Physiol. Res. 48: 445-449, 1999

Hydrogen Peroxide in the Breath of Rats: the Effects of Hypoxia and Paraquat

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Received January 21, 1999
Accepted June 22, 1999

Summary

The hypothesis that oxidative stress can be induced by hypoxia was tested by measuring the concentration of hydrogen peroxide by a luminometric technique in the breath samples of rats exposed to hypoxia and paraquat. The group of animals (n=15) exposed to normobaric hypoxia (10 % O_2) for three days had an increased amount of H_2O_2 (200 %, $P<0.001$) in their breath in comparison to control animals. After 7 days of recovery in air, the exposed animals still produced significantly increased levels of H_2O_2 (152 %, $P<0.001$). Paraquat administration was used as a positive control, since it is a redox cycling compound producing free radicals. In the animals treated with a toxic dose of paraquat, the peak H_2O_2 production was observed 5 h after i.p. injection (156 %, $P<0.02$). Within the next 2 h it decreased to the control level and stayed constant for 48 h, when the animals began to die. It is suggested that H_2O_2, observed in the breath samples, is a product of a metabolic pathway that could itself be sensitive to oxidative damage.

Key words

Hydrogen peroxide • Breath • Hypoxia • Paraquat • Lung

Introduction

Hydrogen peroxide is produced as an intermediate in a whole range of metabolic pathways. It is potentially one of the most harmful species of oxygen metabolism due to its ability to cross the membranes. It is also a precursor of the highly reactive hydroxyl radical which can be formed either by the Fenton-type reaction in the presence of transition metals or *via* the Haber-Weiss reaction in the presence of superoxide and iron (Fong *et al.* 1976). Hydrogen peroxide, formed in mitochondria of different lung cells or by activated phagocytes (Sibille and Reynolds 1990) can mediate oxidative lung damage by initiating membrane lipid peroxidation (Block *et al.* 1989) and by modulating the activity of key enzymes (Meharg *et al.* 1993). It also increases pulmonary vasoconstriction (Wilhelm and Herget 1995) and participates in edema formation (Burghuber *et al.* 1984).

Increased levels of hydrogen peroxide have been found in the breath of smokers (Nowak *et al.* 1996) and in people exposed to ozone (Madden *et al.* 1997). The concentration of hydrogen peroxide also increased for a short time in the breath of dogs after lung transplantation, in patients with a cardiopulmonary bypass or in patients with the adult respiratory distress syndrome (Wilson *et al.* 1993).

We have found increased production of hydrogen peroxide by alveolar and peritoneal macrophages isolated from rats exposed to hypoxia

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(Wilhelm *et al.* 1996, 1997). For this reason, we designed the present study (1) to find out whether hypoxia-induced changes in hydrogen peroxide production could be detected as an increase of its concentration in expired air in *vivo* and (2) to compare the effect of hypoxia with the effect of paraquat, a widely studied initiator of oxidative damage (Frank 1981, Piotrowski *et al.* 1996). Our results have indicated that hypoxia substantially increases the concentration of hydrogen peroxide in expired air probably due to enzymatic production that is itself sensitive to oxidative damage.

Methods

Animals

Wistar male rats (weighing 300 ± 35 g) were divided into three equal groups of 15 animals each. The first group (controls) was kept in normoxia and their H_2O_2 production was measured concomitantly with the experimental groups. The second group was exposed to hypoxia in a normobaric hypoxic chamber (F_{iO2}=0.1) and H_2O_2 production was tested after 3 days of hypoxia and again after 7 days of recovery in air. The third group was given paraquat (Sigma, 45 mg/kg, i.p.) and tested 1, 3, 5, 7, 24, and 48 h later. The animals were killed after the last measurement by an overdose of an anesthetic and the extent of lung inflammation was examined.

H_2O_2 analysis

H_2O_2 production was measured as the concentration of H_2O_2 in the water vapor generated by the rat in a body box in one hour. In a preliminary experiment the hydrogen peroxide concentration was estimated in the expired air of intubated rats anesthetized with pentobarbital (40 mg/kg, i.p.). The tracheal cannula was then attached to a circuit with constant flow of air (0.5 l/min). The outflow of the circuit was connected to a specially constructed glass chamber submerged in acetone cooled to –65 °C with dry ice, where the vapor was frozen out.

Individual awake rats were placed for one hour into a body box (volume 4.5 l) flushed with a constant air flow of 3 l/min. The outlet of the box was connected to the glass chamber submerged in acetone and dry ice. The condensed, frozen water vapor was weighed and analyzed for H_2O_2. The amount of 2.5 ± 0.3 pmol of H_2O_2 per hour did not differ significantly from the amount of 2.4 ± 0.4 pmol of H_2O_2 per hour obtained in intubated animals. Hence, in the following experiments non-intubated animals were used.

