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Production of Hydrogen Peroxide by Alveolar Macrophages From Rats Exposed to Subacute and Chronic Hypoxia

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Summary

We have studied *in vitro* alveolar macrophages (AMs) obtained by tracheobronchial lavage from rats exposed to subacute (3 hours and 3 days) and chronic (3 weeks) hypoxia (F_{iO2} = 0.1) and from rats recovering from chronic hypoxia. Hydrogen peroxide production by AMs was measured by luminol-dependent chemiluminescence after AMs adhered to the walls of the measuring cuvette, after stimulation with phorbol-myristate-acetate (PMA), and when N-formyl-methionyl-leucyl-phenylanine (FMLP) was added subsequently to the cells which had been previously stimulated by adherence or PMA. H_2O_2 production after cell adherence and adherence combined with FMLP stimulation did not differ between the groups. The increase of H_2O_2 production after adding PMA, and FMLP in addition to PMA was significantly higher in AMs from rats exposed to hypoxia for 3 days than in the controls. Other experimental groups did not differ from their controls. It is concluded that 3 days' hypoxia primes AMs for enhanced production of H_2O_2 upon stimulation. The mechanism is probably at the level of synthesis of proteins involved in H_2O_2 production, or the shift to a more reactive phenotype of alveolar macrophages subpopulations.

Key words
Hypoxia – Alveolar macrophages – Hydrogen peroxide – Lung tissue injury – Rat

Introduction

The exposure of animals to chronic hypoxia results in pulmonary hypertension which is mainly due to the morphologic reconstruction of the pulmonary vascular bed (Reid 1986). The typical findings concern stimulation of fibroproduction and growth of smooth muscles in the wall of peripheral lung blood vessels (Herget and Ježek 1989). The structural changes in the wall of pulmonary blood vessels of rats were already observed after 3 days of exposure to hypoxia (Rabinowitch *et al.* 1981), and the lung tissue injury during the first few days of hypoxia may be an initiating signal for the reconstruction of pulmonary blood vessels and development of pulmonary hypertension. Recruitment of macrophages in the lung tissue is an integral part of lung injury (Richards *et al.* 1980). Activated alveolar macrophages (AMs) produce reactive oxygen species and growth stimulating cytokines (Stein-Streiespirlein and Toeews 1989).

AMs similarly as other phagocytic and non-phagocytic cells contain NADPH-oxidase which, upon activation, produces superoxide as the primary product (Maly and Schurer-Maly 1995). Superoxide is probably the precursor of other reactive oxygen species, such as hydrogen peroxide, which can be formed during superoxide dismutation, both spontaneous and enzymatic.

Hydrogen peroxide possesses many biochemical and physiological activities. It has been shown to stimulate membrane associated protein kinase C, phospholipase A_2 and release of arachidonic acid (Chakraborti and Chakraborti 1995). It also increases the tonus of pulmonary vascular smooth muscles and the membrane ion transport (Wilhelm and Herget 1995). In some studies, hydrogen peroxide produces contractions of isolated pulmonary arteries

(Sheehan *et al.* 1993), while in others a vasodilator effect was found on pulmonary arterial rings (Perry and Taylor 1988). It is thus apparent that the effects of hydrogen peroxide are divergent, and its role has not been definitely settled. From this point of view, the changes in hydrogen peroxide production by AMs during hypoxia are of interest.

Therefore, in the present study, we have investigated the ability of AMs obtained from rats exposed to various periods of hypoxia to produce hydrogen peroxide. Four different ways of stimulation of AMs *in vitro* were used with the aim to discern the possible pathways for activation of hydrogen peroxide production.

