Decreased collagen VI in the tunica media of pulmonary vessels during exposure to hypoxia: a novel step in pulmonary arterial remodeling

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Abstract

The development of hypoxic pulmonary hypertension is characterized by the structural remodeling of pulmonary arteries. However, the relationship between changes of arterial cells and the extracellular matrix remains unclear. We focused on the evaluation of the non-fibrillar collagen changes in tunica media induced by a four-day exposure to hypoxia and the correlation of these changes with the pulmonary arterial wall structure modifications. We used 20 adult male Wistar rats. The amount and localization of collagen VI, collagen IV, matrix metalloproteinase (MMP) 2, and MMP9 were tested in pulmonary arteries immunohistochemically. Two-dimensional electrophoresis and messenger RNA (mRNA) expression were used for the subsequent comparison of protein changes in arterial tunica media cells (normoxia/hypoxia). Collagen VI was significantly reduced strictly in the tunica media of conduit arteries of hypoxia-exposed rats; however, its mRNA increased. The amount of collagen IV and its mRNA were not altered. We detected a significant increase of MMP9 strictly in the tunica media. In addition, a significantly increased number of MMP9-positive cells surrounded the arteries. MMP2 and the expression of its mRNA were decreased in tunica media. We conclude that the loss of collagen VI is an important step characterizing the remodeling of pulmonary arteries. It could influence the phenotypic status and behavior of smooth muscle cells and modify their proliferation and migration.

Introduction

Exposure to hypoxia induces the development of hypoxic pulmonary hypertension (HPH) characterized by a sustained elevation of pulmonary arterial pressure resulting from both vasoconstriction and vascular remodeling (reviewed in Pugliese et al.[1]). In particular, the early phase of hypoxia exposure is reported as critical for later HPH development.[2,3] Hypoxia causes changes in both the cellular part of the vascular wall and the composition of the extracellular matrix (ECM) in pulmonary arteries; these changes affect each other.[4] Thus, we focused our interest on these changes in four-day exposure to hypoxia. Although all the layers of the pulmonary arterial wall play specific roles in the remodeling process,[5,6] we focused on the changes of pulmonary arterial tunica media structure, the thickening of which represents a crucial step in the development of high-resistance pulmonary arteries. Tunica media consists of the ECM and cells, mostly smooth muscle cells (SMCs). It has long been known that the hypoxic pulmonary

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vascular remodeling entails both SMC proliferation and migration[7,8] and alteration of the ECM,[9] especially in the early phase of hypoxic exposure;[10] however, the exact mechanisms of these phenomena remain unclear.

Because an important step in the process of vascular remodeling seems to be a switch between the SMCs contractile and synthetic phenotype,[5,11] it is hard to determine whether this switch is the cause or consequence of changes in the ECM.[12] The reason is the fact that SMCs are an important source of both ECM proteins and ECM-cleaving metalloproteinases (MMPs). MMP production, release, and effects can be modulated during the remodeling process and can reversely influence the morphology and functions of SMCs.[13]

Although changes in the protein composition of pulmonary vascular walls accompanying the remodeling have been intensively studied,[14–17] changes in non-fibrillar collagens have not yet been investigated. Moreover, their alteration so far has only been described in a homogenized mix of pulmonary tissues (in several types of pulmonary hypertension (PH)). Analysis focused on the tunica media during HPH has not yet been reported. This is because, as recently reported,[18–20] the non-fibrillar collagens are a group of proteins associated with the remodeling process in various other tissues; because only these collagens (collagens IV and VI) play a role as anchorage proteins of cells, we hypothesize that the early phase of pulmonary vascular wall remodeling is associated with changes in their content in tunica media, which could contribute to the phenotypical switch of SMCs in this location.

Materials and methods

Animals

We used 20 adult male Wistar rats (200–250 g, ANLAB, Czech Republic). The animals lived in normoxia ($n = 10$) or were exposed to isobaric hypoxia ($FiO_2 = 10\%$) in a normobaric hypoxic chamber[21] for four days ($n = 10$). The rats were euthanized by an intraperitoneal injection of thiopental (50 mg/100 g). Whole lungs from five normoxic and five hypoxia-exposed animals were removed and prepared for immunohistochemical staining. Isolated pulmonary arteries (approximately 250–400 μm, 3rd–5th order, classified as conduit arteries) from the remaining five normoxic and five hypoxia-exposed rats were dissected under a microscope. The isolated vessels of each animal were used for further protein and messenger ribonucleic acid (mRNA) analyses of cells isolated from their walls (immunohistochemical staining of one isolated vessel from each animal proved that the changes detected in vascular walls in whole lungs can be correlated with the results of the proteomic and mRNA analyses).

All experimental procedures were performed in accordance with the European Union and NIH guidelines and approved by the Animal Studies Committee of the Second Medical School, Charles University, Prague.

