ORIGINAL RESEARCH

Activation of Calpain-2 by Mediators in Pulmonary Vascular Remodeling of Pulmonary Arterial Hypertension

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Abstract

Calpain mediates collagen synthesis and cell proliferation and plays an important role in pulmonary vascular remodeling in pulmonary arterial hypertension (PAH). In the present study, we investigated whether and how calpain is activated by PAH mediators in pulmonary artery smooth muscle cells (PASMCs). These data show that smooth muscle–specific knockout of calpain attenuated and knockout of calpastatin potentiated pulmonary vascular remodeling and pulmonary hypertension. Treatment of PASMCs with the PAH mediators platelet-derived growth factor (PDGF), serotonin, H_2O_2, endothelin-1, and IL-6 caused significant increases in calpain activity, cell proliferation, and collagen-I protein level without changes in protein levels of calpain-1 and -2. The calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA/AM) did not affect calpain activation, but the extracellular signal–regulated kinase (ERK) 1/2 inhibitor PD98059 and knocking down of calpain-2 prevented calpain activation in PAH mediator–treated PASMCs. Mass spectrometry data showed that the phosphorylation of calpain-2 at serine (Ser) 50 was increased and the phosphorylation of calpain-2 at Ser369 was decreased in PDGF-treated PASMCs. The PDGF-induced increase in Ser50 phosphorylation of calpain-2 was prevented by PD98059, whereas dephosphorylation of calpain-2 at Ser369 was blocked by the protein phosphatase 2A inhibitor fostriecin. Furthermore, smooth muscle of pulmonary arteries in PAH animal models and patients with PAH showed higher levels of phospho-Ser50-calpain-2 (P-Ser50) and lower levels of phospho-Ser369-calpain-2 (P-Ser369). These data support that calpain modulates pulmonary vascular remodeling in PAH. PAH mediator–induced activation of calpain is caused by ERK1/2-dependent phosphorylation of calpain-2 at Ser50 and protein phosphatase 2A–dependent dephosphorylation of calpain-2 at Ser369 in pulmonary vascular remodeling of PAH.

Keywords: pulmonary hypertension; platelet-derived growth factor; serotonin; H_2O_2; vascular smooth muscle cells

Clinical Relevance

The mechanism of calpain activation is not known. This study confirms the mediating role of calpain in pulmonary arterial hypertension (PAH) and identifies two phosphorylation sites in calpain-2 protein that are altered in cultured pulmonary artery smooth muscle cells treated by PAH mediators and in smooth muscle of pulmonary arteries in PAH animal models and in patients with PAH. The data from multidisciplinary approaches provide rationale for manipulating calpain in the treatment of PAH.

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Pulmonary arterial hypertension (PAH) is a severe and progressive disease, a key feature of which is pulmonary vascular remodeling. Pulmonary vascular remodeling is associated with accumulation of extracellular matrix, including collagen, and vascular smooth muscle cell proliferation and hypertrophy. These processes contribute to medial hypertrophy and muscularization, leading to obliteration of precapillary pulmonary arteries and sustained elevation of pulmonary arterial pressure (1, 2). A broad range of mediators, including growth factors (e.g., platelet-derived growth factor [PDGF]), inflammatory cytokines (e.g., IL-6), reactive oxygen species (e.g., H_2O_2), as well as vasoactive compounds, such as serotonin (5-HT) and endothelin (ET) 1, contribute to the process of pulmonary vascular remodeling in patients with PAH and in animal models (3–5). PDGF as a potent mitogen promotes the proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) (6, 7). Moreover, blocking the PDGF signaling by imatinib not only prevents, but also reverses the hypoxia- and monocrotaline (MCT)-induced PAH in animal models (7). Inflammation has an important role in various types of PAH. Patients suffering from PAH have elevated circulating levels of proinflammatory cytokines (e.g., IL-6), which are correlated with the severity of disease, as well as contribute to the proliferation of vascular cells (8). A growing body of evidence indicates that overproduction of reactive oxygen species is associated with the activation of redox-sensitive signaling pathways, causing vascular remodeling and enhanced vasoconstriction in PAH (4, 9). Vasoactive substances (i.e., 5-HT and ET-1) have been shown to elevate pulmonary vascular tone and stimulate PASMC proliferation. Mitogenic effects of 5-HT on PASMCs correlates with increased expression of serotonin transporter (10). Furthermore, inhibition of serotonin transporter prevented the MCT-induced PAH, suggesting that 5-HT has a role in pulmonary vascular remodeling (11). ET-1 exerts its vasoactive and proliferative effects on PASMCs via activation of ET type A and type B receptors (12, 13). In addition, serum-induced PASMC proliferation was diminished by blocking the release or inhibition of ET-1 (12). Altogether, these data provide convincing evidence that PAH mediators, PDGF, 5-HT, H_2O_2, ET-1, and IL-6, play important roles in the pathogenesis of PAH.

Calpain is a conserved family of calcium-mediated, cytosolic, neutral cysteine proteases, which perform controlled proteolysis of substrate proteins in a variety of mammalian cells, including PASMCs (14, 15). There are at least 15 isozymes in the family (16). Calpain-1 and calpain-2 are two major typical calpains. Calpain-1 and calpain-2 isoforms are composed of a distinct 80-kD large catalytic subunit and a common 30-kD small subunit (calpain-4) that helps maintain calpain activity (14). Calpastatin functions as the major specific endogenous inhibitor for calpain-1 and calpain-2 (17). Calpain has been shown to be implicated in numerous pathological processes (18). Calpain activates an intracrine transforming growth factor-1β/Smad signal pathway, leading to collagen synthesis, proliferation, and migration of vascular cells, and plays an important role in pulmonary vascular remodeling in hypoxia- and MCT-induced PAH and human PAH (6, 19). Calpain activation is much higher in the smooth muscle of pulmonary arteries of patients with PAH (6). However, it remains unknown how calpain is activated in PAH.

In the present study, we provide further evidence confirming the mediating role of calpain in pulmonary vascular remodeling of PAH, and explore the mechanisms of calpain activation in PASMCs. We found that smooth muscle–specific knockout of calpain attenuates and knockout of calpastatin potentiates pulmonary hypertension. PAH mediators, PDGF, 5-HT, H_2O_2, ET-1, and IL-6, induce calpain activation in PASMCs. PAH mediator–induced activation of calpain is caused by extracellular signal–regulated kinases (ERKs) 1/2–dependent phosphorylation of calpain-2 at serine (Ser) 50 and protein phosphatase (PP) 2A–dependent dephosphorylation of calpain-2 at Ser369 in pulmonary vascular remodeling of PAH.

Materials and Methods

The online supplement contains a full version of the methods.

Hypoxic Pulmonary Hypertension Model

Smooth muscle–specific calpain-4 knockout mice, calpastatin knockout mice, and their littermate control mice were exposed to room air or 10% oxygen (hypoxia). After 4 weeks, pulmonary hypertension and pulmonary vascular remodeling were assessed.

Assessment of Pulmonary Hypertension and Histological Analysis

Mice were anesthetized and the trachea was intubated. Right ventricle (RV) systolic pressure (RVSP) was measured. The free wall of the RV, left ventricle (LV), and septum (S) were then carefully dissected and individually weighed to calculate the Fulton index (ratio of RV / LV + S) as a parameter of right ventricular hypertrophy.

The right lungs were removed and snap frozen in liquid nitrogen for preparing homogenates, and the left lungs were fixed for morphometric analysis.

Cell Culture

Human PASMCs (Lonza, Walkersville, MD) were cultured according to the manufacturer's instructions. Before all experimentation, the third- to seventh-passage cells were equilibrated in growth factor-free medium for 24 hours.

Calpain Activity Assay

The calpain activity was measured using *tert*-butyloxycarbonyl-L-leucyl-L-methionine amide (*t*-BOC-Leu-Met) and Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-Leu-Leu-Val-Tyr-AMC) peptide as substrates.