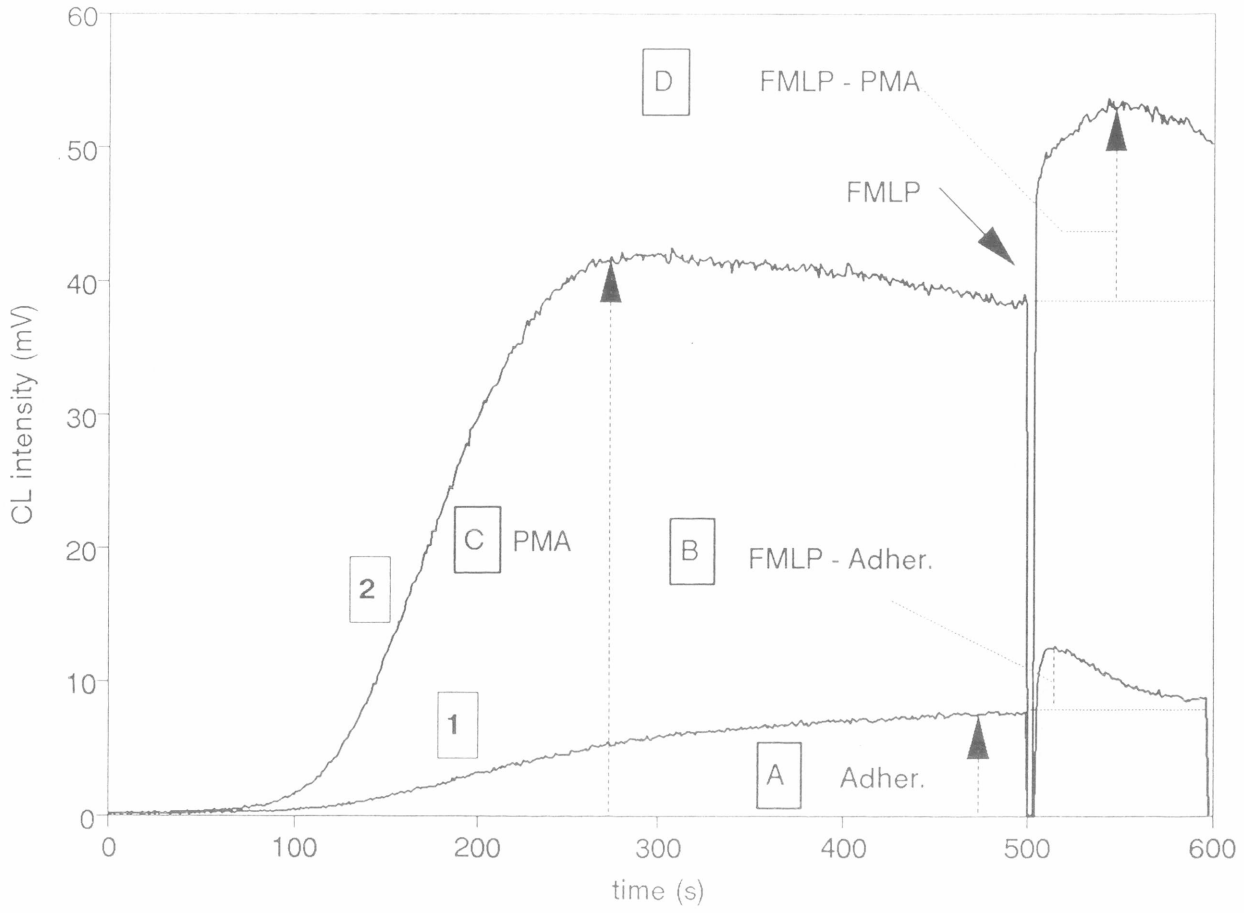

Fig. 1
Schematic presentation of the measured parameters. Tracing 1 represents the situation when AMs produce chemiluminescence during adherence. The maximum of this response is shown as parameter A. Exactly 500 s after the beginning of the reaction, FMLP was added and the net increase of chemiluminescence (i.e. measured value minus chemiluminescence due to adherence) was taken as parameter B. In the case of tracing 2, PMA was present in the reaction mixture from the beginning of the experiment. The maximum response was measured as parameter C. Then at 500 s, FMLP was added and the net increase (i.e. measured value minus effect of PMA and adherence) was taken as parameter D.

