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Proteomic analysis of the lung in rats with hypobaric hypoxia-induced pulmonary hypertension

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Summary. Experimental pulmonary hypertension that develops in hypobaric hypoxia is characterized by structural remodeling of the lung. Proteomics - which may be the most powerful way to uncover unknown remodeling proteins involved in enhancing cardiovascular performance - was used to study 150 male Wistar rats housed for up to 21 days in a chamber at the equivalent of 5500 m altitude level. After 14 days' exposure to hypobaric hypoxia, pulmonary arterial pressure (PAP) was significantly increased. In lung tissue, about 140 matching protein spots were found among 8 groups (divided according to their hypobaric period) by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (pH4.5-pH6.5, 30 kDa-100 kDa). In hypobaric rats, three spots were increased two-fold or more (vs. control rats) in two-dimensional differential in-gel electrophoresis (2D-DIGE). The increased proteins were identified, by matrix-assisted laser desorption ionization time of flight (MALDI-TOF), as one isoform of heat shock protein 70 (HSP70) and two isoforms of protein disulfide isomerase associated 3. This result was confirmed by Western blotting analysis of 2D-PAGE. Conceivably, HSP70 and PDIA3 may play roles in modulating the lung structural remodeling that occurs due to pulmonary hypertension in hypobaric hypoxia.

Key words: Proteomics, Hypobaric hypoxia, Lung, Rat

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Introduction

The experimental pulmonary hypertension that develops in a hypobaric hypoxic environment (HHE) is characterized by structural remodeling of the pulmonary arteries such as intimal thickening and extension of smooth muscle into previously nonmuscular arterioles (Meyrick and Reid, 1978; Heath and Williams, 1981; Reid, 1986; Nakanishi et al., 1996, 1999, 2001, 2002, 2004). Various factors -- such as hypoxia-induced transcription factors (HIF) and several growth factors [including PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), and TGF-ß (transforming growth factor-ß)] - are associated with the vascular remodeling processes that occur in the lung in response to chronic hypoxia (Katayose et al., 1993; Arcot et al., 1995; Tuder et al., 1995; Semenza, 2002; Wenger, 2002). However, examining other proteins may provide insights into these pathobiologic processes.

The proteomic approach has emerged as a powerful tool that can (a) provide protein-expression profiles, which may be useful for the prediction of clinical events or therapeutic responses, or for the unraveling of disease mechanisms, and (b) allow analysis of the quantities of certain proteins present in different conditions (Toda and Kimura, 1997; Toda et al., 2000). Moreover, proteomic studies may well be a key propulsive force favoring the discovery of new biomarkers. In the present study, for a better understanding of the mechanisms underlying the response of the rat lung to HHE, rats were housed for up to 3 weeks in a mechanical chamber at the equivalent of an altitude of 5500 m. We measured pulmonary arterial pressure and then, using proteomic analysis informed by

two-dimensional differential in-gel electrophoresis (2D-DIGE), tried to identify proteins previously unknown to be involved in vascular remodeling in the lung in HHE.

Materials and methods

We used essentially the same methods as in previous reports (Nakanishi et al., 1996, 1997, 1999, 2001, 2002, 2004). Although we intended several experiments to involve 4 to 12 weeks' exposure to HHE, many rats scheduled for 6-12 weeks' exposure became weak and emaciated, or even died, some time after 4 weeks' exposure (Nakanishi et al., 1997). Therefore, in the present study we elected to use a maximum of 21 days' exposure to HHE. Briefly, a total of 150 adult male Wistar rats, approximately 8 weeks old and weighing 190 to 210 g, were divided into 7 groups. Groups of 17 or 18 rats each were housed in a 5.6x3.0x3.0m mechanical chamber (Hitachi Corp.) and exposed to an HHE equivalent to 5500 m in altitude (380 mmHg = 50.6 kPa) for 0.5 to 21 days. The chamber comprised a main room to house the animals and an accessory room. When investigators (with their own oxygen supply) needed to enter the accessory room, it was decompressed to an HHE equivalent to 5500 m in altitude. They then took care of the HHE rats. Tests on the animals in the HHE were conducted in the accessory room. The oxygen content in the main room was maintained constant at an FiO_2 of 0.105 to simulate 5500 m in altitude. The age of the rats when exposure to HHE was initiated was such that all were approximately 11 weeks old at the termination of the experiment. The chamber was kept at an ambient temperature of 23±1°C and a relative humidity of 60-70%, with light exposure daily from 06.00 to 18.00 h. All rats were given commercial chow and tap water ad libitum. After 0.5, 1, 3, 5, 7, 14, or 21 days of exposure to HHE, rats were killed by decapitation. An additional 17 rats kept in the ground-level environment served as controls. Mean pulmonary arterial pressure (PAPm) and mean systemic arterial pressure (SAPm) were evaluated at 0.5, 1, 3, 7, 14, or 21 days of exposure to the HHE condition described above, the technique used and the results obtained having been reported elsewhere (Nakanishi et al., 2004). This experimental study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments in the National Defense Medical College.

Blood sampling and autopsy

Before autopsy, the rats were decapitated and blood samples were taken (from rats not subjected to arterial pressure measurement) for the determination of hematocrit.

At autopsy, lung tissues were frozen in liquid nitrogen, and stored at -80°C until used for proteomic and/or Western blotting analyses.

