Reduction of endoplasmic reticulum stress by 4-phenylbutyric acid prevents the development of hypoxia-induced pulmonary arterial hypertension

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Koyama M, Furuhashi M, Ishimura S, Mita T, Fuseya T, Okazaki Y, Yoshida H, Tsuchihashi K, Miura T. Reduction of endoplasmic reticulum stress by 4-phenylbutyric acid prevents the development of hypoxia-induced pulmonary arterial hypertension. Am J Physiol Heart Circ Physiol 306: H1314–H1323, 2014. First published March 7, 2014; doi:10.1152/ajpheart.00869.2013.—Pulmonary arterial hypertension (PAH) is characterized by vasoconstriction and vascular remodeling of the pulmonary artery (PA). Recently, endoplasmic reticulum (ER) stress and inappropriate adaptation through the unfolded protein response (UPR) have been disclosed in various types of diseases. Here we examined whether ER stress is involved in the pathogenesis of PAH. Four weeks of chronic normobaric hypoxia increased right ventricular (RV) systolic pressure by 63% compared with that in normoxic controls and induced RV hypertrophy and medial thickening of the PA in C57BL/6J mice. Treatment with 4-phenylbutyric acid (4-PBA), a chemical chaperone, significantly reduced RV systolic pressure by 30%, attenuated RV hypertrophy and PA muscularization, and increased total running distance in a treadmill test by 70% in hypoxic mice. The beneficial effects of 4-PBA were associated with suppressed expression of inflammatory cytokines and ER stress markers, including Grp78 and Grp94 in the activating transcription factor-6 branch, sXbp1 and Pdi in the inositol-requiring enzyme-1 branch and Atf4 in the PKR-like ER kinase branch, and reduced phosphorylation of c-Jun NH2-terminal kinase and eukaryotic translation initiation factor-2a in the lung. The pattern of changes in ER stress and inflammatory markers by 4-PBA in the lung of the PAH model was reproduced in PA smooth muscle cells by chronic stimulation of platelet-derived growth factor-BB or hypoxia. Furthermore, knockdown of each UPR branch sensor activated other branches and promoted proliferation of PA smooth muscle cells. The findings indicate that activation of all branches of the UPR and accompanying inflammation play a major role in the pathogenesis of PAH, and that chemical chaperones are potentially therapeutic agents for PAH.

endoplasmic reticulum stress; chemical chaperone; pulmonary arterial hypertension; vascular smooth muscle cell; platelet-derived growth factor; inflammation

PULMONARY ARTERIAL HYPERTENSION (PAH) is characterized by vasoconstriction, micro-thrombosis, and vascular remodeling in the pulmonary vasculature and progresses insidiously with dismal outcomes (26). The pathogenesis of PAH is not fully understood, but growth factor receptors, protease-activated receptor-2, increased elastase activity, dysfunction of ion channels (voltage-dependent K+ and transient receptor potential channels), loss of adenosomatous polyposis coli-α3 integrin interaction, and inflammatory cytokines have been suggested to be involved in proliferation of vascular cells in the lung (2, 5, 15, 24). Vasodilatory agents, including prostanoids, endothelin receptor antagonists, and phosphodiesterase type-5 (PDE5) inhibitors, have been shown to improve symptoms and the prognosis of PAH to some extent. However, the prognosis of PAH still remains poor (9, 31). Several recent studies using animal models of PAH have indicated that proliferation of vascular cells and their escape from apoptosis are therapeutic targets, and that inhibition of the platelet-derived growth factor (PDGF) receptor may prevent and reverse PAH (4, 11, 23). However, current inhibitors of growth factor receptors are, in general, not highly specific and have different efficacies and serious side effects, which make the inhibitors unsuitable for clinical application. A strategy to indirectly inhibit PDGF signaling for PAH treatment has not been systematically explored.

