Physiol. Res. 50: 153-163, 2001

Hyperoxia and Recovery from Hypoxia Alter Collagen in Peripheral Pulmonary Arteries Similarly

J. NOVOTNÁ[1], J. BÍBOVÁ[2], V. HAMPL[2], Z. DEYL[3], J. HERGET[2]

[1]Department of Biochemistry, [2]Department of Physiology, Second Medical School, Charles University, [3]Institute of Physiology, Czech Academy of Sciences and [1,2]Centre for Experimental Cardiovascular Research, Prague, Czech Republic

Received March 20, 2000
Accepted August 4, 2000

Summary

Chronic hypoxia causes pulmonary hypertension, the mechanism of which includes altered collagen metabolism in the pulmonary vascular wall. This chronic hypoxic pulmonary hypertension is gradually reversible upon reoxygenation. The return to air after the adjustment to chronic hypoxia resembles in some aspects a hyperoxic stimulus and we hypothesize that the changes of extracellular matrix proteins in peripheral pulmonary arteries may be similar. Therefore, we studied the exposure to moderate chronic hyperoxia (F_{iO2} = 0.35, 3 weeks) in rats and compared its effects on the rat pulmonary vasculature to the effects of recovery (3 weeks) from chronic hypoxia (F_{iO2} = 0.1, 3 weeks). Chronically hypoxic rats had pulmonary hypertension (Pap = 26±3 mm Hg, controls 16±1 mm Hg) and right ventricular hypertrophy. Pulmonary arterial blood pressure and right ventricle weight normalized after 3 weeks of recovery in air (Pap = 19±1 mm Hg). The rats exposed to moderate chronic hyperoxia also did not have pulmonary hypertension (Pap = 18±1 mm Hg, controls 17±1 mm Hg). Collagenous proteins isolated from the peripheral pulmonary arteries (100-300 μm) were studied using polyacrylamide gel electrophoresis. A dominant low molecular weight peptide (approx. 76 kD) was found in hypoxic rats. The proportion of this peptide decreases significantly in the course of recovery in air. In addition, another larger peptide doublet was found in rats recovering from chronic hypoxia. It was localized in polyacrylamide gels close to the zone of α2 chain of collagen type I. It was bound to anticollagen type I antibodies. An identically localized peptide was found in rats exposed to moderate chronic hyperoxia. The apparent molecular weight of this collagen fraction suggests that it is a product of collagen type I cleavage by a rodent-type interstitial collagenase (MMP-13). We conclude that chronic moderate hyperoxia and recovery from chronic hypoxia have a similar effect on collagenous proteins of the peripheral pulmonary arterial wall.

Key words

Chronic hypoxia • Chronic hyperoxia • Collagen • Metalloproteinases • Vascular remodeling • Pulmonary hypertension • Rat

Introduction

Exposure of rats to chronic hypoxia results in reversible structural remodeling of peripheral pulmonary arteries and in pulmonary hypertension (Herget *et al.* 1978, Reid 1986). Structural changes of peripheral pulmonary arteries and pulmonary hypertension were also described in rats exposed to chronic severe hyperoxia (Jones *et al.* 1984, 1985).

We described recently that the sojourn of rats in chronic hypoxia increases the collagenolytic activity in peripheral pulmonary arteries (Novotná and Herget 1998, Toyyi *et al.* 1998) reported several months later that in the pulmonary artery trunk and its main branches the collagenolytic activity peaks during recovery from chronic hypoxic exposure. An increased expression of interstitial collagenase and gelatinases was also described in the lungs of rats exposed to hyperoxia (Pardo and Selman 1996). Return to air after a hypoxic exposure is interpreted by some authors as further lung vascular hyperoxic injury (Reid 1989). The present study tested the hypothesis whether there are similar collagen changes in the walls of peripheral pulmonary arteries during recovery from hypoxia and during exposure to moderate hyperoxia. Moderate hyperoxia was selected because severe hyperoxia is known to induce inflammation in the lungs (Deneke and Fanburg 1980), while there are almost no inflammatory changes in hypoxic exposure (Meyrick and Reid 1978).

The SDS electrophoresis was used to analyze the collagenous proteins extracted from peripheral pulmonary arteries. In addition, we measured the basic parameters which characterize the presence of pulmonary hypertension.

Methods

Exposure to hypoxia and recovery from hypoxia

Fourteen young male Wistar rats (body weight 182±3 g) were exposed to hypoxia ($F_{iO2} = 0.1$) for 3 weeks in a previously described isobaric hypoxic chamber (Herget *et al.* 1978, Hampl and Herget 1990). Oxygen concentration was continuously monitored and regulated. KOH and soda lime adsorbed CO_2 and excess humidity was condensed in a refrigerator and adsorbed by silicagel. Seven experimental rats were examined immediately (within 4 hours) after exposure. Six rats were examined after 3 weeks of recovery in air. One died during the hypoxic exposure. A control group of seven littermate male rats (body weight 184±2 g) was kept in the same room in atmospheric air.

Exposure to hyperoxia

In a different experiment, seven male Wistar rats (body weight 230±7 g) were kept for 3 weeks in the same environmental chamber as was used for the hypoxic exposure, but the F_{i02} was maintained at 0.35. Littermate control rats (n = 7, body weight 228±10 g) were kept in air.

Hemodynamic measurements and pulmonary artery preparation

At the end of the experiment, the rats were anesthetized by Thiopental (30 mg/kg b.w. i.p.). The right jugular vein was exposed and the pulmonary artery was catheterized without opening the chest (Herget and Paleček 1971). The rats spontaneously breathed atmospheric air. After cannulating the trachea, the lungs were ventilated with air at positive pressure (peak inspiratory pressure 12 cm H_2O; end expiratory pressure 2.5 cm H_2O) and the chest and left heart ventricle were widely opened. The lungs were then perfused with a cold saline containing 4 % bovine albumin (Sigma, fraction V) *via* a cannula inserted into the pulmonary artery. After about 10 min of perfusion, the heart and the lung were separately removed from the chest. The right and left heart ventricles and the septum were weighed (Fulton *et al.* 1952). The lung was placed on ice and the peripheral pulmonary arteries (100-300 μm) were dissected under a dissecting microscope (Novotná and Herget 1998). The length and diameter of each arterial segment *in situ* were measured by an eyepiece micrometer and cumulated samples (16-25 vascular segments) in each rat were weighed (wet weight). Segments of vessels were then cut into small pieces, washed in distilled water, lyophilized and weighed (dry weight).