Protein extraction

Fifteen milligrams of each lung sample was washed with normal saline and then homogenized on ice in 250 μl lysis buffer [8.3 M urea, 0.2% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) Triton X-100, 65 mM DL-dithiothreitol, 2% Pharmalyte (pH 3-10)], containing 10 μl protein inhibitor cocktail (Sigma Chemical Co., St Luis, MO). For this, we used a Sample Grinding Kit (GE Healthcare, Buckinghamshire, UK). After centrifugation (15,000 rpm for 5 min) to collect the supernatant fluid, the fluid was adjusted to pH 8.0-9.0 using 50 mM sodium hydroxide. Protein concentrations were determined using the DC Protein Assay system (Bio-Rad Laboratories, Hercules, CA). The protein concentration of each sample was adjusted to 5-10 mg/ml.

Two dimensional (2D) SDS-polyacrylamide gel electrophoresis

The 2D SDS-polyacrylamide gel electrophoresis (SDS-PAGE) protocol was adapted from Toda et al (Toda and Kimura, 1997; Toda et al., 2000). Briefly, isoelectric focusing (IEF) was carried out using immobilized pH gradient (IPG) strips [18 cm, pH 4-7; Immobiline™ DryStrip (Amersham Bioscience, Uppsala, Sweden)]. Protein IEF was performed at 20°C using a CoolPhoreStar IPG-IEF (Anatech Co., Tokyo, Japan) as follows: step 1 at 500 volts (V) for 2 h, step 2 at 700 V for 1 h, step 3 at 1,000 V for 1 h, step 4 at 1,500 V for 1 h, step 5 at 2,000 V for 1 h, step 6 at 2,500 V for 1 h, step 7 at 3,000 V for 1 h, and step 8 at 3,500 V for 10 h. In this way, a total of 46,700 Volt-hours (Vh) was reached. Strips were washed for 30 min in an equilibration buffer [6 M urea, 32 mM DL-dithiothreitol, 0.025 M Tris-HCl pH 6.8, 2% (w/v) SDS, 0.005% (w/v) BPB, and 30% (v/v) glycerol], followed by washing for 20 min with a reduction buffer [0.025 M Tris-HCl pH 6.8, 2% (w/v) SDS, 0.005% (w/v) BPB, 30% (v/v) glycerol, and 0.25 M iodoacetamide]. SDS-PAGE, which was performed using 10% polyacrylamide gels (18x18 cm), was run at 25 mA for 5 h in a CoolPhoreStar SDS-PAGE Tetra-200 (Anatech Co.). The gels were washed overnight in fix solution [50% methanol (MeOH), 10% acetic acid], and stained with ProteoSilver Plus Silver Stain Kit (Sigma-Aldrich, St Louis, MO) or SYPRO Ruby (Bio-Rad Laboratories), in accordance with the manufacturers' protocols.

Protein labeling

Proteins were minimally labeled according to the manufacturer's instructions (CyDye DIGE fluor minimal labeling kit; GE Healthcare). Briefly, each minimal CyDye was reconstituted in fresh N, N-dimethyl-

formamide (DMF), and a 400 pmol quantity was used to label 50 μg of protein at pH 8.5. Cy3 and Cy5 were used for random labeling of experimental samples (for each exposure time) and control samples. The labeling reaction proceeded on ice in the dark for 30 min, until terminated by the addition of 1 μl 10 mM lysine (on ice in the dark for 10 min).

Two-dimensional differential in-gel electrophoresis (2D-DIGE)

Following the labeling reaction, 160 μg of each of the Cy3- and Cy5-labeled samples were mixed. Samples were actively rehydrated into 18-cm pH 4-7 Immobiline DryStrips, and these were placed in a strip holder and then focused at 20°C using a CoolPhoreStar IPG-IEF as above. The strips were rehydrated in an equilibration buffer, and then proteins were further separated on 10% SDS-PAGE gels casted upon low-fluorescence glass plates, utilizing a CoolPhoreStar SDS-PAGE Tetra-200.

Scanning and image analysis

After 2D-DIGE, the gels were scanned using an Ettan DIGE imager (GE Amersham) at 100 μm resolution, as elaborated in the equipment setup instructions. The Cy3- and Cy5-labeled images for each gel were scanned at excitation/emission wavelengths of 540/595 nm and 635/680 nm, respectively. After scanning the fluorophores in each gel, the images were imported into ImageMaster 2D Platinum (GE Amersham) for spot detection, according to the manufacturer's recommendations. After spot detection, the abundance changes were expressed as the spot-volume ratio for each hypobaric sample over the control sample. When the spot-volume ratio obtained for a given hypobaric sample over the control sample was more than 2.0 or less than 1/2, and p was <0.05 by independent Student's t-test, those protein spots were marked.