Smooth muscle cell isolation

SMCs were obtained from the isolated peripheral pulmonary arteries by collagenase digestion. The arteries were minced into fragments and washed by Dulbecco's modification of Eagle's Minimum Essential Medium (DMEM). Then, 0.2 mL of 0.2% collagenase (clostridial collagenase, Sigma-Aldrich, Cat. No. C0130), dissolved in DMEM to a concentration of 4 mg/mL, was added and digestion was performed for 90 min at 37°C. Supernatant was gently removed after centrifugation for 2 min at 90 g; the remaining partially digested fragments were resuspended in 0.25 mL of 0.4% collagenase and incubated for the next 150 min at 37°C with intermittent gentle trituration every 15 min. The released cells were collected by centrifugation (400 g, 7 min) and washed three times by PBS. The cell pellet was stored at –80°C. The isolated cell type was verified by alpha actin detection.[22]

Immunohistochemistry

Whole lungs and isolated pulmonary arteries were sampled for histological evaluation. The material was fixed in Baker's fluid, dehydrated through a graded alcohol series, embedded in paraffin, and cut into 5-μm sections. A longitudinal midline section of the left lung and a transverse section of the lower right lobe were made in each animal. The method of indirect immunohistochemistry was used for the detection of collagens IV and VI and MMP2 and 9. All special chemicals for the immunohistochemical analysis were purchased from Abcam, Cambridge, UK. The specimens were processed by the EXPOSE rabbit-specific horseradish peroxidase/diaminobenzidine detection IHC kit according to the manufacturer's instructions. Briefly, after blocking of endogenous peroxidase activity, the antigen retrieval and non-specific protein block were performed. The incubation with primary antibody was followed by 30 min of incubation with HRP Conjugate. After a short visualization of the specimens with a mixture of DAB Chromogen and DAB Substrate, the nuclei were briefly counterstained with hematoxylin. In the rabbit polyclonal antibody to rat collagen IV (Ab6586), the antigen was retrieved by 0.1% protease from Bacillus licheniformis (10 min, room temperature, Sigma-Aldrich), the antibody was diluted with PBS 1:500 and the incubation was overnight in a refrigerator (4°C). In the rabbit polyclonal antibody to rat collagen VI (Ab6588), the antigen was retrieved by heating the specimen at a temperature of 96°C for 15 min in citrate buffer (pH 6), the antibody was diluted with PBS 1:200, and the incubation was 1 h at room temperature. In the antibodies to MMPs (MMP2: rabbit polyclonal Ab37150, MMP9: rabbit monoclonal Ab76003), the antigen was retrieved by heating as above,

the antibodies were diluted with PBS 1:1000 and 1:500, respectively, and the incubation was 1 h at room temperature.

Collagens IV and VI were measured in arterial tunica media and adventitia. Images of all arteries found in the specimens of whole lungs as well as images of isolated pulmonary arteries were captured by the light microscope BM53 (Olympus, Tokio, Japan) equipped with a charge-coupled device camera ProgRes (Jenoptic, Jena, Germany) and computer image analysis software NIS Elements AR (Laboratory Imaging, Prague, Czech Republic). The media/adventitia ratio of color density from the vessels of animals living in normoxia and exposed to four days of hypoxia were compared.

The presence of MMPs was tested in arteries with a diameter of approximately $350 \pm 70 \mu m$ (conduit arteries), because the change of collagen VI distribution was detected strictly in this order of vessels. The amount of MMPs in arteries was expressed as the percentage of color-positive areas in the tunica media. Isolated MMP-positive cells, in and around the tunica adventitia of pulmonary arteries, were also counted.

Two-dimensional gel electrophoresis and protein analysis

SMCs proteins were separated by two-dimensional gel electrophoresis (2-DE) and detected by on-line nano-liquid chromatography coupled with tandem mass spectrometry analysis (nLC-MS/MS).

Lyophilized samples of isolated SMCs were sonicated (15 min, 20°C) in 140 mL of lysis buffer. The supernatant was taken for subsequent 2-DE analysis. Isoelectric focusing and separation by 2-DE was performed on homogeneous 10% SDS-polyacrylamide gel as described earlier.[23] Precision Plus Protein[TM] Standards (molecular weight range 10–250 kDa, Bio-Rad) were added at the top end of the gel. Spot normalized volume was used to select statistically significant differential spots by PDQuest Advanced (Bio-Rad). The significance level was set at $P \leq 0.05$. Spots with differential expression were excised from the Coomassie-stained gels and then processed by the methods described in Jágr et al.[23] The tryptic peptides were extracted using Ziptips (Millipore) aided purification of samples. nLC-MS/MS analyses were performed as described in our previous studies[23,24] with updated software. Database searches (UniProt) were performed as described in Eckhardt et al.[25] with the taxonomy restricted to *Rattus norvegicus*. Only significant hits were accepted (http://www.matrixscience.com; MASCOT score ≥ 80 for proteins; ≥ 30 for peptides).

mRNA isolation

mRNA was isolated from dissected pulmonary arteries (approximately 250–400 μm, 3rd–5th order) of five rats exposed to hypoxic conditions for four days and five normoxic controls. The samples were homogenized by MagNA Lyser Instrument (Roche Diagnostic). The mRNA isolation was performed using the Total RNA Purification Micro Kit (Norgen Biotek).