In the present study, we examined the hypothesis that endoplasmic reticulum (ER) stress contributes to the development of PAH. ER stress is referred to as accumulation of unfolded and/or misfolded proteins in the ER, and recent studies have disclosed involvement of ER stress in a variety of diseases, including inflammatory diseases and proliferative diseases (3, 12, 20, 27). Newly synthesized proteins translocate to the ER lumen, where protein molecules undergo folding to proper three-dimensional structures with the help of chaperone proteins and appropriate levels of calcium and ATP. However, various noxious extracellular stimuli induce derangement of protein-folding processes, leading to ER stress, which is reflected as the unfolded protein response (UPR) in cells. The UPR induces activation of three branches of signaling, each triggered by activating transcription factor-6 (ATF6), inositol-requiring enzyme-1 (IRE1), and PKR-like ER kinase (PERK) in the ER membrane (25). Interestingly, ATF6 signaling has been shown to upregulate Nogo, leading to PAH via disruption of the mitochondria-ER unit in vascular smooth muscle cells (28). However, changes in IRE1 and PERK branches of the UPR and their roles in PAH remain unclear. Hence we comprehensively examined the UPR associated with PAH development in vivo and in vitro PAH models and the impact of suppression of UPR by a chemical chaperone on PAH.

MATERIALS AND METHODS

The present study was approved by the Committee for Animal Research, Sapporo Medical University, and was conducted in accordance with the Guidelines of Sapporo Medical University for Animal

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Use in Research. All biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Animals and treatment protocols. Seven-week-old male C57BL/6J mice (oriental Yeast, Tokyo, Japan) were divided into four groups: normoxic control (Normo-Ctrl), normoxic 4-phenylbutyric acid (PBA; Normo-PBA), hypoxic control (Hypo-Ctrl), and hypoxic 4-PBA (Hypo-PBA) groups. The Normo-Ctrl and Normo-PBA groups were kept under room air, and the Hypo-Ctrl and Hypo-PBA groups were kept under hypoxia (normobaric 10% O2 and 90% N2) using hypoxic chambers for 4 wk. The Normo-PBA and Hypo-PBA groups were treated with 4-PBA (Alfa Aesar, Hehsham, UK), a chemical chaperone (17), included in drinking water at 20 mM, and measurements of body weights and water intake indicated that the mice received 350–550 mg kg−1·day−1 of 4-PBA. Systolic and diastolic blood pressures were measured in a conscious state every week by an indirect tail-cuff method (MK-2000ST; Muromachi Kikai, Tokyo, Japan), and data of five repetitive recordings were averaged for each animal. The accuracy, precision, and reproducibility of MK-2000ST compared with invasive direct blood pressure measurements have been described previously (14). A treadmill test and right heart catheterization were performed after 4 wk of treatment. The mice then were killed by an overdose of tribromoethanol, and the lungs, heart, and tibiae were sampled for biochemical and morphometric analyses.

Treadmill test. Exercise capacity under a normoxia condition was measured using a motor-driven treadmill system (MK-680S, Muromachi Kikai, Tokyo, Japan), and data of five repetitive recordings were averaged for each animal. The accuracy, precision, and reproducibility of MK-2000ST compared with invasive direct blood pressure measurements have been described previously (14). A treadmill test and right heart catheterization were performed after 4 wk of treatment. The mice then were killed by an overdose of tribromoethanol, and the lungs, heart, and tibiae were sampled for biochemical and morphometric analyses.

Right heart catheterization. Mice were anesthetized with an intra-peritoneal injection of tribromoethanol (250 mg/kg) before catheterization. A 1.4-Fr catheter-tip pressure transducer catheter (Mikro-tip; Millar Instruments, Houston, TX) was inserted in the right jugular vein and advanced to the right ventricle (RV) via the right femoral vein. The position of the catheter was confirmed by the right ventricular systolic pressure (RVSP) by monitoring waveforms. RVSP was used as an index of pulmonary artery (PA) pressure, since placement of a catheter transducer in the murine PA potentially obstructs PA blood flow. Hemodynamic data were recorded using a PowerLab LabChart system (AD Instruments, Castle Hill, NSW, Australia).

Assessment of remodeling of the heart and PA. The excised heart was divided into the RV and left ventricle (LV) plus interventricular septum (LV+S), and Fulton index (RV/(LV+Si)) was calculated by weights of the RV and LV+S. Extent of RV hypertrophy was also assessed as RV weight normalized by tibia length.

