Alteration of Pulmonary Artery Integrin Levels in Chronic Hypoxia and Monocrotaline-Induced Pulmonary Hypertension

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Key Words

Integrins • Chronic hypoxia • Monocrotaline • Pulmonary hypertension • Pulmonary arteries

Abstract

Background: Pulmonary hypertension is associated with vascular remodeling and increased extracellular matrix (ECM) deposition. While the contribution of ECM in vascular remodeling is well documented, the roles played by their receptors, integrins, in pulmonary hypertension have received little attention. Here we characterized the changes of integrin expression in endothelium-denuded pulmonary arteries (PAs) and aorta of chronic hypoxia as well as monocrotaline-treated rats. **Methods and Results:** Immunoblot showed increased α_1 -, α_8 - and α_v -integrins, and decreased α_5 -integrin levels in PAs of both models. β_1 - and β_3 -integrins were reduced in PAs of chronic hypoxia and monocrotaline-treated rats, respectively. Integrin expression in aorta was minimally affected. Differential expression of α_1 - and α_5 -integrins induced by chronic hypoxia was further examined. Immunostaining showed that they were expressed on the surface of PA smooth muscle cells (PASMCs), and their distribution was unaltered by chronic hypoxia. Phosphorylation of focal adhesion kinase was augmented in PAs of chronic

hypoxia rats, and in chronic hypoxia PASMCs cultured on the α_1 -ligand collagen IV. Moreover, α_1 -integrin binding hexapeptide GRGDTP elicited an enhanced Ca^{2+} response, whereas the response to α_5 -integrin binding peptide GRGDNP was reduced in CH-PASMCs. **Conclusion:** Integrins in PASMCs are differentially regulated in pulmonary hypertension, and the dynamic integrin-ECM interactions may contribute to the vascular remodeling accompanying disease progression.

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Introduction

Pulmonary hypertension is characterized by a sustained rise in the pulmonary arterial pressure that results from pulmonary vasoconstriction and vascular remodeling [1]. Pulmonary vascular remodeling involves increased pulmonary arterial cell proliferation and hypertrophy, leading to thickening of the pulmonary arterial wall. Another underlying feature of pulmonary vascular remodeling that accompanies pulmonary hypertension is an increase in the deposition of extracellular matrix (ECM) components, particularly collagen, elastin, tenascin-C and fibronectin, which have been documented in both human and animal models of the disease [1–4]. The

increased deposition of matrix proteins in the pulmonary vasculature has been attributed to increases in serine elastase activity, as well as a change in the balance of matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase (TIMP) activity [5, 6]. The importance of elastase/proteinase in pulmonary vascular remodeling is exemplified in animal models of pulmonary hypertension. For example, inhibition of serine elastase activity reversed pulmonary hypertension both in rats exposed to chronic hypoxia as well as monocrotaline (MCT) [5, 7], while inhibition of MMPs by gene transfer of a human TIMP1 gene attenuated and aggravated vascular remodeling, respectively, in rats treated with MCT and chronic hypoxia [6]. Additionally, the specific MMP inhibitor Batimastat, which has no influence on systemic circulation, attenuated pulmonary hypertension in chronically hypoxic rats [6].

While the importance of matrix components in pulmonary vascular remodeling has been implicated extensively in the development of pulmonary hypertension, relatively little attention has been paid to the involvement of integrins, the receptors for the ECM proteins. Integrins comprise a superfamily of structurally related heterodimeric transmembrane receptors that mediate cell-cell and cell-matrix interactions. They physically bridge the ECM and the cytoskeleton, acting as transducers of 'outside-in' and 'inside-out' signaling [8]. Of the 18 α - and 8 β -subtypes that have been identified to date, α_{1-9} , α_v , β_1 , β_3 , β_4 and β_5 integrins have been reported in vascular smooth muscle cells (SMCs), and α_{1-5} , α_7 , α_8 , α_v , β_1 , β_3 and β_4 integrins have been detected in the pulmonary vasculature [8–10]. Integrins play important roles in various vascular functions, including mechano-transduction, myogenic response, ECM synthesis, SMC proliferation, apoptosis and migration, neointimal formation, and eutrophic inward remodeling [9, 11]. In the pulmonary vasculature, we showed that integrin-binding hexapeptides are capable of mobilizing intracellular Ca^{2+} in pulmonary arterial smooth muscle cells (PASMCs). We further showed that one of these peptides, namely GRGDSP, mobilizes intracellular Ca^{2+} from ryanodine receptor-gated organelles and lysosome-related acidic organelles, by causing an increase in cyclic ADP ribose [10]. In the context of pulmonary hypertension, the involvement of $\alpha_v\beta_3$ integrin has thus far been documented in MCT-treated rats, where the elastase-mediated activation of MMPs leads to $\alpha_v\beta_3$ integrin clustering and subsequent transcription of tenascin-C and an increase in SMC proliferative response to growth factors [1, 12, 13].

