Metalloproteinase inhibition by Batimastat attenuates pulmonary hypertension in chronically hypoxic rats

Jan Herget, Jana Novotná, Jana Bíbová, Viera Povysilová, Marie Vanková and Václav Hampl

Am J Physiol Lung Cell Mol Physiol 285:199-208, 2003. First published Mar 28, 2003;
doi:10.1152/ajplung.00167.2002

Am J Physiol Lung Cell Mol Physiol 285: L199–L208, 2003.
First published March 28, 2003; 10.1152/ajplung.00167.2002.

Metalloproteinase inhibition by Batimastat attenuates pulmonary hypertension in chronically hypoxic rats

Jan Herget,[1,2] Jana Novotná,[2,3] Jana Bíbová,[1,2] Viera Povýšilová,[4] Marie Vaňková,[2,5] and Václav Hampl[1,2]

Departments of [1]Physiology, [3]Medical Chemistry and Biochemistry, [4]Pathology, and [5]Pathological Physiology, Charles University Second Medical School, 15000 Prague 5; and [2]Centre for Experimental Cardiovascular Research; 14000 Prague, Czech Republic

Submitted 30 May 2002; accepted in final form 24 March 2003

Herget, Jan, Jana Novotná, Jana Bíbová, Viera Povýšilová, Marie Vaňková, and Václav Hampl. Metalloproteinase inhibition by Batimastat attenuates pulmonary hypertension in chronically hypoxic rats. *Am J Physiol Lung Cell Mol Physiol* 285: L199–L208, 2003. First published March 28, 2003; 10.1152/ajplung.00167.2002.—Chronic hypoxia induces lung vascular remodeling, which results in pulmonary hypertension. We hypothesized that a previously found increase in collagenolytic activity of matrix metalloproteinases during hypoxia promotes pulmonary vascular remodeling and hypertension. To test this hypothesis, we exposed rats to hypoxia (fraction of inspired oxygen = 0.1, 3 wk) and treated them with a metalloproteinase inhibitor, Batimastat (30 mg/kg body wt, daily ip injection). Hypoxia-induced increases in concentration of collagen breakdown products and in collagenolytic activity in pulmonary vessels were inhibited by Batimastat, attesting to the effectiveness of Batimastat administration. Batimastat markedly reduced hypoxic pulmonary hypertension: pulmonary arterial blood pressure was 32 ± 3 mmHg in hypoxic controls, 24 ± 1 mmHg in Batimastat-treated hypoxic rats, and 16 ± 1 mmHg in normoxic controls. Right ventricular hypertrophy and muscularization of peripheral lung vessels were also diminished. Batimastat had no influence on systemic arterial pressure or cardiac output and was without any effect in rats kept in normoxia. We conclude that stimulation of collagenolytic activity in chronic hypoxia is a substantial causative factor in the pathogenesis of pulmonary vascular remodeling and hypertension.

chronic hypoxia; vascular remodeling; collagen

BY INDUCING STRUCTURAL REMODELING of peripheral pulmonary vasculature, chronic hypoxia causes pulmonary hypertension (for review, see Ref. 51). Growth and proliferation of vascular smooth muscle cells and fibrotization of the walls of prealveolar vessels reduce both vascular compliance and total cross-sectional area of the pulmonary vascular bed. The resulting rise in pulmonary vascular resistance elevates pulmonary arterial blood pressure (PAP). Right ventricular hypertrophy develops as a consequence of chronic pressure overload.

The pathogenesis of structural remodeling of peripheral lung arteries is closely related to the alteration of collagen metabolism by lung hypoxia (reviewed in Ref. 17). Accumulation of collagen and increase of its turnover in lung conduit vessels during pulmonary hypertension have been recognized for a long time (8, 9). More recently, collagenolytic activity was also found increased in peripheral pulmonary vessels of chronically hypoxic rats (40). The only enzymes capable of initiating the breakdown of collagen are specific matrix metalloproteinases (MMP) (for review, see Ref. 41), and we were able to confirm elevated MMP activity in resistance lung arteries during hypoxia (40).

Although intriguing, these data are not sufficient to determine whether the increased collagenolysis has any pathogenetic role in the mechanism of pulmonary hypertension. In essence, one of three possibilities can be expected. First, collagenolysis occurs in parallel to the mechanism of pulmonary hypertension and has no role in it. Second, elevated collagenolytic activity opposes the increased collagen synthesis and thus acts as a negative feedback mechanism limiting the development of pulmonary hypertension. Third, accelerated collagenolysis promotes proliferation of the vascular wall components and thus contributes to the progression of pulmonary hypertension.

As counterintuitive as this third hypothesis may seem in a condition characterized by extracellular matrix accumulation, a mechanism for such an effect is plausible. Accelerated collagen turnover is known to be capable of activating mesenchymal cell proliferation. This effect may be either direct, via growth-promoting properties of products of collagen degradation (4), or indirect, by releasing growth-stimulating cytokines from their bond to extracellular matrix proteins (56). Moreover, indirect support for the idea that increased collagenolysis promotes pulmonary hypertension development comes from an extensive set of studies of the role of another important component of extracellular matrix, elastin. Elastolytic activity is elevated during the development of chronic hypoxic pulmonary hyper-

Address for reprint requests and other correspondence: J. Herget, Dept. of Physiology, Charles Univ. Second Medical School, Plzeňská 130/221, 15000 Praha 5, Czech Republic (E-mail: jan.herget@lfmotol.cuni.cz).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

tension, and blockade of this activity inhibits pulmonary hypertension (reviewed in Ref. 48).

For these reasons, we hypothesized that an increase in collagen breakdown is an essential event leading to vascular wall remodeling in the chronic hypoxic model of pulmonary hypertension. To test this hypothesis, we used a specific MMP inhibitor, Batimastat (61), in chronically hypoxic rats. Batimastat was previously used for a successful inhibition of injury-induced vascular remodeling in systemic arteries (7, 28, 61). We reasoned that if increased collagenolytic activity is indeed essential in the process of pulmonary vascular remodeling, then indexes of pulmonary hypertension should be diminished by Batimastat treatment. The results were reported in a preliminary form (42).

METHODS

Experiments were performed in accordance with European Community and National Institutes of Health guidelines for using experimental animals. All procedures were approved by our institution's Animal Studies Committee. Four groups of adult male Wistar specific pathogen-free rats (Anlab, Prague, Czech Republic) were used. Two groups were placed in an isobaric hypoxic chamber (fraction of inspired oxygen = 0.1) for 3 wk (16), and two groups were kept in atmospheric air. Rats in one of the hypoxic groups were given intraperitoneal injections of Batimastat (30 mg/kg body wt) on each day of the hypoxic exposure (HB group, $n = 22$). The second hypoxic group served as vehicle control: it was treated identically as the HB group but received only the solvent for Batimastat (phosphate buffer plus 0.01% Tween 80) (61) (HC group, $n = 25$). To ensure exactly identical hypoxic conditions, we exposed all rats of both hypoxic groups to hypoxia together at the same time. Of the normoxic groups kept in air, one was treated with daily injections of Batimastat for 3 wk (NB group, $n = 15$), and another received only the vehicle (NC group, $n = 23$).

After 3 wk of Batimastat or vehicle treatment, the rats were removed from the hypoxic chamber and anesthetized with pentobarbital (25 mg/kg body wt ip). Each group was divided into three subgroups. One subgroup was used for hemodynamic and morphological measurements, another for collagen analysis in peripheral lung vessels, and the third one for collagenolytic activity determination.