The lungs were fixed in 10% neural formaldehyde, embedded in paraffin, and sectioned at 5-μm intervals. Lung sections were stained by the Elastica-Van-Gieson protocol and also immunostained using antibodies against α-smooth muscle actin (Dako, High Wycombe, UK; 1:50) and protein disulfide isomerase (PDI) (Cell Signaling, Danvers, MA). In each sample, full visual fields were scanned at 8-fold magnification by a fluorescent microscope, BIOREVO BZ-9000, and a BZ-II analyzer (Keyence, Osaka, Japan), and then 30–50 transversely sectioned precapillary vessels near the bronchus, ranging from 50 to 100 μm in diameter, were scanned at 80-fold magnification. Medial thickness was measured in each precapillary vessel using ImageJ 1.46 software in a double-blind manner by two different researchers, and thickness data were averaged for each mouse.

Cell culture. Human pulmonary smooth muscle cells (PASMC; Lonza, Walkersville, MD) were grown in smooth muscle cell basal medium (SmBM; Lonza) containing 5% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO2. Cells at passages 4–6 were used for all of the experiments. PASMC were seeded in six-well microplates at ∼1.8 × 10⁴ cells/well. When the cells were 70–80% confluent, cell growth was arrested in FBS-free medium (SmBM + 0.5% BSA + 1% penicillin/streptomycin cocktail) for 18 h. After the medium had been changed to a medium containing 0.2% FBS, cells were treated with 20 ng/ml PDGF-BB for 24 or 48 h, with or without 250 μM 4-PBA (ph (7.4). Cell proliferation was assessed by the MTS assay using a kit of CellTiter 96 Aqueous Assay (Promega, Madison, WI) and immunofluorescence-staining ratio of proliferating cell nuclear antigen (PCNA) (DAKO) and 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). In a series of hypoxia experiments, cells were cultured in SmBM supplemented with 5% FBS and 1% penicillin/streptomycin cocktail at 37°C under 5% O2 and 5% CO2 by use of a Forma Sevis II 3110 Water-Jacketed CO2 incubator (Thermoscientific, Waltham, MA) for 72 h.

Small interfering RNA knockdown. Small interfering RNA (siRNA) knockdown was performed by using Stealth RNAi (Invitrogen, Carlsbad, CA) targeting UPR sensors, Atf6, Ire1, and Pk. PASMC were transfected with specific or control Stealth RNAi using lipofectamine 2000 (Invitrogen) and cultured for 24 h.

Quantitative real-time PCR analysis. Total RNA was isolated from tissues or cell lysates using Trizol reagent (Invitrogen). For reverse transcription, 1 μg of the total RNA was converted to first-strand cDNA in 20 μl of reaction mixture using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was performed using SYBR Green with the ABI Prism 7500 Sequence Detector (Applied Biosystems). The thermal cycling program was 10 min at 95°C for enzyme activation and 40 cycles of denaturation for 15 s at 95°C, 30-s annealing at 58°C, and 30-s extension at 72°C. The mRNA levels of genes of interest were normalized by 18s ribosomal RNA level and expressed in arbitrary units. Primer sequences used in the experiments are shown in Table 1 (for human genes) and Table 2 (for mouse genes).