Immunoprecipitation

The cell lysates were incubated with anti–calpain-2 antibody and Dynabeads Protein G (Life Technologies, Grand Island, NY). Immunoprecipitates were separated by SDS-PAGE and analyzed by matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry.

MALDI-TOF-TOF Mass Spectrometry

Mass spectrometry was performed as described previously (20).

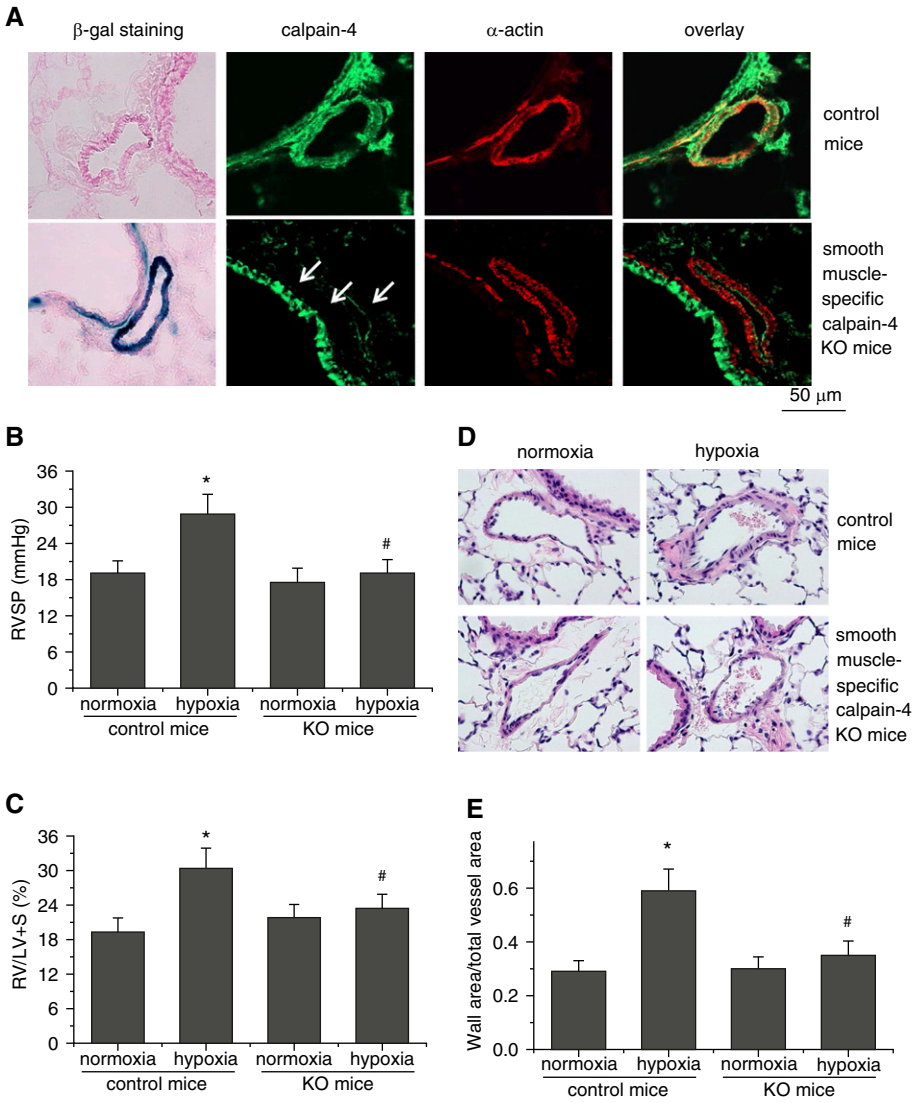

Figure 1. Smooth muscle–specific knockout (KO) of calpain-4 attenuates hypoxia-induced pulmonary hypertension and pulmonary vascular remodeling. At 5 days after a regimen of tamoxifen administration, male control mice and male capn4$^{flox/flox}$;SMMHC-Cre-ER/+;R26R/+ mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 4 weeks. (*A*) Lung slides from capn4$^{flox/flox}$;SMMHC-Cre-ER/+;R26R/+ mice and control mice were stained for β-gal and were also double stained for α-actin (*red*) and calpain-4 (*green*, *arrows*). Original magnification, ×400. (*B*) The changes in right ventricle (RV) systolic pressure (RVSP). (*C*) The changes in RV / LV + S (where LV is left ventricle, and S is septum). (*D*) Representative images of lung sections of control mice and capn4$^{flox/flox}$;SMMHC-Cre-ER/+;R26R/+ mice exposed to normoxia or hypoxia. Original magnification, ×400. (*E*) The changes in ratio of wall area to total vessel area in the lung sections of control mice and capn4$^{flox/flox}$;SMMHC-Cre-ER/+;R26R/+ mice exposed to normoxia or hypoxia. Results are expressed as mean ± SE; $n = 8$ experiments. $^{*}P < 0.05$ versus normoxia; $^{\#}P < 0.05$ versus hypoxia group of control mice.

Determination of Changes in Phosphorylation Status of Calpain-2

After treatments, immunoprecipitation was performed using phospho-specific antibodies against phosphorylated calpain-2 at Ser50 (P-Ser50) or Ser369 (P-Ser369) of calpain-2. The precipitates were analyzed by Western blot using antibody directed against calpain-2.

RNA Interference in PASMCs

Calpain-1 small interfering RNA (siRNA) (target sequence: 5′-AAGCTAGTGTTCGTGCACTCT-3′), calpain-2 siRNA (target sequence: 5′-CTGGAACACTATAGACCCAGA-3′), and the nontargeting siRNA (target sequence: 5′-AACG TACGCGGAATACTTCGA-3′) were used to knock down calpain-1 and calpain-2.

Site-Directed Mutagenesis of Calpain-2

Calpain-2 cDNA was cloned into a pcDNA3.1/V5-His mammalian expression vector to create the pcDNA3.1/V5-His–human calpain-2 wild-type plasmid. Ser50 and Ser369 were mutated into alanine.

Verification of the Specificity of Phospho-Specific Antibodies against P-Ser50 and P-Ser369 of Calpain-2

In order to proof the specificity of the P-Ser50–calpain-2 and P-Ser369–calpain-2 antibodies, wild-type and mutant human calpain-2 were overexpressed in human embryonic kidney (HEK) 293T stable cell line expressing the small hairpin RNAs (shRNAs) targeting the 3′ untranslated region of the calpain-2 mRNAs for specific down-regulation of calpain-2 gene expression without affecting the overexpression of wild and mutant calpain-2 constructs. Calpain-2–silenced HEK 293T cells were transfected with pcDNA3.1/V5-His–human calpain-2 wild-type and mutant plasmids. The cell lysates were then subjected to immunoprecipitation using phospho-specific antibodies against P-Ser50 or P-Ser369 of calpain-2. The precipitates were analyzed by Western blot using antibody directed against calpain-2 and V5 tag.

Determination of Phosphorylation Status of Calpain-2 in Pulmonary Arteries of Lungs from Mice and Patients with PAH

To determine the extent of phosphorylated calpain-2 in pulmonary arterioles, double immunostaining of P-calpain-2/α-actin was performed on lung tissue slides from normoxic and hypoxic mice and from normal subjects and patients with PAH.

Study Approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Georgia Regents

University. In the human lung studies, patient identifiers, including name, age, sex, and ethnic group, were concealed. A waiver of informed consent was approved by the Human Assurance Committee of the Georgia Regents University.

