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Bortezomib alleviates experimental pulmonary hypertension by regulating intracellular calcium homeostasis in PASMCs

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PULMONARY HYPERTENSION (PH) is a fatal disease due to the progressive increase in pulmonary arterial (PA) pressure, pulmonary vascular remodeling, and right ventricular (RV) hypertrophy. In PA smooth muscle cells (PASMCs), the chronic hypoxia (CH)-associated imbalance in Ca2+ homeostasis and abnormally elevated intracellular Ca2+ concentration ([Ca2+]i) play a critical role in the pathogenesis of PH through their contribution to acceleration of PA contraction and PASMC proliferation (9, 17). The rise in [Ca2+]i is mainly induced through three pathways: 1) Ca2+ release from the intracellular Ca2+ store localized on the endoplasmic/sarcoplasmic reticulum, 2) extracellular Ca2+ influx from the plasma membrane ion channels, which comprise L-type voltage-dependent Ca2+ channels (VDCCs), receptor-operated Ca2+ channels (ROCCs), and store-operated Ca2+ channels (SOCCs), or 3) Ca2+ exchange via the membrane Ca2+-ATPases and Na+/Ca2+ exchanger (14, 38). Previously, we and others demonstrated that extracellular Ca2+ influx through SOCCs, termed store-operated Ca2+ entry (SOCE), contributes to hypoxia-induced elevation of [Ca2+]i and, subsequently, accounts for hypoxia-triggered PASMC proliferation (17, 21, 43, 44, 46). It is well accepted that SOCCs are mainly composed of the canonical transient receptor potential (TRPC) proteins (30). Among the seven TRPC isoforms (TRPC1-7), only TRPC1 and TRPC6 expression is upregulated by CH. Using specific knockdown approaches, we further demonstrated that hypoxia-regulated TRPC1 and TRPC6 expression greatly attributes to hypoxia-triggered SOCE and PASMC proliferation (21). Moreover, studies using knockout mouse models further elucidated the critical roles of TRPC1 and TRPC6 in the elevated PA contraction and remodeling during progression of CH-induced PH (CHPH) (24, 52).

The ubiquitin-proteasome system plays a major role in regulation of protein degradation (6–8). A protein substrate conjugated with a polyubiquitin chain could be recognized and then hydrolyzed by the 26S proteasome. The 26S proteasome consists of two subcomplexes, the 20S core particle, in which proteolysis takes place, and the 19S regulatory particle, which binds to the ubiquitinated substrate (41). The 20S proteasome contains six active sites: two chymotrypsin-like, two trypsin-like, and four caspase-like proteases.
like, and two caspase-like (3, 16, 28). Recently, we showed the hypoxia-induced elevation of proteasome activity and expression of the main subunits proteasome subunit-β types 1 and 6 (PSMB1 and PSMB6) in PASMCs. Moreover, specific knockdown of the active subunit PSMB6 largely abolished hypoxia-induced PASMC proliferation, suggesting the potential role of proteasome activity in hypoxic PA remodeling during CHPH pathogenesis (48). Bortezomib (BTZ) is the first proteasome inhibitor approved by the US Food and Drug Administration for treatment of multiple myeloma (2, 13, 26, 29, 33, 34, 40, 51). The functional attenuation of PH pathogenesis in both CPHPH and monocrotaline (MCT)-induced PH (MCT-PH) animal models, which is related to amelioration of endothelial function by upregulation of endothelial nitric oxide (NO) synthase (eNOS), phosphorylated eNOS, and NO, was first reported by Kim et al. (15). In CPHPH or CH/SU-5416-induced PH animal models, BTZ upregulated annexin A1, leading to GATA4-dependent downregulation of Bcl-xl, causing specific apoptotic and autophagic killing of remodeled vascular cells (10). Researchers showed that BTZ could also significantly improve hemodynamic parameters, such as RV hypertrophy and pulmonary vascular remodeling, in a PA hypertension model (4). For experiments with the CHPH control, rats were placed in a hypoxic (10% O2) chamber for 21 days, as described previously (37, 47). For experiments with the CHPH model, rats were randomly divided into four groups: saline control, BTZ rats each received a single injection of MCT (50 mg/kg sc; Sigma-Aldrich, St. Louis, MO), while the saline control and saline treatment. 