Protein extraction and Western blot analysis. Lung tissues or cells scraped from culture dishes were placed in a cold lysis buffer containing 50 mM Tris-HCl (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 10 mM NaVO₃, 10 mM Na₃VO₄, 40 mM Mg-cytrophosphate, 0.5% NP-40, and 1% protease inhibitor cocktail. After homogenization on ice, the lysates were centrifuged at 13,000 g for 30 min, and the supernatants were used for Western blotting. Total protein content of the samples was assessed by a microplate protein assay (Bio-Rad, Hercules, CA), and equal amounts of proteins were applied to SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred onto PVDF membranes (Whatman, Florham Park, NJ) and incubated for 1 h at room temperature with a blocking solution (3% BSA) in Tris-buffered saline buffer containing 0.1% Tween 20 (TBS-T). The blocked membranes were incubated with primary antibodies for c-Jun NH2-terminal kinase (JNK), β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), IRE1, phospho-JNK, (Thr183/Tyr185), eukaryotic translation initiation factor-2α (eIF2α), PDI (Cell Signaling), glucose-regulated protein (GRP) 78 (GRP78) (Enzo Life Sciences, Farmingdale, NY), GRP94 (Stressgen, Ann Arbor, MI), phospho-eIF2α (Ser52) (Invitrogen), phospho-IRE1 (Ser724) (Novus Biologicals, Littleton, CO), ATF6 (Bio Academia, Osaka, Japan), and vinculin overnight at 4°C and then washed three times for 15 min each time using TBS-T. The membranes were incubated with the appropriate secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature and washed. Immunoblotted proteins were visualized by a BM chemiluminescence blotting substrate (Roche Diagnostics, Germany) and a lumino-image analyzer (LAS-2000mini; Fujifilm, Tokyo, Japan). Densitometry was analyzed using ImageJ software.

Statistical analysis. Data are shown as means ± SE. Intergroup differences were tested by one-way ANOVA and Tukey’s post hoc test for multiple comparisons. Two-way repeated-measures ANOVA was used for testing differences in time courses of data between the
study groups. Differences in survival rates were analyzed by the log-rank test in Kaplan-Meier curves. JMP statistical software (version 9.0, SAS Institute, Cary, NC) was used for the statistical analyses, and the difference was considered significant if the P value was <0.05.

RESULTS

Chronic hypoxia-induced PAH and cardiac remodeling in mice. While body weights increased over the 4-wk experimental period in mice under the condition of normoxia (Normo-Ctrl and Normo-PBA groups), significant reduction in body weights was observed in mice under the hypoxia condition (Hypo-Ctrl and Hypo-PBA groups), as shown in Fig. 1A. Treatment with 4-PBA did not affect the body weight changes in either normoxic mice or hypoxic mice. Systolic and diastolic blood pressure levels were similar in all of the study groups. Differences in survival rates were analyzed by the log-rank test in Kaplan-Meier curves. JMP statistical software (version 9.0, SAS Institute, Cary, NC) was used for the statistical analyses, and the difference was considered significant if the P value was <0.05.

Effects of 4-PBA on exercise tolerance and survival in mice with PAH. While there was no significant difference in total running distance in the treadmill test between the Normo-Ctrl and Normo-PBA groups, the distance was significantly reduced by chronic hypoxia and was longer in the Hypo-PBA group than in the Hypo-Ctrl group (519 ± 80 vs. 291 ± 48 mm, P < 0.05) (Fig. 1H). Kaplan-Meier survival curves showed a clear trend toward lower mortality in the Hypo-PBA group than in the Hypo-Ctrl group, although the difference did not reach statistical significance (Fig. 1I).

Chronic hypoxia-induced pulmonary remodeling and ER stress. Histology of the lung showed thickening of the tunica media of pulmonary arterioles (50–100 μm) due to increased smooth muscle cells stained by smooth muscle actin in the Hypo-Ctrl group compared with those in the Normo-Ctrl group (Fig. 2A). The hypoxia-induced medial thickening was significantly attenuated by 4-PBA, although 4-PBA had no effect on medial thickness area of the PA in normoxic animals (Fig. 2B). Labeling of PDI, a marker of ER stress, indicated that ER stress

### Table 2. Primers for human genes in quantitative real-time PCR

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<tr>
<th>Genes</th>
<th>Species</th>
<th>Accession No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>18s</td>
<td>Human</td>
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<td>5’-GGTTCGATGAGAGTGGGCAC-3’</td>
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<td>IGF1</td>
<td>Human</td>
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<td>NM_004083</td>
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<tr>
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<td>5’-GGTTCGATGAGAGTGGGCAC-3’</td>
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in smooth muscle cells of the PA was increased by chronic hypoxia, and that 4-PBA could suppress the hypoxia-induced ER stress (Fig. 2C).