In this study we hypothesized that integrins are regulated concurrently with the increased deposition of ECM components observed in the development of pulmonary hypertension. To test this hypothesis, we took advantage of two different rat models of pulmonary hypertension, namely the chronic hypoxia and MCT-induced models, in order to discriminate between the direct effects of experimental treatments (that is, hypoxia or MCT) versus those related to the resultant vascular remodeling. In doing so, we systematically compared the differences in the expression of a number of α and β integrin proteins specifically in endothelium-denuded pulmonary arteries (PAs) and aorta, in contrast to previous gene expression profiling studies that have taken a global approach using mostly extracts from whole lung tissues [for review, see 14]. We further confirmed the expression of the integrins in small PAs by immunohistochemistry, compared focal adhesion kinase (FAK) phosphorylation in PAs and PASMCs, and correlated the changes in function by monitoring the $[Ca^{2+}]_i$ responses generated by integrin-specific peptide ligands in PASMCs of the chronic hypoxia rat model.

Materials and Methods

Chronic Hypoxia and MCT Treatment

Male Wistar rats (150–200 g) were used for all treatments. Hypoxic pulmonary hypertension was induced by exposure to normobaric hypoxia (10% O_2) for 4 weeks, while normoxic controls were reared in room air for the same period of time. MCT-induced pulmonary hypertension was developed by injection with a single subcutaneous dose of MCT (60 mg/kg; Sigma). Animals were sacrificed 24 days later. All animals were anesthetized with sodium pentobarbital (130 mg/kg intraperitoneally) prior to removing the heart and lungs. Development of pulmonary hypertension was validated in both models by confirming right ventricular hypertrophy, which was done by separating the right ventricle (RV) from the left ventricle plus septum (LV+S), weighing these components and calculating the ratio of RV/(LV+S). All animal procedures were performed in accordance with the guidelines specified by the Johns Hopkins Animal Care and Use Committee.

Isolation of PAs and PASMCs

PAs were dissected and PASMCs were enzymatically isolated as previously described [15]. Briefly, lungs were removed from male Wistar rats (150–200 g) anesthetized with sodium pentobarbital (130 mg/kg intraperitoneally), upon which intrapulmonary arteries of 3rd and higher generations (inner diameter approx. 0.3–1 mm) were dissected in HEPES-buffered salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES and 10 glucose, pH 7.4. PAs were cut open, carefully cleaned of connective tissue, and the endothelial layer was removed by rubbing the luminal surface thoroughly with a cotton swab. Arteries were stored at $-80^\circ C$ for Western blot analysis. For

enzymatic isolation of PSMCs, arteries were incubated in ice-cold HBSS (30 min), and then in reduced- Ca^{2+} (20 μM) HBSS (20 min, room temperature), upon which they were digested in reduced Ca^{2+} HBSS containing collagenase (type I, 1,750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml) and dithiothreitol (1 mM) at 37°C for 18 min. After washing with Ca^{2+} -free HBSS, single SMCs were gently dispersed from the tissues by trituration in Ca^{2+} -free HBSS. PSMCs were plated on 25-mm glass cover slips for Ca^{2+} fluorescence experiments or on 35-mm culture dishes, which were noncoated or coated with human collagen type IV or fibronectin (BD Biosciences), for determination of FAK phosphorylation. PSMCs isolated from normoxic and chronic hypoxic rats and normoxic rats were transiently cultured under normoxic condition (air + 5% CO_2) or in a modular incubator chamber (Billups-Rothenberg) containing 4% O_2 and 5% CO_2 (16–24 h, 37°C).

Preparation of Protein Samples and Immunoblot

Total protein was isolated from PAs, aorta, and cultured PSMCs for Western blot analysis of integrin expression and phosphorylation of FAK. Endothelium-denuded PAs and aorta were frozen in liquid nitrogen, pulverized and homogenized with a Dounce homogenizer (30 strokes) in ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing phenylmethylsulfonyl fluoride (1 mM) and protease cocktail inhibitor (Roche). Homogenized tissues or cultured cell lysate were centrifuged (3,000 g, 4°C, 10 min), upon which the protein concentrations of the postnuclear supernatant were measured with the BCA Protein Assay Kit (Pierce). Protein samples were analyzed by SDS-PAGE and immunoblot. They were treated with Laemmli sample buffer with (for integrin β_3) or without (for all other integrin subtypes) β -mercaptoethanol (100°C, 5 min), separated by an 8% polyacrylamide gel (5 μg per lane), and electrotransferred onto Immobilon P membranes (0.45 mm; Millipore) using a tank transfer system (80 V, 3 h, 4°C). Upon blocking (1 h, room temperature) with 5% skim milk in PBS containing 0.05% Tween-20 (PBST), membranes were incubated with primary antibodies diluted in PBST containing 3% BSA (BSA/PBST) at 4°C overnight. The following primary antibodies were used: integrin α_1 (1:1,000, AB1934; Chemicon International); α_5 (1:1,000, AB1949; Chemicon); α_8 (1:2,500; generous gift from Dr. Lynn Schnapp, University of Washington); α_v (1:250, 611012; BD Biosciences); β_1 (1:2,000, AB1952; Chemicon); β_3 (1:500, 4702; Cell Signaling); total FAK (1:1,000, 06-543; Millipore); phospho-FAK (1:1,000, 44-624G; Invitrogen); Actin (1:5,000, SC-1615; Santa Cruz). After washing in PBST, membranes were incubated with horseradish peroxidase-coupled donkey-anti-rabbit or sheep-anti-mouse secondary antibodies (Amersham Biosciences) diluted in 1% BSA/PBST (1 h, room temperature), and again washed extensively. Protein signal was then detected using enhanced chemiluminescence (Pierce Biotechnologies), and intensity was quantified using a Gel Logic 200 Image System (Kodak).