The hemodynamic study started with tracheal intubation and pulmonary artery catheterization while the rat spontaneously breathed room air. The internal diameter of a 30-mm-long tracheal tube was 2.1 mm. A specially shaped polyethylene catheter (1.1 mm outer and 0.75 inner diameter) was used to measure PAP (21). Another cannula (0.9 mm inner diameter) was introduced into the carotid artery to measure systemic arterial blood pressure and, subsequently, to take a blood sample for hematocrit determination. After stable readings of both pressures were obtained, mechanical ventilation with air was begun at ~60 breaths/min (10 cmH_2O peak inspiratory pressure, 0 cmH_2O end expiratory pressure). The chest was opened at midline with extra care taken to minimize bleeding. Blood flow rate in the ascending aorta was then measured with an ultrasonic flow meter (T106 + 2.5 mm SS-series flow probe with J-reflector; Transonic Systems, Ithaca, NY) as an estimate of cardiac output. This value relative to body weight is referred to as cardiac index. Although the absolute values obtained with this method are lower than cardiac output in vivo due to the anesthesia and especially the thoracotomy, the almost identical values found in the Batimastat-treated and control rats (Table 1) permit exclusion of major differences in cardiac output as a source of the difference in PAP. Numbers of animals with all hemodynamic measurements successfully completed are given in Table 1.

After completion of the hemodynamic measurements, we killed the anesthetized rats by removing the heart and lung. The heart was dissected, and the right and left ventricles and septum were separately weighed (13). Right ventricle to left ventricle plus septum weight ratio (RV/LV+S) was used as an index of right ventricular hypertrophy associated with pulmonary hypertension. The lungs were filled through the trachea with neutral formol solution at a pressure of 12 cmH_2O and then placed in the same solution for several days. Lung sections were then cut and stained by the hematoxylin resorcin fuchsin method. Because one important aspect of lung vascular remodeling in pulmonary hypertension is a rise in proportion of peripheral vessels that contain a muscular layer in their wall, we assessed the remodeling by counting distal vessels bound to alveolar ducts or to alveoli ($\leq$300 μm) on one slide of each rat and determining how many of them were muscularized (23). All peripheral pulmonary blood vessels found in sagittal sections through the hilus region of the right and left lungs were counted. All counts were performed by one person blinded to the denomination of the slides. The number of counted vessels was 74–134 (range) in each rat. The result is reported as percentage of double-laminated peripheral vessels (%DL), reflecting the fact that the muscular layer is enclosed between two elastic laminae, whereas the nonmuscularized vessels have only one lamina (23).

We used six animals of each group to analyze collagen proteins in extracts from peripheral pulmonary arteries. Lungs were removed from anesthetized rats, and peripheral pulmonary arteries (3rd and 4th intrapulmonary branches)

Table 1. *Body weight, hemodynamics, and hematocrit*

Group	n	BW, g		LV+S/BW, mg/g	SAP, mmHg	CO, ml/min	CI, ml·min^{-1}·100 g^{-1}	Hematocrit, %
		Start	End					
NC	9	259 ± 14	341 ± 7	2.1 ± 0.06	66 ± 9	36.7 ± 6.8	10.6 ± 0.9	0.47 ± 0.01
NB	8	254 ± 11	314 ± 10†	1.9 ± 0.13	73 ± 9	32.0 ± 4.5	9.4 ± 0.4	0.42 ± 0.01
HC	7	234 ± 11	234 ± 11*	2.4 ± 0.12	72 ± 6	25.2 ± 2.4*	14.0 ± 1.9*	0.69 ± 0.02*
HB	6	248 ± 19	197 ± 4*	2.5 ± 0.06	82 ± 12	25.4 ± 1.4*	13.9 ± 0.8*	0.62 ± 0.02*†

The data are means ± SE. NC, group kept in normoxia treated with vehicle; NB, group kept in normoxia treated with Batimastat; HC, group exposed to hypoxia treated with vehicle; HB, group exposed to hypoxia treated with Batimastat; n, number of successfully completed measurements; BW, body weight; LV+S/BW, left heart ventricle plus septum wet weight relative to BW; SAP, systemic arterial blood pressure; CO, cardiac output estimated from ascending aorta blood flow; CI, estimate of cardiac index (CO/BW); Start and End, weight at the beginning and end, respectively, of Batimastat or vehicle treatment. *$P < 0.05$ hypoxic groups differs from corresponding normoxic group; †$P < 0.05$ Batimastat-treated group differs from corresponding vehicle-treated group.

were dissected under a dissecting microscope as previously described (40). The collagen fraction was isolated by limited pepsin digestion (40). Pepsin digest solution was centrifuged (15 000 *g*, 30 min), and the supernatant was lyophilized and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (32, 40).

In seven animals of the NC, HC, and HB groups, the collagenolytic enzymes in the dissected peripheral pulmonary arteries were analyzed by zymography (34). Briefly, peripheral pulmonary arteries were extracted in SDS-PAGE sample loading buffer (40% 0.5 M Tris·HCl, 10% SDS, 10% glycerol, and 4% bromphenol blue) for 20 h at 4°C. The extraction volume was 100 μl/μg dry tissue weight. After extraction of the tissue samples in the extracting buffer, they were centrifuged (14 000 *g*, 40 min) and separated on 10% SDS-PAGE containing 0.1% gelatin. To remove SDS, we washed the gels for 30 min in 2.5% (vol/vol) Triton X-100 and removed the Triton by washing the gels with distilled water and incubation buffer (50 mM Tris·HCl, pH 7.8; 10 mM $CaCl_2$; and 10 mM NaCl). The gels were then incubated in the incubation buffer (17 h, 37°C) and then stained with 0.25% Coomassie brilliant blue R in methanol-acetic acid-water (40:10:50 vol/vol/vol). Because gelatin substrate was used, the zymography preferentially targeted MMPs with gelatinolytic activity. The amount of each loaded sample was standardized to the dry weight of lyophilized tissue of peripheral pulmonary arteries. A high-molecular-weight calibration kit from Pharmacia Biotech (Uppsala, Sweden) was used as standard. All individual protein bands loaded in the gels were analyzed densitometrically using custom software (Jiří Semecký, Prague, Czech Republic).

Western blot analysis of collagen proteins and MMP-13. Collagen type I origin of the peptides occurring in extracts from peripheral pulmonary arteries of hypoxic rats was confirmed by Western blot analysis in three additional rats exposed to similar hypoxia (5 days) and one normoxic control rat. After SDS-PAGE electrophoretic separation on discontinuous slab gel using 4% stacking gel and 7.5% separating gel, proteins were electrotransferred to nitrocellulose membrane (Immobilon-NC, Millipore, Bedford, MA) in 15 mM sodium borate buffer, pH 9.3, 4°C, for 24 h in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Starting transfer power conditions were 15 V/250 mA; finishing conditions were 15 V/350 mA. The blots were blocked with 10% skim milk-PBS overnight. Polyclonal rabbit antibodies to rat collagen type I (catalogue no. 2150–1980, ANAWA Switzerland) were diluted 1:50 in 2% skim milk-PBS, and nitrocellulose membrane was incubated in this solution for 1 h at room temperature and then washed with 2% skim milk-PBS. Membrane was then incubated in horseradish peroxidase (HRP)-conjugated anti-swine rabbit antibody solution (USOL, Prague, Czech Republic) 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 methanol, 20 ml 10 mM Tris·HCl, and 0.01% H_2O_2) for 15 min. The reaction was allowed to proceed in the dark for 15 min until all bands were visualized. The membrane was then air-dried.

