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MINIREVIEW

A Possible Role of the Oxidant Tissue Injury in the Development of Hypoxic Pulmonary Hypertension

J. HERGET, J. WILHELM [1], J. NOVOTNÁ[1], A. ECKHARDT, R. VYTÁŠEK, L. MRÁZKOVÁ, M. OŠŤÁDAL

Department of Physiology and [1]Department of Medical Chemistry and Biochemistry, Second Faculty of Medicine, Charles University, Prague, Czech Republic

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Summary

Chronic sojourn in hypoxic environment results in the structural remodeling of peripheral pulmonary arteries and pulmonary hypertension. We hypothesize that the pathogenesis of changes in pulmonary vascular structure is related to the increase of radical production induced by lung tissue hypoxia. Hypoxia primes alveolar macrophages to produce more hydrogen peroxide. Furthermore, the increased release of oxygen radicals by other hypoxic lung cells cannot be excluded. Several recent reports demonstrate the oxidant damage of lungs exposed to chronic hypoxia. The production of nitric oxide is high in animals with hypoxic pulmonary hypertension and the serum concentration of nitrotyrosine (radical product of nitric oxide and superoxide interaction) is also increased in chronically hypoxic rats. Antioxidants were shown to be effective in the prevention of hypoxia induced pulmonary hypertension. We suppose that the mechanism by which the radicals stimulate of the vascular remodeling is due to their effect on the metabolism of vascular wall matrix proteins. Non-enzymatic protein alterations and/or activation of collagenolytic matrix metalloproteinases may also participate. The presence of low-molecular weight cleavage products of matrix proteins stimulates the mesenchymal proliferation in the wall of distal pulmonary arteries. Thickened and less compliant peripheral pulmonary vasculature is then more resistant to the blood flow and the hypoxic pulmonary hypertension is developed.

Key words

Pulmonary hypertension • Chronic hypoxia • Pulmonary vasculature • Structural remodeling • Oxygen free radicals • Nitrotyrosine

Introduction

Two basic mechanisms increase the pulmonary vascular resistance in hypoxic pulmonary hypertension: structural remodeling of peripheral pulmonary vasculature and vasoconstriction. In the developed hypoxic pulmonary hypertension the structural remodeling prevails. It consists of proliferation and hypertrophy of vascular smooth muscle cells in peripheral pulmonary arteries (muscularization) and excessive production of matrix

Fax+4202 24920590
E-mail: physres@biomed.cas.cz http://www.lf2.cuni.cz/physiolres/physiolres.htm

proteins in the vascular wall. Vasoconstricticton may play a role in the early phases of exposure to hypoxia, although pharmacological vasodilatation was documented even in the "steady-state" of the disease (McMurtry *et al.* 1977, Dingemans and Wagenvoort 1978, Emery *et al.* 1981). We hypothesize that hypoxic injury to the pulmonary vascular wall is a mechanism, which triggers vascular remodeling (Herget and Ježek 1989, Hampl and Herget 2000). The production of reactive oxygen metabolites plays a crucial role in lung tissue injury (Kinnula *et al.* 1995). In the present paper, we discuss the evidence that the production of free radicals is increased in chronic hypoxia and that increased radical production may be pathogenetically involved in remodeling of the wall of peripheral pulmonary arteries.

Production of reactive oxygen species (ROS) in lung tissue hypoxia

Several studies have documented that exposure to low partial pressures of oxygen induces rapid changes in membrane lipid composition of a variety of mammalian cell lines (Coleman *et al.* 1976, Johnstone *et al.* 1985). These changes may be caused by a free radical-initiated lipid peroxidation, a view supported by an increase of malonaldehyde, one of the aldehydic products of lipid peroxidation, in the serum, heart, lung, liver and kidney of hypoxic rats (Nakanishi *et al.* 1995). Accordingly, indicators of lipid peroxidation have been observed in cultures of pulmonary artery endothelial cells exposed to hypoxia (Block *et al.* 1989).