Complementary DNA synthesis and real-time polymerase chain reaction (RT-PCR)

For the reverse transcription of individual mRNA samples, the Omniscript Reverse Transcription Kit (Qiagen) with random hexamers (New England Biolabs, Inc.) was used in a final concentration of 10 μM. mRNA levels were quantified by quantitative real-time 5xHOT FIREPol Probe qPCR Mix Plus (ROX) (Solis BioDyne) and with TaqMan Gene Expression Assays (Life Technologies) labelled with FAM reporter dye specific to rat collagen type I (Col1A1, Cat. No. Rn01463848_m1), collagen type III (Col3A1, Cat. No. Rn01437681_m1), collagen type IV (Col4A1, Cat. No. Rn01482927_m1), collagen type VI (COL6A3, Cat. No. Rn01427387_m1), and metalloproteinases MMP2 (Cat. No. Rn01538170_m1) and MMP9 (Cat. No. Rn00579162_m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat. No. Rn01462662_g1) was used as a reference gene.

The RT-PCR experiments were performed on a 96-well optical reaction plate using BioRad iQ5 MultiColor Real-Time PCR Detection System.

Data are the mean of 12–18 experimental points from 5–6 independent samples. The relative gene expression was calculated as $2^{-\Delta Ct}$.

Statistics

Statistical analyses were performed using ANOVA followed by Student–Newman–Keuls method. P values ≤ 0.05 were considered significant. Data are presented as means $\pm$ S.E.M. P values in proteomics (2-DE) were corrected for multiple-testing by false discovery rate (FDR) based on frequency histogram.[26] FDR adjusted P values ≤ 0.05 were considered significant.

Results

Detection of collagen VI and IV by immunohistochemistry

Collagen VI was detected in tunica media. It formed thin envelopes around SMCs. It was also positive in the ECM of pulmonary arterial adventitia. The hypoxic group had significantly decreased color density positivity of collagen VI in tunica media when compared with tunica adventitia, but only of conduit arteries (Fig. 1a and b). The media/adventitia ratio of collagen VI density was 2.046 ± 0.159 in normoxic animals and 0.059 ± 0.009 in the hypoxia exposed group ($P < 0.001$) strictly in the conduit arteries, $324 \pm 60 \mu m$ in diameter (Fig. 1e). Collagen VI was detected as confluent positive envelopes around SMCs in the normoxic animals (Fig. 1a, inset). These structures were
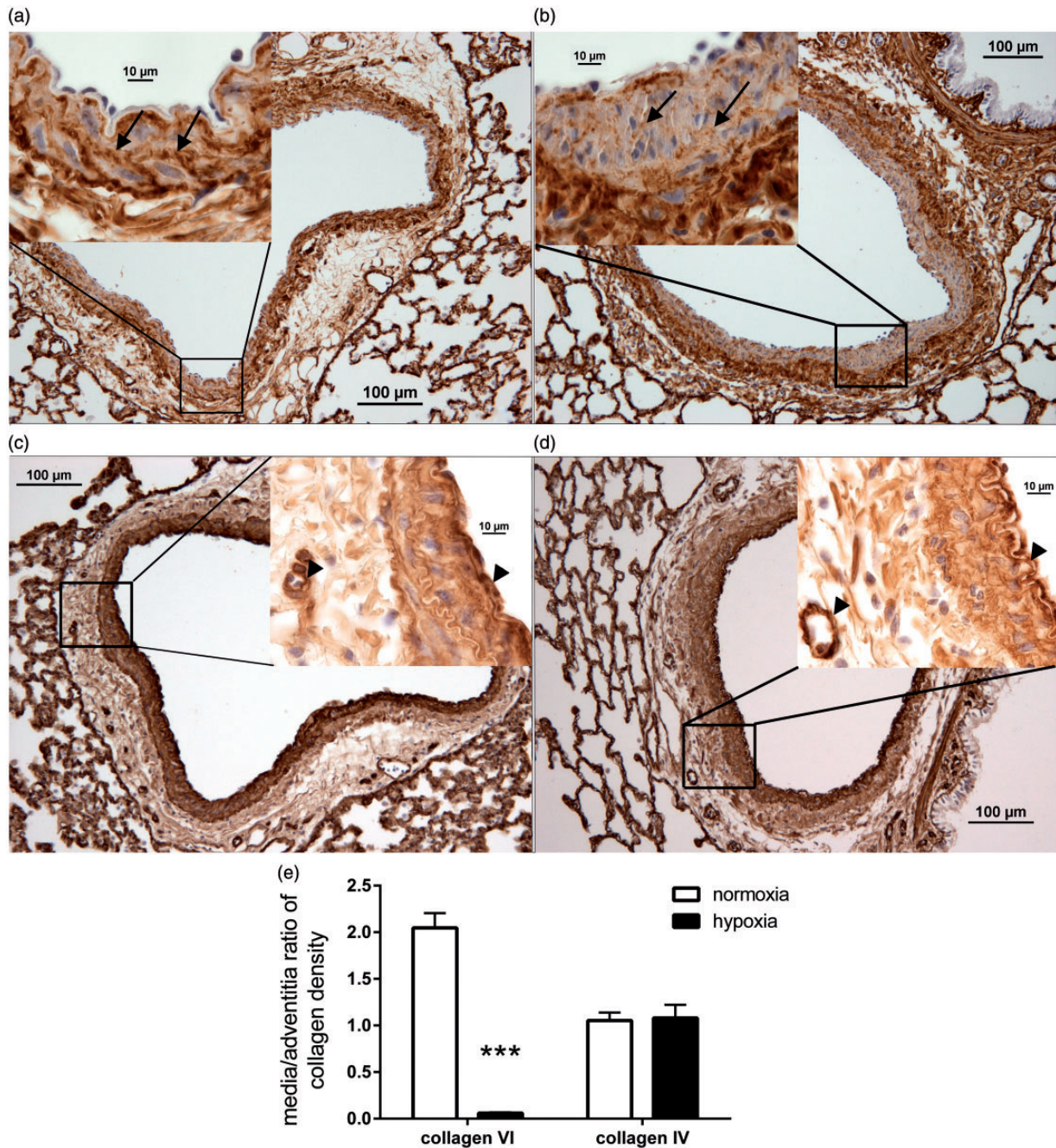
Fig. 1. Immunohistochemical detection of collagen type VI (a, b) and collagen type IV (c, d) in the conduit pulmonary arteries of normoxic (a, c) and hypoxic (b, d) rats. Arrows indicate the difference of collagen type VI distribution in the arterial tunica media of normoxic and hypoxic rats (a, b). Arrowheads indicate collagen type IV in subendothelial basement membranes (c, d). Original magnification 20×, insets 100×. There are visible compact collagen VI envelopes surrounding the cells in normoxic arteries. These are fragmented specifically in tunica media of hypoxic vessels. The fragmentation is not present in collagen IV structure. (e) Media/adventitia ratio of color density of immunohistochemically labeled collagen VI and IV in conduit pulmonary arteries in lungs of normoxic and hypoxic rats. $***P \leq 0.001$.