Results

Smooth Muscle–Specific Knockout of Calpain-4 Attenuates Hypoxia-Induced Pulmonary Hypertension and Pulmonary Vascular Remodeling

We previously reported that calpain activation is much higher in the smooth muscle of pulmonary arteries of hypoxia- and MCT-induced PAH and human PAH, and that global knockout of calpain-4 attenuate hypoxic pulmonary hypertension and vascular remodeling (6). To study whether calpain-mediated pulmonary vascular remodeling is due to calpain activation in PASMCs, calpain activity was inhibited in a mouse line of smooth muscle–specific knockout of calpain-4. As shown in Figure 1A, the lungs from capn4$^{flox/flox}$;SMMHC-Cre-ER/+;R26R/+ mice, not control mice, exhibit high β-gal activity and lower calpain-4 protein in the smooth muscle of airway and pulmonary arteries, suggesting that calpain-4 was successfully knocked out specifically in smooth muscle cells. More importantly, RVSP and Fulton index (RV / LV + S) were much higher in control mice exposed to hypoxia than those exposed to normoxia. However, RVSP and the ratio of RV / LV + S were comparable between capn4$^{flox/flox}$;SMMHC-Cre-ER/+;R26R/+ mice exposed to normoxia and those exposed to hypoxia (Figures 1B and 1C). Moreover, hypoxia for 4 weeks caused significant increases in thickness of the pulmonary vascular walls in control mice. Hypoxia failed to increase medial thickness of the pulmonary vascular walls in the smooth muscle layer of pulmonary arterioles in capn4$^{flox/flox}$;SMMHC-Cre-ER/+;R26R/+ mice (Figures 1D and 1E). Taken together, these results indicate that knockout of calpain-4 specifically in smooth muscle attenuates hypoxia-induced pulmonary hypertension and pulmonary vascular remodeling.

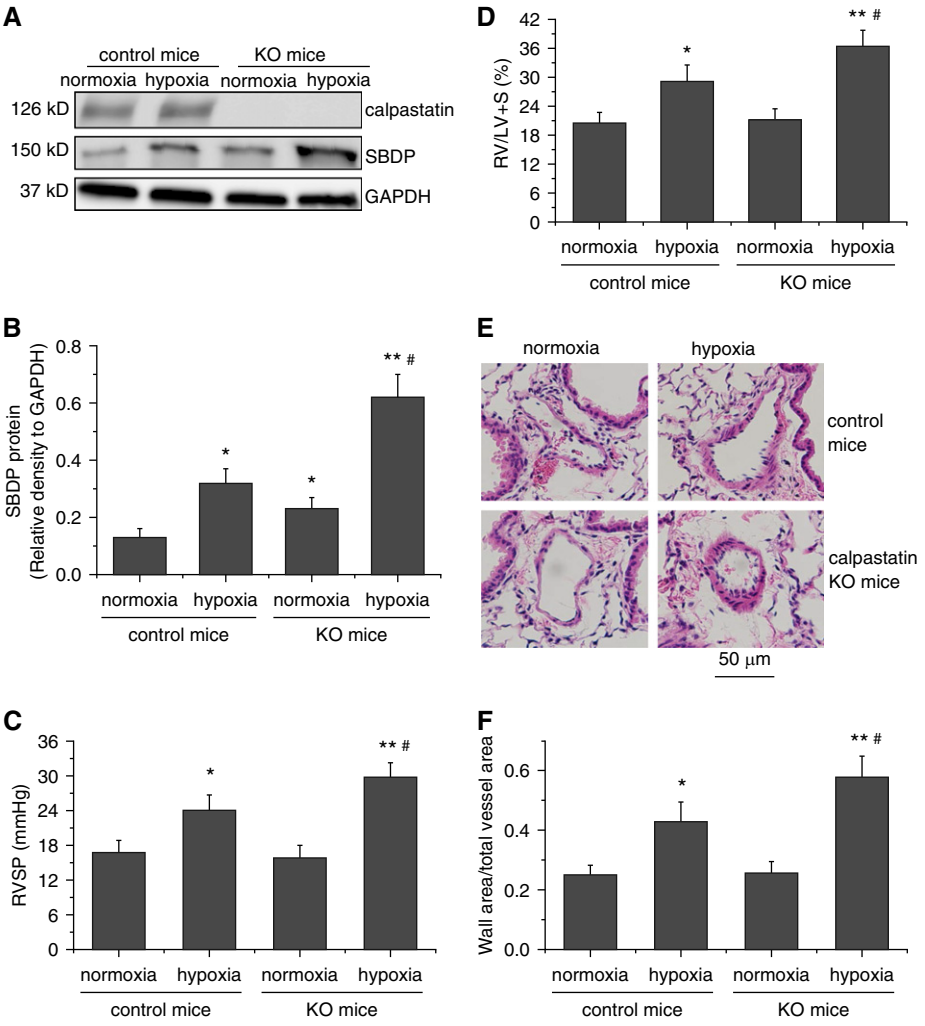

Figure 2. KO of calpastatin potentiates hypoxia-induced pulmonary hypertension and pulmonary vascular remodeling. Male homozygous mice and their littermates were exposed to normoxia or hypoxia for 4 weeks. (*A*) Representative immunoblots of calpastatin and spectrin breakdown product (SBDP) in lung homogenates from 8 experiments. (*B*) *Bar graph* depicting the changes in SBDP protein level quantified by scanning densitometry. (*C*) The changes in RVSP. (*D*) The changes in RV / LV + S. (*E*) Representative images of lung sections of control mice and calpastatin KO mice exposed to normoxia or hypoxia. Original magnification, ×400. (*F*) The changes in ratio of wall area to total vessel area in the lung sections of control mice and calpastatin KO mice exposed to normoxia or hypoxia. Results are expressed as mean ± SE; $n = 8$. $^{*}P < 0.05$ versus normoxia group of control mice; $^{**}P < 0.05$ versus normoxia group of KO mice; $^{\#}P < 0.05$ versus hypoxia group of control mice. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Knockout of Calpastatin Potentiates Hypoxia-Induced Pulmonary Hypertension and Pulmonary Vascular Remodeling

As shown in Figures 2A and 2B, spectrin breakdown product protein levels were much higher in the lungs of calpastatin knockout mice, suggesting that calpastatin knockout increases calpain activity in mouse lungs. Interestingly, RVSP, Fulton index, and medial thickness of the pulmonary vascular walls in the smooth muscle layer of pulmonary arterioles were much higher in calpastatin knockout mice exposed to hypoxia than those in the littermate control mice (Figures 2C–2F).

PAH Mediators, PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6, Increase Calpain Activity and Collagen-I Protein Level without Affecting the Calpain Protein Levels in PASMCs