Protein digestion, mass spectrometry and protein identification

Spots in the maps for which the intensity differed significantly between the control group and a given exposure-time group were selected to be identified by mass spectrometry. Spots were excised with a 1.8 mm core from the SYPRO Ruby-stained gel using FluoroPhoreStar 3000 (Anatech Co.) for digestion. Spots were washed in reduction buffer (0.1 ml of 1.5 mg/ml dithiothreitol in 100 mM ammonium bicarbonate) for 30 min, alkalized in 0.1 ml of 10 mg/ml iodoacetamide in 100 mM ammonium bicarbonate for 30 min, destained in 100 mM ammonium bicarbonate in 50% MeOH for 15 min twice and 100 mM ammonium bicarbonate in 50% acetonitrile for 10 min three times, and finally incubated in 0.1 ml of 100% acetonitrile for 5 min. Gels were then dried completely by vacuum-drying. In-gel digestion was performed at 37°C overnight using 20 μg/ml sequencing grade trypsin (Promega) in 40 mM ammonium bicarbonate. To achieve complete peptide recovery, sequential extraction steps [10% trifluoroacetic acid (TFA), 50% acetonitrile at room temperature for 20 min] were carried out three times using the digested samples. The supernatants containing peptides were collected, and then concentrated and dried. Peptides were mixed with equal amounts of matrix solution [α-cyano-4-hydroxy-cinnamic acid (CHCA) in 0.05% TFA, 50% acetonitrile], immediately loaded onto the target plate, and allowed to air-dry at room temperature. MALDI-TOF mass spectrometry was performed using an AXIMA-CFR Plus (Shimadzu Co., Kyoto, Japan) according to the manufacturer's instructions. Protein identification was carried out by Peptide Mass Fingerprinting (PMF) analysis with the aid of Mascot software, comparisons being made with the NCBInr (National Center for Biotechnology Information non-redundant) sequence databases.

Western blotting analysis

After 2D SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (PVDF, Hybond-N+; Amersham Biosciences). The membranes were blocked with 5% fat-free dry milk (Block Ace, Dainippon Pharmaceutical, Osaka, Japan) in Tris-buffered saline (0.025 M Tris-HCl, pH 7.4) containing 0.1% Tween 20 (TBST) for 1 h at room temperature, and incubated overnight either with polyclonal rabbit heat shock protein 70 (HSP70) antibody (Nobus Biologicals, Littleton, CO) diluted at 1:1500 or with polyclonal goat protein disulfide isomerase associated 3 (PDIA3) antibody (Ray Biotech, Inc, Norcross, GA) diluted to 2.5 μg/ml in TBST with 1% bovine serum albumin. After being washed with TBST three times, the membranes were further incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody against goat immunoglobulins (Histofine, Simple Stain MAX-PO; Nichirei Bioscience, Japan) at an appropriate dilution, and then washed with TBST three times. The immunoreactive protein spots were visualized by means of an ECL Plus Western Blotting Detection System (GE Healthcare) and an LAS 3,000 system (Fuji Photo Film, Tokyo, Japan).

Immunohistochemistry

The indirect immunoperoxidase method was applied to 4 μm thick formalin-embedded paraffin sections. The antibodies used were as follows: primary rabbit polyclonal rabbit heat shock protein 70 (HSP70) antibody (Nobus Biologicals) diluted at 1:100 or polyclonal goat PDIA3 antibody (Ray Biotech) diluted to 10 μg/ml, and also horseradish peroxidase-labeled secondary antibody against rabbit or goat immunoglobulins [Histofine simplex MAX-PO(Multi), ready to use; Nichirei] or goat immunoglobulins [Histofine simplex MAX-PO(G), ready to use; Nichirei].

For immunohistochemistry of PDIA3, sections were boiled in 0.05 M Tris-0.001 M EDTA, pH9.0, for 40 min. For the negative control, the incubation step with the primary antibody was omitted.

Data analysis

Statistical analysis of the difference in incidence between two groups was performed using an independent Student's t-test. A p value of less than 0.05 was considered significant.

Results

Rats exposed to a hypobaric hypoxic environment (HHE)

The body weight of rats exposed to HHE decreased from 321.5±6.0 g (control rats, n=13) to 267.8±4.7 g (n=14) on day 21 of HHE exposure, while their hematocrit increased from 42.2±0.1% (control rats, n=6) to 60.4±0.5% (n=6) on day 21. The PAPm increased significantly from 14.7±1.0 mmHg (control rats, n=6) to 23.8±1.6 mmHg (n=6) on day 14 and 24.5 + 2.7 mmHg (n=6) on day 21. However, SAPm did not change, as described elsewhere (Nakanishi et al., 2004).

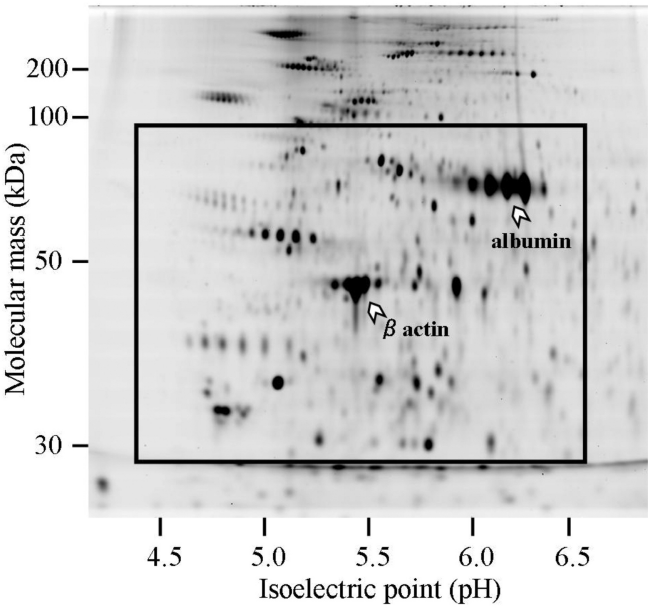

Fig. 1. Two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) of rat lung tissues in control group. Proteins were extracted and separated in pH 4-7 of 18-cm immobilized pH gradient (IPG) strips for the first dimension and in 10% polyacrylamide gel for the second dimension. The gel images obtained from 4 samples of lung tissues (one sample from each of 4 rats) in the control group were not significantly different from each other, particularly within the IEF range pH 4.5 to pH 6.5 and the molecular weight range 30,000 to 100,000 kDa (boxed area). The gel was stained with silver.