Effects of 4-PBA on ER stress markers in the lungs. Figure 3A shows mRNA levels of molecules in UPR signaling in the lung. Gene expression levels of ATF6, GRP78, and GRP94 in the ATF6 branch, IRE1 and spliced form of X-box binding protein 1 (sXBP1) in the IRE1 branch, and PERK and C/EBP homologous protein (CHOP) in the PERK branch were increased after 4-wk chronic hypoxia, although changes in gene expression levels of total form of X-box binding protein 1 (tXBP1), PDI, ER degradation-enhancing/H9251mannosidase-like protein (EDEM) and activating transcription factor-4 (ATF4) did not reach statistical significance. Treatment with 4-PBA did not significantly change mRNA levels of ER stress sensors, Atf6, Ire1, and Perk, in hypoxic mice, but reduced mRNA levels of downstream genes, Grp78 and Grp94 in the ATF6 branch, sXbp1 and Pdi in the IRE1 branch, and Atf4 in the PERK branch (Fig. 3B). No significant effect of 4-PBA on tXbp1, Edem, or Chop was observed.

Consistent with mRNA data, chronic hypoxia increased protein levels of phospho-JNK and PDI in the IRE1 pathway and phospho-eIF2-α in the PERK pathway (Fig. 3C). Hypoxia-induced activation of those UPR molecules was significantly reduced in the Hypo-PBA group compared with that in the Hypo-Ctrl group (Fig. 3D).

Since ER stress contributes to inflammatory reactions, mRNA levels of inflammatory cytokines were determined. Gene expression levels of monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were significantly increased in the lungs of hypoxic animals compared with those in the lungs of normoxic controls.
(Fig. 3E). Treatment with 4-PBA significantly reduced gene expression levels of Il6 and Tnfa, although the effect of 4-PBA on Mcp1 was not statistically significant (Fig. 3F).

**Effects of 4-PBA on ER stress markers and cell proliferation upon chronic stimulation of PDGF-BB or hypoxia in PASMC.** In preliminary experiments, we found that long-term (48 h), but not relatively short-term (24 h), treatment with 20 ng/ml PDGF-BB started to induce activation of ER stress markers (data not shown). After treatment with 20 ng/ml PDGF-BB for 48 h, mRNA levels of Grp78 and Grp94 in the ATF6 branch of the UPR were significantly increased in PASMC (Fig. 4, A and B), although the change in tXbp1 did not reach statistical significance (data not shown). As for the IRE1 branch, the expression level of Pdi tended to increase, and the expression levels of sXbp1 and Edem significantly increased after PDGF-BB treatment (Fig. 4, C–E). Change in the level of Chop expression in the PERK branch after PDGF-BB treatment was not statistically significant (Fig. 4F). Treatment with 4-PBA did not affect UPR signaling in the basal condition (data not shown), but significantly suppressed chronic stimulation of PDGF-BB-induced upregulation of Grp78, Grp94, sXbp1, Pdi, and Edem (Fig. 4, A–F). Significant suppression of the UPR signaling by 4-PBA was also observed in Western blot analysis. Protein levels of cleaved ATF6 (54 kDa), GRP94, phospho-IRE1, phospho-JNK, and PDI were increased by chronic stimulation of PDGF-BB, and those changes were suppressed by 4-PBA treatment (Fig. 4G).

Treatment with PDGF-BB for 48 h increased proliferation of PASMC determined by the MTS assay by 17% (Fig. 4H) and increased the ratio of PCNA-positive cells to DAPI-stained nuclei (PCNA/DAPI) by threefold (Fig. 4I). Treatment with 4-PBA did not affect cell proliferation in the absence of PDGF-BB stimulation (data not shown), but significantly suppressed the effect of PDGF-BB on proliferation of PASMC (Fig. 4, H and I).

Expression levels of Mcp1, Il6, and Tnfa were higher in PDGF-BB-treated PASMC than in untreated controls, although the change in Mcp1 was not statistically significant (Fig. 4, J–L). Treatment with 4-PBA significantly suppressed the expression of Mcp1, Il6, and Tnfa in PDGF-BB-treated cells.