Analysis of collagenous proteins in peripheral pulmonary arteries

Noncollagenous proteins were removed after incubation of samples in 15 volumes of 4 M guanidine-HCl in 0.05 M $(CH_3COO)_2Na$ buffer, pH 5.8, for 48 h at 4 ^oC. After washing in distilled water, the remaining tissue was pepsinized by 10 volumes of 1 : 10 ratio between the weight of pepsin and dry weight of the tissue in 0.5 M CH_3COOH, pH 2.5, for 4 h at room temperature

and then for 20 h at 4 °C and centrifuged (8000x*g*, 30 min) (Novotná and Herget 1998). The supernatant was lyophilized.

Gel electrophoretic separations (SDS-PAGE) were performed by the method of Laemmli (1970) on discontinuous slab gel using 4 % stacking gel and 7.5 % separating gel. Samples were dissolved in a sample buffer in a concentration 4 g/ml and 30 μg of the collagenous fraction were loaded per line. The collagen standard from Sigma was loaded in a concentration 10 μg per line. The electrophoretic separation was run in a Tris-glycine buffer system without reduction in Mini-PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories, USA). The gels were stained for proteins with 0.25 % Coomassie Brilliant Blue R in methanol - acetic acid - water (40 : 10 : 50 v/v/v). Destaining was performed with methanol-acetic acid-water (40 : 10 : 50 v/v/v).

Immunoblotting detection of collagenous proteins

After electrophoretic separation, proteins were electrotransferred to a nitrocellulose membrane in 15 mM sodium borate buffer, pH 9.2, 4 °C for 24 h in Mini Trans-Blott Electrophoretic Transfer Cell (Bio-Rad Laboratories, USA). The starting transfer power conditions were 25 V/250 mA and finishing conditions were 25 V/350 mA. Polyclonal rabbit antibodies to rat collagen type I (ANAWA Switzerland, cat. No. 2150-1908) were diluted 1:50 in 2 % skimmed milk-PBS and the nitrocellulose membrane was incubated in this solution for 1 h at room temperature, and washed with 2 % skimmed milk-PBS. The membrane was then incubated in a horseradish peroxidase (HRP)-conjugated swine antirabbit antibodies solution diluted 1:500 for 1 h at room temperature. After washing, the membrane was stained with HRP substrate, 4-chloro-1-naphthol (15 mg in 5 ml of methanol, 20 ml of 10 mM Tris-HCl, 0.04 % H_2O_2). The reaction was allowed to proceed in the dark for 15 min until all bands were visualized. The membrane was then air-dried.

Chemicals

All chemicals were of the highest available purity; collagen type I (from the rat tail), bovine albumin (fraction V), acrylamide, ammonium persulfate, EDTA, SDS, N,N,N',N',-tetramethylenediamine (TEMED), mercaptoethanol, glycine, Tris base, Coomassie Brilliant Blue R, Triton X-100 and 4-chloro-1-naphtol were obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). SDS-PAGE molecular mass standards, high molecular weight-SDS calibration kit (Pharmacia Biotech, USA) and the HRP-conjugated swine immunoglobulins against rabbit immunoglobulins (SwAR/Px) were from USOL (Prague, Czech Republic).

Statistical analyses

The results are presented as means ± S.E.M. They were statistically evaluated by ANOVA and Scheffe´s test. The statistical analyses were performed using the statistical software StatView 5.0, SAS Institute Inc (Cary, North Carolina). Differences were considered significant at $P<0.05$.

Table 1. Body weight, pulmonary artery mean blood pressure and right ventricle weight in rats exposed to chronic hypoxia and after two weeks of recovery in air.

Group	n	Body weight (g)	Pap (mm Hg)	RV/BW (mg/100 gb.w.)	(LV+S)/BW (mg/100 gb.w.)	RV/(LV + S) ratio
Controls	7	236 ± 2	15.5 ± 0.6	66.4 ± 2.5	228.1 ± 6.6	0.29 ± 0.01
Hypoxia	7	203 ± 4*	26.0 ± 3.1*	101.6 ± 3.7*	223.2 ± 4.9	0.46 ± 0.02*
Hypoxia + recovery	6	238 ± 7#	18.7 ± 1.0#	74.1 ± 8.1#	233.4 ± 18.1	0.31 ± 0.01#

*Controls – rats kept in atmospheric air. Hypoxia – rats kept in isobaric hypoxic chamber ($F_{iO\check{e}}$ = 0.1, 3 weeks) Hypoxia + recovery – rats of the Hypoxia group examined after two weeks of recovery in air after exposure to hypoxia. Pap – pulmonary artery mean blood pressure, RV/BW – relative weight of the right heart ventricle, (LV+S)/BW – relative weight of the left heart ventricle plus heart septum, RV/(LV + S) – ratio of the weight of the right heart ventricle to the sum of the weights of left ventricle and septum. * significantly different (p<0.05) from the Control group, # significantly different (p<0.05) from the Hypoxia group.*

Table 2. Body weight, pulmonary artery mean blood pressure and right ventricle weight in rats exposed to chronic hyperoxia and in the controls.

Group	n	Body weight (g)	Pap (mm Hg)	RV/BW (mg/100 gb.w.)	(LV+S)/BW (mg/100 gb.w.)	RV/(LV + S) ratio
Controls	7	290 ± 11	17 ± 1	52 ± 2	207 ± 12	0.26 ± 0.01
Hyperoxia	17	209 ± 7*	18 ± 1	67 ± 3*	222 ± 6	0.31 ± 0.01*

Controls – rats kept in atmospheric air. Hyperoxia – rats kept in an isobaric hypoxic chamber (F_{iO2} = 0.35, 3 weeks), For other legend see Table 1.