Identification of differentially expressed proteins by 2D-DIGE

The 2D-PAGE gel images obtained from 4 samples of lung tissues (one sample from each of 4 rats) in the control group were not significantly different from each other, particularly within the IEF range pH 4.5 to pH 6.5, and molecular weight range 30,000 to 100,000 kDa (Fig. 1). When we analyzed within those ranges, we found about 140 matching protein spots among the 8 groups (divided according to their hypobaric period).

The 2D-DIGE procedure was carried out 3 times for each lung sample (Fig. 2). When the spot-volume ratio obtained for a given hypobaric sample over the control sample was more than 2.0 or less than 1/2, three protein-spot features (Nos. 323, 375, and 382) were found to be significantly upregulated, and five (Nos. 288, 348, 350, 471, and 516) were significantly down-regulated (Fig. 3, Table 1). No. 323 was significantly increased on both day 14 and day 21 (vs. the control group), while Nos. 375 and 382 were significantly increased on both day 1 and day 3. In contrast, Nos. 288 and 516 were significantly decreased on days 14 and 21, No. 348 on day 3, No. 350 on days 5 and 7, and No. 471 on day 14.

Protein validation by Western blot analysis

When the three upregulated protein spots were analyzed by MALDI-TOF mass spectrometry, No. 323 was identified as heat shock 70kDa protein (HSP70) 1B, while Nos. 375 and 382 were identified as two subtypes of protein disulfide isomerase associated 3 (PDIA3) (Table 2). To evaluate the expressions of these two proteins in the lungs of rats exposed to HHE, we examined the 2D-PAGE gels by Western blotting analysis using two antibodies (HSP70 and PDIA3). In the case of HSP70, we could not find Spot no. 323 in the control group, although we found three other isoforms of

Table 1. Upregulated and down-regulated proteins in hypobaric hypoxic rat lung (compared with control lung tissue), as revealed by two-dimensional fluorescence difference gel electrophoresis.

Spot No.	Hypobaric period (days)						
	0.5	1	3	5	7	14	21
323						↑	↑
375		↑	↑				
382		↑	↑				
288						↓	↓
348			↓				
350				↓	↓		
471						↓	
516						↓	↓

↑ or ↓: When spot-volume ratio for a given hypobaric sample over control sample was more than 2.0 or less than 1/2, respectively, and p value was less than 0.05 vs. value on day 0 (n=4).

HSP70 in the control group. Spot no. 323 was detected on days 5, 7, 14, and 21, and it appeared to peak on days 14 and 21 (Fig. 4).

In the Western blotting analysis of PDIA3, we detected expressions of at least 3 spots in the control group (Fig. 4). Spots nos. 375 and 382, however, were not detected in the control group, although they were detected on days 0.5, 1, 3, 5, 14, and 21. These two spots were increased on days 1 and 3 (vs. the other groups, including the control group). Moreover, at least 14 isoforms of PDIA3 that were strongly expressed on day 1, and 10 isoforms that were strongly expressed on day 3 were not expressed in the control group.

Immunohistochemistry for HSP70 and PDIA3 Proteins

In the normal condition of the lung, very weak reactions for HSP70 protein were found within the smooth muscle cells in the bronchioles and bronchi, and also within the smooth muscle cells of small- and medium-sized arteries and veins, but not within alveolar cells. In rats exposed to HHE, it was detected within bronchial epithelial cells as well as in all the locations at which it was found in the normal lung. Its staining was intense throughout all phases of HHE (Fig. 5A). It was sometimes detected in vascular endothelial cells in HHE rats.

Table 2. Proteins upregulated in hypobaric hypoxic rat lung versus control lung tissue (as identified by MALDI-TOF MS).

Spot No.	Protein name	Accession No.	MW (kDa)	pI	Protein score	Sequence coverage (%)
323	Heat shock 70kD protein 1B	gi)47059179	70.1	5.6	171	37
375	Protein disulfide isomerase associated 3	gi)149023097	53.6	7.1	234	52
382	Protein disulfide isomerase associated 3	gi)149023097	53.6	7.1	212	49