The underlying mechanism of hypoxic oxidative damage may be multifactorial. The prerequisite of oxidative damage is the formation of oxidants and these may originate from several processes:

1. Superoxide leakage from mitochondrial respiration may play an important role. Under hypoxic conditions the electron transport chain may become more reduced, resulting in an increased rate of non-enzymatic autoxidation of electron transport components, producing superoxide (Cadenas *et al.* 1977).
2. Certain oxygen utilizing enzymes which use reduced flavins or semiquinones produce more superoxide at low oxygen tensions than at normal oxygen tension (Misra and Fridovich 1972).
3. Another possible source of increased radical production with hypoxia is xanthine oxidase, as both this enzyme and its substrate, hypoxanthine, are increased under hypoxic conditions (Fried *et al.* 1973).
4. The important source of reactive oxygen species is NADPH oxidase of phagocytes, which produces superoxide and hydrogen peroxide. White cells may be attracted to hypoxic tissue and activated by products of hypoxic tissue injury. NADPH oxidase was found in smooth muscle cells of pulmonary arteries and was activated by hypoxia to produce superoxide (Marshall *et al.* 1996).

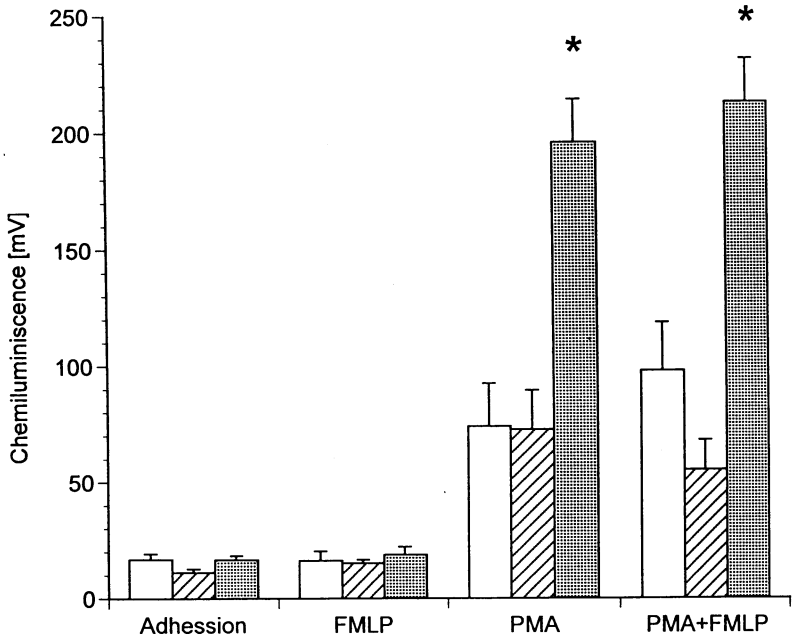

Fig. 1. *The increase of H_2O_2 production (chemiluminiscence) by alveolar macrophages isolated from control rats and from rats exposed to hypoxia. Adhesion – production of H_2O_2 after the cells adhered to measuring cuvette surface. FMLP – surface receptor mediated stimulation by chemotactic peptide N-formyl-methionyl-leucyl-phenylalanin. PMA – direct stimulation of protein kinase C by phorbol myristate acetate. *$P<0.05$. (Reproduced with permission from Wilhelm et al. 1996.)*

We have found an increased production of hydrogen peroxide by alveolar macrophages isolated from rats exposed to hypoxia (Wilhelm *et al.* 1996). Hydrogen peroxide production was measured by luminol-dependent chemiluminescence after stimulation of the macrophages by adherence, phorbol myristate acetate, or by chemotactic peptide. It was observed that a 3-day hypoxia primes alveolar macrophages for an enhanced production of H_2O_2 upon stimulation (Fig. 1). It is interesting that such an effect of hypoxia was not found in peritoneal macrophages (Wilhelm *et al.* 1997).