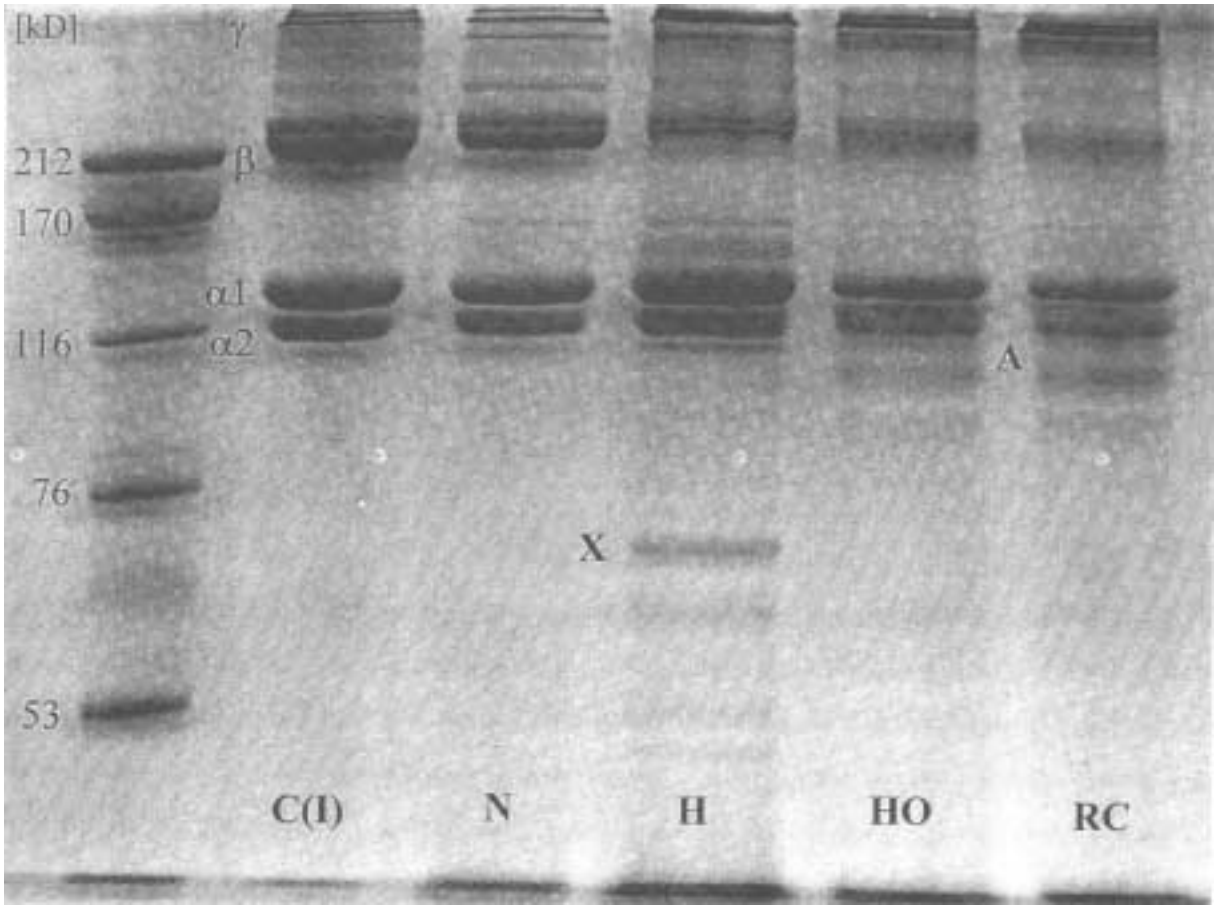

Fig. 1. *Typical gel electrophoresis profile: C(I) – collagen type I standard from rat tail (Sigma), collagenous fraction extracted from peripheral pulmonary arteries of normal control rat (N), rat exposed for 3 weeks to hypoxia (H), rat exposed to chronic hyperoxia (HO), and rat recovering from hypoxic exposure (RC). 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); A – dominant peptide. X – small peptide present predominantly in the hypoxic peripheral pulmonary arteries (Novotná and Herget 1998). Electrophoresis was run in unreduced conditions.*

Results

Rats exposed both to chronic hypoxia and to chronic hyperoxia gained body weight more slowly than the relevant controls, and consequently, their body weight at the end of the exposure was significantly less compared to controls (Tables 1 and 2).

Chronic hypoxia induced pulmonary hypertension characterized by a significant increase of pulmonary artery mean blood pressure and by right ventricular hypertrophy (Table 1). Pulmonary hypertension induced by chronic hypoxia was reversible and both pulmonary arterial pressure and the weight of the right ventricle significantly decreased after 3 weeks´ recovery period towards the values found in the control group.

In contrast to the hypoxic exposure, the sojourn in a moderate hyperoxic environment did not increase pulmonary arterial mean blood pressure. The relative increase of the right heart ventricle was smaller than in hypoxic rats (Table 2). The absolute weight of the left ventricle + heart septum was lower in hyperoxic rats (596±23 mg in the controls, 461±11 mg in hyperoxic rats, $P<0.001$), whereas the absolute weight of the right ventricle was not affected by exposure to hyperoxia (155±6 mg in the controls, 139±5 mg in hyperoxia).

The dry weight of dissected peripheral pulmonary arteries related to their length (pooled samples in individual rats) was significantly larger in hypoxic rats (1.33±0.22 μg/mm) than in the controls (0.74±0.06 μg/mm, $P<0.005$). The relative weight of the dissected peripheral pulmonary arteries remained high even after 3 weeks of recovery in air (1.42±0.23, $P<0.01$ compared to controls). The relative weight of the vessels dissected from rats exposed to hyperoxia (0.89±0.6 μg/mm) did not differ from the controls.