PASMCs were incubated with PDGF-BB (10 ng/ml), 5-HT (5 μM), H_2O_2 (10 μM),

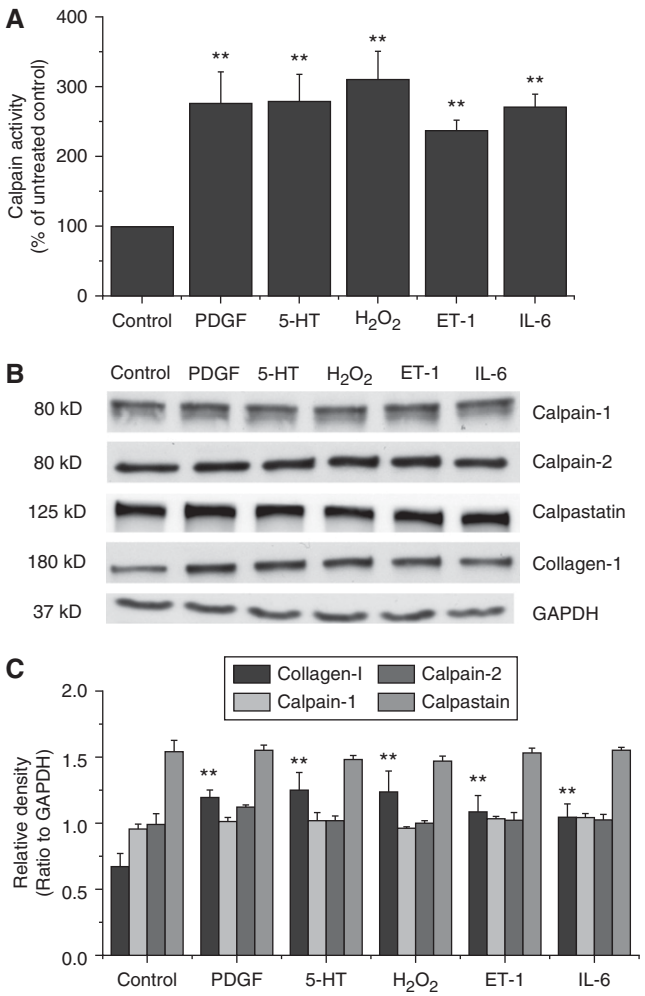

Figure 3. Effect of platelet-derived growth factor (PDGF)-BB, serotonin (5-HT), H_2O_2, endothelin (ET) 1, and IL-6 on the calpain activities and collagen I protein level in pulmonary artery smooth muscle cells (PASMCs). PASMCs were incubated with PDGF-BB (10 ng/ml), 5-HT (5 μM), H_2O_2 (10 μM), ET-1 (10 nM), and IL-6 (10 ng/ml) for 10 minutes to 24 hours, then calpain activity was determined by fluorescence microscopy and protein levels of collagen-I, calpain-1, calpain-2, and calpastatin were measured. (*A*) *Bar graph* depicting the changes in calpain activity. (*B*) Representative immunoblots of collagen-I, calpain-1, calpain-2, and calpastatin from three experiments. (*C*) *Bar graph* showing the changes in protein levels of collagen-I, calpain-1, calpain-2, and calpastatin quantified by scanning densitometry. Results are expressed as mean ± SE; $n = 4$. $^{**}P < 0.01$ versus control.

ET-1 (10 nM), and IL-6 (10 ng/ml) for 10 minutes, after which calpain activity was measured. As shown in Figure 3A, treatment of PASMCs with PAH mediators for 10 minutes significantly increased the calpain activities. PASMCs were also treated with these stimulators for 24 hours and then protein levels of calpain-1, calpain-2, calpastatin, and collagen-I were measured. Incubation of cells with these mediators caused significant increases in collagen-I protein level (Figures 3B and 3C), but the protein levels of calpain-1, calpain-2, and calpastatin were not changed in cells incubated with these PAH mediators (Figures 3B and 3C).

The ERK1/2 Inhibitor, PD98059, Prevents Increases in Calpain Activities and Cell Proliferation Induced by PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6 in PASMCs

PASMCs were incubated with or without the calpain inhibitor, MDL28170 (20 μM), or the ERK1/2 inhibitor, PD98059 (10 μM), for 30 minutes before the addition of t-BOC substrate (10 μM). The cells were further incubated for 20 minutes and then treated with PAH mediators for 10 minutes after which calpain activities were measured. The calpain activation was abolished in the presence of calpain inhibitor or ERK1/2 inhibitor compared with the untreated control (*see* Figures E1A and E1B in the online supplement), suggesting that activation of calpain induced by PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6 requires ERK1/2-mediated phosphorylation. In addition, incubation of PASMCs with calpain inhibitor, MDL28170 (20 μM), or ERK1/2 inhibitor, PD98059 (10 μM), reduced increases in cell proliferation induced by PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6 (Figure E2), indicating that calpain and ERK1/2 are involved in the cell proliferation induced by PAH mediators.

Increases in Calpain Activity Induced by PDGF-BB, 5-HT, H_2O_2, ET-1 and IL-6 Are Not due to Increases in Intracellular Calcium in PASMCs

We evaluated the effect of the 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM), an intracellular calcium chelator, on the PDGF-BB–, 5-HT–, H_2O_2-, ET-1–, and IL-6–induced increases in calpain activities. PASMCs were incubated with or without BAPTA/AM (50 μM) for 30 minutes before the addition of t-BOC substrate (10 μM). The cells were further incubated for 20 minutes and then treated with PAH mediators for 10 minutes. Pretreatment of PASMCs with the calcium chelator, BAPTA/AM, did not alter the mediator-induced increases in calpain activity (Figure E1C).

Down-Regulation of Calpain-2 Diminishes PDGF-BB–, 5-HT–, H_2O_2-, ET-1–, and IL-6–Mediated Increases in Calpain Activities

As shown in Figure E3, transfection of PASMCs with siRNA against the mRNAs of calpain-1 and calpain-2 markedly reduced the protein levels of calpain-1 and calpain-2, respectively. Moreover, silencing of calpain-2 expression, but not calpain-1, prevented the enhanced calpain activity induced by the mediators. These data indicate that calpain-2 rather than calpain-1 is responsible for increases in calpain activities induced by PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6.

Mass Spectrometry of Calpain-2 Phosphorylation in PASMCs Treated with and without PDGF

Recent studies revealed that the mutation of residue Ser50 to alanine in calpain-2 prevents calpain activation and phosphorylation induced by epidermal growth factor (EGF), and mutation of Ser369 causes an increase in calpain activity independently of calcium (21, 22). To examine whether PDGF-BB induces changes in the phosphorylation status of these sites in calpain-2, the lysates obtained from PDGF-BB–treated and untreated PASMCs were immunoprecipitated using calpain-2 antibody, then analyzed by SDS-PAGE and mass spectrometry. The results showed that both Ser50 and Ser369 residues are phosphorylated in calpain-2 for the mass spectra of the phosphopeptides at m/z 1,655 and m/z 944, respectively. Phosphorylation at Ser50 was clearly identified from the fragment observed at m/z 1,655 in the mass spectrum corresponding to ^{47}QDPS(p)FPAIPSALGFK^{61} (parent peptide with 1,574.82 m/z +80 Da of phosphate group). The Ser369 phosphorylation was revealed from the mass spectrometry of the fragment at m/z 944 representing ^{367}RGS(p)TAGGCR^{375} (parent peptide with 864.41 m/z +80 Da of phosphate group). More importantly, our data demonstrate that Ser50 phosphorylation of calpain-2 is increased and Ser369 phosphorylation of calpain-2 is decreased in PDGF-BB–treated cells (Figure E4), suggesting that calpain phosphorylation at these residues plays a crucial role in the regulation of calpain activity in PASMCs.

PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6 Alter the Phosphorylation Level of Calpain-2 at the Main Phosphorylation Sites

To further investigate the phosphorylation of Ser50 and Ser369 in calpain-2, we made antibodies against P-Ser50 and P-Ser369. The lysates of PASMCs treated with and without PAH mediators were immunoprecipitated using these antibodies. The immunoprecipitated proteins were analyzed by Western blot using calpain-2 antibody. As shown in Figure 4, PAH mediators enhanced the phosphorylation level of calpain-2 at Ser50 and reduced the phosphorylation level of calpain-2 at Ser369. These data are consistent with the findings in the mass spectrometry and provide additional evidence that PDGF, 5-HT, H_2O_2, ET-1, and IL-6 modify the phosphorylation status of calpain-2.

In addition, PASMCs were stained using antibodies against P-Ser50–calpain-2 and P-Ser369–calpain-2. We found that PDGF, 5-HT, H_2O_2, ET-1, and IL-6 induced significant increases in P-Ser50–calpain-2 and remarkable decreases in P-Ser369–calpain-2 (Figure E5).

The Specificity of Phospho-Specific Antibodies against P-Ser50 and P-Ser369 of Calpain-2

Similar to the native calpain-2 in PASMCs, the overexpressed calpain-2 in HEK cells can also be phosphorylated at Ser50 and dephosphorylated at Ser369 that was detected in the immunoprecipitates by Western blot using antibodies against calpain-2 and V5 tag. Replacing Ser50 and Ser369 for alanine prevented PDGF-induced alterations of P-Ser50–calpain-2 and P-Ser369–calpain-2 (Figure E6). These results indicate that the antibodies against P-Ser50 and P-Ser369 of calpain-2 are specific for respective phosphorylated residues.