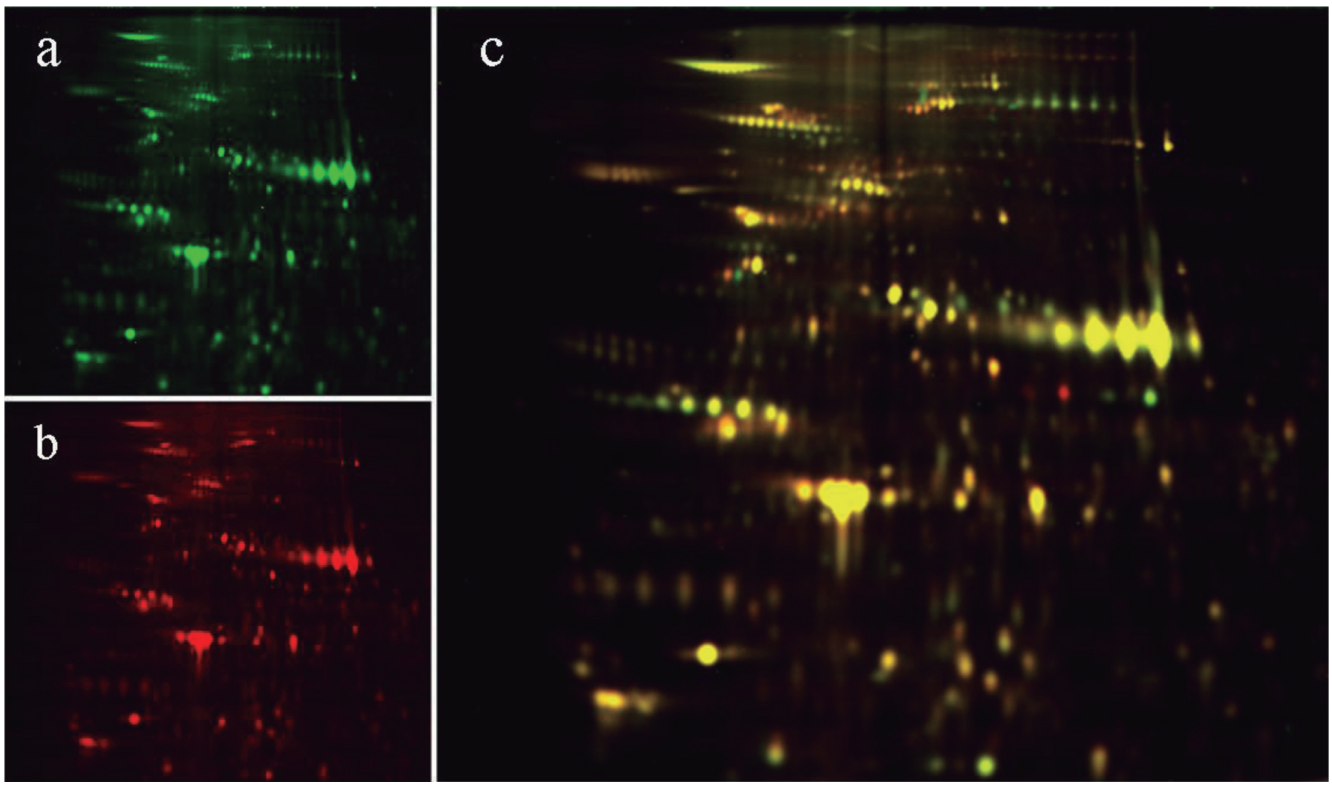

Fig. 2. Representative two-dimensional differential in-gel electrophoresis (2D-DIGE) pictures of scans performed using two dyes: in control lung (Cy3), and in hypobaric hypoxic lung (Cy5), together with an overlay image of the two-dye scans. Proteins were extracted and separated in pH 4-7 of 18-cm immobilized pH gradient (IPG) strips for the first dimension and in 10% polyacrylamide gel for the second dimension. Images were acquired using an Ettan DIGE imager scanner. Dots represent spots detected by the ImageMaster 2D Platinum. **a.** Cy3 (green) image of proteins from control lung tissue. **b.** Cy5 (red) image of proteins from lung tissue at 0.5 day's exposure to hypobaric hypoxic environment. **c.** Overlay image of two-dye scans of the control and hypobaric hypoxic lungs.

Concerning PDIA3 protein, weak reactions were found in the normal lung condition within bronchial epithelial cells and within smooth muscle cells in the bronchioles and bronchi, and also within the endothelial cells and smooth muscle cells of small- and medium-sized arteries and veins, but not within the alveolar cells. In rats exposed to HHE, it was detected in all the locations at which it was found in the normal lung, and its staining was intense in the early phase (day 0.5 to day 5) of HHE (Fig. 5B).

It should be noted that the lung displayed some regional variations in staining intensity for both proteins (HSP70 and PDIA3).

Discussion

Previous experimental studies have characterized some of the morphologic and functional changes that occur in the pulmonary vasculature in rats exposed to prolonged hypobaric hypoxia (Meyrick and Reid, 1978; Heath and Williams, 1981; Reid, 1986; Katayose et al., 1993; Arcot et al., 1995; Tuder et al., 1995; Nakanishi et al., 1996, 1997, 1999, 2001, 2002, 2004; Semenza, 2002; Wenger, 2002). However, little use has been made so far of 2D-DIGE-based proteomics to identify the pulmonary proteins expressed in rats during exposure to HHE. In the present study, we examined the lungs of rats exposed to HHE for up to 21 days using proteomic analysis informed by 2D-DIGE and MALDI-TOF mass spectrometry. Our results suggest that up-regulation of HSP70 1B and of two isoforms of PDIA3 may be characteristic changes during such exposure to HHE. In our Western blotting analysis, increases in HSP70 (including HSP70 1B) and PDIA3 (2 isoforms) were found on days 14 and 21, and days 1 and 3, respectively. Immunohistochemical findings for HSP70 and PDIA3 were consistent with those Western blotting results. On the basis of the above findings, we suggest that HSP70 1B and PDIA3 may be involved in the late phase and initial phase, respectively, in modulating the structural remodeling that occurs in the lung due to pulmonary hypertension in the course of prolonged hypobaric hypoxia.

It is well known that heat-shock proteins are classified into four major families according to their molecular size (HSP90, HSP70, HSP60, and the small HSPs). HSP70 IB is in the HSP70 family, the members of which act as molecular chaperones and are involved in many cellular functions, such as protein folding, transport, maturation, and degradation. In various stress conditions, the synthesis of stress-inducible HSP70 enhances the ability of cells to respond to unfolded proteins (Nollen et al., 1999). During hypoxic stress, neuroprotection due to an induction of HSP70 has been demonstrated both in animal stroke studies and in cell-culture models of ischemia (Plumier et al., 1997; Xu and Giffard, 1997). Moreover, heat shock pretreatment has been shown to protect thyroid FRTL-5 cells against the effects of hypoxia, suggesting that HSP70 may play an essential protective role against hypoxic injury (Kiang et al., 1996). A possible explanation for these findings may be that the increase in HSP70 blocks the apoptosis signal-transduction pathway.