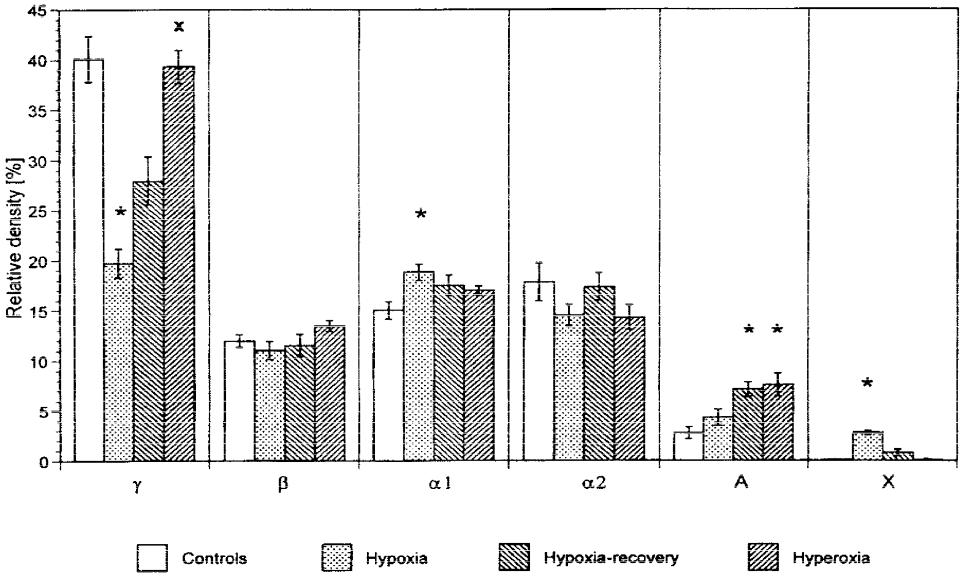

Fig. 2. *Relative density of the main peaks separated by gel elecrophoresis of collagenous proteins isolated from peripheral pulmonary arteries of normoxic rats (Controls, n = 6), rats exposed to chronic hypoxia (Hypoxia, n = 6), rats recovering from hypoxic exposure (Hypoxia-recovery) and rats exposed to chronic hyperoxia (Hyperoxia, n = 5). The isolated peripheral pulmonary arteries from individual rats were pooled. For the identification of columns see Fig. 1. * P<0.02 compared to samples from normoxic rats.*

Figure 1 shows typical gel electrophoretic profiles of the collagen type I standard and the collagenous fraction isolated from peripheral pulmonary arteries of control and experimental rats. The results are summarized in Figure 2. Chronically hypoxic rats showed a significantly lower proportion of the fraction γ in comparison with the controls and rats exposed to hyperoxia. The chronically hypoxic group revealed the presence of an additional low molecular mass peptide X with an approximate molecular weight of 76 kD, as described in our previous study (Novotná and Herget 1998). Peptide X was present in all samples isolated from

rats studied at the end of chronic hypoxic exposure and in 3 out of 7 samples from rats recovering from hypoxia. It was not detected in the extracts from control or hyperoxic rats. In hyperoxic rats and rats recovering from hypoxic exposure, however, we found significantly larger densities of the peptide A doublet (molecular mass slightly lower than collagen fraction α2). Peptide A doublet was recognized by anticollagen type I antibodies in an immunoblot analysis (Fig. 3).

The next experiment was aimed to prove that protein A is not an artifact arising during the isolation procedure. Four samples of peripheral arteries from rats recovering from hypoxia were divided into two parts: one was treated from the beginning of the isolation procedure with 20 mM EDTA to inhibit the metalloproteinase activity in the sample. The second was processed without EDTA. The resulting gels were comparable and the peptide A doublet was present in all samples treated with EDTA (Fig. 4).