**Methods and Materials**

Establishment of CHPH and MCT-PH rat models and BTZ treatment. Sprague-Dawley rats (295–352 g body wt; Guangdong Provincial Medical Experimental Animal Centre) were raised in a specific pathogen-free-grade animal room of the State Key Laboratory of Guangzhou Medical University (GMU). All procedures were approved by the Animal Care and Use Committee of GMU. For the PH model, rats were placed in a hypoxic (10% O2) chamber for 21 days, as described previously (37, 47). For experiments with the CHPH model, rats were randomly divided into four groups: 1) normoxia control, 2) normoxia + BTZ, 3) hypoxia control, and 4) hypoxia + BTZ. For experiments with the MCT-PH model, rats were randomly assigned to one of four groups: 1) saline control, 2) saline + BTZ, 3) MCT control, and 4) MCT + BTZ. The MCT control and MCT + BTZ rats each received a single injection of MCT (50 mg/kg sc; Sigma-Aldrich, St. Louis, MO), while the saline control and saline + BTZ rats were subcutaneously injected with isotonic saline on the first day to establish the model. Rats in saline + BTZ and MCT + BTZ groups were injected subcutaneously with BTZ (1.3 mg/mg of body surface area (catalog no. S1014, Selleckchem) dissolved in DMSO to achieve a concentration of 10 mg/ml and then diluted in 0.9% saline for a final concentration of 0.053 mg/ml) on Monday and Thursday over the 3-wk study (27). Saline control and MCT control groups were given an equivalent volume of saline and DMSO.

**Hemodynamic parameter measurements and lung histochromy.** RV pressure and RV hypertrophy were measured as described previously (21). Intrapulmonary vessels were visualized by hematoyxin-eosin staining on 5-μm-thick formalin-fixed paraffin-embedded lung cross sections. Pulmonary vascular remodeling, indicated by the thickening of the smooth muscle layer of distal small PA, was measured using Aperio software.

**Culture of rat primary distal PASMCs.** As previously described (44), distal (>4th generation) intrapulmonary arteries were dissected from lungs of rats anesthetized with pentobarbital sodium (65 mg/kg ip). The adventitia was removed, the vessel was dissected longitudinally, and the luminal surface was rubbed carefully with a swab to denude the epithelium. PASMCs were harvested from the PA smooth muscle enzymatically, seeded in cell culture dishes (for measurement of [Ca2+]i), cells were seeded onto 25-mm coverslips, and incubated for 3–4 days in 10% FBS-DMEM culture medium (Biological Industries, Israel) in a humidified atmosphere of 5% CO2-95% O2 at 37°C. Cell purity was assessed by morphological appearance under phase-contrast microscopy and by immunofluorescence staining for α-actin under fluorescence microscopy (44, 47). For prolonged hypoxia and BTZ treatment, the medium was replaced with 0.5% FBS-DMEM for 24 h to achieve growth arrest before exposure to hypoxia (4% O2, 60 h) or normoxia in the presence or absence of BTZ for 60 h before various assessments. The O2 concentration in the culture incubator was continuously monitored and modulated in real time with an O2 sensor and a switch controlling nitrogen gassing.

**Cell proliferation assay.** Cell proliferation was evaluated according to the operation manual of a CytoSelect bromodeoxyuridine (BrdU) cell proliferation ELISA kit (Roche).

**Cytotoxicity assay.** Cell cytotoxicity was assessed using the lactate dehydrogenase (LDH) cytotoxicity assay kit (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions.

**Measurement of [Ca2+]i.** [Ca2+]i and SOCE were measured with fura 2-AM (Molecular Probes, Eugene, OR) and fluorescence microscopy, as previously described (21, 44, 47). To obtain statistically convincing results, the fluorescence intensity was determined in at least 20 cells for each sample. Briefly, coverslips with PASMCs were incubated with 7.5 μM fura 2-AM (Invitrogen) for 60 min at 37°C with 5% CO2-95% O2, mounted in a closed polycarbonate chamber, and clamped to a heated aluminum platform (model PH-2, Warner Instruments, Hamden, CT) on the stage of an inverted microscope (TSE 1000-Eclipse, Nikon, Melville, NY). Cells were perfused with 0.5–1 ml/min with Krebs-Ringer bicarbonate (KR) solution (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 0.57 MgSO4, 1.18 KH2PO4, 25 NaHCO3, and 10 mM glucose) equilibrated in heated reservoirs with 5% CO2 and 16% O2. Before the perfusate entered the cell chamber, it traversed the stainless steel tubing and was maintained at 37°C with an in-line heat exchanger and dual-channel heater controller (models SF-28 and TC-344B, Warner Instruments). After 10 min of initial perfusion to remove extracellular dye, [Ca2+]i was measured using a xenon arc lamp, interference filters, an electronic shutter, a ×20 fluorescence objective, and a cooled charge-coupled device imaging camera and determined at 12-s intervals from the ratio of fura 2 fluorescence emitted at 340 nm to that emitted at 380 nm (F340/F380). Data were collected online with InCyte software (Intracellular Imaging, Cincinnati, OH). Background images were acquired by unfocusing the imaging field. The background values were subtracted from the mean fluorescence intensities at each wavelength before the ratio was calculated. [Ca2+]i was estimated by F340/F380.