fragmented in the hypoxic rats (Fig. 1b, inset). We did not observe any difference in color density in the vascular walls of arteries <250 μm between the hypoxic and control rats. In the isolated arteries, the same findings were observed as in whole lungs.

Immunostaining for collagen type IV in pulmonary arteries of whole lung slides as well as in isolated pulmonary arteries was strongly positive in the area of subendothelial basement membranes of both normoxic and hypoxic animals. Fine collagen type IV-positive layers were found on the surfaces of vascular SMCs, corresponding to the location of laminae around the SMCs. The density of collagen IV did not vary depending on exposure to hypoxia or vessel size (media/adventitia ratio 1.053 ± 0.085 in normoxic

vessels, 1.180 ± 0.143 in hypoxia exposed animals) (Fig. 1c–e).

Two-dimensional gel electrophoresis

The samples of cellular suspensions used for the proteomic analyses were alpha actin-positive ($\geq 92\%$), confirming that the suspensions contained a vast majority of SMCs or myofibroblasts. We detected four significant differences in the protein composition of SMCs isolated from rats living in normoxia and those exposed to four days of hypoxia (Fig. 2). Three spots were identified as collagen VI alpha 3 chain (significance of all three spots FDR adjusted P values were under threshold 0.05). The intensity of all three spots
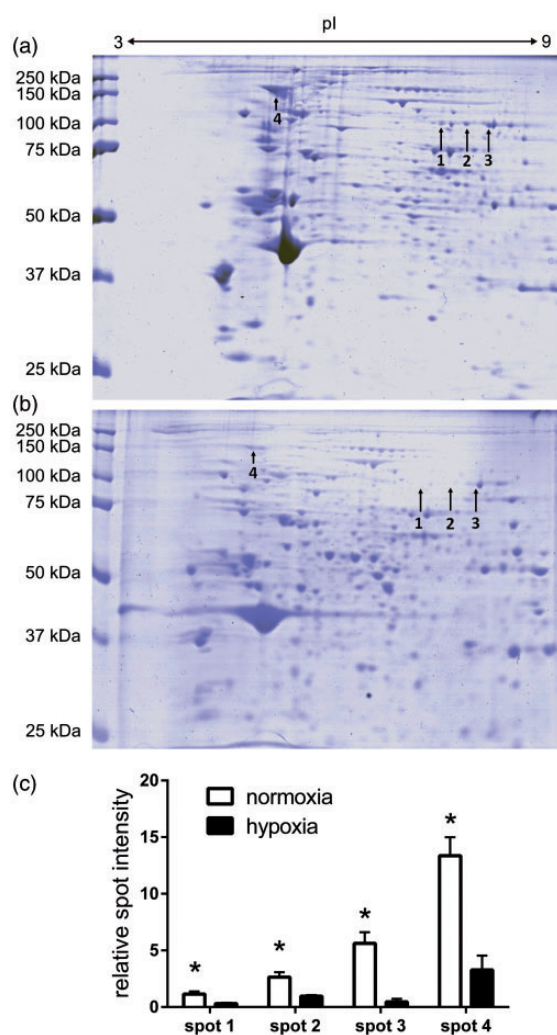

