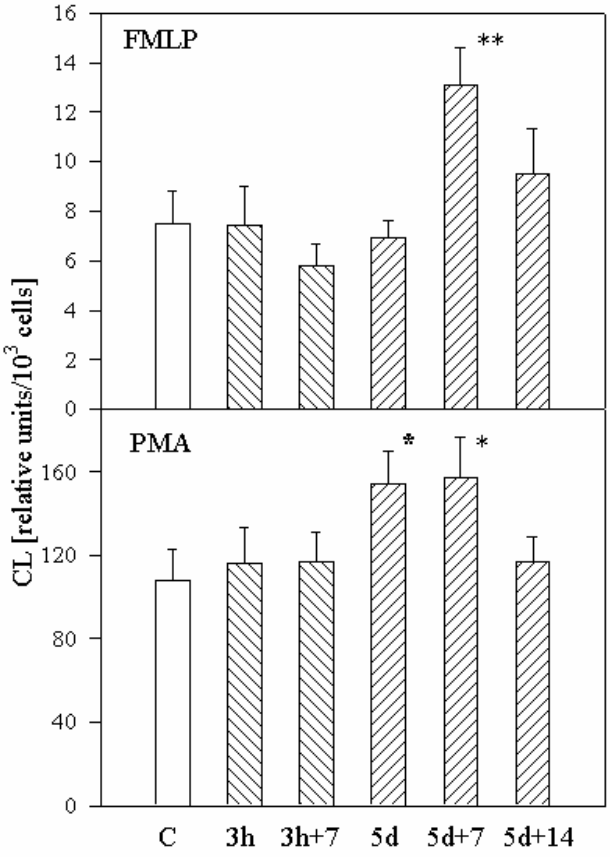

Fig. 2. *Chemiluminescence produced by isolated alveolar macrophages. FMLP – activation with chemotactic peptide, PMA – activation with phorbol ester. For other legend see Fig. 1. Significantly different from controls: * P<0.05, ** P<0.01.*

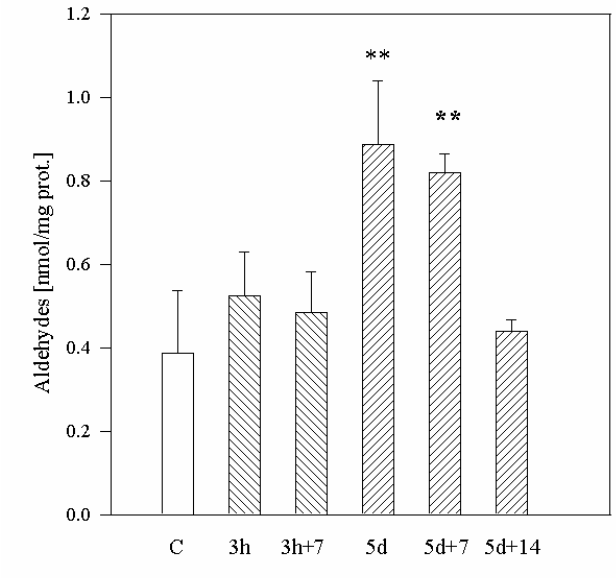

Fig. 3. *Aldehydic products of lipid peroxidation in lung homogenates. For other legend see Fig. 1. Significantly different from controls: ** P<0.01.*

Discussion

Exposure of experimental animals to hypoxia primes alveolar macrophages to higher production of hydrogen peroxide (Tuohy *et al.* 1993, Wilhelm *et al.* 1995). This priming builds up a cell potential that is realized during subsequent cell activation. Non-stimulated macrophages do not produce elevated amounts of H_2O_2, however, upon activation *in vivo* the production will increase as in *ex vivo* conditions. There are several indications that hypoxic exposure involves leukocyte activation *in vivo* (Sobin *et al.* 1983, Burghuber *et al.* 1984).

The different response to PMA and FMLP of alveolar macrophages isolated from the animals exposed to hypoxia (Fig. 2) indicates the specificity of the changes induced by hypoxia. PMA activates protein kinase C directly (Alvarez *et al.* 1993) and hypoxia probably increases concentration of this enzyme (Wilhelm *et al.* 1995). This could explain the increase in H_2O_2 production after 5 days of hypoxia when the cells were stimulated by PMA. The buildup of the overall pathway beginning with the membrane receptor for FMLP takes a longer time because we observed increased H_2O_2 production with this activator after a subsequent period of 7 days of air breathing. During this period the response to PMA was also increased, which suggests that the effects of short-term hypoxia on macrophages prevails at least a week after the end of hypoxic exposure.

Alveolar macrophages represent a potential source of H_2O_2 found in the expired breath. The observation of increased H_2O_2 level in the breath after 5 days of hypoxic exposure and during the following 7 days of air breathing (Fig. 1) supports this idea. On the other hand, H_2O_2 in the breath can originate from other processes as in the case of increased H_2O_2 production 7 days after hypoxic exposure for 3 hours, when no increase in macrophage H_2O_2 production was observed.