To assess possible hypoxic alterations of the principal enzyme initiating collagen breakdown, the interstitial collagenase (MMP-13 in the rat), we used Western blot to detect MMP-13 protein in extracts from peripheral pulmonary arteries of three per group. One microgram of MMP-13 proenzyme (Calbiochem, La Jolla, CA) was used as a standard. After SDS-PAGE separation on a discontinuous slab gel using 4% stacking gel and 10% separating gel, proteins were electrotransferred to a nitrocellulose membrane in 15 mM sodium borate buffer, pH 9.3, 4°C, for 1 h. Starting transfer power conditions were 35 V/350 mA; finishing conditions were 35 V/250 mA. The blots were blocked with 10% skim milk-PBS overnight. Monoclonal antibody to MMP-13 (MMP-13 Ab-1, Calbiochem) was diluted 1:100 in 2% skim milk-PBS, and the nitrocellulose membrane was incubated in this solution overnight at 4°C. The blots were then washed with 2% skim milk-PBS and incubated with secondary antibody (HRP-conjugated swine anti-mouse antibodies solution; USOL) diluted 1:250 for 1 h at room temperature. After washing, the membrane was stained with HRP substrate for 15 min.

Chemicals. Batimastat [BB-94, (4-*N*-hydroxyamino)-2R-isobutyl-3S-(thienyl-thiomethyl)-succinyl-L-phenylalanine-*N*-methylamide] was provided by British Biotechnology (Oxford, UK). Other reagents and drugs were from Sigma (Prague, Czech Republic) unless specified otherwise. All chemicals were of a highest available purity.

Statistical analyses. The data were statistically evaluated by ANOVA and Scheffé's post hoc test (StatView 5.0; SAS Institute, Cary, NC). The results are presented as means ± SE. Differences were considered significant at $P < 0.05$.

RESULTS

At the beginning of treatment, the groups did not differ in body weight (Table 1). During the 3 wk of Batimastat or vehicle treatment, both groups kept in normoxia gained weight. In contrast, both groups exposed to hypoxia lost weight (Table 1). Although the rats of the NB group were slightly lighter on the day of the measurements than the NC group, there was no significant difference in body weight between the hypoxic groups (Table 1). One rat of the HB group died after 9 days of exposure to hypoxia; there was no mortality in other groups.

PAP was significantly higher in rats exposed to hypoxia than in rats kept in air (Fig. 1*A*). In normoxia, there was no difference in PAP between Batimastat- and vehicle-treated rats. In contrast, the HB group had significantly lower PAP than the HC rats (Fig. 1*A*). This effect of Batimastat in hypoxia was selective for the pulmonary circulation, as the systemic arterial pressure did not differ among the groups (Table 1) and was not due to alterations in cardiac output or index, since our estimates of either did not differ between the HC and HB groups (Table 1).

The reducing effect of Batimastat on PAP in hypoxia was in accord with its effect on pulmonary vascular remodeling. Batimastat treatment during exposure to hypoxia partly prevented the typical thickening of the walls of peripheral pulmonary arteries: %DL was elevated significantly less in the HB than in the HC group (Fig. 1*B*).

Right ventricular hypertrophy accompanying pulmonary hypertension of chronic hypoxia was markedly smaller in rats treated with Batimastat than in vehicle-treated ones (Fig. 1*C*). In fact, RV/LV+S was not significantly higher in the HB group than in the normoxic controls (Fig. 1*C*). Left ventricle plus septum weight was not affected by Batimastat treatment (Table 1).

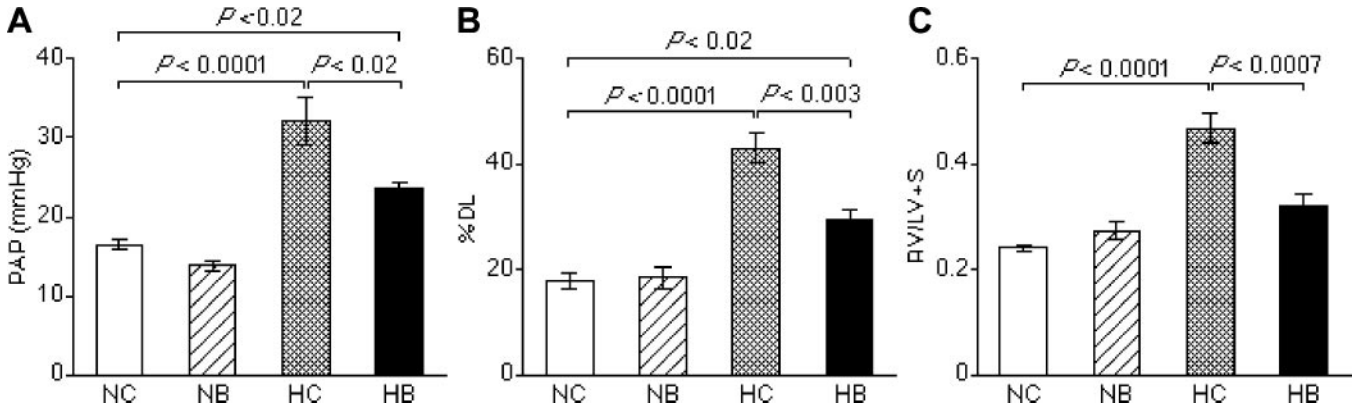

Fig. 1. Hypoxic pulmonary hypertension is attenuated by metalloproteinase inhibition by Batimastat. Pulmonary arterial blood pressure (PAP, *A*), percentage of muscularized (double-laminated) peripheral pulmonary vessels (%DL, *B*), and relative weight of the right heart ventricle [expressed as right ventricle to left ventricle plus septum weight ratio (RV/LV+S), *C*] are all elevated by chronic hypoxia. These increases are significantly reduced by concomitant treatment with a synthetic inhibitor of matrix metalloproteinases, Batimastat. HB, hypoxia and Batimastat treatment group; HC, hypoxic control group; NB, normoxia and Batimastat treatment group; NC, normoxic control group. None of the parameters was affected by Batimastat treatment in normoxia. The data are means ± SE.

Both of the hypoxic groups had higher values of hematocrit than their corresponding normoxic controls (Table 1). The normoxic groups did not differ significantly in hematocrit one from another. Of the hypoxic groups, hematocrit was slightly but significantly lower in rats treated with Batimastat than in the vehicle-treated animals (Table 1).

Analysis of collagenous proteins in the peripheral pulmonary arteries confirmed our previous finding (40) of a typical 76-kDa collagen breakdown product in rats exposed to hypoxia (HC group), which was not present in any of the normoxic groups (Fig. 2). Western blot analysis of extracts from peripheral pulmonary arteries isolated from rats exposed to hypoxia (for 5 days) identified the low-molecular-weight peptide seen in the group exposed to hypoxia as a collagen type I product (Fig. 3). The appearance of this breakdown product in small lung arteries during chronic hypoxia was prevented by Batimastat treatment (Fig. 2).