Materials and Methods

Effect of chronic hypoxia

From a group of 19 male Wistar rats (average body weight 257±12 g) six rats were kept in air (control group), thirteen were exposed to chronic hypoxia for 3 weeks in an isobaric hypoxic chamber (F_{iO2}=0.1) (Hunter *et al.* 1974). Five of these rats exposed to chronic hypoxia were studied immediately after they had been taken out of the hypoxic chamber. The remaining 8 rats were studied 3 days after recovery in air. During exposure to hypoxia, the experimental rats had free access to food and water.

Effect of subacute hypoxia

Seventeen Wistar male rats of average body weight 293±14 g were used. Six were kept in air (control group), five were exposed to hypoxia

(F_{iO2}=0.1) in an isobaric hypoxic chamber for 3 hours and remaining 6 were studied after 3 days' exposure to a similar level of hypoxia.

Isolation of alveolar macrophages

Experimental rats were anaesthetized by halothane. We have recently found that halothane has the smallest effect on hydrogen peroxide production by AMs compared with other routinely used anaesthetics (Wilhelm and Herget 1995b). We then adapted the isolation procedure of Lavnikova *et al.* (1993). After thoracotomy, the lungs were ventilated *via* the trachea with a normoxic gas mixture (21 % O_2 + 5 % CO_2 + 74 % N_2) at a positive pressure of 12 cm H_2O and end expiratory pressure 2.5 cm H_2O at a rate of 40 ml/min. After cannulation of the pulmonary artery an incision was made in the apex of the left ventricle. Then the lungs were perfused with 50 ml at a rate 0.06 ml/min/g body weight of Hank's buffered salt solution without Ca^{2+} and Mg^{2+} (HBSS) containing 0.6 mM EDTA. The lungs and trachea were then excised and lavaged 8 times by slowly instilling and withdrawing 8 ml of warm HBSS. Lungs were gently massaged during the procedure. Thereafter the cells were collected by centrifugation (400 x *g*, 10 min, 20 °C), washed in HBSS and sedimented under the same conditions. The number of cells was counted under a light microscope and cells were kept at room temperature and in atmospheric air.

Measurements of the production of hydrogen peroxide

Hydrogen peroxide production was measured by luminol-dependent chemiluminescence (LDCL) originating in the reaction between hydrogen peroxide and luminol catalyzed by horseradish peroxidase. HRP is essential in this system, as luminol does not produce light with hydrogen peroxide in the absence of HRP (Cormier and Prichard 1968, Sejm 1983). Hydrogen peroxide was assayed after AM adherence to plastic measuring cuvettes, after stimulation with N-formyl-methionyl-leucyl-phenylalanine (FMLP) or with phorbol-myristate-acetate (PMA). 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 dissolving it in 5 mM NaOH, the pH was adjusted to 7.4 with HCl. Horseradish peroxidase (HRP) from Boehringer (Mannheim, Germany) was diluted to 250 U/ml with Ca^{2+}/Mg^{2+}-free HBSS (pH=7.4). FMLP (Sigma) was made into a 0.1 mM stock solution by dissolving it in 0.5 mM NaOH. A 160 μM PMA stock solution was prepared by dissolution in dimethylsulfoxide (Sigma). LDCL was measured on Luminometer 1250 (LKB-Wallac Oy, Finland), equipped with a thermostated cell holder. The instrument was coupled through a custom-made analogical-digital converter to a computer, where the data were collected at 1 s periods. The intensity of LDCL was expressed in mV of the photomultiplier response. The measurements were carried out at 37 °C in a plastic measuring cuvette. The reaction mixture of total volume of 1 ml contained 100 μl of luminol (final concentration 0.1 mM), 10 μl of HRP (2.5 U/ml), 50 μl FMLP (5 μM), 10 μl PMA (1.6 μM). A cell suspension was added as the last to start the reaction. The final cell concentration was $1x10^6$ per ml.

Statistics

Differences between the groups were compared using one-way ANOVA and the Scheffé test (Steel and Torrie 1960). The values of $P<0.05$ were considered significant. The results are presented as means ± S.E.M.

Results

Macrophages ($10\pm2x10^6$ per ml) were obtained by lavage from individual rats of control and experimental groups. There were no significant differences in the number of alveolar macrophages between the two groups of rats.