Immunostaining of Lung Section and PSMCs

Lung tissues were fixed in 4% formaldehyde in PBS (0.05 M phosphate buffer, 0.9% sodium chloride, pH 7.4), rinsed in PBS and cryoprotected with 18% sucrose in PBS (24 h, 4°C). Alternate cryostat sections (10 μm) were collected on lysine-coated slides, dried briefly, and blocked with 1% BSA and 10% normal goat serum (60 min, room temperature). For immunostaining α_8 integrin, a tyramine signal amplification kit (Perkin Elmer) was used; for these

sections, prior to blocking with goat serum, endogenous peroxidase activity was blocked with hydrogen peroxide (3 in 50% methanol in PBS, 30 min). The sections were then incubated overnight (4°C) in a rabbit antibody recognizing individual integrins (same antibodies as used for immunoblotting) and mouse monoclonal antibody recognizing smooth muscle α -actin (Abcam Inc.) diluted in PBS containing 1% BSA, 0.5% Triton X-100). Separate sections were processed similarly for negative control except the primary antibody was replaced with rabbit IgG to evaluate nonspecific staining. For tyramine signal amplification, sections were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin (1 $\mu\text{g}/\text{ml}$) and then with Alexa Fluor 488-conjugated tyramide (Molecular Probes) in amplification solution; otherwise, sections were incubated with goat anti-rabbit conjugated with Alexa Fluor 488 and goat anti-mouse conjugated with Alexa Fluor 594. Washed slides were mounted in Tris-buffered glycerol (pH 8.6).

PSMCs from normoxic and hypoxic rats were placed on poly-L-lysine (0.01% w/v in H_2O ; Sigma) coated 25 mm cover glass and incubated at 37°C in Ham's F-12 medium under air and 5% CO_2 or 4% O_2 and 5% CO_2 in a modular incubator chamber (Billups-Rothenberg), respectively, for 5 h. Cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 10 min and incubated overnight at 4°C with primary antibodies for integrin α_1 (1:400 dilution; Chemicon AB1934) and α_5 (1:100 dilution; Chemicon AB1949) in the presence of 1% normal goat serum. CyTM3-conjugated goat anti-rabbit IgG (1:800 dilution, 111-165-144; Jackson Immuno) was used for secondary antibody incubation for 1 h at room temperature. After mounting the cover glass, images were captured using a Carl Zeiss LSM510 confocal microscope.

Calcium Imaging

PSMCs were loaded with fluo-3 AM dissolved in DMSO containing 20% pluronic acid (10 μM) for 45 min at room temperature (Molecular Probes). Upon washing thoroughly with Tyrode solution containing (in mM) 137 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 D-glucose and 10 NaHEPES (pH 7.4 adjusted with NaOH), the cytosolic dye was allowed to de-esterify for 20 min. Fluo-3 fluorescence of PSMCs were detected under a Nikon Diaphot microscope equipped with epifluorescence attachments and a microfluorometer (model D-104; PTI). After a stable resting $[\text{Ca}^{2+}]_i$ was attained for more than 10 min, the integrin-specific ligands GRGDTP and GRGDNP were applied to PSMCs and the fluorescent signal was recorded for 15 min. The Ca^{2+} response of both normoxic and hypoxic PSMCs was examined under normoxic conditions for the comparison of integrin-dependent response in the absence of acute influence of hypoxia intracellular $[\text{Ca}^{2+}]_i$. Protocols were executed and data collected on-line with a Digi-data analog-to-digital interface and the pClamp software package (Axon Instruments Inc.). Fluorescence intensity (F) was used to calculate the intracellular concentrations of Ca^{2+} : $[\text{Ca}^{2+}]_i = [\text{K}_D \cdot (F - F_{bg})] / (F_{max} - F)$, where F_{bg} is the background fluorescence and F_{max} is the maximum fluorescence. Values for F_{max} were determined in situ by superfusing the cells with the 10 μM Ca^{2+} ionophore 4-Br-A23187 (EMD Biosciences), and values for F_{bg} were obtained in an area devoid of cells upon Mn^{2+} quenching.