Fig. 2. Proteomic changes of cells in media of peripheral pulmonary arteries. Proteome analysis of the cellular lysates obtained from the tunica media of peripheral pulmonary arteries: (a) from the normoxic rats; (b) from the rats exposed to hypoxia for four days. Spot numbers 1–3 indicate a decrease of collagen type VI alfa 3 quantity in lysates from the rats exposed to hypoxia compared with the normoxic animals; spot number 4 symbolizes myosin 11 (significance FDR adjusted P value was under threshold 0.05). pI, isoelectric point.

of collagen VI was lower in the hypoxic samples than in the normoxic group (Fig. 2). Spot number 4 was identified as myosin heavy chain M11. Its intensity was lower in the hypoxic samples (significance FDR adjusted P value was under threshold 0.05) (Fig. 2).

Detection of MMP changes by immunohistochemistry

The expression of both MMP2 and 9 was exclusively intracellular both in normoxic and hypoxic lungs. MMP2 positivity was detected in arterial SMCs, endothelium, and some adventitial cells (mast cells, granulocytes, some fibroblasts, macrophages) (Fig. 3). In hypoxic animals, a mixture of MMP2-positive and MMP2-negative cells was found in the arterial tunica media. The area of positivity in tunica media of conduit arteries was 0.380 ± 0.02 in normoxia and 0.106 ± 0.006 in hypoxia ($P < 0.001$) (Fig. 3a, b, and e). The highest positivity of MMP2 was found in macrophages and in some other interstitial single cells, as well as in the bronchial and alveolar epithelium.

After four days of hypoxia, tunica media of the same vessel category contained more MMP9-positive SMCs – its area of positivity significantly increased (0.053 ± 0.012 in normoxia, 0.138 ± 0.013 in hypoxia, $P < 0.01$) (Fig. 3c–e). The MMP9 positivity appeared in the arterial endothelium and in some cells of the subendothelial layer (probably myofibroblasts). There were also significantly more MMP9-positive cells in and around the tunica adventitia (0.455 ± 0.119 in normoxia, 4.907 ± 0.777 in hypoxia per one vessel, $P < 0.01$) (Fig. 3c, d, and f).

mRNA

The mRNA expression of collagen types I, III, IV, and VI was measured under identical conditions. The expression of two of the monitored genes was significantly changed after the exposure to hypoxia (Fig. 4). Collagen VI and collagen I mRNA expression increased significantly. On the contrary, the mRNA expression of collagen III was significantly decreased. The mRNA expression of collagen IV (a protein bound directly to cells) remained unchanged.

The mRNA expression of MMP2 was decreased in SMCs on the fourth day of hypoxia (Fig. 4).

The degree of mRNA expression of all the measured genes was at a similar level comparable with the reference gene (GAPDH), with the exception of the MMP9 mRNA measurements, which were $>100 \times$ lower than the reference gene, just at the detection level (non-significant ratio normoxia/hypoxia).