The production of H_2O_2 was measured on the basis of HRP-catalyzed LDCL. No luminol chemiluminescence was observed in the absence of HR. However, when HRP was added to the incubated cells a burst of light production ensued after 500 s due to H_2O_2 released extracellularly, as was shown in our previous report (Wilhelm *et al.* 1995).

Figure 1 schematically illustrates the four parameters we have measured. Tracing 1 shows LDCL produced during cell adherence. The maximum chemiluminescence intensity induced by cell adherence was measured as parameter A. Exactly 500 s after the beginning of the measurement, FMLP was added and the increase in chemiluminescence intensity was taken as parameter B. Tracing 2 represents the situation when PMA was added to the reaction mixture at the beginning of the reaction. The maximum LDCL elicited by the combined effects of PMA and adherence was measured as parameter C. At 500 seconds from the beginning of reaction FMLP was added again and its net stimulation denoted as parameter D.

When isolated AMs adhered to the measuring plastic cuvette, LDCL in the presence of HRP ensued. This effect was prevented by repeated agitation of the cell suspension. The reaction was not affected either by the duration of hypoxia or by recovery from chronic hypoxia (Parameter A, Figs 2 and 3). Subsequent injection of FMLP to the reaction mixture resulted in an additional rise of LDCL, which was again not significantly different in all groups of the rats studied (Parameter B).

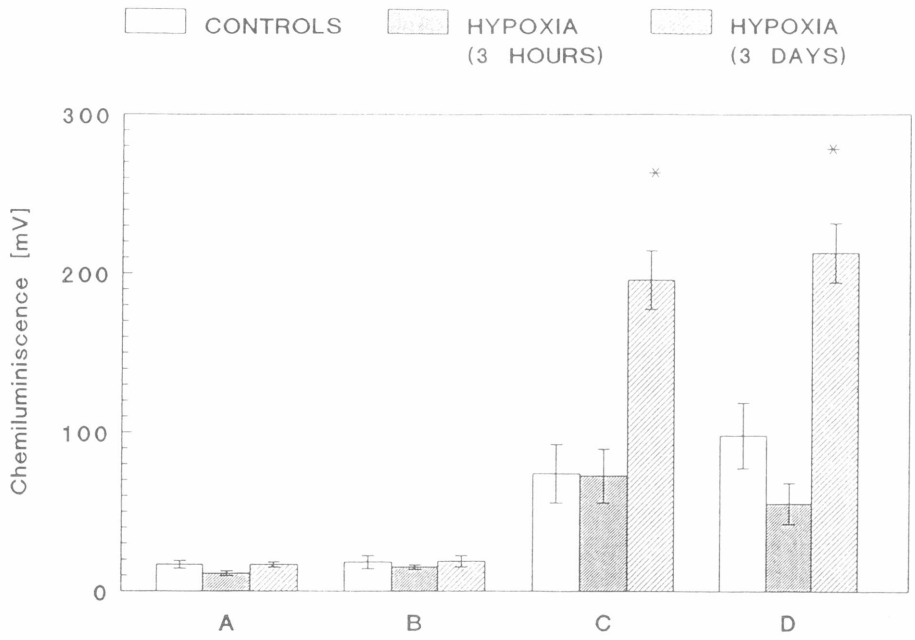

Fig. 2

*The increase of chemiluminescence of alveolar macrophages obtained from control rats and from rats exposed to subacute hypoxia after different stimuli. For explanation of parameters A, B, C and D see Fig. 1. * = $P<0.05$, vertical bars represent S.E.M.*