The amount of hydrogen peroxide in the condensate was measured on the basis of chemiluminescence originating from the reaction between hydrogen peroxide and luminol, catalyzed by horseradish peroxidase (Wilhelm *et al.* 1996). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Sigma (St. Louis, MO). A stock solution of 1 mM luminol was prepared by dissolution in 5 mM NaOH, the pH being adjusted to 8.0 by HCl. Horseradish peroxidase from Boehringer (Mannheim, Germany) was diluted to a final concentration of 3 U/ml. The chemiluminescence measurements were carried out on a Luminometer 1250 (LKB-Wallac Oy, Finland). The chemiluminescence counts were calibrated with standard hydrogen peroxide (Sigma) the concentration of which was assayed by spectrophotometry (Aebi 1984).

The results (means ± SD) were evaluated by ANOVA with Fisher's PLSD post-hoc test.

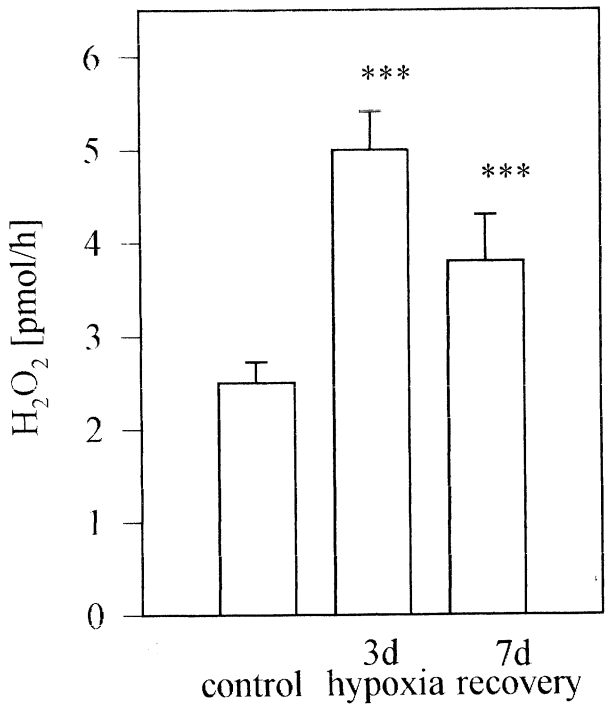

Fig. 1. *The concentration of H_2O_2 in the breath of control animals, after 3 days of hypoxia or after 7 days of recovery in air. The three asterisks indicate the statistical significance ($P<0.001$).*

Results

The amounts of hydrogen peroxide produced after three days of exposure to hypoxia, and after 7 days of subsequent recovery in air were compared to controls in Figure 1. The control value of 2.5 pmol of H_2O_2 produced per hour in the controls increased to 200 % after 3 days of hypoxia, this difference being significant ($P<0.001$). After 7 days of recovery in air the amount of produced H_2O_2 was still increased (152 %, $P<0.001$).

As it is known that paraquat induces lung inflammation by the generation of free radicals, the animals were treated with a toxic dose of paraquat and the time-course of hydrogen peroxide production was followed. The results are summarized in Figure 2. The peak value of H_2O_2 production was observed 5 h after paraquat injection. This was also the only significant increase (156 %, $P<0.02$). The animals started to die after 48 h.