Discussion

First, we verified that changes detected by immunohistochemical staining in a defined type of isolated vessels (the type also used for proteomics and mRNA analysis) and in the same degree of arteries in histological sections of the
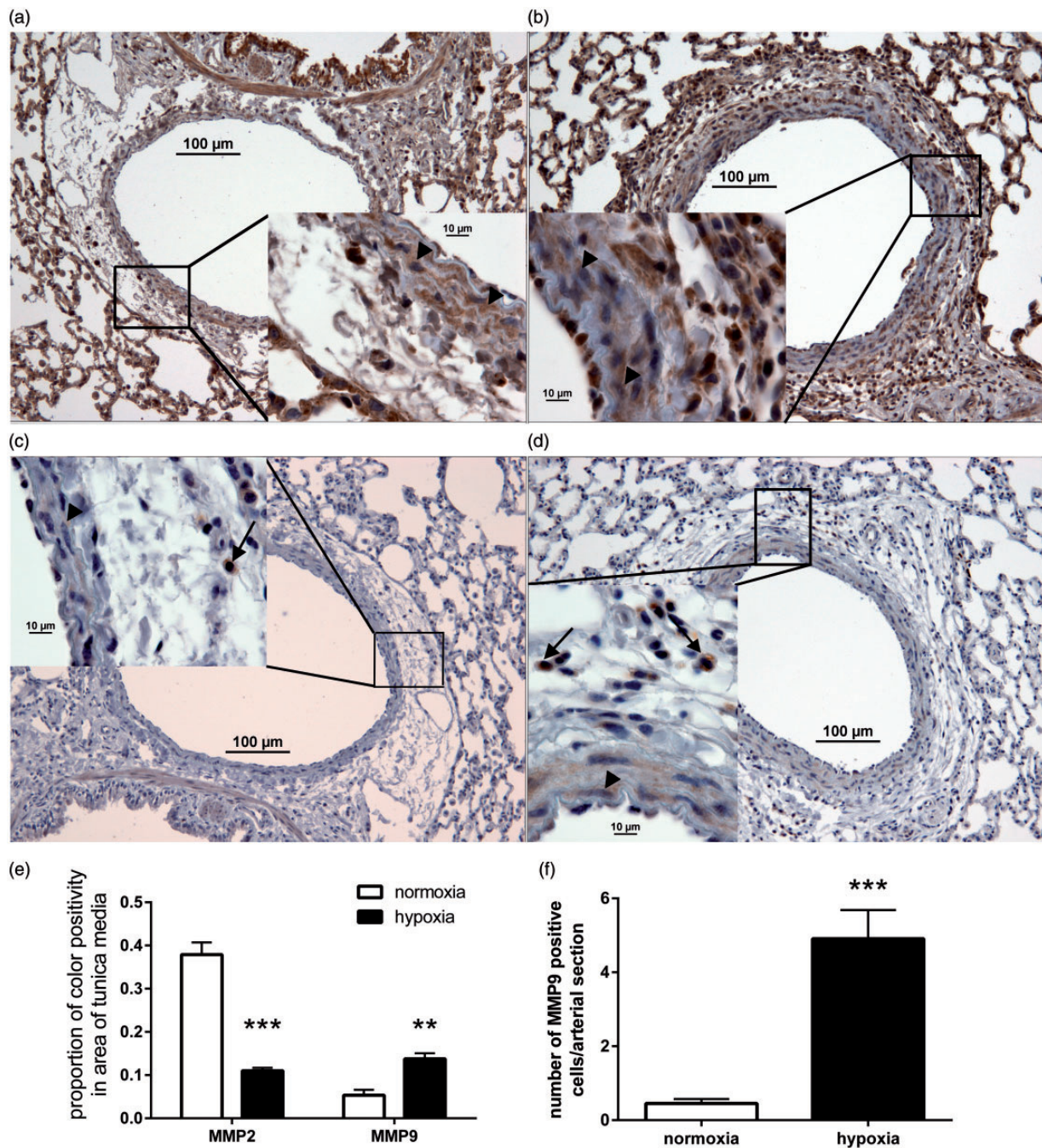
Fig. 3. Immunohistochemical detection of MMP2 (a, b) and MMP9 (c, d) in the conduit pulmonary arteries of normoxic (a, c) and hypoxic (b, d) rats. Arrowheads indicate SMCs in the tunica media, arrows indicate MMP9-positive free cells (c, d). Original magnification 20×, insets 100×. The sum of MMP9-positive area is significantly higher in tunica media of hypoxic vessels and more MMP9-positive single cells appear in tunica adventitia and in the vicinity of vessels. On the other hand, MMP2-positive areas were lager in tunica media of normoxic vessels. (e) Proportion of MMP-positive areas in the tunica media of large conduit pulmonary arteries in lungs of normoxic and hypoxic rats. $**P \leq 0.01$, $***P \leq 0.001$. (f) Number of MMP9-positive cells in and around the tunica adventitia of large conduit pulmonary arteries in lungs of normoxic and hypoxic rats, $***P \leq 0.001$.

whole lungs are identical. Thus, we believe that the results from all the methods we used are comparable to each other. Moreover, we know from previous experiments that the type of isolated vessels, on which we centered our interest, is reactive to hypoxia and thus they are really involved in the process of the development of HPH.[27]

Collagen VI concentration was reduced by four days of hypoxia exclusively around the cells in the tunica media of conduit pulmonary arteries. This specific local loss of collagen VI was confirmed by two independent methods: immunohistochemistry and 2-DE (Figs. 1 and 2). Structural changes of the arterial wall characteristic for the remodeling
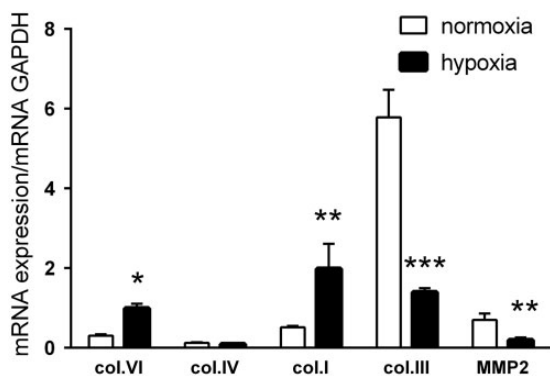
Fig. 4. Relative mRNA expression of collagen type I, III, IV, VI and of MMP2 in isolated pulmonary arteries from rats exposed to hypoxia for four days determined by real-time PCR. GAPDH was used as reference gene. Col. I, collagen I alpha 1; Col. III, collagen III alpha 1; Col. IV, collagen IV alpha 1; Col. VI, collagen VI alpha 3. $*P \leq 0.03$, $**P \leq 0.01$, $***P \leq 0.001$.

process during hypoxia typically begin only in this size of vessels (approximately 250–400 μm) and then extend to the periphery.[1] However, four of our findings are surprising: (1) the loss of collagen VI was localized strictly in tunica media of vascular walls; (2) the amount of collagen IV was unchanged in all of the vascular layers; (3) none of these protein changes were observed in adventitia; and (4) the concentrations of MMPs and their mRNA in the early phase of hypoxia differ.