Statistical Analysis

Data are expressed as means $\pm$ SEM. The numbers of cells are specified in the text. Statistical significance ($p < 0.05$) was assessed by paired or unpaired Student's t tests or ANOVA with

Newman-Keuls post hoc analyses wherever applicable. For Western blot analysis of integrin subtypes, each sample represented protein isolated from one animal and was normalized to the average intensities of control samples within each blot. Control-normalized values were then averaged between replicate blots. FAK phosphorylation was quantified by the ratio of the phospho-FAK signal over total FAK signal for each sample. Conventional house-keeping genes, such as smooth muscle α -actin, β -actin, GAPDH and cyclophilin are regulated with hypoxia [16, 17]. Immunoblot analysis of smooth muscle α -actin, and β -actin were consistently regulated in these experiments, and were therefore not used as loading controls. Instead, protein concentration was used to ensure even loading, and a large sample size was used to minimize random errors resulting from pipetting.

Results

Validation of the Rat Models of Pulmonary Hypertension

Right ventricular hypertrophy as measured by comparing the mass ratio, RV/(LV+S), was used to confirm the development of pulmonary hypertension in chronic hypoxia and MCT-treated animals. RV/(LV+S) was significantly elevated in rats exposed to 4 weeks of chronic hypoxia (normoxia: 0.276 ± 0.004 , $n = 48$; hypoxia: 0.52 ± 0.01 , $n = 46$, $p < 0.001$) and in rats 3 weeks after MCT injection (control: 0.265 ± 0.005 , $n = 27$; MCT: 0.53 ± 0.03 , $n = 25$, $p < 0.001$).

Effect of Chronic Hypoxia on Integrin Protein Levels

We investigated the effect of chronic hypoxia on selected integrin subtypes at the protein level by immunoblot analysis. Chronic hypoxia significantly elevated α_8 integrin protein levels by $51.7 \pm 14.9\%$ compared to normoxic controls ($p < 0.007$, $n = 12$ animals), along with the levels of integrins α_1 and α_v ($45.6 \pm 6.4\%$, $p < 0.001$, $n = 12$ animals for α_1 ; $45.1 \pm 10.0\%$, $p < 0.002$, $n = 15$ animals for α_v) (fig. 1a, b). On the other hand, integrin α_5 and β_1 protein levels were decreased in PAs isolated from chronically hypoxic rats ($-36.6 \pm 6.1\%$, $p < 0.001$, for α_5 ; $-35.5 \pm 6.7\%$, $p < 0.001$, for β_1 ; $n = 15$ animals each) (fig. 1a, b). In contrast to the significant changes in protein expression observed in the PA, chronic hypoxia affected integrin levels minimally in the aorta (fig. 1c, d), in which only the expression of α_8 integrin was reduced to statistically significant levels by chronic hypoxia ($-16.6 \pm 3.5\%$, $p < 0.001$, $n = 16$ animals).

Effect of MCT Treatment on Integrin Protein Levels

To distinguish whether the changes in integrin expression were due to direct effects of hypoxia or to pul-

monary vascular remodeling, we turned to the MCT model of pulmonary hypertension. As shown in figure 2a and b, the changes in integrin protein expression were more pronounced in the PAs of MCT-treated animals. α_1 integrin expression was most dramatically increased, by $149 \pm 36\%$ ($p = 0.004$, $n = 11$ animals), when compared to controls. This was followed by α_8 and α_v integrins, which were increased by $71.4 \pm 14.8\%$ ($p < 0.001$, $n = 11$ animals) and $65.1 \pm 19.5\%$ ($p = 0.006$, $n = 11$ animals), respectively. Levels of α_5 and β_3 integrins were reduced in PAs isolated from MCT-treated animals by $24.7 \pm 6.7\%$ ($p = 0.006$, $n = 11$ animals) and $26.2 \pm 10.8\%$ ($p = 0.034$, $n = 11$ animals) respectively. Protein levels of integrin β_1 , while unaffected in PAs, were increased by $37.8 \pm 13.6\%$ ($p = 0.015$, $n = 11$ animals) in aorta isolated from MCT-treated animals (fig. 2c, d).