**Measurement of SOCE.** PASMCs were perfused for ≥10 min with Ca2+-free KR solution containing 1 mM EGTA to chelate residual Ca2+ 5 μM nifedipine (Sigma-Aldrich) to prevent Ca2+ from entering through L-type VDCCs, and 10 μM cyclopiazonic acid (Sigma-Aldrich) to deplete sarcoplasmic reticulum Ca2+ stores. SOCE was assessed in two ways. J) [Ca2+]i, was measured before and after restoration of extracellular Ca2+, and SOCE was evaluated by the difference between the maximum and minimum F340/F380. 2) Fura 2 fluorescence excited at 360 nm was monitored every 30 s before and after addition of MnCl2 (200 μM) to the cell perfusate, and SOCE was measured using Aperio software.
assessed from the rate at which fura 2 fluorescence was quenched by Mn2+, which entered the cell as a Ca2+ surrogate and reduced fura 2 fluorescence upon binding to the dye. Fluorescence excited at 360 nm (F360) was the same for Ca2+-bound and Ca2+-free fura 2; therefore, changes in fluorescence were caused by Mn2+ alone.

RNA extraction and measurement by RT-quantitative PCR. Total RNA from distal PA smooth muscle and PASMCs of rats was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA) method, as previously described (23, 44, 47). RT and real-time quantitative PCR (qPCR) were also performed as described previously (20, 21). The reactions were assembled following the manufacturer’s recommendation. Briefly, 15 μl of reaction mixture contained 7.5 μl of buffer (iQ SYBR Green Supermix, Bio-Rad), 3 μl of 200 nM forward and reverse primers, 3.9 μl of PCR water, and 3 μl of cDNA template from the sample. The PCR conditions are as follows: 3 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at annealing temperature, and 30 s at 72°C. We verified the specificity of PCR by measuring the melting curve of the PCR products at the end of the reaction. Fluorescence data were specified for collection during primer extension. The relative cDNA ratio was calculated using the value of threshold cycles (19). The PCR primer sequences for the rat were as follows: TGCTGGTGGGTATTTG-GAAC and CTGTCTGTTGGTAGCTGTC for HIF-1α, CA-GAGCCAACACTGTTAGGA and GGAATTGCTGTGGTT-TAAA for BMP4, GACACCTCCTTTCTTATTGA and GAAACAT TGTTGACACGCTT for PPARy, AGCCTCTTACAAACGGA and ACCTGTACATGTTGGCAACC for TRPC1, TACTGTTGT-GCTCTTCAG and GAGCTTTGTGCTCCATAC for TRPC6, and GCAATTATCCATAGACG and GGCGTACTAAGAC-CATCCAA for GAS.

Protein analysis by Western blotting. Rat distal PA smooth muscle and PASMCs were sonicated and lysed in tissue protein extraction buffer (T-per, Pierce, Rockford, IL) with a protease inhibitor cocktail tablet, and the immunoblot assay was performed as described previously (17, 21, 47). Total lysate proteins were loaded on NuPAGE Novex gels (Invitrogen), and the separated proteins were transferred to polyvi-nyliden fluoride membranes (pore size 0.45 μm; Bio-Rad). The membrane was blocked for nonspecific binding by incubation with 5% fat-free dry milk in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 60 min. Primary antibody was diluted in blocking buffer and incubated with the membrane overnight at 4°C. The membrane was washed in TBST (10 min, 5 times), incubated with secondary antibody for 60 min at room temperature, and washed again in TBST (10 min, 5 times), and the antibody-detected protein bands were visualized by ECL reagent (Amersham).

Statistical analysis. Values were means ± SE; n is the number of experiments, which equals the number of rats providing PAS or PASMCs. Statistical analyses were conducted using one-way ANOVA for multiple groups and Student’s t-test for groups of two. All data were tested and found fit for normal distribution. When F ratios obtained with ANOVA were significant, Fisher’s protected least significant difference was used for pair-wise comparisons of means. Differences were considered significant at \( P < 0.05 \).