Collagenolytic activity and its inhibition by Batimastat were documented by zymographic analysis of extracts from peripheral pulmonary arteries on gelatin substrate (Fig. 4). The lytic zones were ascribed to gelatinase A (MMP-2) and its proenzyme on the basis of substrate specificity and molecular weight. Densitometric analysis of the lytic zones showed that chronic hypoxia markedly stimulated MMP-2 (HC group greater than NC group), but this effect was completely

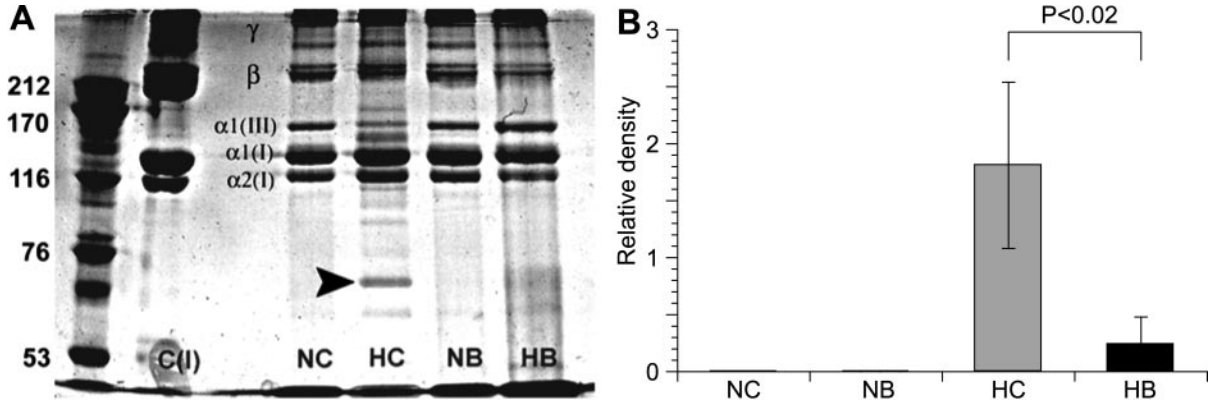

Fig. 2. Collagenous extracts from peripheral pulmonary arteries of hypoxic rats contain a low-molecular-weight peptide that is not present in extracts of normoxic rats or of hypoxic rats treated with Batimastat. *A*: an example of electrophoretic profile representative of 6 similar ones; each lane contains extract from 1 rat's peripheral pulmonary arteries. *Lane 1*, molecular mass markers; *lane 2*, C (I) = collagen type I standard; *lane 3*, normal control rat (NC group); *lane 4*, hypoxic vehicle-treated rat (HC group); *lane 5*, normoxic rat treated with Batimastat (NB group); *lane 6*, hypoxic rat treated with Batimastat (HB group). γ, γ-fraction (chain polymers, collagen type I and III); β, β-fraction (chain dimers, collagen type I and III); α_1, mixture of individual α_1-chains (collagen type I and III); α_2, α_2-chain (collagen type I); arrow, small peptide present in extracts from hypoxic, but not normoxic, peripheral pulmonary arteries. *B*: relative density of the low-molecular-mass collagenous peptide (molecular mass 76 kDa, arrow in *A*). The peptide was undetectable in both of the normoxic groups. The data are means ± SE (n = 6/group).

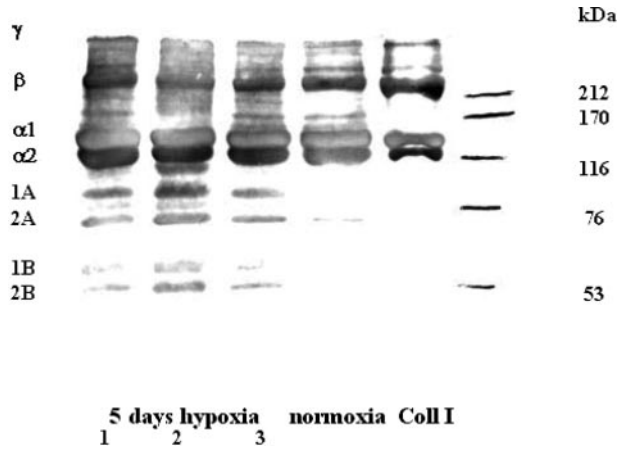

Fig. 3. Western blot analysis demonstrates collagenous origin of peptides found in extracts from peripheral pulmonary arteries of hypoxic rats. Western blot analysis of collagen protein in the extracts from the peripheral pulmonary arteries (PPA) isolated from rats exposed to hypoxia for 5 days (*lanes 1–3*) and from a normoxic rat (*lane 4*). Coll I, collagen type I standard (Sigma); γ, chain polymers; β, collagen chain dimers; α_1 and α_2, individual collagen chains; 1A and 2A, ¾ fragments of α_1 and α_2 chains; 1B and 2B, ¼ fragments of α_1 and α_2 chains. The lane at far *right* is a molecular mass standard.

prevented by Batimastat (Fig. 4*B*). Pro-MMP-2 was higher in the HC group than in normoxic controls (Fig. 4*B*). The density of lytic zones in the HB group did not significantly differ from both the HC and NC groups (Fig. 4*B*). Compared with the normoxic controls, pro-MMP-9 was elevated in the hypoxic control rats but not in the hypoxic, Batimastat-treated group. MMP-9 was not detected.

Both chronic hypoxic exposure and Batimastat treatment altered the expression of rat interstitial collagenase, MMP-13, in peripheral pulmonary arteries (Fig. 5). MMP-13 was the most abundant in vehicle-treated rats exposed to hypoxia. Batimastat treatment reduced MMP-13 expression.

DISCUSSION

The main finding of this study is that MMP inhibition by Batimastat treatment markedly attenuates the development of hypoxic pulmonary hypertension. Chronic hypoxia-induced increases in PAP, peripheral pulmonary vascular muscularization, and right ventricle weight were all significantly smaller in rats treated with Batimastat than in rats treated with vehicle (Fig. 1). Importantly, we were able to document the effectiveness of the chosen Batimastat dose and route of administration in inhibiting collagenolysis. Together, these data show that the previously reported stimulation of collagen breakdown by MMPs in hypoxic pulmonary vessels (40) plays a key pathogenetic role in the mechanism of pulmonary hypertension.

We reported previously that collagen extracts from peripheral pulmonary arteries of hypoxic rats contain a low-molecular-weight peptide that is not present in peripheral pulmonary vessels of normoxic rats (40). This finding was confirmed in the present study (Fig. 2*A*, arrow). The molecular mass of this peptide (~76 kDa) corresponds to three-fourths of the molecule of native collagen and thus suggests that it is a product of cleavage by interstitial collagenase, because this MMP cleaves collagen at approximately one-fourth of the collagen molecule length (reviewed in Ref. 41). The collagen type I origin of this peptide was confirmed by immunoblotting (Fig. 3) and by amino terminal peptide sequencing (12, 40). The increased collagenolytic activity, strongly implicated by those data, was subsequently directly proved by zymography (40). These findings were confirmed in the present study by showing the appearance of a collagen breakdown product (Fig. 2) and increased collagenolytic activity (Fig. 4) in peripheral pulmonary arteries of the hypoxic, vehicle-treated rats but not of the normoxic groups. Importantly, the collagen breakdown peptide was absent, and the hypoxic increase in collagenolytic activity was markedly and significantly reduced in hypoxic rats treated with Batimastat, attesting to the effectiveness of Batimastat administration.