An increase in HSP70 is also found in vascular smooth muscle cells in which hyperplasia and hypertrophy have been induced by growth factors (Patton et al., 1995). Here, using 2D-DIGE, we found an increase in HSP70 1B in the late phase of 21 days' HHE. In our Western blotting analysis, increases in HSP70 isoforms were detected on days 14 and 21 of HHE exposure. In our immunohistochemistry, HSP70 protein was detected within the smooth muscle cells of small-

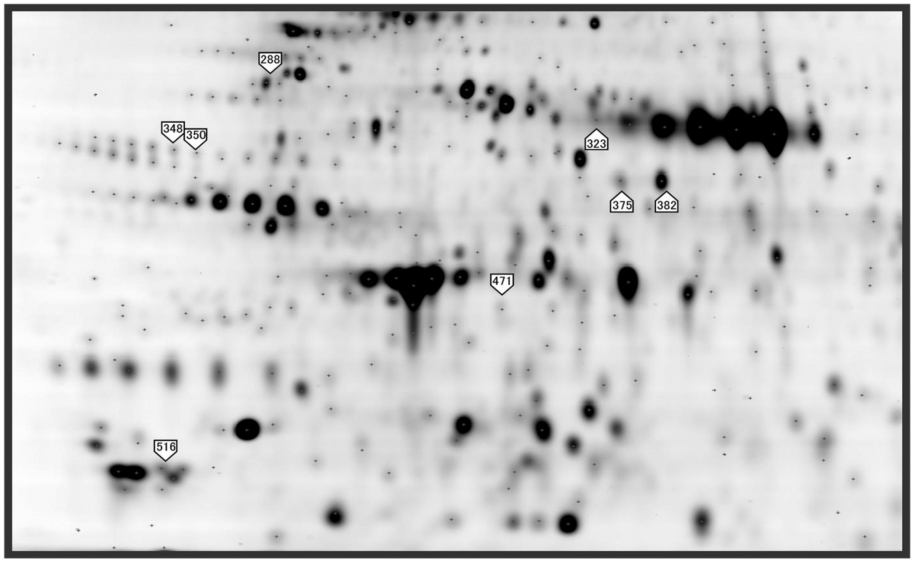

Fig. 3. Upregulated and down-regulated proteins (vs. control lungs) in two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) of rat hypobaric hypoxic lung tissue. Three protein spots were found to be upregulated significantly (volume ratio, >2.0; $p<0.05$) and five were down-regulated significantly (volume ratio, <1/2; $p<0.05$). Upregulated protein: ⌂, down-regulated protein: ▽. Spots were numbered according to two-dimensional SDS-PAGE of control lung. The gel was stained with silver.

and medium-sized arteries, as well as within both bronchial epithelial cells and smooth muscle cells in the bronchioles and bronchi, and its staining was more intense in the late phase of HHE exposure. These increases in HSP70 1B and in several isoforms of HSP70, and the increase in immunoreactive intensity seen in arterial smooth muscle cells, were quite well paralleled by the increases in PAPm detected on days 14 and 21. In our previous study, we found muscularization of pulmonary non-muscular arteries and partially muscular arteries after one week of HHE exposure, as well as medial thickening in muscular arteries after 2 weeks of HHE (same hypobaric hypoxic condition as in the present study) (Nakanishi et al., 1996). Moreover, a few years ago HSP70 was shown to protect against the angiotensin II-induced hypertrophy of isolated vascular smooth muscle cells (Zheng et al., 2006). On the basis of the above findings, we suspect that HSP70 may play an important protective role against pulmonary hypertension, particularly the rapidly increasing pulmonary arterial pressure and structural remodeling of pulmonary arteries that occurs in rats in the late phase of 21 days' HHE. On the current evidence, however, we cannot exclude the increase in HSP70 protein representing a late-phase marker of the hypoxia resulting from pulmonary arterial muscularization with beneficial effects at sites outside the pulmonary vasculature.