The Specific ERK1/2 Inhibitor, PD98059, Prevents the PDGF-BB–Induced Increase in Calpain Activity and Phosphorylation of Calpain-2 at Ser50

Incubation of PASMCs with PDGF-BB (10 ng/ml) for 10 minutes significantly increased phosphorylation of ERK1/2 (Figures 5A and 5B) and calpain activity (Figure 5C). Pretreatment of the cells with PD98059 (10 μM) for 30 minutes prevented the PDGF-BB–induced increases in ERK1/2 phosphorylation (Figures 5A and 5B) and calpain activity (Figure 5C). More importantly, PD98059 (10 μM) abolished the PDGF-BB–induced increase in Ser50 phosphorylation of calpain-2, whereas it did not affect the decrease in phosphorylation of Ser369 induced by PDGF-BB (Figures 5D and 5E). Taken together, these data indicate that PDGF-BB activates calpain through phosphorylation of calpain-2 at Ser50, which is mediated by ERK1/2. PDGF-BB–induced dephosphorylation of calpain-2 at Ser369 is independent of ERK1/2 in PASMCs.

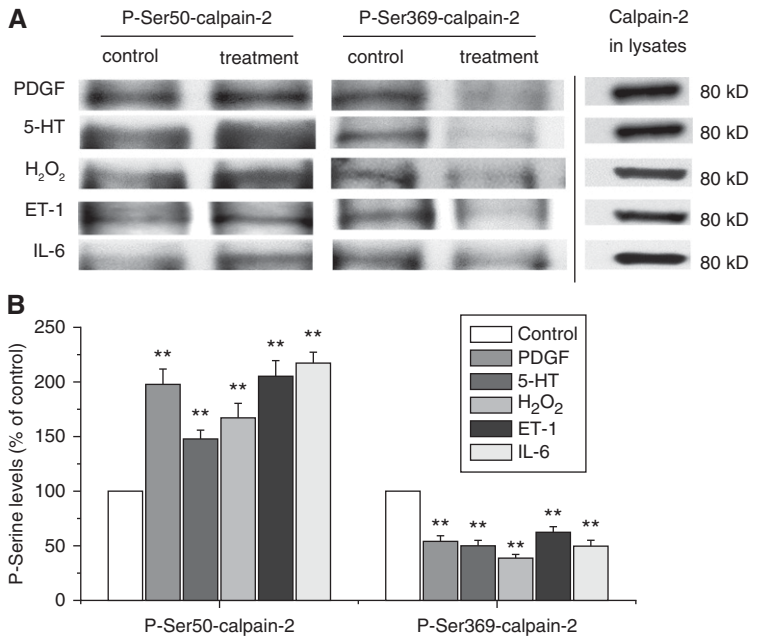

Figure 4. Effect of PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6 on the phosphorylation status of calpain-2. PASMCs were incubated with PDGF-BB (10 ng/ml), 5-HT (5 μM), H_2O_2 (10 μM), ET-1 (10 nM), and IL-6 (10 ng/ml) for 10 minutes, after which cells were lysed and immunoprecipitated using specific antibodies against P-serine (Ser) 50–calpain-2 or P-Ser369–calpain-2. The immunoprecipitates were subjected to immunoblots against calpain-2. (*A*) Representative immunoblots of the immunoprecipitates. (*B*) *Bar graph* showing the changes in the levels of P-Ser50–calpain-2 or P-Ser369–calpain-2. Results are expressed as mean ± SE; $n = 3$. $^{**}P < 0.01$ compared with untreated control.

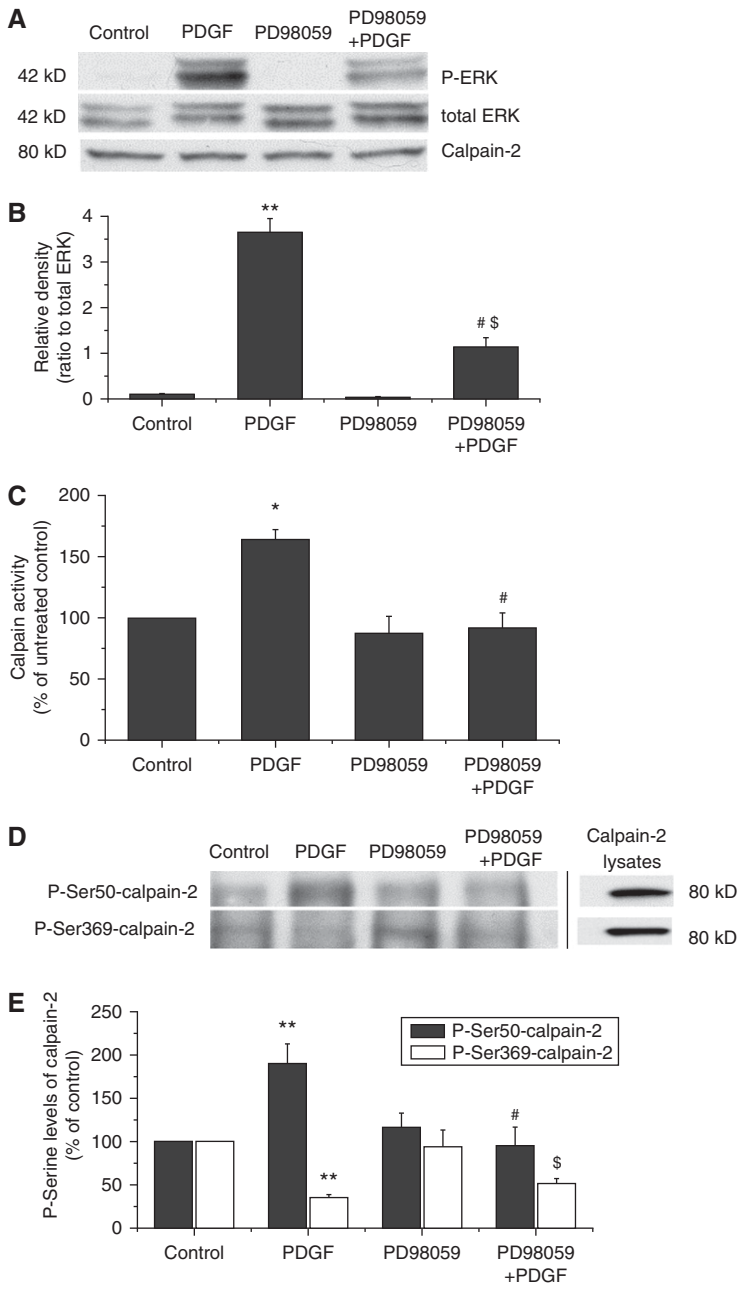

Figure 5. Effect of the extracellular signal–regulated kinase (ERK) 1/2 inhibitor PD98059 on PDGF-BB–induced increase in calpain activity and changes in the phosphorylation status of calpain-2. PASMCs were pretreated with PD98059 (10 μM) for 30 minutes and then incubated with PDGF-BB (10 ng/ml) for 10 minutes, after which calpain activity was determined by spectrofluorometry and protein levels of calpain-2, p-ERK1/2, and total ERK1/2, as well as phosphorylation status of calpain-2, were measured as described in Materials and Methods. (*A*) Representative immunoblots of p-ERK1/2, total ERK1/2, and calpain-2 from three experiments. (*B*) *Bar graph* showing the changes in ERK1/2 phosphorylation quantified by scanning densitometry. (*C*) *Bar graph* depicting the changes in calpain activity. (*D*) Representative immunoblots against calpain-2 using the immunoprecipitates pulled down by antibodies against P-Ser50–calpain-2 or P-Ser369–calpain-2. (*E*) *Bar graph* depicting changes in P-Ser50– and P-Ser369–calpain-2 protein levels quantified by scanning densitometry. Results are expressed as mean ± SEM; $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated control; $^{\#}P < 0.05$ versus PDGF-BB treatment; $^{\$}P < 0.001$ versus PD98059 without PDGF-BB.