The production of reactive oxygen species by animals exposed to hypoxia was directly detected by measuring H_2O_2 in the expired breath (Wilhelm *et al.* 1999). Animals exposed to hypoxia for 3 days had an increased amount of H_2O_2 in their breath by 100 % in comparison to control animals. After 7 days of recovery in air, the exposed animals still produced significantly increased levels of H_2O_2 (Fig. 2). It was hypothesized that H_2O_2 is a product of enzymic reactions that are themselves sensitive to oxidative damage, because the production of H_2O_2 was inhibited under the conditions of severe oxidative stress induced by paraquat administration.

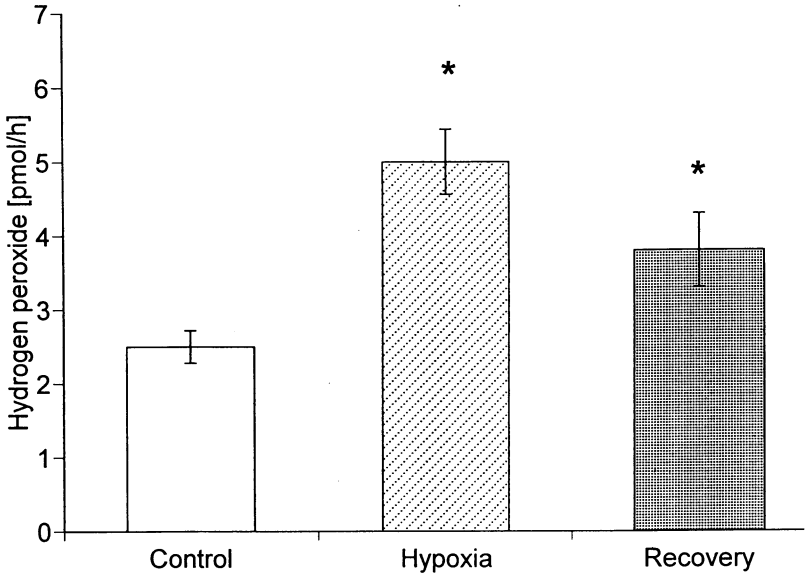

Fig. 2. *Production of hydrogen peroxide in the breath of rats exposed to and recovering from hypoxia. Ordinate shows the amount of hydrogen peroxide collected from the expired breath during 1 hour. Hypoxia indicates the group exposed to 10% oxygen for 3 days, recovery represents the measurement of the hypoxic group after 7 days of air breathing. * $P<0.001$. (Reproduced with permission from Wilhelm et al. 1999.)*

Oxidative damage to various tissues can be detected by measuring the concentration of fluorescent end products of lipid peroxidation. These are usually termed lipofuscin-like pigments (LFP). The exposure of rats to hypoxia for various time periods induced formation of LFP in both erythrocytes and the spleen (Wilhelm and Herget 1999a). The concentration of LFP depended on the duration of hypoxic exposure both in erythrocytes and the spleen. The higher concentration of LFP (compared with normoxia) was found in erythrocytes after 3 days of hypoxia. On the contrary, a decrease in LFP concentration was observed 3 h and 21 days after exposure. The decrease of LFP in erythrocytes was paralleled by an increase in the spleen.

In the next study, we focused on the effects of hypoxia on the lung tissue (Wilhelm and Herget 1999b). The LFP concentration was measured in relation to the activity of superoxide dismutase (SOD). This enzyme is known to be inducible by its substrate. This implies that the increase in its activity is to be expected under conditions of enhanced free radical production. There are principally two types of mammalian SOD that differ by the metal ion in their active center and by their subcellular localization. Cu,Zn-SOD is preferentially found in the cytoplasm, while Mn-SOD is an enzyme localized in the mitochondrial matrix. The experiment was organized in the following way. Adult male rats were exposed to normobaric hypoxia (10 % O_2) for 3 h, 1 day, 10 days and 21days. After 21 days of hypoxic treatment, samples were also taken after 3 and 14 days of recovery in the air. LFP were assayed in lipophilic extracts of the lungs. Four types of fluorophores were observed, labeled according to their excitation/emission maxima F263/376, F287/330, F330/370, and F345/458. These fluorophores had a different time-course of concentration during hypoxia and recovery in air. F287/330 increased after 3 h