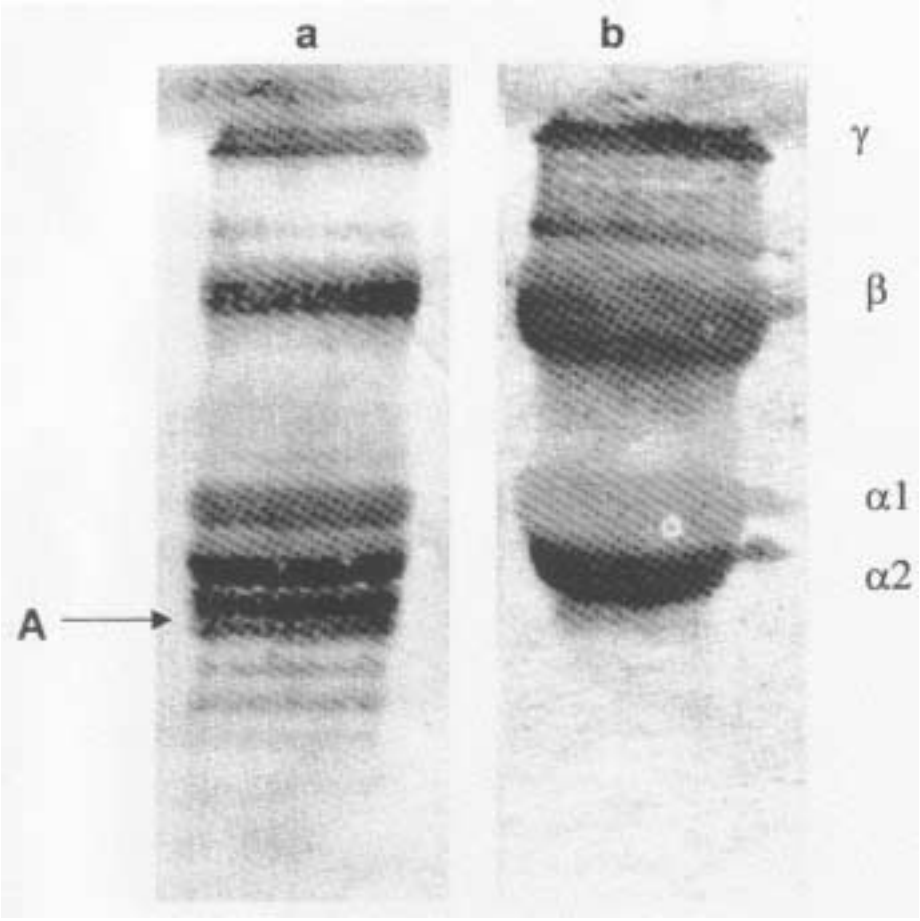

Fig. 3. *Protein transfer to nitrocellulose filter and specific staining of collagen peptides with antibodies to collagen type I (ASC) from calf skin: a) collagenous fraction extracted from peripheral pulmonary artery of rat exposed to hyperoxia, b) collagen type I (ASC) standard from calf skin. For identification of bands see Fig. 1.*

Discussion

The exposure of rats to chronic hypoxia results in pulmonary hypertension which was described in detail in a number of studies (for review see Reeves and Herget 1984, Voelkel and Tuder 1995). Thus, pulmonary hypertension and right heart hypertrophy resulting from the chronic hypoxic exposure was expected in the present study. The relative dry weight of isolated peripheral pulmonary arteries was significantly higher in hypoxic rats than in the controls, which is consistent with a proliferative remodeling of peripheral arteries. The relative dry weight of isolated peripheral pulmonary arteries stayed increased after 3 weeks of recovery in a normoxic environment when both pulmonary artery mean blood pressure and weight of the right ventricle returned towards normal values. This finding is in agreement with our previous observation that the structure of peripheral pulmonary blood vessels recovers more slowly than the lung hemodynamics or right ventricular hypertrophy (Herget *et al.* 1978).

Moderate chronic hyperoxia (35 % O_2) did not produce pulmonary hypertension. Pulmonary arterial blood pressure and the relative weight of the dissected peripheral pulmonary arteries did not differ from the controls. We also searched for right ventricular hyper-

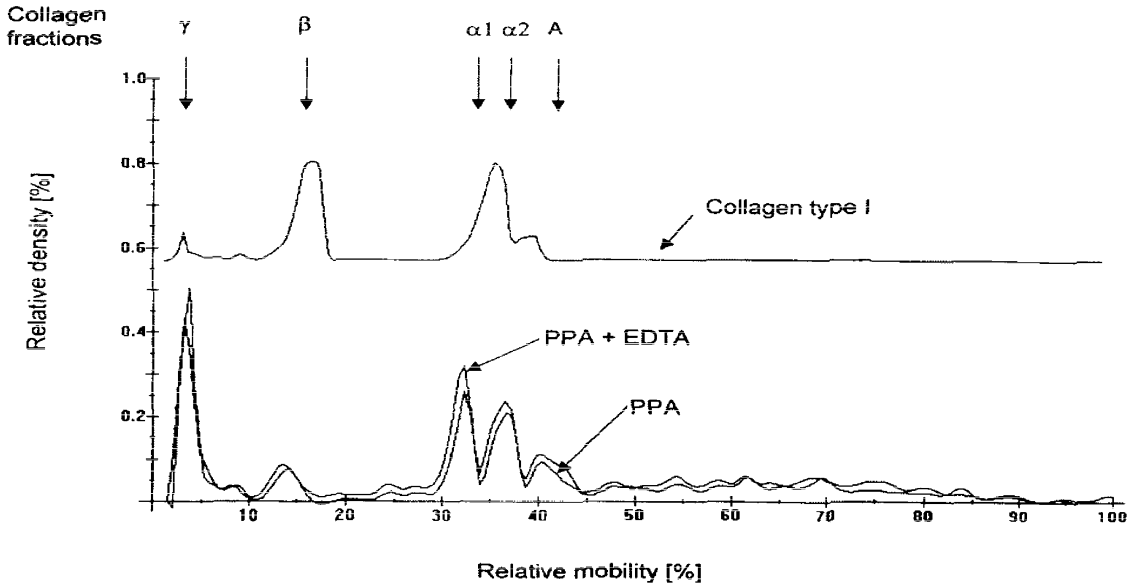

Fig. 4. *Relative density of the main peaks separated by gel electrophoresis of collagenous proteins isolated from peripheral pulmonary arteries of rat recovering from chronic hypoxia. PPA – densitogram of collagenous proteins from peripheral pulmonary arteries. PPA + EDTA – collagenous proteins from peripheral pulmonary arteries extracted in the presence of 20 mM EDTA. For denomination of collagen fractions see legend to Fig. 1.*

trophy in our experimental groups because, aside from the increased pulmonary arterial pressure, right ventricular hypertrophy is usually a good indicator of chronic pulmonary hypertension. However, our data on ventricular weight are not easy to interpret. The right ventricular weight increased in relation to body weight and to the left ventricular weight, suggesting a chronic increase in the right ventricular afterload. However, the increase in the relative right ventricular weight was not due to an actual increase in its absolute value, but merely due to an unchanged absolute right ventricular weight in relation to a decreased body weight, whereas left ventricular weight was reduced in proportion to body weight. Thus, chronic hyperoxia did not cause hypertrophy of the right ventricle; it only prevented a reduction of right ventricular wall in proportion to the loss of the body weight and left ventricular weight. Whether such an effect could be produced by an increased pressure load to the right ventricle is not known. It is unlikely that the absence of increased pulmonary arterial pressure in chronically hyperoxic rats is the result of a decreased cardiac output after the return to normoxia. In a pilot study, we used an ultrasonic flow probe (Transonic Systems, Ithaca, NY) to measure blood flow in the ascending aorta of open-chest rats ventilated with air at positive pressure immediately after the hyperoxic exposure. The observed values were 38±2 ml/min in ex-hyperoxic rats (n=6) and 42±4 ml/min in normoxic controls (n=5, n.s.). In these open-chest rats, the pulmonary arterial blood pressure measured by a needle inserted in the trunk of the pulmonary artery did not differ significantly from that measured by a catheter in closed-chest rats.