Inhibition of PP2A Using Fostriecin Diminishes Calpain Activation Induced by PDGF

To identify the mechanism responsible for the PDGF-induced dephosphorylation of calpain-2 at Ser369, we examined the effects of the inhibitors of PP1 (tautomycin), PP2A (fostriecin), or PP2B (cyclosporin A) on the PDGF-BB–induced increase in calpain activity in PASMCs. As shown in Figures 6A and 6B, incubation of the cells with tautomycin (10 nM) or cyclosporin A (10 μM) for 30 minutes did not affect the PDGF-BB–induced increase in calpain activity, suggesting that PP1 and PP2B are not involved in the PDGF-induced calpain activation and phosphorylation. However, pretreatment of the cells with fostriecin (200 nM) for 30 minutes prevented the increase in calpain activity induced by PDGF (Figure 6C), suggesting that PP2A is associated with calpain activation and phosphorylation.

Next, we investigated the effect of fostriecin on the PDGF-BB–induced ERK1/2 activation and the phosphorylation of calpain-2. As shown in Figures 6D and 6E, preincubation of the PASMCs with fostriecin did not affect the PDGF-BB–induced phosphorylation and activation of ERK1/2 and the increase in the phosphorylation of calpain-2 at Ser50. However, fostriecin prevented the reduction of calpain-2 phosphorylation at Ser369 induced by PDGF-BB (Figures 6F and 6G). Collectively, these results indicate that PP2A is responsible to the PDGF-BB–induced dephosphorylation of calpain-2 at Ser369 in PASMCs.

Higher Levels of P-Ser50–Calpain-2 and Lower Levels of P-Ser369–Calpain-2 in Smooth Muscle Cells of Pulmonary Arteries of Mice with Hypoxic Pulmonary Hypertension and Patients with PAH

We have previously reported that calpain activity is higher in smooth muscle cells of pulmonary arterioles from PAH animal models and from patients with PAH (6). To investigate whether increased calpain activation in smooth muscle cells of pulmonary arteries in PAH is associated with alterations in the phosphorylation status of Ser50– and Ser369–calpain-2, we determined the levels of P-Ser50–calpain-2 and P-Ser369–calpain-2 in smooth muscle of pulmonary arteries of mice with hypoxic pulmonary hypertension and patients with PAH. As shown in Figure 7, the smooth

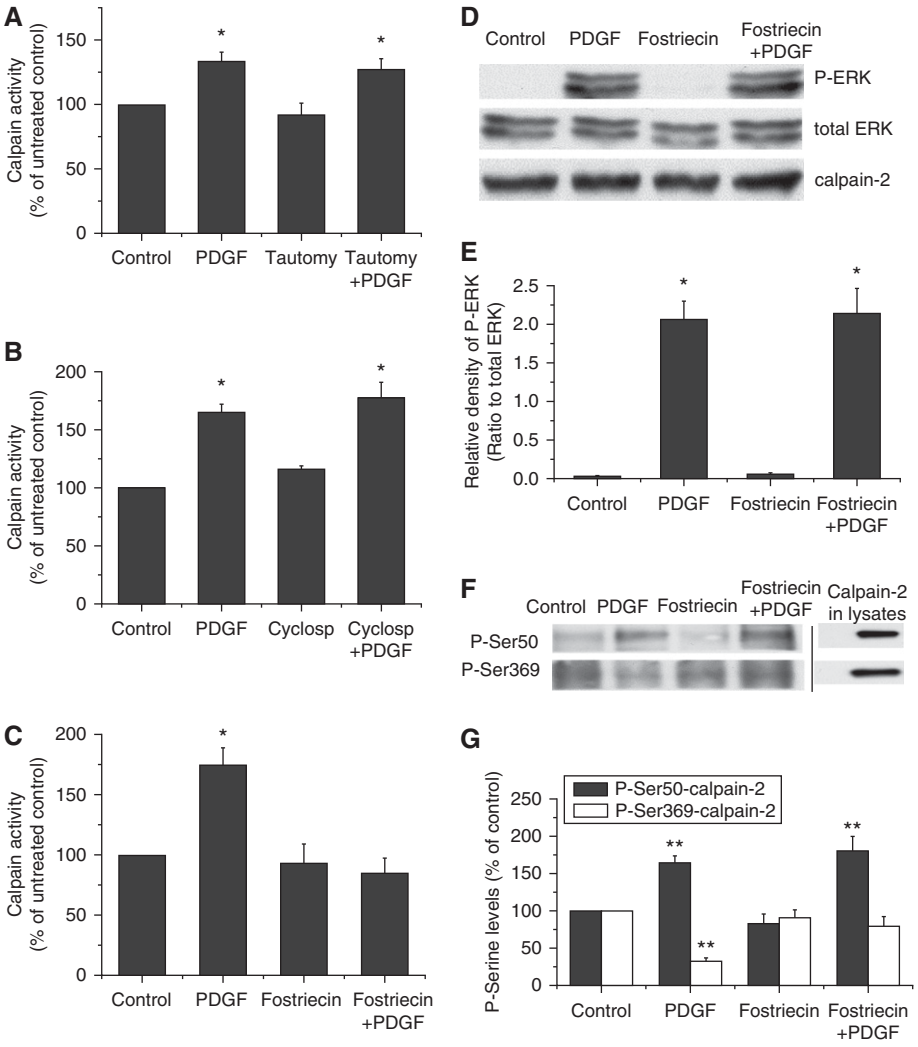

Figure 6. Effect of protein phosphatase (PP) 2A inhibitor, fostriecin, on PDGF-BB–induced increase in calpain activity and changes in the phosphorylation status of calpain-2. PASMCs were preincubated with the PP1 inhibitor tautomycin (tautomy, 10 nM), the PP2A inhibitor fostriecin (200 nM), or the PP2B inhibitor cyclosporin A (cyclosp, 10 μM), for 30 minutes and then treated with PDGF-BB (10 ng/ml) for 10 minutes, after which calpain activity was measured by spectrofluorometry. In addition, protein levels of calpain-2, p-ERK1/2, and total ERK1/2, as well as phosphorylation status of calpain-2, were measured in fostriecin- and PDGF-BB–treated PASMCs. (*A–C*) *Bar graphs* depicting the changes in calpain activity. (*D*) Representative immunoblots of p-ERK1/2 and total ERK1/2, as well as calpain-2 from four experiments. (*E*) *Bar graph* showing the changes in ERK1/2 phosphorylation quantified by scanning densitometry. (*F*) Representative immunoblots against calpain-2 using the immunoprecipitates pulled down by antibodies against P-Ser50–calpain-2 or P-Ser369–calpain-2. (*G*) *Bar graph* depicting changes in P-Ser50– and P-Ser369–calpain-2 protein levels quantified by scanning densitometry. Results are expressed as mean ± SE; $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated control.

muscle of pulmonary arteries of mice with hypoxic pulmonary hypertension and patients with PAH contained higher levels of P-Ser50–calpain-2 and lower levels of P-Ser369–calpain-2. These results indicate that higher levels of P-Ser50–calpain-2 and lower levels of P-Ser369–calpain-2 may contribute to higher calpain activity in smooth muscle cells of pulmonary arteries in PAH animal models and in patients with PAH.

Discussion

We previously reported that global inhibition of calpain prevents pulmonary vascular remodeling in hypoxia-induced PAH and the progression of established PAH induced by MCT (6). Lung tissues from patients with PAH and from hypoxia- and MCT-induced PAH animal models demonstrated higher levels of calpain activation in smooth muscle of pulmonary arterioles (6). To further study whether calpain-mediated pulmonary vascular remodeling is due to calpain activation in PASMCs, calpain activity is inhibited in a mouse line of smooth muscle–specific knockout of calpain-4. We found that smooth muscle–specific knockout of calpain attenuated pulmonary vascular remodeling and pulmonary hypertension induced by chronic hypoxia. Moreover, increase in calpain activity by knockout of calpastatin potentiated pulmonary vascular remodeling and pulmonary hypertension. These data establish that calpain is activated in smooth muscle cells of pulmonary arterioles and modulates pulmonary remodeling associated with PAH. In the present study, knockouts of calpain-4 and calpastatin do not affect RVSP and pulmonary vascular thickness at basal condition, suggesting that calpain is not of primary importance in maintaining the low vascular resistance of the pulmonary circulation. Nevertheless, our data show that the calpain activation may be an important pathway common to various extracellular mitogenic factors involved in PAH pathogenesis. The mechanism of calpain activation in PAH is not clear yet. Here, we show that PAH mediators, PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6, markedly increased the calpain activities and collagen-I protein levels in PASMCs. The protein levels of calpain-1 and calpain-2 were not altered in PASMCs treated with these PAH mediators. Furthermore, knocking down of calpain-2, not calpain-1, abolished increases in calpain activities induced by PAH mediators. Together, PAH mediator-induced calpain activation in PASMCs is caused by a posttranslational modification of calpain-2.