The conclusions of this study fit with previous reports showing the importance of extracellular matrix alterations in the mechanism of pulmonary hypertension. Increased pulmonary vascular collagen synthesis and collagen accumulation in pulmonary hypertension have been well documented (8–10, 29, 30, 46, 47, 54). This process plays a causative role in the pathogenesis of pulmonary hypertension, as shown by studies where inhibition of collagen synthesis (by β-aminopropionitrile or cis-4-hydroxy-L-proline) partly reduced chronic hypoxic pulmonary hypertension (29, 30, 46). Chronic hypoxia stimulates not only synthesis but also metabolic turnover of collagen (8). In addition to collagen, synthesis of other components of extracellular matrix, including elastin, is also accelerated in pulmonary hypertension (10, 37, 48). Elevated elastin synthesis in chronic hypoxic pulmonary hypertension is associated with accelerated elastolysis, and pulmonary hypertension can be reduced by elastase blockade (reviewed in Ref. 48). It is also useful to note that there is extensive evidence from systemic vessels that extracellular matrix degradation in general and collagenolysis by MMPs in particular have an essential role in vascular remodeling after injury (6, 53, 61). Pulmonary vascular remodeling in chronic hypoxia has many features of injury repair (reviewed in Ref. 16).

Our results differ in several important ways from those recently reported by Vieillard-Baron et al. (59). In that study, viral transfer of the human gene for tissue inhibitor of MMPs (TIMP-1), as well as treatment with a broad-spectrum MMP blocker, doxycycline, augmented several, but not all, indexes of hypoxic pulmonary hypertension in rats. PAP was not significantly altered by TIMP-1 transfection, whereas right ventricular hypertrophy and lung vascular remodeling were augmented. Conversely, doxycycline increased PAP by ~20% but did not significantly affect right ventricular hypertrophy. Remodeling of peripheral pulmonary vasculature was enhanced by doxycycline. Importantly, although collagenolytic activity appeared reduced by TIMP-1 transfection and doxycy-

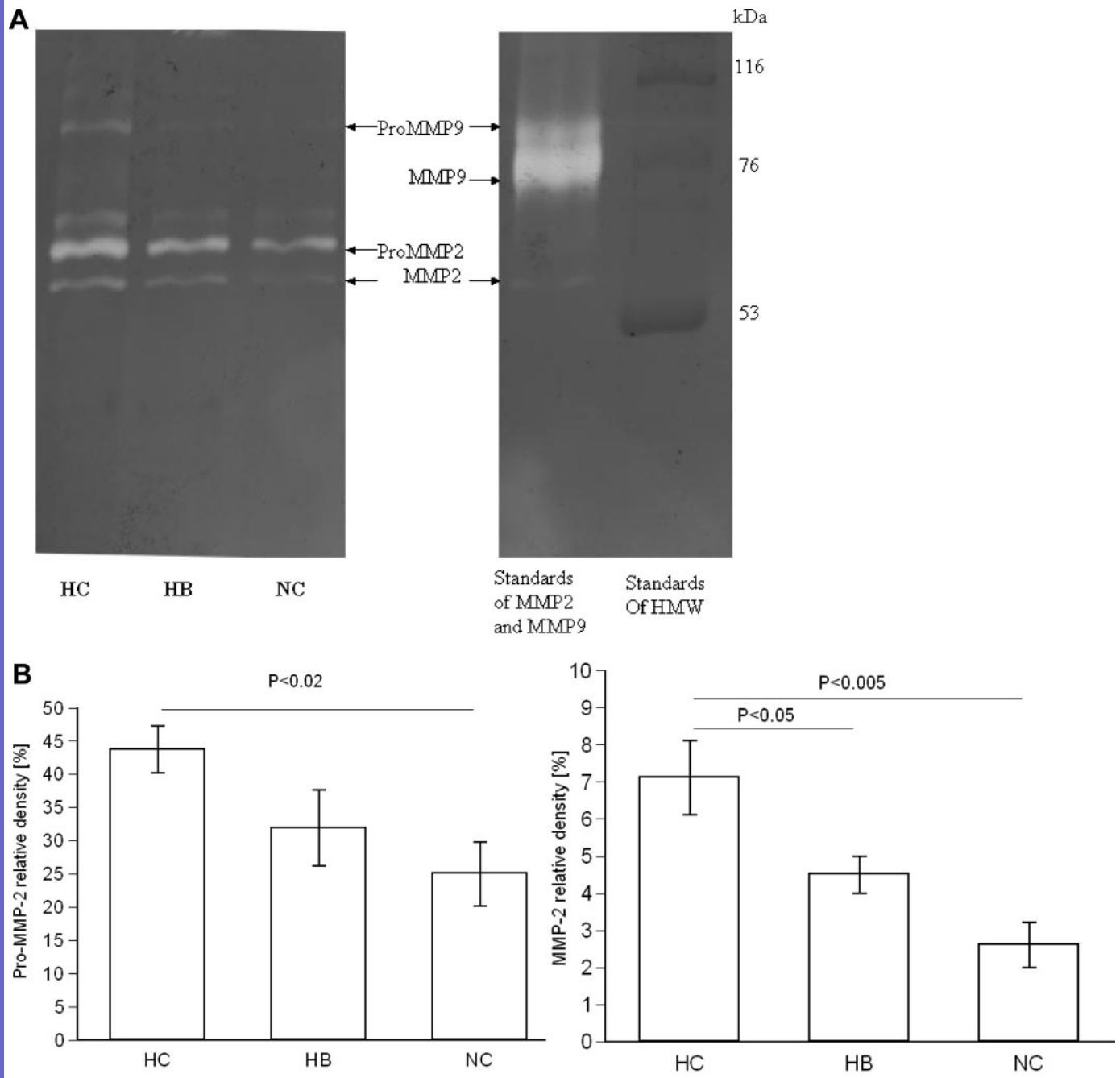

Fig. 4. Gelatin zymography of extracts from peripheral lung arteries. *A*: an example of gelatin zymography representative of 7 similar ones; each lane contains extract from 1 rat's peripheral pulmonary arteries. Lanes are, from the *left*: hypoxic vehicle-treated rat (HC group), hypoxic rat treated with Batimastat (HB group), normal control rat (NC group), standards of matrix metalloproteinase (MMP)-9 and MMP-2, and high-molecular-weight (HMW) standards. Bands were identified as gelatinase A (MMP-2) and its proenzyme (pro-MMP-2) and proenzyme of gelatinase B (pro-MMP-9) on the basis of substrate specificity and molecular weight. *B*: relative densities of lytic zones corresponding to pro-MMP-2 (*left*) and MMP-2 (*right*) in zymographs of extracts from peripheral pulmonary arteries. Note that absolute values of density are shown, meaning that higher numbers represent higher MMP activity. All data are means ± SE (n = 7/group).

cline treatment when evaluated with nonquantitative in situ zymography, several quantitative methods showed that collagenolytic activity was not significantly reduced by either treatment. Although we do not know why our findings so sharply contradict those of Vieillard-Baron et al. (59), we believe MMP inhibition was better defined in our experiment, as shown by our analyses of collagen breakdown products and collagenolytic activity.