The absence of pulmonary hypertension after moderate chronic hyperoxia is in agreement with the report of Hill *et al.* (1989). In their study, the peak right ventricular blood pressure in rats exposed to hyperoxia did not differ from the controls and they did not find right ventricular hypertrophy. A decrease of left ventricle weight and no change of the right ventricle weight was also reported after 3-weeks' exposure to severe hyperoxia (87 % O_2) (Jones *et al.* 1985). Rats in this study, however, showed obvious structural changes of pulmonary vasculature typical for pulmonary hypertension.

The increase of lung vascular resistance in chronic hypoxia is caused by fibroproduction and proliferation of vascular smooth muscle cells in the prealveolar portion of pulmonary vasculature (Reid 1986). The amount of collagen and elastin in the walls of conduit pulmonary arteries increases (Parks *et al.* 1989, Poiani *et al.* 1990, Tozzi *et al.* 1994) and the turnover of matrix proteins is enhanced (Bishop *et al.* 1990). Our study provides evidence that the increase in the

metabolism of matrix proteins also takes place in the peripheral pulmonary arteries. The γ fraction on the gel electrophoresis profile represents highly cross-linked and poorly soluble chain polymers of mature collagen molecules. We interpret the relative decrease of the γ fraction in our hypoxic rats as a result of expansion in the portion of the newly formed, less cross-linked and, consequently, more soluble collagen molecules.

The present results confirm our previous observation (Novotná and Herget 1998) that the exposure to chronic hypoxia results in the presence of a low molecular weight peptide (approximate molecular mass, 76 kD) in extracts from the peripheral pulmonary arteries. Using immunoblotting, we identified this peptide as a collagen type I cleavage (Novotná and Herget 1998). This conclusion was further supported by aminoterminal peptide sequencing (Deyl *et al.* 1998). The present study demonstrates that this collagen-derived peptide decreases significantly after 3 weeks of recovery from hypoxic exposure. Small molecular mass products of collagen degradation stimulate lung collagen metabolism (Gardi *et al.* 1994) and provide signals for smooth muscle cell migration and proliferation (Zempo *et al.* 1996). We hypothesize that these small molecular weight collagen fragments may participate in the initiation of a remodeling of peripheral pulmonary arteries in hypoxic pulmonary hypertension (Hampl and Herget 2000).

In the present study, we found an additional new peptide (peptide A doublet) in the collagenous matrix of the peripheral pulmonary arteries isolated from rats recovering from chronic hypoxia and from rats exposed to chronic moderate hyperoxia. Its molecular mass is close to the molecular mass of α1 (I) and α2 (I) collagen chains. Peptide A also appears to be of collagen origin as it binds the anticollagen type I antibodies in immunoblotting. It is unlikely that the peptide A doublet is an artifact resulting from collagen degradation during the isolation procedure, because an identical protein was present in samples where metalloproteinase activity during the protein isolation procedure was inhibited by EDTA. The presence of peptide A cannot be a result of increased hemodynamic stress of the vascular wall, because peptide A was found in chronically hyperoxic rats, who had no pulmonary hypertension. Thus, it is likely that protein A is a collagen cleavage product resulting from increased collagenolytic activity induced by oxidant tissue stress. This view is supported by findings that exposure to severe hyperoxia results in a reduction of total lung collagen (Riley *et al.* 1987) including type I collagen (Bhatnagar *et al.* 1978). This is partly due to increased collagen degradation (Riley *et al.* 1987).

Both superoxide and hydrogen peroxide are produced in reperfused and reoxygenized organs including lungs (Ryrfeldt *et al.* 1993). Exposure to hyperoxia increases oxygen radical production (Freeman and Crapo 1981, Ho *et al.* 1996). Reactive oxygen metabolites are potent activators of gelatinase A activity *in vitro* (Owens *et al.* 1997). In addition, matrix metalloproteinase activity may be modulated by products of nitric oxide-superoxide interaction (peroxynitrite and its derivatives) (Rajagopalan *et al.* 1996). Nitric oxide production is increased in lung vessels of rats with chronic hypoxic pulmonary hypertension (Hampl *et al.* 1995). We hypothesize that radicals released in chronic hypoxia may play an important role in the pathogenesis of hypoxic pulmonary hypertension (Hampl and Herget 2000, Herget *et al.* 2000).

In a previous study, we demonstrated increased collagenolytic activity in extracts from peripheral pulmonary arteries of chronically hypoxic rats (Novotná and Herget 1998). In the next study we succeeded to inhibit the development of hypoxic pulmonary hypertension by pharmacological inhibition of collagenolysis (Novotná and Herget 2000). Based on the position of the lytic zones in lung samples analyzed by zymography (Novotná and Herget 1998) and on aminoterminal peptide sequencing (Deyl *et al.* 1998) we proposed that hypoxia increases the activity of the rodent-type interstitial collagenase (MMP-13). As other collagenases, MMP-13 can cleave off three quarters of a collagen molecule, resulting in a characteristic 3/4 and 1/4 fragment size (Knauper *et al.* 1996) and might also act as a gelatinase (Welgus *et al.* 1985, Knauper *et al.* 1997). The collagenous peptide X found in the peripheral pulmonary arteries of chronically hypoxic rats (Novotná and Herget 1998) corresponds in size to the larger cleavage product. The collagenous peptide doublet A, found in the present study in the peripheral pulmonary arteries of chronically hyperoxic rats and of rats recovering from chronic hypoxia, cannot be a product of the 3:1 cleavage catalyzed by interstitial collagenases; its molecular weight is too high. The high molecular weight of the peptide A is consistent with its cleavage at the aminotelopeptide site. Unlike other MMPs, the MMP-13 can cleave native collagens type I, II, III not only at the usual 3:1 site, but also at the aminotelopeptide site (Krane *et al.* 1996, Knauper *et al.* 1997). The expression and