Detection of Integrin Expression by Immunohistochemistry

We further examined the expression of integrins in small PA in lung sections using double immunofluorescent staining. Positive signals were detected in small PAs using specific antibodies against α_1 , α_5 , α_8 , β_1 , and β_3 integrins, which overlapped with the signals of smooth muscle-specific α -actin (fig. 3), indicating that they originated from SMCs of small PAs. These results are consistent with the immunoblot studies using large PAs demonstrating integrin expression in PA smooth muscle. Immunoreactivities of various integrin subtypes were also detected in cells other than smooth muscle, but their signal levels were generally higher in actin-positive cells. However, immunohistochemistry was unsuitable for quantitative comparison of integrin expression in PAs of the chronic hypoxia and MCT models due to possible variability introduced by sectional plains, size of smooth muscle layer and sample processing. Differential expression of α_1 and α_5 integrins was further investigated in PASMCs of chronic hypoxia rat. Confocal imaging detected clear immunoreactivity of α_1 and α_5 integrins in the peripheral regions of PASMCs isolated from normoxic and chronic hypoxic rats (fig. 4a, b), suggesting that the integrin proteins were expressed predominantly on the cell surface. Cell surface expression of integrin was similar in normoxic and chronic hypoxic cells. The signals for α_1 integrin were generally stronger, while those for α_5 integrin were weaker, in the hypoxic PASMCs when they were recorded using the same settings for confocal imaging. However, variability of immunofluorescence signals in different samples precluded quantitative comparison.

Fig. 1. Representative immunoblots of integrin proteins in endothelium-denuded PA (a) and aorta (c) of rats exposed to chronic hypoxia and normoxia. Each lane represents protein isolated from an individual animal. **b, d** Densitometric data obtained from immunoblots (n = 12–15 animals). Twelve animals per treatment group were used to compile the data in **b** for α_1 and α_8 integrins, while 15 were used for all others. Data are normalized to the average of the normoxic controls. Asterisks show significant changes in integrin protein expression upon exposure to chronic hypoxia (* p < 0.05, ** p < 0.01 vs. control).

Effect of Chronic Hypoxia on FAK Phosphorylation in PAs and PSMCs

To examine if the alterations in integrin expression were evidenced in integrin-dependent signaling pathways, phosphorylation of FAK in endothelium-denud-

ed PAs of control and chronic hypoxic rats was determined by immunoblotting with antibodies specific for phosphorylated and total FAK. The signal ratio of phosphorylated over total FAK was significantly higher ($181.4 \pm 29.7\%$ of normoxic control, n = 6, p < 0.025) in

Fig. 2. Representative immunoblots of integrin proteins in endothelium-denuded PA (a) and aorta (c) of rats treated with MCT (60 mg/kg) for 24 days versus control. **b, d** Densitometric data obtained from immunoblots (n = 11–12 animals). Data are normalized to the average of the controls. Asterisks show significant changes in integrin protein expression upon treatment with MCT (* p < 0.05, ** p < 0.01 vs. control).

the hypoxic PAs (fig. 5). FAK phosphorylation was also higher in PSMCs isolated from hypoxic rats ($180.8 \pm 34.0\%$ of normoxic control, n = 4), when transiently cultured (24 h) on surfaces coated with type IV collagen, a ligand for the α_1 integrin whose expression was elevated

in hypoxic PSMCs (fig. 1a, b). In contrast, the differences of FAK phosphorylation between normoxic and hypoxic PSMCs disappeared when cells were cultured on fibronectin, a typical ligand for α_5 integrin, whose expression was reduced by hypoxia (fig. 1a, b).

Fig. 3. Immunostaining of integrin and smooth muscle α -actin in small PAs in lung sections of control rats. Confocal images show fluorescent signals from lung sections double-stained with a α_1 (a), α_5 (b), α_8 (c), β_1 (d) and β_3 (e) integrin-specific antibody, and an α -actin-specific antibody. Transmission images show the lung

structures, while the yellow color indicates the regions in which there were overlaying of signals of integrin protein and α -actin in PAs. The images were taken with a Plan Neofluar $\times 20$ objective (numerical aperture = 0.5), pinhole size = 1 airy, and zoom was between 2.7 and 3.6. Scale bars = 20 μm (a, b); 10 μm (c–e).

Fig. 4. Confocal images of immuno-fluorescent signals (red) of α_1 (a) and α_5 integrin (b) in PSMCs isolated from normoxic and chronic hypoxic rats. Transmitted light images (right panels) were included for reference. The images were taken with a Plan Neofluar $\times 40$ oil objective (numeric aperture = 1.3), pinhole size = 1 airy, and zoom = 4. Laser power, sensitivity and gain were set at the same level for confocal imaging of the normoxic and hypoxic cells.

RGD-Induced Calcium Signals

To test if the changes in hypoxia-induced integrin expression are translated into altered physiological function, we monitored the $[Ca^{2+}]_i$ response to RGD peptides in PSMCs isolated from normoxic and chronic hypoxic rats. Integrin α_1 , whose expression was significantly upregulated upon exposure to chronic hypoxia, recognizes collagen types I and IV when dimerized with β_1 integrin. We therefore compared the intracellular Ca^{2+} response of PSMCs to GRGDTP, which preferentially interacts with receptors for collagen, including $\alpha_1\beta_1$ integrin [18].