Although the paper of Vieillard-Baron et al. (59) favors the concept that collagenolysis opposes the development of hypoxic pulmonary hypertension, our

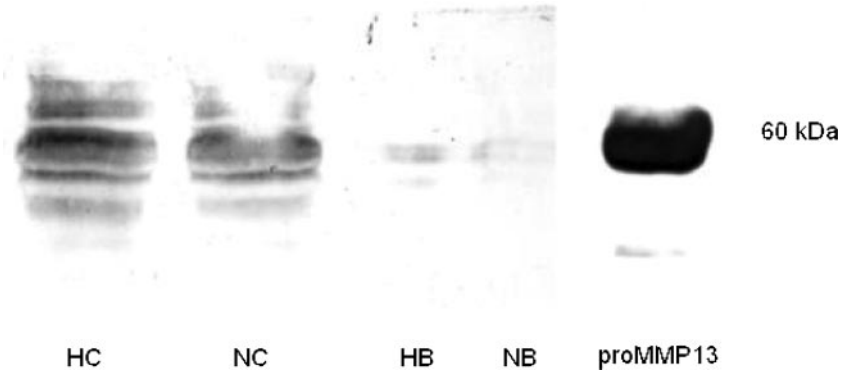

Fig. 5. Interstitial collagenase, MMP-13, detected by immunoblotting in extracts from peripheral pulmonary arteries, is elevated by hypoxia and reduced by Batimastat. Each lane contains a sample from 1 rat and is representative of 3 similar immunoblots for each group.

current results support an opposite view, i.e., that collagenolysis is one of the causes promoting structural remodeling of peripheral pulmonary arteries in pulmonary hypertension. This latter concept is indirectly supported by numerous reports showing that transfecting injured systemic arteries with genes for TIMPs inhibits rather than stimulates their remodeling (for review, see Ref. 34).

Although there is consensus that hypoxia stimulates MMP activity, the mechanism of this effect remains to be elucidated. As discussed extensively elsewhere (17, 24), an intriguing possibility involves MMP activation by peroxynitrite. Initial stages of hypoxic injury to pulmonary vascular tissue are associated with augmented production of reactive oxygen species (such as superoxide) (24, 25, 39) and nitric oxide (for review, see Ref. 17). Superoxide and nitric oxide interact extremely rapidly to form peroxynitrite, which is capable of activating tissue collagenolytic metalloproteinases (49). Serum nitrotyrosine levels, used as a marker of peroxynitrite production (5), are elevated in rats exposed to several days of hypoxia (24). In addition, reactive oxygen species themselves can also control MMP production (31).

We tested the effectiveness of our Batimastat dose and route of administration against MMPs by zymography. This method demonstrated that hypoxia elevated and Batimastat reduced the lytic zones produced on zymography by MMP-2 and, in part, pro-MMP-2 (Fig. 4). It is generally accepted that Batimastat inhibits the activity of various metalloproteinases by binding to their active site (11). It is unlikely, however, that this binding would resist the zymography procedure, particularly the SDS-PAGE. Therefore our data suggest that Batimastat treatment may affect not only the enzymatic activity but also the expression, secretion, and/or degradation of metalloproteinases. In support of this view, Makela et al. (35) reported a positive correlation between zymography and Western blot analysis of MMP-2 and MMP-9 in isolated keratinocytes treated with various synthetic metalloproteinase inhibitors. Li et al. (33), on the other hand, found MMP-2 and -13 inhibition on zymography after Batimastat but no change on Western blot. Although MMP-2 has been shown to be capable of cleaving native collagen molecules in vitro (1, 44), the consensus is that the principal enzyme responsible for initiation of collagen breakdown in vivo is interstitial collagenase, i.e., MMP-13 (rodent-like interstitial collagenase) in the case of the rat (36). Assessment of MMP-13 by zymography was not possible in our study due to the inherently low yield from small pulmonary arteries of individual rats. However, as Batimastat is well known as a broad-spectrum MMP inhibitor, the finding of its effectiveness against MMP-2 can be extrapolated to other MMPs. Using Western blot analysis, we found that MMP-13 protein levels were reduced by Batimastat in both normoxic and hypoxic rats. Thus both our zymography and immunoblotting data support our view that chronic hypoxia increases MMP-2, -9, and -13 protein levels and that this elevation can be partly prevented by Batimastat treatment.

The cellular sources of MMP activity involved in the mechanism of pulmonary vascular remodeling and hypertension may include vascular smooth muscle and mast cells (26, 57). We have preliminary data from quantitative histology showing accumulation of mast cells with massive expression of MMP-13 in the walls of prealveolar arteries of hypoxic rats (58). Accumulation of vascular smooth muscle has been recognized as a typical feature of hypoxic pulmonary vascular remodeling for a long time (51). Because collagenolysis is an important part of cell migration, we reasoned that MMP production by migrating smooth muscle and/or mast cells in hypoxia may be reduced by Batimastat. Indeed, that is what our immunoblotting data indicate (Fig. 5). Although these data are not quantitative, MMP-13 expression appears elevated by chronic hypoxia and reduced by Batimastat. Therefore, one mechanism whereby collagenolysis contributes to pulmonary vascular remodeling in hypoxia may be by enabling mast cells and/or mesenchymal cells to migrate into the walls of peripheral pulmonary arteries injured by hypoxia, thus promoting mesenchymal proliferation. In addition, collagen breakdown may be related to cellular proliferation through stimulatory effects of low-molecular-mass collagen breakdown products (2, 4, 14, 15), altered adherence of mesenchymal cells to the modified collagen (3), or lower binding of cytokines and growth factors to altered matrix proteins (56, 60). Moreover, proteolysis of collagen by MMPs is essential for initiating alternative forms of cell behavior, includ-

ing increased production of extracellular matrix components that stimulate smooth muscle growth and migration (27, 55).

Besides inhibition of MMP activity, additional mechanisms may contribute to the observed reduction of pulmonary hypertension by Batimastat treatment. In a complementary experiment, we excluded the possibility that Batimastat treatment alters hypoxic vasoreactivity of pulmonary blood vessels. We used isolated blood perfused lungs (19) from control rats ($n = 5$) and rats given Batimastat for 10 days ($n = 6$) in the same dose as in the main experiment (both lung and blood donors). We did not find any significant effect of Batimastat on the magnitude of hypoxic vasoconstrictor responses to acute hypoxic challenges (5 or 3% O_2) (data not shown).

Batimastat can inhibit the secretase responsible for proteolytic cleavage of membrane-bound angiotensin-converting enzyme (43), possibly resulting in lower local angiotensin II concentration. Angiotensin II involvement in the mechanism of pulmonary hypertension is supported by several lines of evidence (38, 62), including a demonstration that inhibition of angiotensin-converting enzyme attenuates experimental pulmonary hypertension (22).

Elevated blood viscosity due to marked polycythemia should contribute to the increased PAP in chronic hypoxia, and thus the slightly lower hematocrit in the Batimastat-treated hypoxic rats compared with the vehicle-treated hypoxic group may have contributed to their diminished PAP. However, although statistically significant, the difference in hematocrit between the HC and HB groups does not appear large enough to account for most of the difference in PAP. We found no significant correlation between hematocrit and PAP or RV/LV+S in our experimental groups (analysis not shown). Numerous experiments with animals made polycythemic show that hematocrit has only a minor importance in setting the magnitude of pulmonary hypertension (for review, see Ref. 20). Petit and coworkers (45) demonstrated that augmentation of polycythemia does not worsen hypoxic pulmonary hypertension in rats.