Similarly, the pattern of changes in ER stress markers upon chronic stimulation of PDGF-BB in PASMC was largely reproduced in PASMC exposed to chronic hypoxia. Effects of 72-h hypoxia on expression levels of ER stress markers in PASMC and modification of the hypoxia-induced changes by 4-PBA were similar to those after PDGF-BB treatment except for Chop mRNA level, which was significantly increased after hypoxia (Fig. 5, A–F).

**Effect of knockdown of UPR sensors on PDGF-BB-induced proliferation of PASMC.** To further investigate the association between ER stress and proliferation of PASMC, genetically modified models of UPR sensors, ATF6, IRE1, and PERK, in PASMC were investigated. In PASMC, siRNA-mediated knockdown of Atf6, Ire1, and Perk resulted in efficient suppression by −84, −67, and −73%, respectively (Fig. 6, A–C). Atf6 knockdown in PASMC increased gene expression of the other sensors, Ire1 by 57% and Perk by 47% (Fig. 6, B and C). Knockdown of Ire1 or Perk in PASMC caused a compensated

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**Fig. 2.** Remodeling of the pulmonary artery (PA) and endoplasmic reticulum (ER) stress in chronic hypoxia-induced PAH. A: representative PAs stained with the Elastica-Van-Gieson (EVG) protocol and immunostained with anti-α-smooth muscle actin (SMA) antibody. Scale bars indicate 50 µm. B: medial thickness areas of the PA (50–100 µm) in the Normo-Ctrl (n = 3), Normo-PBA (n = 3), Hypo-Ctrl (n = 6), and Hypo-PBA (n = 6) groups. Values are means ± SE.

**Fig. 4.** Effects of 4-PBA on ER stress markers and cell proliferation in PASMC. A: media thickness areas of the PASMC determined by the MTS assay by 17% (Fig. 4H) and increased the ratio of PCNA-positive cells to DAPI-stained nuclei (PCNA/DAPI) by threefold (Fig. 4I). Treatment with 4-PBA did not affect cell proliferation in the absence of PDGF-BB stimulation (data not shown), but significantly suppressed the effect of PDGF-BB on proliferation of PASMC (Fig. 4, H and I).

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gene induction of the other sensor Atf6 by 29 and 20%, respectively. MTS assay showed that cell proliferation was significantly increased by knockdown of UPR sensors, Atf6, Ire1, and Perk, in PASMC in both the absence and presence of 20 ng/ml PDGF-BB stimulation for 48 h compared with that in the controls (Fig. 6D).

DISCUSSION

We hypothesized that ER stress contributes to the development of PAH and its functional outcomes. In a mouse model of PAH, suppression of ER stress by 4-PBA, which was reflected by reduced expression levels of UPR signaling molecules (Fig. 3),
Fig. 4. Effects of 4-PBA on UPR molecules, cell proliferation, and inflammatory cytokines in platelet-derived growth factor (PDGF)-BB-stimulated pulmonary smooth muscle cells (PASMC). A–F: relative gene expression of ER stress markers, Grp78 (A), Grp94 (B), sXbp1 (C), Pdi (D), Edem (E), and Chop (F), in 20 ng/ml PDGF-BB-treated PASMC with and without 250 μM 4-PBA for 48 h (n = 3 in each group). Levels of mRNA were normalized by 18s rRNA. G: representative Western blots for signal molecules downstream of three UPR sensors in PASMC after 24- or 48-h treatment with a vehicle, with 20 ng/ml PDGF-BB or with 20 ng/ml PDGF-BB and 250 μM 4-PBA. H and I: cell proliferation was assessed by the MTS assay (n = 6 in each group; H) and by the ratio of the number of proliferating cell nuclear antigen (PCNA)-stained cells to the number of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei (n = 3 in each group; I) in 20 ng/ml PDGF-BB-treated PASMC with and without 250 μM 4-PBA for 48 h. J–L: relative gene expression of inflammatory cytokines, Mcp1 (J), Il6 (K), and Tnfa (L), in 20 ng/ml PDGF-BB-treated PASMC with and without 250 μM 4-PBA for 48 h (n = 3 in each group). Levels of mRNA were normalized by 18s rRNA. Values are means ± SE. *P < 0.05 vs. PDGF (−) PBA (−). **P < 0.05 vs. PDGF (48 h) PBA (−).
significantly attenuated elevation of RVSP, prevented RV remodeling and improved exercise tolerance (Fig. 1). These observations corroborate the results of a recent study by Dromparis et al. (6) in a murine model of hypoxia-induced PAH, the model adopted in the present study. They reported that protein levels of ATF6 and GRP78 in the PA were reduced by 4-PBA (~500 mg·kg⁻¹·day⁻¹) in PAH, findings that are consistent with our data (Fig. 3). However, the present study showed for the first time that, not only the ATF6 branch, but also IRE1 and PERK branches, of the UPR were modulated by PAH in a