of hypoxia ($P<0.0001$), then decreased deeply below control value after one day ($P<0.0001$), and with minor fluctuations remained at this level for up to 21 days of hypoxia. During recovery in air it returned to the control level. The other fluorophores were significantly reduced during hypoxia and they either reached the initial values during recovery (F345/458), or rose to significantly higher levels (F263/376, F330/370). The maximum increase was observed on the 14th day of recovery. The time-course of one of these fluorophores (F330/370) is illustrated in Figure 3 together with the changes of Cu,Zn-SOD and Mn-SOD activity. Its concentration slightly increased (to about 160 %) immediately after transition of the animals to hypoxia and then again after transition from hypoxia to normoxia. Its concentration was decreased throughout the 21 days of hypoxia, indicating attenuated production of free radicals during prolonged hypoxia. This view is supported by low levels of Cu,Zn-SOD. The activity of Mn-SOD started to rise immediately after transition to hypoxia and reached its maximum after one day of hypoxia. It then oscillated around the control value and started to rise significantly after transition of the animals to air, reaching the maximum activity after 14 days of recovery in air (Fig. 3). These data, taken together, indicate that free radicals are actively produced during the transition periods from air to hypoxia and *vice versa*. The site of their production appears to be localized in the mitochondria. We assume that F330/370 might represent a fluorophore produced during oxidative damage to lung mitochondria

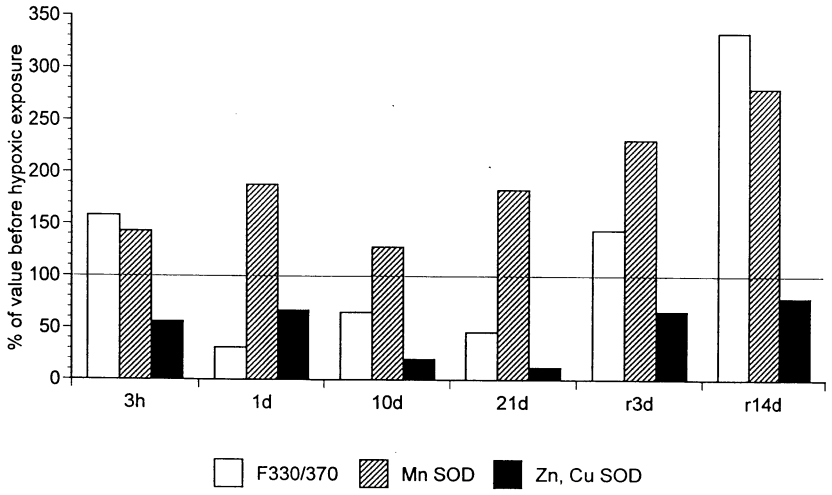

Fig. 3. *The time course of the fluorophore F330/370 in relation to the activity of Cu,Zn-SOD and Mn-SOD during hypoxia and return to air breathing. All the data are expressed in percentage of control values. The time of sampling is indicated below abscissa: 3h – 3 hours of hypoxia, 1d – 1 day of hypoxia, 10d – 10 days of hypoxia, 21d – 21 days of hypoxia, r3d – recovery in air for 3 days, r14d – recovery in air for 14 days.*

Collagen represents a possible target for free radicals in the extracellular matrix. It is difficult, however, to prove collagen oxidation *in vivo* by standard methods. We therefore initiated a study that aimed at the detection of oxidized collagen by means of its fluorescence. In a model oxidation of collagen initiated by UV irradiation *in vitro* we found new specific fluorophores which might be potentially used for the estimation of collagen damaged by free radical attack *in vivo* (Wilhelm *et al.* 1998).

The oxidative damage to the extracellular matrix might represent a factor responsible for the initiation of the remodeling of the vascular wall triggered by chronic hypoxia. We studied the effects of collagen type I oxidation on the proliferation of smooth muscle cells (VSMC) obtained from the rat aorta in culture (Bačáková *et al.* 1997). VSMC proliferated more rapidly on UV-irradiated collagen than on normal collagen type I. The oxidation of UV-irradiated collagen was detected by the assay of specific fluorophores described above. As we have previously observed lung macrophage activation by hypoxia (Wilhelm *et al.* 1996), we tested the effects of collagen type I exposed to activated macrophages *in vitro* on the proliferation of VSMC in culture (Bačáková *et al.* 1999). The responses were complex, as collagen exposed to activated macrophages was both slightly cytotoxic and also stimulated VSMC proliferation.