Our observation of the slightly reduced hematocrit in hypoxic rats treated with Batimastat corresponds to a similar observation in cancer patients treated with another MMP inhibitor, BAY12–9566 (18). This effect of MMP inhibitors may be related to the evidence that secretion of MMPs by differentiating erythroid cells is important in their maturation (50). MMP secretion is stimulated by erythropoietin (52), possibly explaining why we observed an effect of Batimastat on hematocrit only in hypoxia, whereas a similar tendency in normoxic rats did not reach statistical significance.

In conclusion, the present study shows that treatment of rats with a synthetic MMP inhibitor, Batimastat, during their exposure to chronic hypoxia prevents most of a typical increase in collagenolytic activity in peripheral pulmonary arteries. It also prevents much of the usual pulmonary vascular remodeling and PAP elevation. Increased MMP activity thus represents a substantial factor mediating the effect of hypoxia on the development of pulmonary hypertension.

Batimastat was a gift from British Biotech Pharmaceuticals (Oxford, UK). The work was supported by the Grant Agency of the Czech Republic (grants 305/97/S070 and 305/00/1432), by the Internal Grant Agency of the Czech Ministry of Health (grant NA/5681–3/99), and by Second Medical Faculty Research Project 111300002.

REFERENCES

1. **Aimes RT and Quigley JP.** Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem* 270: 5872–5876, 1995.
2. **Bačáková L, Herget J, and Wilhelm J.** Influence of macrophages and macrophage-modified collagen I on the adhesion and proliferation of vascular smooth muscle cells in culture. *Physiol Res* 48: 341–351, 1999.
3. **Bačáková L, Lisá V, Kubínová L, Wilhelm J, Novotná J, Eckhart A, and Herget J.** Ultraviolet light-irradiated collagen III modulates expression of cytoskeletal and surface adhesion molecules in rat aortic smooth muscle cells in vitro. *Virchows Arch* 440: 50–62, 2002.
4. **Bačáková L, Wilhelm J, Herget J, Novotná J, and Eckhart A.** Oxidized collagen stimulates proliferation of vascular smooth muscle cells. *Exp Mol Pathol* 64: 185–194, 1997.
5. **Beckman JS and Koppenol WH.** Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am J Physiol Cell Physiol* 271: C1424–C1437, 1996.
6. **Bendeck MP, Zempo N, Clowes AW, Galardy RE, and Reidy MA.** Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circ Res* 75: 539–545, 1994.
7. **Bigatel DA, Elmore JR, Carey DJ, Cizmeci-Smith G, Franklin DP, and Youkey JR.** The matrix metalloproteinase inhibitor BB-94 limits expansion of experimental abdominal aortic aneurysms. *J Vasc Surg* 29: 130–138, 1999.
8. **Bishop JE, Guerreiro D, and Laurent GJ.** Changes in the composition and metabolism of arterial collagens during the development of pulmonary hypertension in rabbits. *Am Rev Respir Dis* 141: 450–455, 1990.
9. **Botney MD, Liptay MJ, Kaiser LR, Cooper JD, Parks WC, and Mecham RP.** Active collagen synthesis by pulmonary arteries in human primary pulmonary hypertension. *Am J Pathol* 143: 121–129, 1993.
10. **Crouch EC, Parks WC, Rosenbaum JL, Chang D, Whitehouse L, Wu L, Stenmark KR, Orton EC, and Mecham RP.** Regulation of collagen production by medial smooth muscle cells in hypoxic pulmonary hypertension. *Am Rev Respir Dis* 140: 1045–1051, 1989.
11. **Denis LJ and Verweij J.** Matrix metalloproteinase inhibitors: present achievements and future prospects. *Invest New Drugs* 15: 175–185, 1997.
12. **Deyl Z, Novotná J, Mikšík I, and Herget J.** Micropreparation of tissue collagenase fragments of type I collagen in the former surfactant-peptide complexes and their identification by capillary electrophoresis and partial sequencing. *J Chromatogr A* 796: 181–193, 1998.
13. **Fulton RM, Hutchinson EC, and Jones AM.** Ventricular weight in cardiac hypertrophy. *Br Heart J* 14: 413–420, 1952.
14. **Gardi C, Pacini A, de Santi MM, Calzoni P, Viti A, Corradeschi F, and Lungarella G.** Development of interstitial lung fibrosis by long-term treatment with collagen breakdown products in rabbits. *Res Commun Chem Pathol Pharmacol* 68: 238–250, 1990.
15. **Gardi D, Calzoni P, Marcolongo P, Vavarra E, Vanni L, and Lungarella G.** Collagen breakdown and lung collagen metabolism: an in vitro study on fibroblast cultures. *Thorax* 49: 312–318, 1994.
16. **Hampl V and Herget J.** Perinatal hypoxia increases hypoxic pulmonary vasoconstriction in adult rats recovering from