Fig. 5. Effects of 4-PBA on UPR molecules in PASMC exposed to 72-h hypoxia. Relative gene expression is shown of ER stress markers, Grp78 (A), Grp94 (B), sXbp1 (C), Pdi (D), Edem (E), and Chop (F), in PASMC exposed to normoxia or hypoxia with and without 250 μM 4-PBA for 72 h (n = 3 in each group). Levels of mRNA were normalized by 18s rRNA. Values are means ± SE. *P < 0.05 vs. Normo-PBA (--). **P < 0.05 vs. Hypo-PBA (--).

Fig. 6. Effects of knockdown of UPR sensors on cell proliferation in PDGF-BB-stimulated PASMC. A–C: relative gene expression of UPR sensors, Atf6 (A), Ire1 (B), and Perk (C), in PASMC with small interfering RNA (siRNA)-mediated knockdown of each UPR sensor. Sc, scramble control. *P < 0.05 vs. Sc. D: Cell proliferation was assessed by the MTS assay in PASMC with siRNA-mediated knockdown of each UPR sensor that was treated with 20 ng/ml PDGF-BB together with or without 250 μM 4-PBA for 48 h (n = 6 in each group). *P < 0.05, **P < 0.05 vs. Sc-PDGF (--). †P < 0.05 vs. Sc-PDGF (+).
4-PBA-sensitive manner (Fig. 3, A–D). It has been reported that signaling downstream of IRE1 contributes to cell proliferation, and that signal pathways in the IRE1 and PERK branches are involved in inflammatory response (32, 34). Furthermore, cross talk between the branches of the UPR has been demonstrated (30, 33). Hence, suppression of PAH by 4-PBA might be due to not only inhibition of ATF6-Nogo signaling shown in a previous study (6), but also modification of IRE1 and PERK signaling accompanied by inflammatory responses, such as activation of JNK and induction of inflammatory cytokines. The novel findings of the present study are that activation of all branches of the UPR, including ATF6, IRE1, and PERK pathways, and accompanied inflammation play a major role in the pathogenesis of chronic hypoxia-induced PAH.

Of the various factors involved in the pathogenesis of PAH, the PDGF-β receptor has received attention as a molecule responsible for increase in vascular smooth muscle cells and neointima proliferation of the PA (2, 24, 35). Since contribution of ER stress to the development of PAH was indicated by results of in vivo experiments (Figs. 1–3), we examined whether ER stress plays a role in PDGF-β receptor signaling in PASMC in vitro. Exposure of PASMC to PDGF-BB for 48 h induced upregulation of Grp78, Grp94, sXbp1, and Edem, and the expression level of Pdi also tended to increase (Fig. 4). Activation of signal pathways downstream of ATF6 and IRE1 branches in the UPR was indicated by increased protein expression of GRP94 and phosphorylation of IRE1 and JNK. Treatment with 4-PBA significantly suppressed both the upregulation of these UPR-related genes and phosphorylation of IRE1 and JNK. Collectively, these findings support the hypothesis that ER stress is involved in the pathogenesis of PAH in relation to chronic stimulation of PDGF-β receptor signaling.