Effects of antioxidants on the development of hypoxic pulmonary hypertension

Several reports have documented inhibition of the development of experimental pulmonary hypertension by antioxidants in laboratory animals. Already 10 years

ago, the group of Dr. Reid reported that antioxidant dimethythiourea inhibits the structural reconstruction of peripheral blood vessels and right heart hypertrophy induced by 10-day exposure to 10% oxygen in rats (Langleben *et al.* 1989). Similar results have been reported recently (Lai *et al.* 1998). Furthermore, the latter authors measured the pulmonary arterial blood pressure and found that it was lower in hypoxic rats treated with dimethylthiourea than in not-treated hypoxic rats. Bottje *et al.* (1995) successfully treated the pulmonary hypertension syndrome in broilers by vitamin E implants applied after hatching. The incidence of the pulmonary hypertension syndrome in chickens was decreased and the authors reported a significant increase in the antioxidant capacity in the vitamin E-treated group.

Another antioxidant used for influencing experimental pulmonary hypertension was N-acetyl-L-cysteine (NAC), a precursor of reduced glutathione. Hoshikawa *et al.* (1995) reported attenuation of pulmonary hypertension in rats by NAC treatment during exposure to hypoxia. In addition, they estimated phosphatidylcholine hydroperoxide as a measure of the presence of lipid peroxidation. It was increased in rats exposed to chronic hypoxia and not treated with NAC. This increase was reduced by NAC treatment.

In our experiments, we exposed young male rats to chronic isobaric hypoxia and the animals were treated with 20 g NAC per 1 liter of drinking water. Compared with hypoxic rats offered tap water without NAC, their pulmonary arterial mean blood pressure and right ventricle heart weight related to the body weight were significantly reduced (Herget *et al.* 1999). However, they were still higher than in normoxic controls (both treated and not-treated with NAC) (Table 1). Hoshikawa *et al.* (1995) reported that NAC treatment in hypoxia inhibited even the muscularisation of peripheral pulmonary arteries. This was not the case in our study and the relative number of peripheral pulmonary arteries with smooth muscle cells in their media was not significantly different in NAC-treated hypoxic rats from that in non-treated hypoxic controls (Table 1).

Table 1. Effects of NAC treatment in rats with experimental hypoxic pulmonary hypertension

	Body weight (g)	P_{AP} (mm Hg)	P_S (mm Hg)	Cardiac output (ml)	RV/BW (mg/100 g)	(LV+S)/BW (mg/100 g)	DL vessels (%)
Hypoxic	$263 \pm 21^{**}$	$27 \pm 0.9^{1,**}$	111 ± 6	28 ± 5	$73 \pm 5^{*,**}$	208 ± 8	$34.5 \pm 3.4^{**}$
Hypoxic + NAC	$250 \pm 17^{**}$	$21 \pm 0.8^{1,**}$	91 ± 5	23 ± 3	$60 \pm 4^{*,**}$	196 ± 6	$27.8 \pm 1.1^{**}$
Normoxic	354 ± 20	15 ± 0.4	107 ± 4	29 ± 4	46 ± 6	187 ± 9	12 ± 1.6
Normoxic + NAC	305 ± 15	14 ± 0.4	103 ± 5	24 ± 2	55 ± 2	203 ± 5	13.4 ± 1.2

*P_{AP} – pulmonary arterial mean blood pressure, P_S – mean blood pressure in the aorta, RV/BW – relative weight of the right heart ventricle, LV+S/BW – relative weight of the left heart ventricle plus septum, DL – % of muscularized distal pulmonary arteries. * Hypoxic vs. Hypoxic + NAC, ** hypoxic group vs. relevant normoxic control group.*

Role of radical lung injury in the pathogenesis of hypoxic pulmonary hypertension