The period of increased H_2O_2 production by alveolar macrophages corresponded to the initiation of lung lipid peroxidation (Fig. 3) indicating a causal relationship. The only exception to the general scenario of increased H_2O_2 production in alveolar macrophages accompanied by lung oxidative damage and increased H_2O_2 level in the breath is the posthypoxic H_2O_2 increase 7 days after exposure to 3 hours of hypoxia (Fig. 1).

We should realize that the increase in H_2O_2 expressed in percentages need not necessarily be related to the oxidative damage. We believe that the key to the explanation of this phenomenon consists in the measured

concentrations. The amount of H_2O_2 in the breath is expressed in pmols produced per hour. Taking into consideration the volume of the condensate (typically less than 1 ml) it can be deduced that the lung concentration might be in the femtomolar range. Such low concentrations are beyond the sensitivity of decomposing enzymes (K_m of glutathione peroxidase is about 10 μM) (Jenkinson *et al.* 1984) and most probably are not able to induce oxidative damage.

It appears that measurements of breath H_2O_2 levels cannot serve for the assessment of lung oxidative damage. It might be used, together with other biomarkers found in the exhaled breath, to monitor respiratory diseases (Kharitonov and Barnes 2002). Here we document that an increased level of H_2O_2 is not accompanied by oxidative damage. Moreover, we have demonstrated in the recent study (Wilhelm *et al.* 1999) that extensive lung damage was not accompanied by an increase in H_2O_2 breath concentration. It can therefore be concluded that the use of H_2O_2 in the breath as the sole indicator of lung oxidative damage is questionable.

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References

ALVAREZ E, SANTA MARIA C, MACHADO A: Respiratory burst reaction changes with age in rat peritoneal macrophages. *Biochim Biophys Acta* **1179**: 247-252, 1993.

BAUERLE PA, RUPEC RA, PAHL HL: Reactive oxygen intermediates as second messengers of a general pathogen response. *Pathol Biol*: **44**: 29-35, 1996.

BLOCK ER, PATEL JM, EDWARDS D: Mechanism of hypoxic injury to pulmonary artery endothelial cell plasma membranes. *Am J Physiol* **257:** C223-C231, 1989.

BURGHUGER O, MATHIAS MM, MCMURTRY IF, REEVES JT, VOELKEL NF: Lung edema due to hydrogen peroxide is independent of cyclooxygenase products. *J Appl Physiol* **56:** 900-905, 1984.

HAMPL V, HERGET J: Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from chronic exposure to hypoxia. *Am Rev Respir Dis* **141:** 2830-2842, 1990.

JENKINSON SG, LAWRENCE RA, TUCKER WY: Glutathione peroxidase, superoxide dismutase, and glutathione S-transferase activities in human lung. *Am Rev Respir Dis* **130:** 302-304, 1984.

KHARITONOV SA, BARNES PJ: Biomarkers of some pulmonary diseases in exhaled breath. *Biomarkers* **7:** 1-32, 2002.

KINNULA VL, CRAPO JD, RAIVIO KO: Biology of disease. Generation and disposal of reactive oxygen metabolites in the lung. *Lab Invest* **73:** 3-19, 1995.

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265-275, 1951.

MADDEN MC, HANLEY N, HARDER S, VELEZ G, RAYMER JH: Increased amounts of hydrogen peroxide in the exhaled breath of ozone-exposed human subjects. *Inhal Toxicol* **9:** 317-330, 1997.

MARSHALL CA, MAMARY J, VERHOEVEN AJ, MARSHALL BE: Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. *Am J Respir Cell Mol Biol* **15:** 633-644, 1996.

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 .

RUSSELL WJ, JACKSON RM: Hydrogen peroxide release by mitochondria from normal and hypoxic lungs. *Am J Med Sci* **308:** 239-243, 1994.

SOBIN SS, TREMENR HM, HARDY JO, CHIODI HP: Changes in arterioles in acute and chronic hypoxic pulmonary hypertension and recovery in rat. *J Appl Physiol* **55:** 1445-1455, 1983.

TUOHY MB, BAIN MH, GREENING AP: Rat alveolar function is primed by exposure to hypoxia. *Eur Respir J* **6:** 267, 1993.

VANDEN HOEK TL, BECKER LB, SHAO Z, LI C: Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* **273:** 18092-18098, 1998.

VESELÁ A, WILHELM J: The role of carbon dioxide in free radical reactions of the organism. *Physiol Res* **51**: 335-339, 2002.

VISSERS MCM, WINTERBOURN CC: Oxidative damage to fibronectin. II. The effect of H_2O_2 and the hydroxyl radical. *Arch Biochem Biophys* **285:** 357-364, 1991.

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, SOJKOVÁ J, HERGET J: Production of hydrogen peroxide by alveolar macrophages from rats exposed to subacute and chronic hypoxia. *Physiol Res* **45:** 185-191, 1995.

WILHELM J, FRYDRYCHOVÁ M, VÍZEK M: Hydrogen peroxide in the breath of the rats: the effects of hypoxia and paraquat. *Physiol Res* **48:** 445-449, 1999.

WILSON WC, LABORDE PR, BENUMOF JL, TAYLOR R, SWETLAND JF: Reperfusion injury and exhaled hydrogen peroxide. *Anesth Analg* **77:** 963-970, 1993.

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