RESULTS

**BTZ inhibited characteristic changes in CHPH rat models.** After exposure to CH (10 ± 0.5% O2 for 21 days), rats developed typical PH. As shown in Fig. 1, A and B, RV systolic pressure (RVSP) in the hypoxia control group was markedly increased to 42.78 ± 6.4 mmHg compared with 30.52 ± 6.25 mmHg in the normoxia control group. However, treatment with BTZ (1.3 mg/m2 sc, twice a week) resulted in a remarkable restoration of hypoxia-elevated RVSP (Fig. 1, A and B; \( P < 0.01, n = 6 \)). In parallel with the changes in RVSP, BTZ also significantly inhibited the hypoxia-upregulated ratio of RV to left ventricle (LV) plus septum ([RV/(LV + S)] and the ratio of RV to body weight (RV/BW, g/kg; Fig. 1, C and D; \( P < 0.01, n = 6 \)).

Lung histological and morphometric analyses were performed to evaluate pulmonary vascular remodeling in all groups of rats. Data showed that CH greatly induced PA wall thickening and remodeling, while BTZ treatment markedly suppressed the hypoxia-induced increase in PA wall thickness in the CH group (Fig. 1, E–J; \( P < 0.01, n = 6 \)).

During the continuous 21-day exposure to normoxia or hypoxia, body weight of all rats was monitored every 3 days. In the hypoxia group, we observed a marked (~9%) body weight loss at 3 days after hypoxic exposure that recovered at 6 days posthypoxia. However, all rats in the normoxia-exposed group demonstrated persistent body weight gain. Moreover, BTZ treatment did not have obvious effects on the body weight of normoxic or hypoxic rats. We confirmed that injection of BTZ (1.3 mg/m2 sc, twice a week) resulted in attenuation of pulmonary vascular resistance and remodeling as well as a remarkable reduction of RV hypertrophy in the CHPH model, represented as a decrease in RV systolic pressure, media wall thickening of small pulmonary vasculature, and a decrease in RV/(LV + S) and RV/BW. These results are consistent with previously published findings (10, 15).

**BTZ suppressed CH-elevated basal [Ca2+], and SOCE in primary PASMCs freshly isolated from CHPH rats.** After PASMCs were freshly isolated from rats exposed to normoxia or CH with or without BTZ, they were seeded onto coverslips for [Ca2+] measurement after 12–24 h of stabilization in basal culture medium. Basal [Ca2+] in PASMCs from the hypoxia control group increased to 1.34 ± 0.09 compared with 1.03 ± 0.06 in PASMCs from the normoxia control group (\( n = 4, 0.06 \)).
However, this upregulation could be suppressed by BTZ treatment (Fig. 1, K and L). We assessed SOCE in two ways: 1) Mn²⁺ quenching of F₃₆₀ and 2) change in F₃₄₀/F₃₈₀ during a 10-min perfusion with Ca²⁺-free physiological salt solution (PSS), followed by a 10-min perfusion with Ca²⁺-containing PSS. Consistent with the changes in basal [Ca²⁺]ᵢ, BTZ restored hypoxia-enhanced SOCE in PASMCs (Fig. 1, K, M, N, and O).
BTZ inhibited hypoxia-upregulated TRPC1 and TRPC6 expression in distal PAs and PASMCs. Expression of TRPC1 and TRPC6 in distal PAs and PASMCs was evaluated at the mRNA and protein levels. Consistent with our previous findings, we confirmed that hypoxia elevated TRPC1 and TRPC6 expression in PAs and PASMCs. Treatment with BTZ reversed this hypoxic upregulation. Moreover, BTZ did not affect the expression of TRPC1 and TRPC6 in normoxic PAs (Fig. 2, n = 4, P > 0.05) and PASMCs (Fig. 3; n = 4, P > 0.05).

BTZ normalized hypoxia-upregulated HIF-1α and BMP4 expression and hypoxia-downregulated PPARγ expression in distal PAs and PASMCs. According to previous studies from our laboratory and many others, the HIF-1α signaling axis has been proven to be a key contributor to the abnormal intracellular Ca²⁺ homeostasis during hypoxic exposure, while the transcriptional factor PPARγ exerts a protective effect on hypoxia-induced [Ca²⁺]ᵢ and SOCE, likely by inhibiting the HIF-1α-mediated signaling axis (40, 41). Similarly, our results indicate that hypoxia exposure induced a marked increase in the HIF-1α-BMP4 signaling axis but decreased PPARγ expression at the mRNA and protein levels. However, treatment with BTZ eliminated the hypoxia-induced changes in the expression of these proteins without affecting the normoxic controls, suggesting a specific role in hypoxia (Figs. 4 and 5; n = 4, P > 0.05).