We hypothesized that the proliferative process in the walls of peripheral pulmonary arteries is triggered by an alteration of matrix proteins in their walls. Exposure to hypoxia increases collagenolytic activity in the peripheral pulmonary vasculature, which results in a presence of specific, low molecular weight cleavage products of collagen type I (Novotná and Herget 1998). It has been shown repeatedly that the presence of collagen cleavage turns on mesenchymal proliferation (Gardi *et al.* 1990, 1994, Bačáková *et al.* 1997). In our experiment with NAC treatment (Herget *et al.* 1999) collagenous proteins were extracted from isolated peripheral pulmonary arteries. The extracts were analyzed by SDS PAGE electrophoresis. A characteristic, ~76 kD cleavage product occurred in all extracts from non-treated rats exposed to hypoxia. However, this protein was not present in samples from hypoxic rats treated with NAC (Fig. 4).

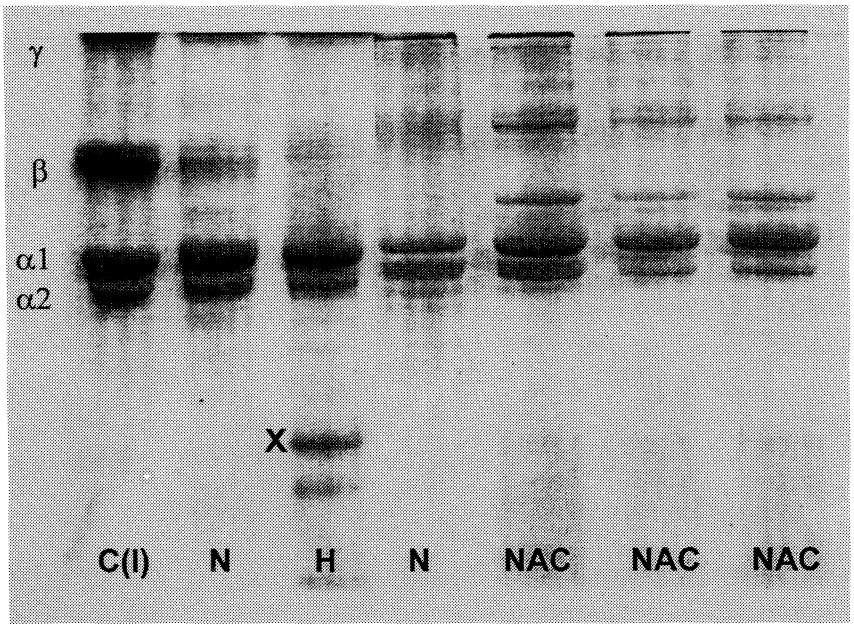

Fig. 4. *Gel electrophoresis profile of the collagenous fraction isolated from peripheral pulmonary arteries of rats exposed to hypoxia and of rats exposed to hypoxia and treated with N-acetyl-L-cysteine. C(I) – collagen type I standard from rat tail (Sigma), collagenous fraction extracted from peripheral pulmonary arteries of normal control rats (N), rats exposed for 3 weeks to hypoxia (H), rats exposed to chronic hypoxia and treated with N-acetyl-L-cysteine (NAC). γ – γ-fraction (chain polymers, collagens type I + III); β – β-fraction (chain dimers, collagens type I + III); α1 – mixture of individual α1 chains (collagens type I + III); α2 – α2 chains (collagen type I); X – small peptide present predominantly in the hypoxic peripheral pulmonary arteries (Novotná and Herget 1998).*