chronic exposure to hypoxia. *Am Rev Respir Dis* 142: 619–624, 1990.
17. **Hampl V and Herget J.** Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. *Physiol Rev* 80: 1337–1372, 2000.
18. **Heath EI, O'Reilly S, Humphrey R, Sundaresan P, Donehower RC, Sartorius S, Kennedy MJ, Armstrong DK, Carducci MA, Sorensen JM, Kumor K, Kennedy S, and Grochow LB.** Phase I trial of the matrix metalloproteinase inhibitor BAY12–9566 in patients with advanced solid tumors. *Cancer Chemother Pharmacol* 48: 269–274, 2001.
19. **Herget J and McMurtry IF.** Dexamethasone potentiates hypoxic vasoconstriction in salt solution-perfused rat lungs. *Am J Physiol Heart Circ Physiol* 253: H574–H581, 1987.
20. **Herget J and Paleček F.** Experimental chronic pulmonary hypertension. *Int Rev Exp Pathol* 18: 347–406, 1978.
21. **Herget J and Paleček F.** Pulmonary arterial blood pressure in closed chest rats. Changes after catecholamines, histamine and serotonin. *Arch Int Pharmacodyn Ther* 198: 107–117, 1972.
22. **Herget J, Pelouch V, Kolář F, and Ošťádal B.** The inhibition of angiotensin converting enzyme attenuates the effects of chronic hypoxia on pulmonary blood vessels. *Physiol Res* 45: 221–226, 1996.
23. **Herget J, Suggett AJ, Leach E, and 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.
24. **Herget J, Wilhelm J, Novotná J, Eckhardt A, Vytásek R, Mrázková L, and Ošťádal M.** A possible role of the oxidant tissue injury in the development of hypoxic pulmonary hypertension. *Physiol Res* 49: 493–501, 2000.
25. **Hoshikawa Y, Ono S, Tanita S, Sakuma T, Noda M, Tabata T, Ueda S, Ashino Y, and Fujimura S.** Contribution of oxidative stress to pulmonary hypertension induced by chronic hypoxia. *Nippon Kyobu Shikkan Gakkai Zasshi* 33: 1169–1173, 1995.
26. **Jacob MP, Badier-Commander C, Fontaine V, Benazzoug Y, Feldman L, and Michel JB.** Extracellular matrix remodeling in the vascular wall. *Pathol Biol (Paris)* 49: 326–332, 2001.
27. **Jones PL, Crack J, and Rabinovitch M.** Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the $\alpha_v\beta_3$ integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol* 139: 279–293, 1997.
28. **Kenagy RD, Vergel S, Mattsson E, Bendeck M, Reidy MA, and Clowes AW.** The role of plasminogen, plasminogen activators, and matrix metalloproteinases in primate arterial smooth muscle cell migration. *Arterioscler Thromb Vasc Biol* 16: 1373–1382, 1996.
29. **Kerr JS, Riley DJ, Frank MM, Trelstad RL, and Frankel HM.** Reduction of chronic hypoxic pulmonary hypertension in the rat by beta-aminopropionitrile. *J Appl Physiol* 57: 1760–1766, 1984.
30. **Kerr JS, Ruppert CL, Tozzi CA, Neubauer JA, Frankel HM, Yu SY, and Riley DJ.** Reduction of chronic hypoxic pulmonary hypertension in the rat by an inhibitor of collagen production. *Am Rev Respir Dis* 135: 300–306, 1987.
31. **Kheradmand F, Werner E, Tremble P, Symons M, and Werb Z.** Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. *Science* 280: 898–902, 1998.
32. **Laemmli VK.** Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* 227: 680–685, 1970.
33. **Li YY, Kadokami T, Wang P, McTiernan CF, and Feldman AM.** MMP inhibition modulates TNF-α transgenic mouse phenotype early in the development of heart failure. *Am J Physiol Heart Circ Physiol* 282: H983–H989, 2002.
34. **Li YY, McTiernan CF, and Feldman AM.** Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling. *Cardiovasc Res* 46: 214–224, 2000.
35. **Makela M, Larjava H, Pirila E, Maisi P, Salo T, Sorsa T, and Uitto VJ.** Matrix metalloproteinase 2 (gelatinase A) is related to migration of keratinocytes. *Exp Cell Res* 251: 67–78, 1999.
36. **Massova I, Kotra LP, Fridman R, and Mobashery S.** Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J* 12: 1075–1095, 1998.
37. **Mecham RP, Whitehouse LA, Wren DS, Parks WC, Griffin GL, Senior RM, Crouch EC, Stenmark KR, and Voelkel NF.** Smooth muscle-mediated connective tissue remodeling in pulmonary hypertension. *Science* 237: 423–426, 1987.
38. **Morrell NW, Atochina EN, Morris KG, Danilov SM, and Stenmark KR.** Angiotensin converting enzyme expression is increased in small pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. *J Clin Invest* 96: 1823–1833, 1995.
39. **Nakanishi I, Tajima F, Nakamura A, Yagura SY, Ookawara T, Yamashita H, Suzuki K, Taniguchi N, and Ohno H.** Effects of hypobaric hypoxia on antioxidant enzymes in rats. *J Physiol* 489: 869–876, 1995.
40. **Novotná J and Herget J.** Exposure to chronic hypoxia induces qualitative changes of collagen in the walls of peripheral pulmonary arteries. *Life Sci* 62: 1–12, 1998.
41. **Novotná J and Herget J.** Possible role of matrix metalloproteinases in reconstruction of peripheral pulmonary arteries induced by hypoxia. *Physiol Res* 51: 323–334, 2002.
42. **Novotná J, Herget J, Bíbová J, and Hampl V.** Inhibition of collagenolytic activity suppresses hypoxic pulmonary hypertension in rats (Abstract). *Physiol Res* 46: 45P, 1999.
43. **Parvathy S, Oppong SY, Karran EH, Buckle DR, Turner AJ, and Hooper NM.** Angiotensin-converting enzyme secretase is inhibited by zinc metalloprotease inhibitors and requires its substrate to be inserted in a lipid bilayer. *Biochem J* 327: 37–43, 1997.
44. **Patterson ML, Atkinson SJ, Knauper V, and Murphy G.** Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS Lett* 503: 158–162, 2001.
45. **Petit RD, Warburton RR, Ou LC, and Hill NS.** Pulmonary vascular adaptations to augmented polycythemia during chronic hypoxia. *J Appl Physiol* 79: 229–235, 1995.
46. **Poiani GJ, Tozzi CA, Choe JK, Yohn SE, and Riley DJ.** An antifibrotic agent reduces blood pressure in established pulmonary hypertension in the rat. *J Appl Physiol* 68: 1542–1547, 1990.
47. **Poiani GJ, Tozzi CA, Yohn SE, Pierce RA, Belsky SA, Berg RA, Yu SY, Deak SB, and Riley DJ.** Collagen and elastin metabolism in hypertensive pulmonary arteries of rats. *Circ Res* 66: 968–978, 1990.
48. **Rabinovitch M.** EVE and beyond, retro and prospective insights. *Am J Physiol Lung Cell Mol Physiol* 277: L5–L12, 1999.
49. **Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, and 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.
50. **Ratajczak J, Majka M, Kijowski J, Baj M, Pan ZK, Marquez LA, Janowska-Wieczorek A, and Ratajczak MZ.** Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. *Br J Haematol* 115: 195–204, 2001.
51. **Reid LM.** Structure and function in pulmonary hypertension: new perceptions. *Chest* 89: 279–288, 1986.
52. **Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, and Dammacco F.** Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Blood* 93: 2627–2636, 1999.
53. **Southgate KM, Davies M, Booth RF, and Newby AC.** Involvement of extracellular-matrix-degrading metalloproteinases in rabbit aortic smooth-muscle cell proliferation. *Biochem J* 288: 93–99, 1992.
54. **Stenmark KR, Fasules J, Hyde DM, Voelkel NF, Henson J, Tucker A, Wilson H, and Reeves JT.** Severe pulmonary hypertension and arterial adventitial changes in newborn calves at 4,300 m. *J Appl Physiol* 62: 821–830, 1987.

55. **Stringa E, White D, Tuan RS, Knauper V, and Gavrilovic J.** Role of newly synthesized fibronectin in vascular smooth muscle cell migration on matrix-metalloproteinase-degraded collagen. *Biochem Soc Trans* 30: 102–111, 2002.
56. **Taipale J and Keski-Oja J.** Growth factors in extracellular matrix proteins. *FASEB J* 11: 51–59, 1997.
57. **Tozzi CA, Thakker-Varia S, Yu SY, Bannett RF, Peng BW, Poiani GJ, Wilson FJ, and Riley DJ.** Mast cell collagenase correlates with regression of pulmonary vascular remodeling in the rat. *Am J Respir Cell Mol Biol* 18: 497–510, 1998.
58. **Vajner L, Herget J, Uhlík J, and Konrádová V.** MMP-13 production by rat pulmonary mast cells in pulmonary hypertension (Abstract). *Biomed Papers* 145: 48, 2001.
59. **Vieillard-Baron A, Frisdal E, Eddahibi S, Deprez I, Baker AH, Newby AC, Berger P, Levame M, Raffestin B, Adnot S, and d'Ortho MP.** Inhibition of matrix metalloproteinases by lung TIMP-1 gene transfer or doxycycline aggravates pulmonary hypertension in rats. *Circ Res* 87: 418–425, 2000.
60. **Werb Z and Chin JR.** Extracellular matrix remodeling during morphogenesis. *Ann NY Acad Sci* 857: 110–118, 1998.
61. **Zempo N, Koyama N, Kenagy RD, Lea HJ, and 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.
62. **Zhao L, al-Tubuly R, Sebkhi A, Owji AA, Nunez DJ, and Wilkins MR.** Angiotensin II receptor expression and inhibition in the chronically hypoxic rat lung. *Br J Pharmacol* 119: 1217–1222, 1996.