activity of MMP-13 and of gelatinases A (MMP-2) and B (MMP-9) is increased in hyperoxic rats (100 % O_2 for 60 h) (Pardo and Selman 1996). It is possible that during chronic hypoxia MMP-13 cleaves pulmonary arterial collagens by triple helicase activity at the 3:1 site and during reoxygenation/ hyperoxia at the telopeptidase site by its peptidolytic activity (Knauper 1996, 1997, Krane *et al.* 1996). Knauper *et al.* (1997) prepared the C-terminal truncated from recombinant MMP-13 ($\alpha_{249\text{-}451}$) collagenase 3) which failed to cleave native triple helical collagens and cleaved only α1,2 (I) chains of collagen I. It generated α1,2 (I) chains somewhat smaller than is typical. This α1,2 (I) chain was in the similar position as the collagenous peptide A doublet found in our experiments. The triple helicase activity of MMP-13 is dependent on the C-terminal domain. On the contrary, the telopeptidase activity of MMP-13, which produces large α1,2 (I) chains, is independent of the C-terminal domain and depends on the catalytic site only. We assume that under certain conditions (hyperoxia, reoxygenation) the C-terminal domain may be altered and the telopeptidase activity of MMP-13 prevails. The resulting α1,2 (I) chains are probably too large to stimulate mesenchymal proliferation as in the case of small 3:1 triple helicase collagen fagments (peptide X in hypoxia).

Tozzi *et al.* (1998) studied the collagenolytic activity in large conduit pulmonary blood vessels (pulmonary artery trunk and its first branches) and found that it was enhanced after several days of recovery from chronic hypoxia, but not immediately after the exposure. The apparent contradiction with our results may be explained by the difference in the size and type of the vessels studied. In the pathogenesis of pulmonary hypertension, the small, peripheral arteries (used in our study) are probably exposed to different mechanical forces than the large, conduit arteries studied by Tozzi *et al.* (1998). Chronic hypoxia elicits fibroproduction, vascular smooth muscle proliferation and vasoconstriction in the pulmonary arterial tree, predominantly in its peripheral portion. There is evidence that the mechanism initiating these changes includes hypoxic injury to the walls of peripheral pulmonary arteries in the early stages of the hypoxic exposure (see Herget and Ježek 1989 for review). The morphological remodeling and vasoconstriction of the peripheral pulmonary vascular bed increases its resistance to flow and thus elevates the intravascular pressure in the larger upstream pulmonary arteries. Unlike the small peripheral arteries, the large, upstream arteries are therefore exposed to increased passive distension. Increased tension in the walls of pulmonary arteries stimulates collagen synthesis (Rilley and Gullo 1988). Hence, it is plausible that the increased collagenolytic activity in the large pulmonary arteries during recovery from chronic hypoxia is simply a reversal of stretch-induced fibroproduction, while the increased collagenolysis in the peripheral arteries during the hypoxic exposure is more directly related to the hypoxic injury to the vascular wall.

Acknowledgements

The work was supported by the grants of GAČR 203/96/K128, 305/97/S070, 306/97/0854 and GAUK 190/1998/C/2.LF. Hardware and software for the measurement of densitograms was designed by ing. Jiří Semecký, Prague, CR.

References

BHATNAGAR RS, HUSSAIN MZ, STREIFEL JA, TOLENTINO M, ENRIQUEZ B: Alteration of collagen synthesis in lung organ cultures by hyperoxic environments. *Biochem Biophys Res Commun* **83**: 392-397, 1978.

BISHOP JE, GUERREIRO D, LAURENT J: Changes in the composition and metabolism of arterial collagens during the development of pulmonary hypertension in rabbits. *Am Rev Resp Dis* **141**: 450-455, 1990.

DENEKE SM, FANBURG BL: Normobaric oxygen toxicity of the lung. *N Engl J Med* **303**: 76-86, 1980.

DEYL Z, NOVOTNÁ J, MIKŠÍK I, HERGET J: Micropreparation of tissue collagenase fragments of type I collagen in the form of surfactant-peptide complexes and their identification by capillary electrophoresis and partial sequencing. *J Chromatogr A* **796**: 181-193, 1998.

FREEMAN BA, CRAPO JD: Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem* **256**: 10986-10992, 1981.

FULTON RM, HUTCHINSON EC, JONES AM: Ventricular weight in cardiac hypertrophy. *Br Heart J* **14**: 413-420, 1952.

GARDI C, CALZONI P, MARCOLONGO P, CAVARRA E, VANNI L, LUNGARELLA G: Collagen breakdown products and lung collagen metabolism: an in vitro study on fibroblast cultures. *Thorax* **49**: 312-318, 1994.

HAMPL V, CORNFIELD DN, COWAN NJ, ARCHER SL: Hypoxia potentiates nitric oxide synthesis and transiently increases cytosolic calcium levels in pulmonary artery endothelial cells. *Eur Respir J* **8**: 515-522, 1995.

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.

HAMPL V, HERGET J: Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. *Physiol Rev* **80**: 1337-1372, 2000.

HERGET J, JEŽEK V: Pulmonary hypertension in chronic lung disease. In: *Pulmonary Hypertension. Problems and Controversies*. H DENOLIN, CA WAGENVOORT (eds), Elsevier, Amsterdam, 1989, pp 142-162.

HERGET J, PALEČEK F: Catheterization of the pulmonary artery in closed chest rats. *Physiol Bohemoslov* **20**: 373, 1971.

HERGET J, SUGGETT AJ, LEACH E, BARER GR: Resolution of pulmonary hypertension and other features induced by chronic hypoxia in rats during complete and intermittent normoxia. *Thorax* **33**: 468-473, 1978.

HERGET J, WILHELM J, NOVOTNÁ J, ECKHARDT A, VYTÁŠEK R, MRÁZKOVÁ L, OŠŤÁDAL M: A possible role of oxidant tissue injury in the development of pulmonary hypertension. *Physiol Res* **49:** 493-501, 2000.