Exogenous application of GRGDTP (1 mM) to PSMCs isolated from normoxic control rats elicited a maximum increase in $[Ca^{2+}]_i$ of 73 ± 16 nM (fig. 6a, b; at 10 min, $n = 30$ dishes). When applied to PSMCs isolated from rats exposed to chronic hypoxia, GRGDTP caused $[Ca^{2+}]_i$ changes of a larger magnitude ($\Delta[Ca^{2+}]_i = 114 \pm 24$ nM, at 3.5 min, $n = 29$ dishes) than in normoxic PSMCs. Moreover, the time course of the $[Ca^{2+}]_i$ transient induced by GRGDTP was faster in the hypoxic cells, where the peak $\Delta[Ca^{2+}]_i$ was reached by 3.5 min after GRGDTP application, as opposed to 8 min in normoxic controls. At 3.5 min after GRGDTP application, the Ca^{2+} response observed in PSMCs isolated from chronic hypoxic rats

was significantly larger than the normoxic controls (fig. 6b, $p < 0.001$).

To test if the decreased α_5 integrin expression in the PAs of chronic hypoxic rats affected $[Ca^{2+}]_i$ signaling, we utilized the hexapeptide GRGDNP, which predominantly targets the fibronectin receptor including $\alpha_5\beta_1$ integrin [19]. GRGDNP (0.5 mM) elicited a biphasic Ca^{2+} response with an initial peak followed by a sustained plateau phase (fig. 6c, d). The initial response in PSMCs isolated from normoxic and chronic hypoxic rats were similar, at 153 ± 21 and 125 ± 21 nM, respectively. However, the sustained phase of the GRGDNP-induced Ca^{2+} response was significantly reduced in PSMCs isolated from chronic hypoxic rats (34 ± 8 nM, $n = 27$ dishes) as compared to those isolated from normoxic controls (79 ± 12 nM, $n = 26$ dishes, $p = 0.003$).

Discussion

The present study extends our previous report on the characterization of integrin expression and Ca^{2+} response in rat PA smooth muscle [10]. Immunostaining shows that integrin proteins are expressed in α -actin-positive smooth muscle layer in small PAs and localized in the surface of

Fig. 5. **a** Representative immunoblots of phosphorylated FAK and total FAK in endothelium-denuded PAs from normoxic and hypoxic animals. Each lane represents protein isolated from an individual animal. **b** A bar graph summarizes the averaged signal ratio of phosphorylated FAK over total FAK in PAs ($n = 6$ animals in each group). **c** The signal ratio of phosphorylated FAK over total FAK in PASMCs isolated from normoxic and hypoxic rats that were transiently cultured in culture dish coated with collagen IV or fibronectin ($n = 4$ experiments with cells from 4 different animals in each group). Asterisks show significant changes in increase in FAK phosphorylation upon exposure to chronic hypoxia (* $p < 0.05$ vs. normoxia).

PASMCs. The expression of integrin proteins is altered in endothelium-denuded PAs in chronic hypoxia and MCT-induced pulmonary hypertensive rats. Both models displayed significant increases in α_1 , α_8 and α_v integrins as well as a downregulation of α_5 integrin. Integrin β_1 and β_3 protein levels were also significantly suppressed in chronic hypoxia and MCT-treated rats, respectively. Downstream integrin signaling was also affected in congruence with the altered integrin expression, such that the levels of FAK phosphorylation were augmented in PAs of chronically hypoxic rats, and the enhanced phosphorylation was further maintained in hypoxic PASMCs when cultured on the α_1 integrin ligand collagen IV. Moreover, the upregulation of α_1 integrin and the downregulation of α_5 integrin in chronic hypoxic PAs were correlated with increased and decreased Ca^{2+} mobilization in PASMCs induced by RGD peptides that specifically target these in-

tegrins. The effects of both chronic hypoxia and MCT were specific to the pulmonary vasculature, as endothelium-denuded aorta from the same animals displayed virtually no change in integrin expression, except a small reduction of α_8 integrin in the hypoxic animals and a minor increase in β_1 integrin in MCT treated rats. Our results therefore indicate that integrin expression and associated signaling pathways are altered in pulmonary hypertension and partake in pulmonary vascular remodeling that accompanies disease progression.