The posttranslational modification of calpain-2 induced by PAH mediators in PASMCs is not due to the increase in intracellular calcium. We found that the presence of the calcium chelator, BAPTA/AM, did not affect the calpain activation evoked by PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6. Interestingly, incubation of PASMCs with the ERK1/2 inhibitor, PD98059, prevents the PAH mediator–induced increases in calpain activity, suggesting that ERK1/2 is involved in calpain activation induced by PAH mediators.

Phosphorylation as a post-translational modification is one of the

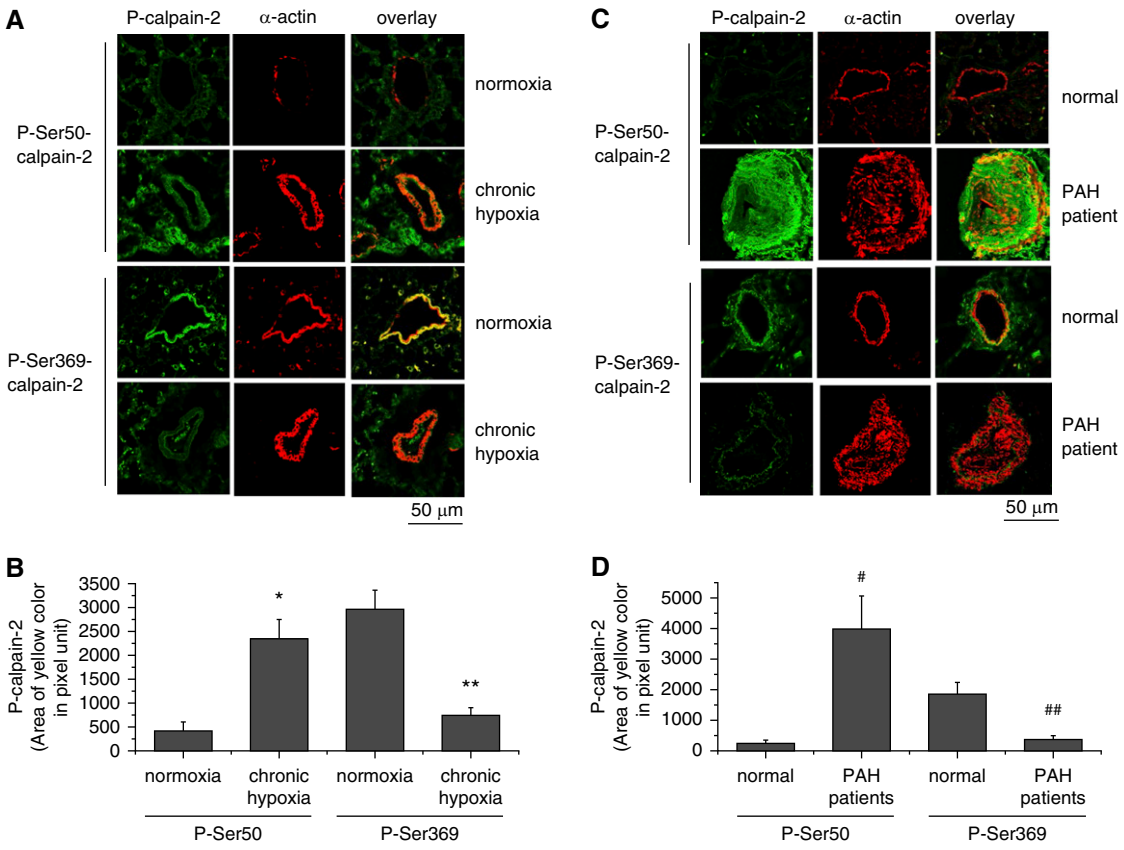

Figure 7. Higher levels of P-Ser50–calpain-2 and lower levels of P-Ser369–calpain-2 in smooth muscle cells of pulmonary arteries of mice with hypoxic pulmonary hypertension and patients with pulmonary arterial hypertension (PAH). Mouse and human lung slides were double stained for P-Ser50–calpain-2 (*green*) and P-Ser369–calpain-2 (*green*) and α-actin (*red*). (*A* and *C*) Representative images of lung tissues from normoxic and hypoxic mice (*A*) and from patients with PAH (*C*). Original magnification, ×400. (*B* and *D*) *Bar graph* depicting the changes in the levels of P-Ser50–calpain-2 and P-Ser369–calpain-2 in smooth muscle cells of mouse and human pulmonary arteries. Results are expressed as mean ± SE; $n = 8$ (for mice) and 20 (for patients). $^{*}P < 0.01$ versus normoxia for P-Ser50; $^{**}P < 0.01$ versus normoxia for P-Ser369; $^{\#}P < 0.01$ versus normal for P-Ser50; $^{\#\#}P < 0.01$ versus normal for P-Ser369.

mechanisms that may modulate calpain activity (23–25). Phosphorylation of calpain-2 by ERK may activate calpain directly or reduce calcium requirement for calpain activation (22, 25). Our data show that ERK1/2 inhibition reduces increases in cell proliferation induced by PAH mediators, indicating that ERK1/2-induced calpain-2 activation leads to functional consequence of cell proliferation. It has been shown that mutation of residue Ser50 in calpain-2 prevents calpain activation and phosphorylation induced by ERK (22). Mutation of Ser369 blocks phosphorylation of calpain-2 induced by protein kinase A and causes an increase in calpain activity (21). However, there is no direct evidence showing that Ser50 and Ser369 in calpain-2 are phosphorylated. Using mass spectrometry, we found that Ser50 and Ser369 in calpain-2 are phosphorylated. More importantly, Ser50 phosphorylation of calpain-2 is increased and Ser369 phosphorylation of calpain-2 is decreased in PDGF-treated PASMCs. To further examine the calpain-2 phosphorylation induced by PAH mediators, we then made antibodies against P-Ser50- and P-Ser369-calpain-2. The site-directed mutagenesis study confirms the specificity of these antibodies for P-Ser50- and P-Ser369-calpain-2. Consistent with the mass spectrometry results, the Western blot analysis and fluorescence staining using these antibodies showed that the Ser50 phosphorylation of calpain-2 was increased and the Ser369 phosphorylation of calpain-2 was decreased in PASMCs treated with PDGF-BB, 5-HT, H_2O_2, ET-1, and IL-6. More importantly, we found that the ERK1/2 inhibitor, PD98059, prevented the increase in the calpain-2 phosphorylation at Ser50, but did not influence the reduction of phosphorylation at Ser369 induced by PDGF-BB, suggesting that ERK1/2 causes the calpain-2 phosphorylation at Ser50 and that ERK1/2 is not involved in the dephosphorylation of calpain-2 at Ser369.