HILL NS, JEDERLINIC P, GAGNON J: Supplemental oxygen reduces right ventricular hypertrophy in monocrotaline-injected rats. *J Appl Physiol* **66**: 1642-1648, 1989.

HO YS, DEY MS, CRAPO JD: Antioxidant enzyme expression in rat lungs during hyperoxia. *Am J Physiol* **14**: L810-L818, 1996.

JONES R, ZAPOL WM, REID L: Pulmonary artery remodeling and pulmonary hypertension after exposure to hyperoxia for 7 days. A morphometric and hemodynamic study. *Am J Pathol* **117**: 273-285, 1984.

JONES R, ZAPOL WM, REID L: Oxygen toxicity and restructuring of pulmonary arteries – a morphometric study. The response to 4 weeks' exposure to hyperoxia and return to breathing air. *Am J Pathol* **121**: 212-223, 1985.

KNAUPER V, LOPEZ-OTIN C, SMITH B, KNIGHT G, MURPHY G: Biochemical characterization of human collagenase-3. *J Biol Chem* **271**: 1544-1550, 1996.

KNAUPER V, COWELL S, SMITH B, LOPEZ-OTIN C, O'SHEA M, MORRIS H, ZARDI L, MURPHY G: The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction. *J Biol Chem* **272**: 7608-7616, 1997a.

KRANE SM, BRYNE MH, LEMAITRE V, HENRIET P, JEFFREY JJ, WITTER JP, LIU X, WU H, JAENISCH R, EECKHOUT Y: Different collagenase gene products have different roles in degradation of type I collagen. *J Biol Chem* **271**: 28509-28515, 1996.

LAEMMLI VK: Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* **227**: 680-685, 1970.

MEYRICK B, REID L: The effect of continued hypoxia on rat pulmonary arterial circulation. *Lab Invest* **38**: 188-200, 1978.

NOVOTNÁ J, HERGET J: Exposure to chronic hypoxia induces qualitative changes of collagen in the walls of peripheral pulmonary arteries. *Life Sci* **62**: 1-12, 1998.

NOVOTNÁ J, HERGET J: Suspenssion of hypoxic pulmonary hypertension by the inhibitor of collagenolytic activity (Batimastat) in rats. *Physiol Res* **49:** P12, 2000.

OWENS MW, MILLIGAN SA, JOURDHEUIL D, GRISHAM MB: Effects of reactive metabolites of oxygen and nitrogen on gelatinase A activity. *Am J Physiol* **17**: L445-L450, 1997.

PARDO A, SELMAN M: Matrix metalloproteinases and lung injury. *Braz J Med Biol Res* **29**: 1109-1115, 1996.

PARKS WC, MECHAM RP, CROUCH EC, ORTON EC, STENMARK KR: Response of lobar vessels to hypoxic pulmonary hypertension. *Am Rev Resp Med* **140**: 1455-1457, 1989.

POIANI GJ, TOZZI CA, YOHN SA, PIERCE RA, BELSKY SA, BERG RA, YU SY, DEAK SB, RILEY DJ: Collagen and elastin metabolism in hypertensive pulmonary arteries of rats. *Circ Res* **66**: 968-978, 1990.

RAJAGOPALAN S, MENG XP, RAMASAMY S, HARRISON DG, GALIS ZS: Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* **98**: 2572-2579, 1996.

REEVES JT, HERGET J: Experimental models of pulmonary hypertension. In: *Pulmonary Hypertension*. EK WEIR, JT REEVES (eds), Futura Publ Co, New York, 1984, pp 361-391.

REID LM: Structure and function in pulmonary hypertension. New perceptions. *Chest* **89**: 279-288, 1986.

REID LM: The control of cellular proliferation in the pulmonary circulation. *Am Rev Resp Dis* **140**: 1490-1493, 1989.

RILEY DJ, KRAMER MJ, KERR JS, CHAE CU, YU SY, BERG RA: Damage and repair of lung connective tissue in rats exposed to toxic levels of oxygen. *Am Rev Resp Dis* **135**: 441-447, 1987.

RILLEY D, GULLO J: Pressure applied to cultured pulmonary artery endothelial calls causes release of fibroblast mitogen and induces a proto-oncogene. *FASEB J* **2**: A300, 1988.

RYRFELDT A, BANNEBERG G, MOLDEUS P: Free radicals and lung disease. *Br Med Bull* **49**: 588-603, 1993.

TOZZI CA, CHRISTIANSEN DL, POIANI GJ, RILEY DJ: Excess collagen in hypertensive pulmonary arteries decreases vascular distensibility. *Am J Resp Crit Care Med* **149**: 1317-1326, 1994.

TOZZI CA, THAKKER-VARIA S, YU SY, BANNETT RF, PENG BW, POIANI GJ, WILSON FJ, RILEY DJ: Mast cell collagenase correlates with regression of pulmonary vascular remodeling in the rat. *Am J Resp Cell Mol Biol* **18**: 497-510, 1998.

VOELKEL NF, TUDER RM: Cellular and molecular mechanisms in the pathogenesis of severe pulmonary hypertension. *Eur Resp J* **8**: 2129-2138, 1995.

WELGUS HG, GRANT GA, SACCHETTINI JC, ROSWIT WT, JEFFREY JJ: The gelatinolytic activity of rat uterus collagenase. *J Biol Chem* **260**: 13601-13606, 1985.

ZEMPO N, KOYAMA N, KENAGY RD, LEA HJ, CLOWES AW: Regulation of vascular smooth muscle cell migration and proliferation in vitro and in injured rat arteries by a synthetic matrix metalloproteinase inhibitor. *Arterioscler Thromb Vasc Biol* **16**: 28-33, 1996.

Reprint requests

Prof. Jan Herget, MD, Department of Physiology, Second Medical School, Charles University, Plzeňská 221, 150 00 Prague, Czech Republic. Jan.Herget@lfmotol.cuni.cz