Like HSP70, PDI is a component of the

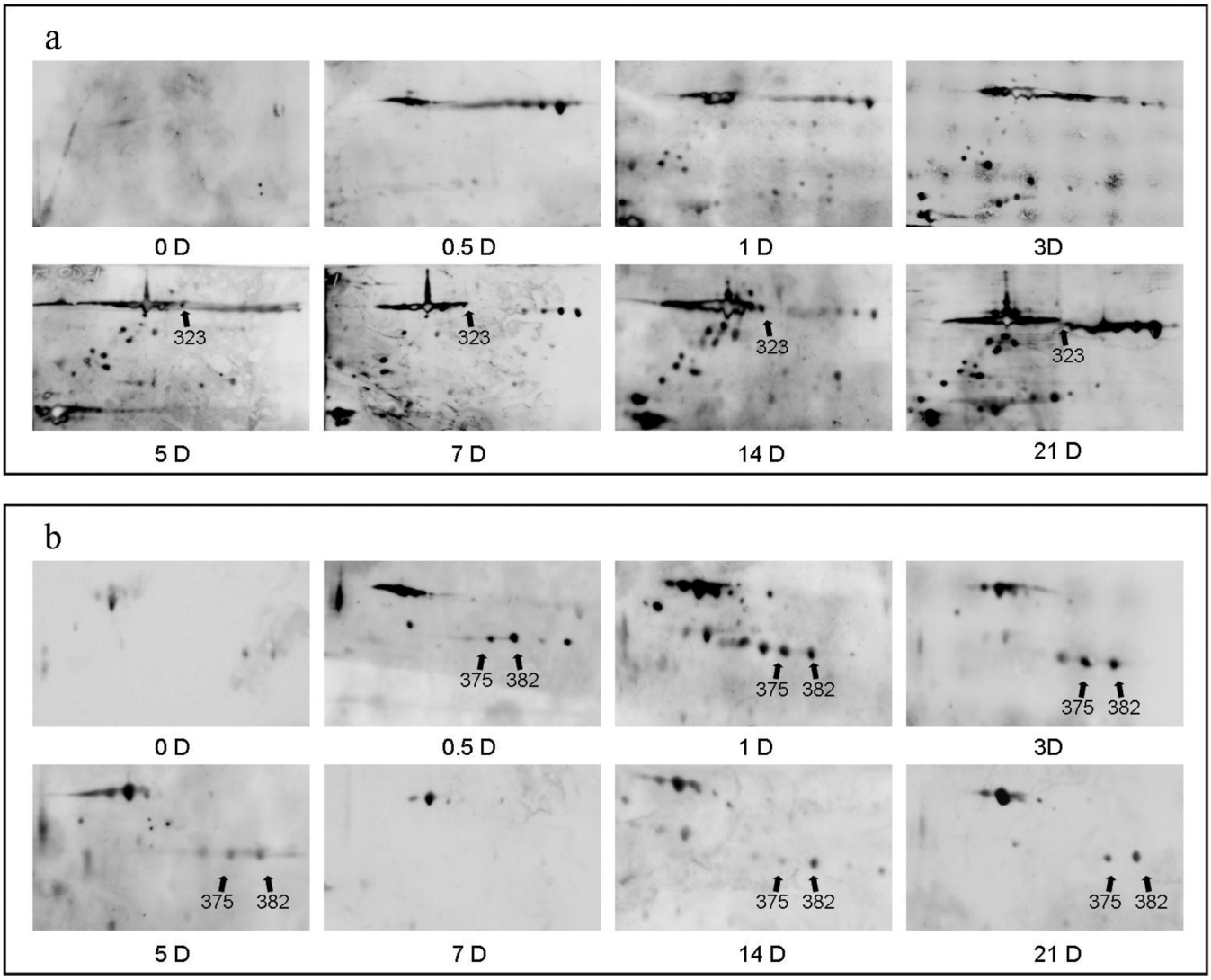

Fig. 4. Western blotting analysis of two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) of heat shock protein 70 **(a)** and protein disulfide isomerase associated 3 **(b)** in hypobaric hypoxic rat lungs. Spots were numbered according to two-dimensional SDS-PAGE of control lung.

endoplasmatic reticulum-stress signaling pathway, also known as the unfolded protein response (UPR). PDIA3 (also known as ERp60, ERp57, Grp58, and 1,25-MARRS) is a member of the PDI family, and specifically interacts with glycoproteins such as calnexin and calreticulin, playing an important role as a molecular chaperone during glycoprotein biosynthesis and folding (Jessop et al., 2007). Concerning hypoxic stress, an increase in PDI has been detected by 2D-PAGE in the myocardium of mice exposed to 3 weeks' hypoxia (Tian et al., 2009), and PDI has been shown to be upregulated in endothelial cells exposed to 18 h of hypoxia (0% O_2) (Graven et al., 2002). Moreover, increases in two structural homologs of PDIA3 (PDI and endothelial PDI) have been reported to have a neuroprotective effect in rat brain exposed to ischemia (Tanaka et al., 2000). Recently, an anti-apoptotic effect of PDIA3 was demonstrated in the melanoma cell-line A375 after the induction of ER stress (Corazzari et al., 2007). In the present study, several isoforms of PDIA3, including the two isoforms we detected by 2D-DIGE, were increased on days 1 and 3, but decreased on day 5 of HHE exposure. Moreover, the immunohistochemical intensity for PDIA3 protein was increased in the early phase of HHE in the smooth muscle cells of arteries, bronchioles, and bronchi. Since it is known that hypoxia induces apoptosis via ER stress (Kim et al., 2008), we suggest that PDIA3 may play a key protective role in the initial response to the ER stress resulting from the hypoxic stress that occurs during HHE.

Structurally, pulmonary hypertension is characterized by vascular remodeling in pulmonary arteries (Meyrick and Reid, 1978; Heath and Williams, 1981; Reid, 1986; Nakanishi et al., 1996, 1999, 2001, 2002, 2004). Concerning the examination by proteomics of proteins associated with smooth muscle cells in the pulmonary arteries of animals with pulmonary hypertension, Kwapiszewska et al. (2008), who studied