Collagen VI is an abundant anchorage protein connecting collagen IV of the external lamina to fibrillar components of the ECM (collagen I, III) (for review, see Fitzgerald et al.[28]). It affects cellular adhesion, spreading, and migration in various tissues.[29] It is also an important factor regulating the invasion of various tumor cells,[30,31] but its role in the vascular wall has not yet been studied. While the filamentous collagen VI is an important part of the three-dimensional structure of the ECM, the soluble collagen VI (after enzymatic cleavage) plays a role in the signaling of tissue damage and also modulates cellular proliferation, survival, and wound healing (for review see Mak et al.[18]). It has been described that the process of tissue remodeling, especially its early phase, is associated with changes of collagen VI in various tissues (liver fibrosis, myopathies).[18–20] Decreased quantity of collagen VI is related to a loss of the three-dimensional organization of the fibronectin molecules, which could affect various cellular functions.[32] A downregulated collagen VI gene expression was detected in patients with chronic obstructive pulmonary disease (COPD).[33] The lack of collagen VI density in the ECM of skeletal muscles was observed as part of myodystrophic diseases, such as Bethlem myopathy and Ullrich congenital muscular dystrophy.[19,34,35] However, some positive effects of collagen VI loss to stimulation of tissue regeneration and wound healing were also demonstrated, e.g. the increased spreading and migration of human intestinal epithelial cells[36] as well as of human skin fibroblasts.[37]

Other reports demonstrated that pro-proliferative effects of collagen VI are accompanied with an increased amount of collagen VI in the tissue, e.g. in the development of the liver[20,38] or in lung fibrosis.[38] Collagen VI usually showed increased turnover in these tissues. It was associated with its degradation by metalloproteinases and the presence of collagen VI fragments. These fragments were reported to stimulate cell proliferation (for a review see Rasmussen et al.[39]). The block of MMPs attenuates SMC migration and/or proliferation.[40] Thus, cleavage of some part(s) of the ECM seems essential for migration and proliferation. We propose that this important cleavage step could be based on the collagen VI envelope disruption (Fig. 5). In particular, one of these fragments, endotrophin (C-terminal fragment of collagen VI alpha 3), is known to promote phenotypic modulation and proliferation of cells, particularly epithelial-to-mesenchymal transition of cells and aggressive growth of tumor cells.[41]

We assume that the loss of bounds around SMCs could contribute to their phenotype switch (described in the literature[1,11]). This change is consistent with our finding of a significantly lower amount of myosin heavy chain M11 protein (Fig. 2) in the cellular digest of tunica media on the fourth day of hypoxia compared to normoxic controls. M11 is one of the most accepted markers of SMC contractile phenotype.[42] The arterial tunica media SMCs comprise: (1) well differentiated contractile cells that are myosin heavy chain-positive and resistant to proliferation; and (2) highly proliferative, myosin heavy chain-negative SMC-like cells.[43] Their ratio is changed during vascular remodeling because of phenotypic plasticity. It is a consequence of switching between contractile and synthetic phenotype of SMCs.[42] Thus, the decrease of myosin M11 amount in samples from hypoxia-exposed animals could demonstrate an increased amount of highly proliferative SMC-like cells. Hyperproliferative SMC-like cells were also detected in pulmonary vascular walls of patients with COPD.[44]

We suppose that our observation of the increases of collagen VI and collagen I mRNA and the parallel decrease of collagen III mRNA (Fig. 4) are additional proof of the phenotypical change of SMCs in the tunica media. Thus, these mRNA changes in SMCs could be interpreted as a further sign of their phenotype switch. Although further increase of collagen VI may represent an important step resulting in cellular re-anchoring to ECM. The remodeling during pulmonary hypoxia exposure is a dynamic process. The loss of collagen VI around SMCs fits with the hypothesis relating to their phenotypical status change and their tendency to the peripheral migration. These cells will adhere in different, more peripheral locations of pulmonary arterial tree (this migration was recently described in Sheikh et al.[45]). In addition, it seems necessary to start the new protein production (e.g. collagen VI) for the re-anchoring of these cells. This explains the collagen VI loss, and, on the other side, the reboot of its mRNA expression.

Our observation of the collagen VI loss in arterial walls is consistent with the previously described increase in collagenolytic activity in the early phase of exposure to hypoxia.[9,46]

It is known that collagen VI is degraded by serine proteases and MMPs (MMP2 and MMP9).[20] The increased activity of MMPs in the lungs was also observed in hypoxia.[13,47] We detected an increase of MMP9 and decrease of MMP2-positive areas, but, again, strictly in the tunica media of large conduit arteries (Fig. 3). Although collagen IV is cleaved by the same types of MMPs, we did not observe any attenuation of its density in any vascular layer. Collagen IV is a stable part of the external lamina;[48] thus, we assume that it is not attenuated because of steric factors: collagen IV is incorporated deeper into the external lamina (it is the anchor for collagen VI), while collagen VI creates the upper sandwich layer, better accessible by metalloproteinases.