The differential regulation of integrins observed in this study underscores the complex interplay of ECM/integrin-dependent mechanisms, which depends on the temporal alterations in synthesis, deposition and reorganization of various ECM components, as well as the expression of specific integrins at different stages of disease progression. In the chronic hypoxia model, increased de-

Fig. 6. $[Ca^{2+}]_i$ transients elicited by integrin-binding peptides in PSMCs from normoxic and chronic hypoxic rats. **a** Time course of GRGDTP-induced $[Ca^{2+}]_i$ response in normoxic (black squares) or chronic hypoxic (gray circles) PSMCs. $n = 29$ dishes of PSMCs, from 3 rats on 3 separate days. **b** Bar graph summarizing the peak and plateau phases of $\Delta[Ca^{2+}]_i$ at 3.5 and 10 min

after application of GRGDTP. **c** Time course of GRGDNP-elicited $[Ca^{2+}]_i$ response in normoxic (black squares) or chronic hypoxic (gray circles) PSMCs. $n = 30$ dishes of PSMCs, from 3 rats on 3 separate days. **d** Bar graph summarizing the peak and plateau phases of $\Delta[Ca^{2+}]_i$ at 3.5 and 13 min postapplication of GRGDNP.

position of ECM components including collagen type I, III and IV, elastin, laminin as well as fibronectin has been reported [20–23]. Transcripts of type IV collagen increase within 6 h and decline after 10 days, whereas type I and III collagen as well as fibronectin mRNA increase after 3 days and then decline to the control level after 10 days of exposure to hypoxia [22]. Elevated levels of ECM proteins

could be observed in 4 days of hypoxia and progress with time [23]. Similar increases in collagen, elastin, laminin, tenascin and fibronectin occur in the MCT model [24–27]. Elevated mRNA levels of laminin and tenascin could be detected within one day, and the increases in immunolocalizable fibronectin, laminin, perlecan and type IV collagen protein around the vasculature could be ob-

served 4 days after MCT treatment [24, 26]. ECM components continue to increase as the disease develops. The present study at 4 weeks of chronic hypoxia and 24 days of MCT treatment, therefore, is a snapshot view of the alterations in integrin expression in established pulmonary hypertension.

Amongst the integrins surveyed in this study, α_1 integrin was most prominently increased in both chronic hypoxia and MCT models of pulmonary hypertension. α_1 integrin, when dimerized to β_1 integrin is a major receptor for collagen [4, 28], with a preference for type IV collagen, the basement membrane collagen [29]. In addition to type IV collagen adhesion, $\alpha_1\beta_1$ integrin mediates feedback regulation of type I collagen synthesis and collagen-dependent proliferation [29]. Its expression is associated with the differentiated phenotype of SMCs, where transition from a contractile to synthetic phenotype results in its downregulation at both transcript and protein levels [28, 30]. Levels of the ligand, type IV collagen, are increased in PAs of animals with chronic hypoxia and MCT-induced pulmonary hypertension [3, 24], along with the expression and activity of MMP-2, which degrades collagen type IV [1, 6]. Loss of intact type IV collagen accompanying basement membrane degradation is associated with SMC dedifferentiation and a correlative increase in SMC migration [31]. We hypothesize that the upregulation of α_1 integrin in hypertensive PAs increases the sensitivity of PASMCs to intact type IV collagen, in order to maintain SMCs in a differentiated state counteracting the dedifferentiation process involved with the progression of pulmonary hypertension.

In addition to the α_1 integrin, α_v and α_8 integrins are upregulated in PAs of chronic hypoxia and MCT-treated rats. α_v -protein dimerizes with β_1 , β_3 , β_5 , β_6 , and β_8 integrins to recognize RGD-containing ligands, including vitronectin, fibronectin, fibrinogen, von Willebrand factor, thrombospondin, osteopontin and collagen [8]. Its increased protein levels in PAs isolated from chronic hypoxia and MCT-treated rats is consistent with previous studies in the systemic vasculature demonstrating, for example, its upregulation in small mesenteric arteries of hypertensive rats [32, 33] as well as in the neointima of various animal models of vascular injury [11, 32, 34]. $\alpha_v\beta_3$ integrin is furthermore involved in PASMC hyperplasia in the MCT model of pulmonary hypertension [1, 12, 13]. Many studies including those in the pulmonary vasculature show reduced vascular response to injury via decreased SMC proliferation and migration, MMP production and increased SMC apoptosis upon $\alpha_v\beta_3$ inhibition [34–37]. The increase in α_v integrin observed in this

study may contribute to PASMC proliferation and migration in pulmonary hypertension as a part of the vascular remodeling process.

α_8 integrin is highly expressed in vascular smooth muscle and dimerizes with β_1 integrin to bind ligands such as tenascin-C, vitronectin and fibronectin [8, 38, 39]. Expression of $\alpha_8\beta_1$ integrin has been proposed as required for maintaining the contractile, differentiated phenotype of vascular SMCs. Indeed, α_8 integrin expression is decreased during neointimal formation. α_8 integrin gene silencing increases vascular SMC migration and expression of SMC de-differentiation markers. α_8 integrin overexpression attenuates SMC migratory activity and restores contractile phenotypes [11, 38, 39]. On the other hand, integrin $\alpha_5\beta_1$, the prototypical receptor for fibronectin, acts as a signal for cell proliferation [40, 41]. It is involved in the polymerization of fibronectin, which promotes SMC replication, migration and survival, and the overall maintenance of a synthetic phenotype [41]. α_5 integrin was decreased in both models of pulmonary hypertension. Thus, the upregulation of α_8 integrin and downregulation of α_5 integrin PAs obtained from both models of pulmonary hypertension may provide feedback signals to maintain the contractile phenotype of PASMCs and offset pulmonary vascular remodeling.