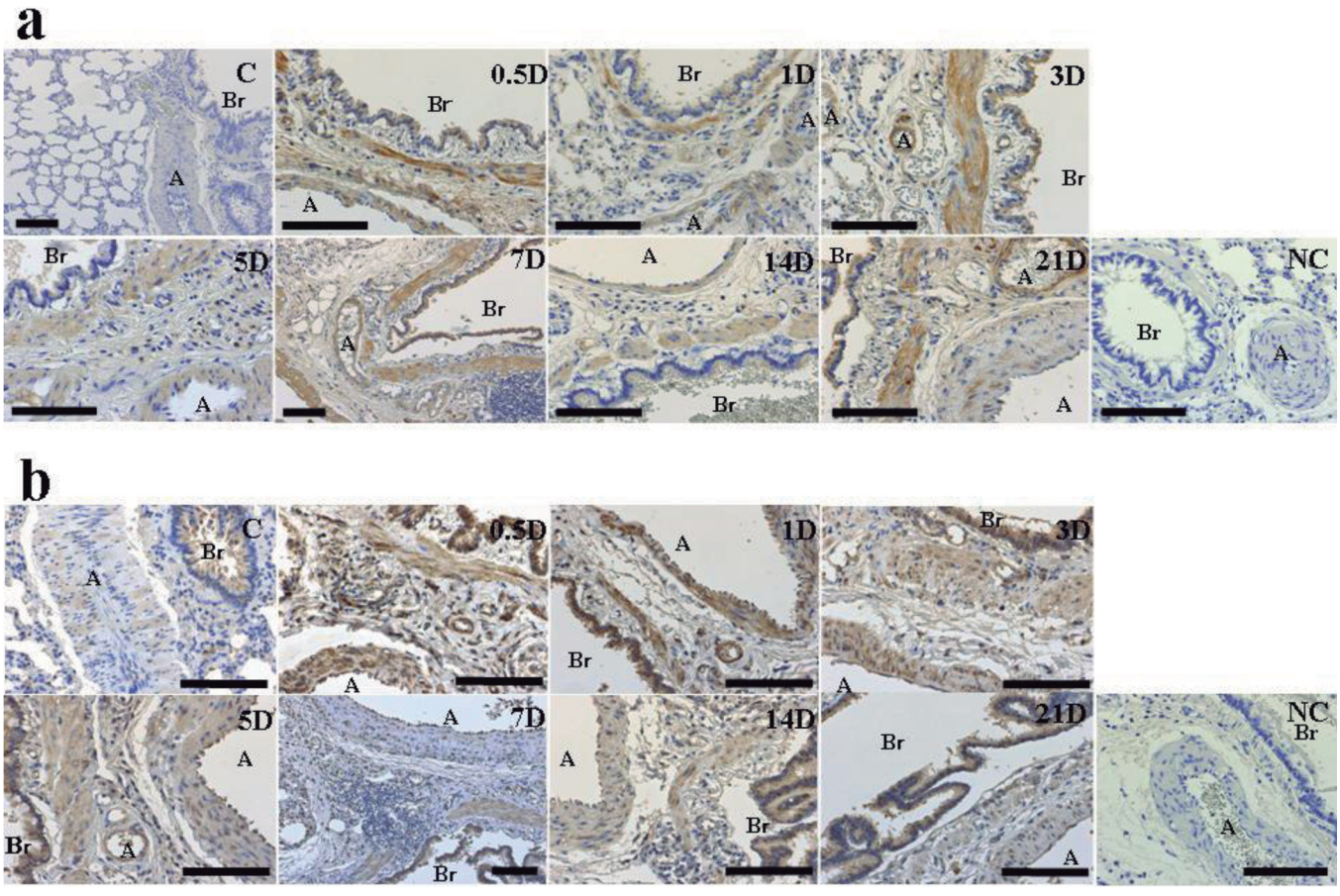

Fig. 5. Immunohistochemistry for HSP70 protein **(a)** and PDIA3 protein **(b)** in the lungs of hypobaric hypoxic environment (HHE) rats. In control rats, HSP70 and PDIA3 protein reactions are very weak within the smooth muscle and bronchial epithelial cells of the bronchus (Br), and within the smooth muscle cells of an artery (A). In HHE rats, each protein is detected within the smooth muscle and bronchial epithelial cells of a bronchus, and within the smooth muscle cells of an artery. C, control rat; 0.5D, 1D, 3D, 5D, 7D, 14D, and 21D indicate number of days' exposure to hypobaric hypoxia; NC, negative control. Scale bars: 100 μm.

mice exposed to 24 hours' hypoxia, demonstrated that one of the prominent upregulated proteins identified by 2D-PAGE was Fh1-1, which belongs to the "muscle development" group. They indicated that this is a new protein involved in hypoxia-induced proliferation and migration of pulmonary artery smooth muscle cells. In an in vitro 2-DE DIGE examination of pulmonary artery smooth muscle cells, Zhang et al. (2009) reported that the expressions of gelsolin-like actin-capping protein and transgelin were higher under hypoxia than in normoxia, suggesting modulations of the motility of cells by different interactions with the actin cytoskeleton. In the present study, unfortunately, we were unable to detect characteristic proteins associated with smooth muscle cells in pulmonary vessels.

In conclusion, we have obtained evidence suggesting that HSP70 1B and PDIA3, as identified by 2D-PAGE (pH4.5-pH6.5, 30kDa-100kDa) and MALDI-TOF may play roles in the response to HHE in rats. In particular, we speculate (a) that HSP70 1B may be involved in important protective roles that involve modulation of the structural remodeling that occurs in the lung due to the pulmonary hypertension occurring during hypobaric hypoxia, while (b) PDIA3 may be involved in the response to ER stress that occurs in the initial phase of hypoxia. In this study, we used lung tissues from rats exposed to HHE. This represents a limitation of the study in that we cannot exclude the influence of several cells present in the lung outside the pulmonary arteries, nor can we exclude species differences between rats and humans. Furthermore, the precise roles played by these proteins in vascular smooth muscle cells during remodeling of the pulmonary vasculature remain uncertain. Therefore, further studies will be needed to establish the pathobiologic relevance of the above findings.

Conflict of interest. The authors declare that they have no conflict of interest.

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