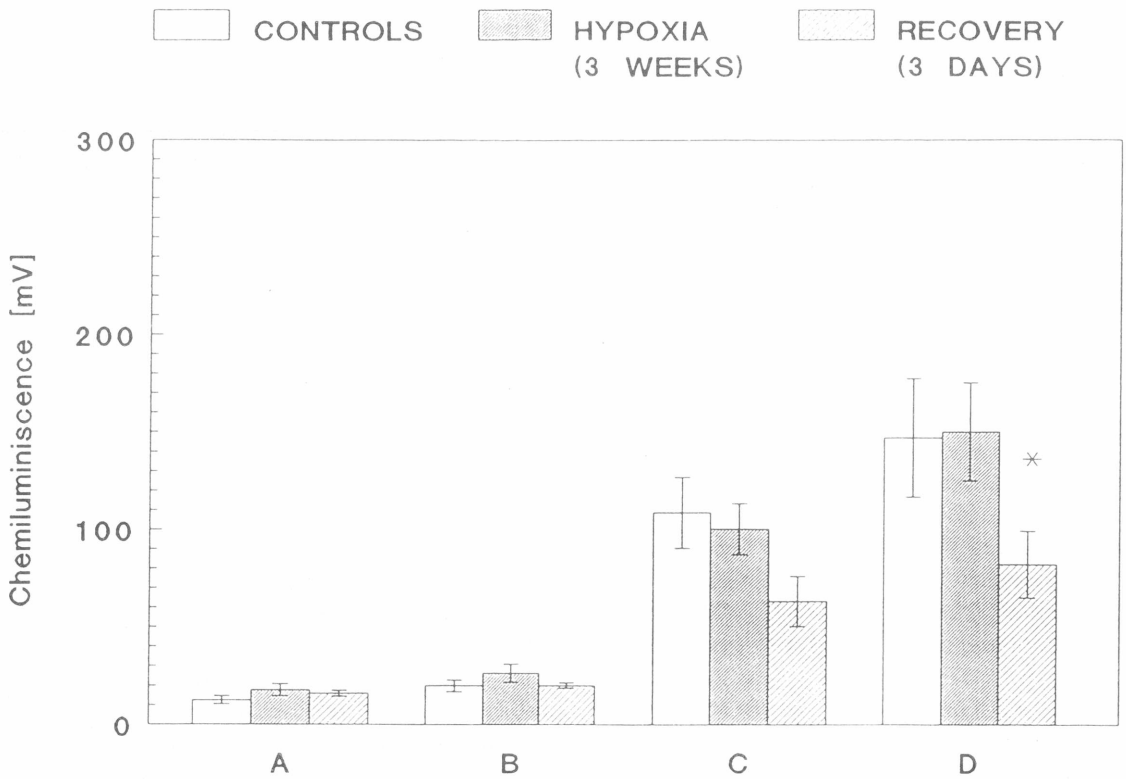

Fig 3

*The increase of chemiluminescence of alveolar macrophages obtained from control rats, rats exposed to chronic hypoxia and from rats recovering from this exposure. For explanation of parameters A, B, C and D see Fig. 1. ** = $P<0.05$, vertical bars represent S.E.M.*

When PMA was added in the presence of HRP, the rise of LDCL was not affected by exposure to chronic hypoxia or by the recovery from chronic hypoxia (Parameter C, Fig. 3). In subacute hypoxia, however, the reaction was significantly greater in rats exposed to hypoxia for 3 days. Similar potentiation was not found in rats exposed for 3 hours (Parameter C, Fig. 2). The additional rise in LDCL was seen after the subsequent addition of FMLP (Parameter D). This rise was again significantly greater in rats exposed for 3 days to hypoxia than in all the other groups in which the increase was not significant. A significant decrease of parameter D was observed in the recovery group compared with rats living in hypoxia for 3 weeks (Fig. 3).

Discussion

There is evidence that lung tissue injury occurs at the beginning of a sojourn in a hypoxic environment. The lung lymph drainage is increased (Erdman *et al.* 1975) and fluid accumulates in the perivascular tissue (Martin *et al.* 1986, Sugita *et al.* 1989). Sobin *et al.* (1983) found perivascular lung oedema even after less than one hour of hypoxic exposure. Scott *et al.* (1978), however, did not observe lung interstitial oedema in rats exposed for 8 hours to severe hypoxia but they showed signs of capillary endothelial damage with the formation of granular blebs in the capillary lumen. Also Heath *et al.* (1973) reported ultrastructural changes in the lungs of rats exposed for several hours to severe hypoxia.