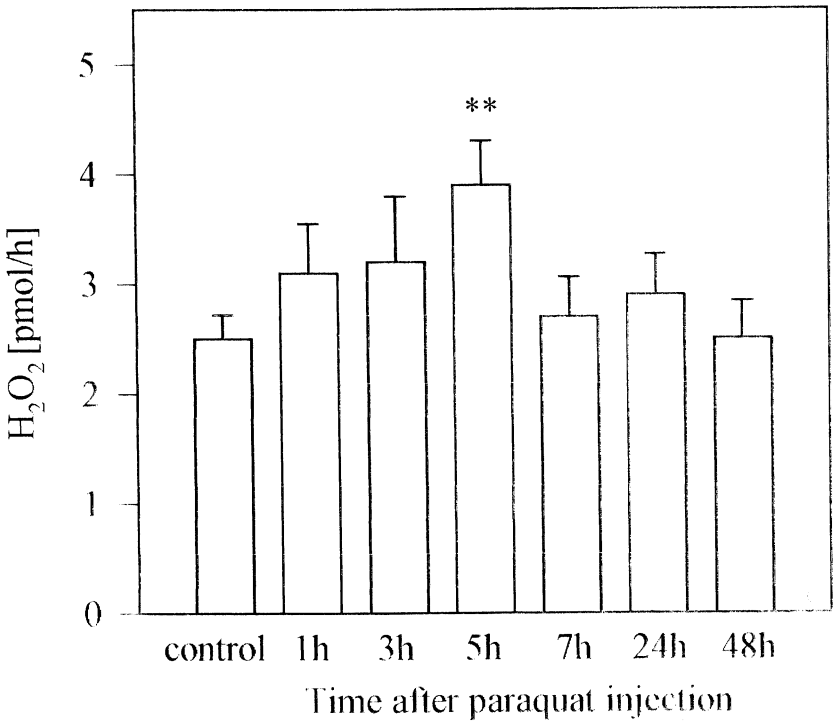

Fig. 2. *The time course of H_2O_2 concentration in the breath of animals injected with paraquat. The two asterisks indicate the statistical significance ($P<0.02$).*

Discussion

The first attempts to measure hydrogen peroxide in breath samples began in the eighties (Williams and Chance 1983). More recently, several reports dealing with this subject have been published. This assay is relatively simple and, being noninvasive, it may be of considerable diagnostic value. However, the interpretation of the measured data is not simple because of unknown factors influencing the levels of hydrogen peroxide in the breath samples.

Although, theoretically, the expired air is not the only possible source of H_2O_2 in our rats, the fact that the H_2O_2 concentrations in awake and intubated control rats were similar, suggests that it is the most important one.

Significantly increased levels of hydrogen peroxide were observed in patients undergoing pulmonary thromboendarterectomy but not earlier than on the postoperative day 2. Another significant increase was observed in hydrochloric acid-induced canine lung injury after 0.5 h, however, the expired H_2O_2 returned to control values after 2 h (Wilson *et al.* 1993).

Other studies documenting a significant increase of hydrogen peroxide in the breath samples involved relatively mild treatments such as exposure of humans to low levels of ozone (Madden *et al.* 1997) or smoking (Nowak *et al.* 1996). The present results are in agreement with the above mentioned examples.

Hypoxia used in our experiments corresponds to a 3-day-stay at 5000 m altitude, which is not a lethal treatment, so that the life-span of treated animals is not shortened. Nevertheless, the level of H_2O_2 in the breath after this treatment is elevated for at least 7 days of recovery in air and might represent the oxidative stress induced by the hypoxic treatment. Though not directly lethal, it can induce metabolic changes associated with the pathophysiology of hypoxia. Persisting elevated levels of H_2O_2 might be a manifestation of these metabolic

changes. Thus, our experiments may serve as confirmation for the seemingly paradoxical hypothesis of oxidative damage produced by hypoxia. This question has been widely studied. Hypoxic conditions accelerate the production of superoxide (and consequently also of H_2O_2) in the electron transport chain of mitochondria (Cadenas *et al.* 1977) and by some oxygen-utilizing enzymes that use reduced flavins or semiquinones as cofactors (Misra and Fridovich 1972). Xanthine oxidase is another possible source of increased superoxide production in hypoxia, because both the enzyme and its substrate, hypoxanthine, may be increased under hypoxic conditions (Fried *et al.* 1973, Granger *et al.* 1981). However, the increased production of reactive oxygen species does not necessarily lead to oxidative damage if the protective mechanisms are adequate.

On the other hand, treatment of animals with paraquat induces very harsh oxidative damage. The lungs of animals treated with paraquat showed signs of severe damage 48 h later and, the mortality after such treatment is high (Calderbank 1968). It is not known if there is a direct relationship between the paraquat dose and H_2O_2 concentration in breath. Nevertheless, the increased production of hydrogen peroxide, which was observed only 5 h after paraquat injection, then sharply decreased. This is a similar situation as in the above mentioned canine model, where hydrochloric acid increased the production of H_2O_2 only temporarily (Wilson *et al.* 1993). It could thus be hypothesized that H_2O_2 is produced by a mechanism which is itself sensitive to oxidative damage.

The likely enzymatic source of H_2O_2 in the lung is represented by NADPH oxidase of alveolar macrophages. This suggestion is supported by our previous observation of increased production of hydrogen peroxide by alveolar macrophages isolated from animals exposed to hypoxia for 3 days (Wilhelm *et al.* 1996).

Acknowledgements

This study was supported by a grant GAČR 305/97/S070.

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Reprint requests

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