In PASMC, siRNA-mediated Atf6 knockdown increased gene expression of the other sensors, Ire1 and Perk (Fig. 6, B and C). Knockdown of Ire1 or Perk in PASMC also caused a compensated gene induction of the other sensor Atf6 (Fig. 6, A–C). Furthermore, the proliferation of PASMC with each UPR sensor knockdown was significantly increased in the absence and presence of chronic stimulation of PDGF-BB (Fig. 6D). These results suggest that UPR-compromised models, such as knockdown of UPR sensors, including ATF6, IRE1, and PERK, induce more ER stress through compensative activation of other branches and proliferation of PASMC, possibly contributing to vascular remodeling.

Chronic hypoxia induced upregulation of UPR-related gene expression in PASMC (Fig. 5), as did PDGF-BB treatment. Although there were differences in the extents of changes in mRNA levels, patterns of the gene upregulation and their responses to 4-PBA were similar with chronic hypoxia and PDGF-BB stimulation in PASMC. These findings are consistent with results of earlier studies showing that chronic hypoxia induced activation of the PDGF-β receptor by NADPH oxidase-derived ROS (19, 21). It is unlikely that PDGF is solely responsible for ER stress relevant to PAH, but the results of the present study suggest that PDGF receptor signaling is associated with enhanced ER stress in the mechanism of chronic hypoxia-induced PAH.

In the present study, we used 4-PBA as a chemical chaperone for reducing ER stress. Efficacy of 4-PBA in alleviation of ER stress has been demonstrated in a variety of tissues in earlier studies, including ours (13, 17, 29). On the other hand, 4-PBA is also known to suppress histone deacetylase (HDAC) activity (1, 16). Recently, Zhao et al. (36) found that HDAC activity in lung tissue was significantly increased in patients with end-stage idiopathic PAH and in a rat model of hypoxia-induced PAH. Inhibition of HDAC activity by pharmacological inhibitors attenuated elevation of PA pressure, PA muscularization, and RV hypertrophy in rats exposed to chronic hypoxia. Interestingly, HDAC inhibitors suppressed PDGF-β expression in media cells of the hypertensive bovine PA and proliferative response of PASMC to PDGF in vitro (36). Thus we cannot exclude the possibility that protective effects of 4-PBA in the present murine model of PAH were partly attributable to inhibition of HDAC activity. However, IC50 of 4-PBA for inhibition of HDAC was reported to be 620 μM in DS19 cells (16) and millimolar ranges of 4-PBA have been used for inhibiting HDAC in cell culture experiments (1). Thus it is unlikely that the effects of 250 μM 4-PBA in the present experiments using PASMC are mainly due to HDAC inhibition.

A prostanoid, an endothelin receptor blocker, and a PDE5 inhibitor are currently used as monotherapy or combined therapy for treatment of PAH with limited success (7, 8). As a novel approach to PAH, PDGF inhibitors have received attention (4, 10, 23). However, toxicities of PDGF inhibitors and heterogeneous actions of the agents are obstacles in their translation to clinical use. The present study showed that chronic activation of PDGF-BB-induced ER stress could be suppressed by 4-PBA, a chemical chaperone that is in current clinical use for treatment of urea cycle disorders without severe adverse effects (18, 22). In a recent study by Dromparis et al. (6), two structurally different chemical chaperones, 4-PBA and tauroursodeoxycholic acid, prevented and reversed PAH induced by chronic hypoxia or monocrotaline in rodents. Collectively, these observations strongly indicate that chemical chaperones are novel promising agents for treatment of PAH, although pharmacological interactions of chemical chaperones with prostanoids, endothelin receptor blockers, or PDE5 inhibitors remain to be examined.

In conclusion, development of PAH by chronic hypoxia was associated with upregulation of signaling molecules in all three branches of UPR signaling accompanied by activation of inflammatory responses in the lung in vivo, and the pattern of upregulation of ER stress and inflammatory markers was largely mimicked by chronic stimulation of PDGF-BB or hypoxia in PASMC. Treatment with 4-PBA prevented hypoxia-induced PAH, RV remodeling, and PA muscularization and suppressed expression of UPR signaling molecules in the lung in vivo and in PDGF- or hypoxia-stimulated PASMC in vitro. These findings indicate that all branches of the UPR under ER stress play a major role in the pathogenesis of PAH, and that chemical chaperones are potentially therapeutic agents for PAH.

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