Our experiments confirmed SMCs as an important source of MMP2 and MMP9. We detected a lower expression of mRNA for pro-MMP2 in isolated cells (Fig. 4) in hypoxia than in normoxic controls, which is in agreement with the downregulated protein concentration of MMP2 in the tunica media (Fig. 3). A similar decrease of mRNA for pro-MMP2 (and pro-MMP9) was observed by Ye et al. in endothelial and SMCs after only one day of hypoxia.[49] The mRNA for pro-MMP9 was just at the detection limit on the fourth day of hypoxia in our experiment, but the enzyme MMP9 was upregulated in the tunica media (Fig. 3). These results document different roles of both gelatinases (MMP2 and MMP9) during the development of PH caused by hypoxia. We suppose that concentrations of MMPs vary specifically during the first days of hypoxia and we assume that MMP2 has a shorter action time during hypoxia than MMP9. This is supported by the finding of the significantly increased number of cells packed with MMP9 not only in the arterial wall but also in the vicinity of arteries (about 300 µm diameter) on the fourth day of hypoxia. These cells and their MMPs will probably contribute to the cleavage of ECM in the later phases of vascular remodeling.

MMPs are released from cells as non-active precursors, the final action of which depends not only on their total amount but also on the ratio between activating and inhibitory factors regulating their local activity. Thus, the discrepancy of collagen VI density in various vascular layers can depend also on those ratios.[50] We suppose that this local blockade of proteolytic enzyme activity, however, may only participate in the final difference of collagen VI density in the tunica adventitia and media in our experiment, because the variance in MMP amount was detected directly between those two vascular layers. Thus, the collagen VI disruption observed specifically around the cells of tunica media is in agreement with the speculation that the cells of tunica media represent the essential source of MMPs and thus also the main cause of collagen VI cleavage in this phase of hypoxia (Fig. 5).

This is supported by the fact that their activity is accompanied with their phenotypical switching.[51]

The specific local loss of collagen VI in pulmonary arteries during hypoxia is a novel observation. Its role in the remodeling process accompanied with the development of HPH should be studied further.

Limitations of the study

We focused on protein changes in tunica media induced by a four-day exposure to hypoxia. We know that it is only a
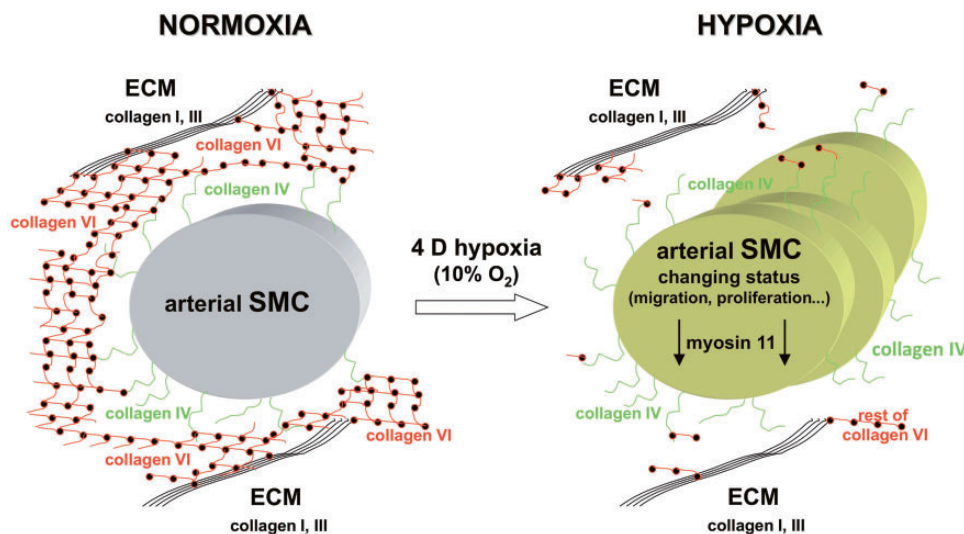

Fig. 5. Our results demonstrate a marked reduction of collagen VI content (red lines with black dots: collagen VI forms "beaded" fibrils) in the tunica media of pulmonary arteries (about 300 µm diameter) during hypoxia. On the contrary, the amount of collagen type IV (in green on the surface of SMCs) was unaltered. Fibrillar collagens (type I and III) are in black and collagen VI anchorages them to collagen IV. The status of some SMCs during HPH development is changed (decreasing concentration of protein myosin 11: marker of SMC status). The loss of collagen VI around cells in media (disorganization of chemical and physical contacts) could be a new step in this process.

view on the "particular time period" of the remodeling process of the pulmonary arteries, which cannot bring insight to the whole hypoxic remodeling process. However, we also brought a description about past (the detection of MMPs, which cleaved ECM before) and also future events (mRNAs of proteins which will translate into proteins). This limitation concerning the length of time of observation is common in basic research and subsequent analysis of the mechanisms that regulate ECM remodeling during hypoxia will be important for a better understanding of this process.

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