To identify which PP is responsible for the dephosphorylation of calpain-2 at Ser369, the effects of the PP1 inhibitor, tautomycin, the PP2A inhibitor, fostriecin, and the PP2B inhibitor, cyclosporin A, on the PDGF-induced increase in calpain activity were assessed. We found that tautomycin and cyclosporin A did not affect the PDGF-induced increase in calpain activity. Importantly, our data indicate that the PP2A inhibitor, fostriecin, prevents the PDGF-induced increase in calpain activity, suggesting that PP2A might be involved in the dephosphorylation of calpain-2 at Ser369. Furthermore, the PP2A inhibitor, fostriecin, prevents the decrease in the Ser369 phosphorylation, but does not affect ERK1/2 activation and the Ser50 phosphorylation of calpain-2 in PDGF-treated PASMCs. Taken together, our data support that PDGF-induced dephosphorylation of calpain-2 at

Ser369 is caused by PP2A. Phosphorylation of the Ser369 may limit the domain movements, freeze the enzyme in an inactive conformation, and subsequently block calpain activation (21). We postulate that removal of the phosphate group from the Ser369 residue is required for the activation of calpain-2 induced by PAH mediators. Interestingly, we observed that P-Ser50 and P-Ser369 in calpain-2 protein respond differently to PP2A. This might be caused by a difference in the recognition motif for phosphatase in these phosphorylation sites. Further studies are needed to test these speculations.

We also determined the levels of P-Ser50– and P-Ser369–calpain-2 in smooth muscle of pulmonary arterioles of mice with hypoxic pulmonary hypertension and patients with PAH. We observed higher levels of P-Ser50–calpain-2 and lower levels of P-Ser369–calpain-2 in smooth muscle of pulmonary arteries in PAH animal models and in patients with PAH. Therefore, increased phosphorylation of calpain-2 at Ser50 and decreased phosphorylation of calpain-2 at Ser369 contribute to higher calpain activation in smooth muscle cells of pulmonary arterioles of PAH.

The vascular remodeling is a complex process involving extracellular matrix synthesis and turnover in the inflammatory environment. Notably, calpain-1 regulates matrix metalloproteinase-2 activity, facilitating age-associated aortic wall calcification and fibrosis (26, 27). Thus, other calpain isoforms such as calpain-1, cannot be excluded in the mechanisms of pulmonary vascular remodeling of PAH.

In summary, we have demonstrated that calpain is activated in smooth muscle of pulmonary arterioles and modulates pulmonary vascular remodeling associated with PAH, and that PAH mediators, PDGF, 5-HT, H_2O_2, ET-1, and IL-6, activate calpain via ERK1/2-dependent phosphorylation of calpain-2 at Ser50 and PP2A-dependent dephosphorylation of calpain-2 at Ser369 in PASMCs in pulmonary vascular remodeling of PAH. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Tuder RM. Pathology of pulmonary arterial hypertension. *Semin Respir Crit Care Med* 2009;30:376–385.
2. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest* 2008;118:2372–2379.
3. Csiszar A, Labinskyy N, Olson S, Pinto JT, Gupte S, Wu JM, Hu F, Ballabh P, Podlutsky A, Losonczy G, *et al*. Resveratrol prevents monocrotaline-induced pulmonary hypertension in rats. *Hypertension* 2009;54:668–675.
4. Nozik-Grayck E, Suliman HB, Majka S, Albietz J, Van Rheen Z, Roush K, Stenmark KR. Lung EC-SOD overexpression attenuates hypoxic induction of Egr-1 and chronic hypoxic pulmonary vascular remodeling. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L422–L430.
5. Han W, Dong Z, Dimitropoulou C, Su Y. Hydrogen sulfide ameliorates tobacco smoke-induced oxidative stress and emphysema in mice. *Antioxid Redox Signal* 2011;15:2121–2134.
6. Ma W, Han W, Greer PA, Tuder RM, Toque HA, Wang KKW, Caldwell RW, Su Y. Calpain mediates pulmonary vascular remodeling in rodent models of pulmonary hypertension, and its inhibition attenuates pathologic features of disease. *J Clin Invest* 2011;121:4548–4566.
7. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, *et al*. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 2005;115:2811–2821.
8. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB. Interleukin-6 overexpression induces pulmonary hypertension. *Circ Res* 2009;104:236–244.
9. Wedgwood S, Lakshminrusimha S, Fukai T, Russell JA, Schumacker PT, Steinhorn RH. Hydrogen peroxide regulates extracellular superoxide dismutase activity and expression in neonatal pulmonary hypertension. *Antioxid Redox Signal* 2011;15:1497–1506.
10. MacLean MR, Deuchar GA, Hicks MN, Morecroft I, Shen S, Sheward J, Colston J, Loughlin L, Nilsen M, Dempsie Y, *et al*. Overexpression of the 5-hydroxytryptamine transporter gene: effect on pulmonary hemodynamics and hypoxia-induced pulmonary hypertension. *Circulation* 2004;109:2150–2155.
11. Guignabert C, Raffestin B, Benferhat R, Raoul W, Zadigue P, Rideau D, Hamon M, Adnot S, Eddahibi S. Serotonin transporter inhibition prevents and reverses monocrotaline-induced pulmonary hypertension in rats. *Circulation* 2005;111:2812–2819.
12. Davie N, Haleen SJ, Upton PD, Polak JM, Yacoub MH, Morrell NW, Wharton J. ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 2002;165:398–405.
13. Galié N, Manes A, Branzi A. The endothelin system in pulmonary arterial hypertension. *Cardiovasc Res* 2004;61:227–237.
14. Suzuki K, Sorimachi H, Yoshizawa T, Kinbara K, Ishiura S. Calpain: novel family members, activation, and physiologic function. *Biol Chem Hoppe Seyler* 1995;376:523–529.
15. Fujitani K, Kambayashi J, Sakon M, Ohmi SI, Kawashima S, Yukawa M, Yano Y, Miyoshi H, Ikeda M, Shinoki N, *et al*. Identification of mu-, m-calpains and calpastatin and capture of mu-calpain activation in endothelial cells. *J Cell Biochem* 1997;66:197–209.
16. Suzuki K, Hata S, Kawabata Y, Sorimachi H. Structure, activation, and biology of calpain. *Diabetes* 2004;53:S12–S18.
17. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev* 2003;83:731–801.
18. Kovacs L, Su Y. The critical role of calpain in cell proliferation. *J Biomol Res Ther* 2014;3:112-1–112-7.
19. Su Y, Cui Z, Li Z, Block ER. Calpain-2 regulation of VEGF-mediated angiogenesis. *FASEB J* 2006;20:1443–1451.
20. Rafikov R, Rafikova O, Aggarwal S, Gross C, Sun X, Desai J, Fulton D, Black SM. Asymmetric dimethylarginine induces endothelial nitric-oxide synthase mitochondrial redistribution through the nitration-mediated activation of Akt1. *J Biol Chem* 2013;288:6212–6226.
21. Shiraha H, Glading A, Chou J, Jia Z, Wells A. Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Mol Cell Biol* 2002;22:2716–2727.
22. Glading A, Bodnar RJ, Reynolds IJ, Shiraha H, Satish L, Potter DA, Blair HC, Wells A. Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal–regulated kinase–mediated phosphorylation. *Mol Cell Biol* 2004;24:2499–2512.
23. Glading A, Uberall F, Keyse SM, Lauffenburger DA, Wells A. Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. *J Biol Chem* 2001;276:23341–23348.
24. Zadran S, Jourdi H, Rostamiani K, Qin Q, Bi X, Baudry M. Brain-derived neurotrophic factor and epidermal growth factor activate neuronal m-calpain via mitogen-activated protein kinase–dependent phosphorylation. *J Neurosci* 2010;30:1086–1095.
25. Kovács L, Alexa A, Klement E, Kókai E, Tantos A, Gógl G, Sperka T, Medzihradszky KF, Tözsér J, Dombrádi V, *et al*. Regulation of calpain B from *Drosophila melanogaster* by phosphorylation. *FEBS J* 2009;276:4959–4972.
26. Jiang L, Wang M, Zhang J, Monticone RE, Telljohann R, Spinetti G, Pintus G, Lakatta EG. Increased aortic calpain-1 activity mediates age-associated angiotensin II signaling of vascular smooth muscle cells. *PLoS One* 2008;3:e2231.
27. Jiang L, Zhang J, Monticone RE, Telljohann R, Wu J, Wang M, Lakatta EG. Calpain-1 regulation of matrix metalloproteinase 2 activity in vascular smooth muscle cells facilitates age-associated aortic wall calcification and fibrosis. *Hypertension* 2012;60:1192–1199.