Oxygen radicals produced by activated AMs and probably also by other activated leukocytes may contribute to the development of hypoxic lung injury and transvascular leakage. Hypoxia may enhance their production. Tuohy *et al.* (1993) showed that hypoxia primes the AMs for a large release of hydrogen peroxide under the *in vitro* conditions. Rat AMs cultured for several hours in a hypoxic environment produced more hydrogen peroxide when stimulated by PMA in the presence of endotoxin.

After one-electron oxidation, luminol can produce light by direct reaction with superoxide (Lind *et al.* 1983). Due to diffusion of luminol into the cytoplasm both intracellular and extracellular superoxide can be assayed. In our present measurements, we did not find any chemiluminescence in the absence of HRP which indicates that the superoxide was not produced. It is probably the consequence of the specific way of activation, as in our previous report we found that superoxide production by alveolar macrophages was stimulated by mineral dust (Wilhelm *et al.* 1987). In the present study, we have studied hydrogen peroxide production after stimulation of three independent pathways of extracellular and/or intracellular signalling. The H_2O_2 production after cell adherence to plastic measuring cuvettes might represent an entirely artificial condition. It is mediated by receptors for foreign surfaces which are probably not encountered during hypoxia *in vivo*. Nevertheless, it is an expression of the changes in cell reactivity produced by hypoxia.

FMLP-triggered hydrogen peroxide production is another receptor-mediated process. It was dependent on the preceding state of cell activation. The same dose of FMLP induced a much higher net chemiluminescence increase when it had been applied to cells previously stimulated by PMA than in cells activated by adherence only.

PMA affects H_2O_2 production by the direct stimulation of intracellular protein kinase C. Since exposure to hypoxia produced the greatest effects on H_2O_2 production stimulated by PMA and by FMLP following PMA, while H_2O_2 production induced by cell adherence and by FMLP following cell adherence was not changed, we can hypothesize that the signalling pathway in the region of protein kinase C is the key element sensitive to hypoxic exposure.

The production of H_2O_2 by AMs obtained from rats exposed for 3 hours and 3 weeks to hypoxia did not differ in AMs from lungs of the controls. The first period is probably too short. The process of vascular injury and reconstruction is probably terminated after 3 weeks of hypoxia and the reconstructed vessels are in the steady state. Because of the above discussed morphological evidence concerning the reconstruction of the vascular wall during the second and third day of hypoxia, the increased ability of AMs to produce hydrogen peroxide found in this period may be an important factor. The hydrogen peroxide released in excess from AMs primed by hypoxia may participate in the mechanisms of lung injury and/or vascular proliferation of vascular wall mesenchymal cells.

It is important to note that we did not observe increased hydrogen peroxide production in unstimulated AMs – the basal levels of H_2O_2 production were similar in the control and all hypoxic groups. What we have observed is the buildup of the machinery producing hydrogen peroxide upon specific signals. We can only speculate whether these signals are present during hypoxia and whether a potential of higher production of hydrogen peroxide is involved. In addition, the possible enhanced hydrogen peroxide release may be balanced with a change of antioxidant enzymic activity in the lung tissue. Actually, this problem may be quite complicated as the increase in superoxide dismutase activity would enhance the amount of hydrogen peroxide produced in the case when catalases and peroxidases were damped by hypoxia. Therefore, future attempts to elucidate the effects of hypoxia on hydrogen peroxide production by

AMs should include the assay of individual antioxidant enzymes.

The mechanism of the described changes in AMs induced by hypoxia possibly acts at the level of protein synthesis because of the observed time factor. Three hours of exposure is probably a time too short for the synthesis of new proteins, and 21 days is long enough for adaptive mechanisms to occur. This view is also supported by the probable decrease in H_2O_2 production in the groups activated by PMA and FMLP after 3 days of recovery in atmospheric air that followed 3 weeks' exposure to hypoxia. Alternatively, it is possible that subacute hypoxia induces a shift in the different subpopulations of AMs to a more reactive phenotype.

The fact that different pathways of induction of H_2O_2 production are influenced by hypoxia to various levels might indicate that the mechanism by which hypoxia acts is not of a general nature, but that it rather triggers the formation of certain proteins specifically.

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Reprint Requests

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