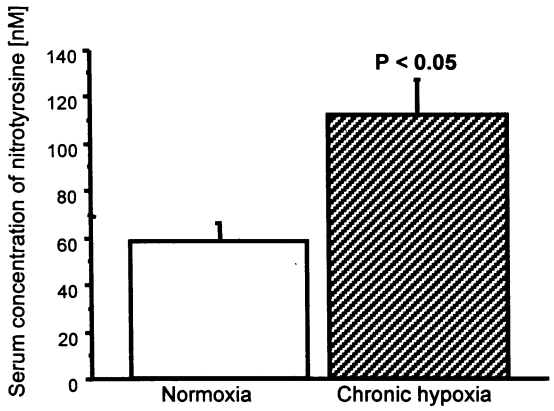

Fig. 5. *Concentration of nitrotyrosine in serum of normoxic and hypoxic rats. Nitrotyrosine was measured by inhibition ELISA. Polystyrene plates (Maxisorp, Nunc) were coated with nitrated serum albumin. Plates were incubated under gentle shaking for 90 min with monoclonal antibody (NO-60-E3, prepared in our laboratory) and diluted serum samples. After washing, the plates were then incubated with anti-mouse IgG conjugated with peroxidase (A-8924, Sigma) for another 90 min and developed with o-phenylenediamine.*

We hypothesize that the activation of collagenolysis in the peripheral pulmonary arteries is related to radical injury to the vascular wall (Hampl and Herget 2000). Increased NO production in the lungs after exposure to chronic hypoxia is well documented (Hampl and Herget 2000). Nitric oxide can react with superoxide yielding peroxynitrite and other radical products. Nitration of tyrosine in proteins to nitrotyrosine can be used as a marker of peroxynitrite production (Beckman 1996). We have recently found that the blood serum nitrotyrosine concentration is increased in rats exposed to chronic hypoxia (10 % O_2 for 4 days) (Fig. 5). This documents a production of peroxynitrite in the early phases of hypoxic exposure. Nitric oxide, superoxide and also peroxynitrite are potent activators of interstitial matrix metalloproteinase (Rajagopalan *et al.* 1996). The importance of increased collagenolytic activity for vasculature remodeling was further supported by our recent findings that pharmacological inhibition of collagenolysis by Batimastat suppressed the pulmonary vascular changes induced by chronic hypoxia (Novotná *et al.* 1999).

Hydrogen peroxide probably also plays a physiological role in the regulation of vascular tone. Vascular endothelial cells in culture release H_2O_2 into the extracellular space. This production has a constant rate under oxygen concentrations between 100 and 10 % and decreases when oxygen concentration is lowered below 10 %, reaching one third of the original activity at 0 % oxygen (Kinnula *et al.* 1993). H_2O_2 induces

vasoconstriction in preparations of isolated pulmonary arteries (Sheenan *et al.* 1993) as well as in isolated lungs (Burghuber *et al.* 1984, 1985, Tate *et al.* 1984). However, a vasodilator action of H_2O_2 was reported in other studies (Burke and Wolin 1987, Burke-Wolin and Wolin 1990, Monaco and Burke-Wolin 1995, Burke-Wolin *et al.* 1997)

We have found that high doses of hydrogen peroxide injected into the inflow cannula of isolated ventilated lungs produced a dose-dependent vasoconstriction in the range of 0.25-10 mM, with a maximum response between 2-5 mM. The effects of H_2O_2 were modulated by ionophores or specific inhibitors of ionic channels or pumps. A key role was deduced for sodium ions that regulate the subsequent inflow or outflow of calcium, i. e. an ion-mediated vasoconstriction (Wilhelm and Herget 1995). Therefore the participation of released ROS on changes of pulmonary vascular tone in hypoxic pulmonary hypertension cannot be excluded, although a direct experimental evidence of this action has not been well established yet.

In conclusion, there is growing evidence that the release of free radicals due to hypoxic lung injury participates in the onset of hypoxic pulmonary hypertension. The probable source includes NADPH oxidase of activated macrophages. The possibility that oxygen radicals are released from other cells due to the effects of hypoxia on their energy metabolism pathways has still not gained sufficient experimental support. The important pathogenetic mechanism probably concerns the role of radical substances, oxygen radicals and peroxynitrite, in the mesenchymal proliferation in peripheral pulmonary arteries.

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Reprint requests

Dr. Jan Herget, Department of Physiology, Second Faculty of Medicine, Charles University, Prague, Plzeňská 221, 150 06 Praha 5, Czech Republic, e-mail: Jan.Herget@lfmotol.cuni.cz