Compared to the large repertoire of α -subunits, the number of β integrins is limited. Only 8 β integrins are available to heterodimerize with 18 α -subunits. Of the 24 $\alpha\beta$ heterodimers identified to date, 12 contain the β_1 , while the major partner for β_3 integrin is α_v [8]. β_1 and β_3 integrins were decreased in PAs of chronic hypoxia and MCT models of pulmonary hypertension, respectively. Inhibition of β_1 integrin has been shown to reduce vascular SMC migration and adhesion [42, 43], and knockout as well as blockade of β_3 integrin decreased neointimal thickening and SMC migration in injured arteries [37, 44]. Since β integrins heteromerize with various α -subtypes, the modest decrease in β_1 and β_3 levels may limit the availability of functionally active heterodimeric integrins, although its significance within the context of pulmonary hypertension is presently unclear.

The changes in integrin protein expression detected by immunoblot in PA of pulmonary hypertensive rat models are likely translated into alterations of integrin-dependent signaling. This is evidenced by further examining the functional consequences of differential α_1 and α_5 integrin expression in the chronic hypoxia model. α_1 and α_5 integrins were chosen due to the availability of RGD peptides and ECM proteins that target these integrins relatively specifically. The predominant α_1 and α_5

integrin immunoreactivity on the surface of PSMCs of chronically hypoxic rats suggest that the integrin proteins are incorporated into the sarcolemma, precluding the possibility that cytosolic accumulation of nonfunctional integrin proteins may contribute to the immunoblot data. A higher integrin-dependent activity is supported by the elevated level of FAK phosphorylation, a major downstream signaling pathway, in PASM of hypoxic rats. The enhanced phosphorylation could be related in part to the upregulated α_1 integrin because it was maintained in PSMCs isolated from hypoxic rats that were cultured on the $\alpha_1\beta_1$ ligand type IV collagen, but not on the $\alpha_5\beta_1$ ligand fibronectin. Upregulation of α_1 integrin in the chronic hypoxic PSMCs was furthermore reflected in the increased Ca^{2+} mobilization elicited by the α_1 integrin-binding peptide, GRGDTP, in a manner similar to the collagen IV-induced Ca^{2+} response observed in other cell types [45, 46]. Likewise, the decreased α_5 integrin expression correlated with the reduced sustained Ca^{2+} response of chronic hypoxic PSMCs to the α_5 integrin-binding hexapeptide, GRGDNP. It has to be mentioned, however, that the Ca^{2+} response induced by soluble RGD peptide ligands may only partially reflect the signaling induced by the immobile ligands within the native tissue.

The unique kinetic profiles of the intracellular Ca^{2+} transients elicited by the hexapeptides, GRGDTP and GRGDNP, suggest that integrins mobilize intracellular Ca^{2+} through subtype-specific pathways. Indeed, although both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins are necessary for myogenic constriction in cremaster arterioles [47], $\alpha_5\beta_1$ integrin activation causes vasoconstriction through L-type Ca^{2+} channel potentiation, while $\alpha_v\beta_3$ ligands induce vasodilation, K^+ current activation and L-type Ca^{2+} channel inhibition [9]. We have also shown that the com-

mon integrin ligand GRGDSP mediates Ca^{2+} release from ryanodine-gated Ca^{2+} stores and lysosome-related acidic organelles in rat PSMCs [10]. The diverse modes of Ca^{2+} mobilization transduced by the various integrins are likely linked to the ultimate downstream effect and function of the specific integrins. It will be important in the future to address the Ca^{2+} signaling pathways linked to the specific integrins that are altered in pulmonary hypertension.

In summary, we quantified the integrin levels in PAs of chronic hypoxia and MCT-induced pulmonary hypertensive rats. The similarity in the regulation of α -integrin expression in the two models suggest that they are related generally to pulmonary hypertension, and not to the direct effects of hypoxia or MCT on the pulmonary vasculature. The differential regulation of integrins in the PAs of pulmonary hypertensive animals exemplifies the complex nature of ECM/integrin-mediated signaling, and underscores the multifactorial nature of the mechanisms involved in pulmonary hypertension. Although the exact mechanisms leading to the development of pulmonary hypertension are a topic of active debate, the results of this study highlighting the involvement of a new player, namely integrins, in pulmonary hypertension along with its ECM ligands that play a major role in disease pathogenesis.

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