Fig. 2. Effect of BTZ on expression of canonical transient receptor potential (TRPC) isoforms 1 and 6 in distal pulmonary arteries (PAs) from rats exposed to hypoxia. A and B: mRNA levels of TRPC1 and TRPC6 relative to 18S determined by RT-quantitative PCR. C: representative Western blot showing protein levels of TRPC1 and TRPC6. D and E: mean intensity of TRPC1 and TRPC6 bands relative to β-actin. Values are means ± SE; n = 6 in each group. #P < 0.05, ##P < 0.01 vs. respective normoxia control. *P < 0.05, **P < 0.01 vs. respective hypoxia control. P = 0.94 and P = 0.89 for TRPC1 and TRPC6 mRNA, respectively, and P = 0.44 and P = 0.53 for TRPC1 and TRPC6 protein, respectively, in normoxia + BTZ vs. normoxia control.
BTZ dose-dependently inhibited hypoxia-induced proliferation in cultured rat distal PASMCs. To explore the function of BTZ, rat distal PASMCs were treated with 1, 5, 10, and 20 nM BTZ. The proliferation rate of cells from different treatment groups was assessed by a BrdU incorporation assay. We found that prolonged hypoxia (4% O₂, 60 h) promotes PASMC proliferation. However, this effect could be inhibited by BTZ in a dose-dependent manner (P < 0.01, n = 4). Notably, we also detected a dose-dependent suppression of PASMC proliferation in PASMCs exposed to normoxia, but the inhibitory effect was less evident than in the hypoxia control group (P < 0.01, n = 4). Death occurred in some PASMCs treated with 20 nM BTZ in both hypoxia and normoxia. To verify the toxic effect of BTZ on PASMC survival, we then tested LDH activity. Results showed a marked increase in LDH activity at 20 nM BTZ (P < 0.01, n = 8), in correlation with the cell death phenomenon indicated by the cell survival data. We chose 10 nM BTZ, which induces high inhibition of hypoxia-induced proliferation without significant cell toxicity. The PASMCs were treated with 10 nM BTZ for 0, 2, 6, 12, 24, and 60 h, and the most dramatic inhibition of BTZ on PASMC proliferation was obtained at 60 h (n = 4, P < 0.01; Fig. 6). Therefore, 10 nM BTZ for 60 h was used in the following in vitro experiments.
Fig. 4. Effect of BTZ on hypoxia-inducible factor (HIF)-1α, bone morphogenetic protein 4 (BMP4), and peroxisome proliferator-activated receptor-γ (PPARγ) expression in distal PAs from rats exposed to hypoxia. A–C: mRNA levels of HIF-1α, BMP4, and PPARγ relative to 18S. D: representative Western blot showing protein levels of HIF-1α, BMP4, and PPARγ, and PPARγ bands relative to β-actin. Values are means ± SE; n = 6 in each group. #P < 0.05, ##P < 0.01 vs. respective normoxia control. *P < 0.05, **P < 0.01 vs. respective hypoxia control. P = 0.93, P = 0.12, and P = 0.81 for HIF-1α, BMP4, and PPARγ mRNA, respectively, and P = 0.78, P = 0.35, and P = 0.88 for HIF-1α, BMP4, and PPARγ protein, respectively, in normoxia + BTZ vs. normoxia control.
Fig. 5. Effect of BTZ on expression of HIF-1α, BMP4, and PPARγ in rat distal PASMCs. PASMCs isolated from normoxic rats were exposed to normoxia or prolonged hypoxia with or without 10 nM BTZ. A–C: HIF-1α, BMP4, and PPARγ mRNA relative to 18S determined by RT-quantitative PCR. D: Western blot of protein levels of TRPC1 and TRPC6. E–G: mean intensity of TRPC1 and TRPC6 bands normalized to β-actin. Values are means ± SE; n = 6 in each group. #P < 0.05, ##P < 0.01 vs. respective normoxia control. *P < 0.05, **P < 0.01 vs. respective hypoxia control. P = 0.55, P = 0.77, and P = 0.99 for HIF-1α, BMP4, and PPARγ mRNA, respectively, and P = 0.75, P = 0.92, and P = 0.69 for HIF-1α, BMP4, and PPARγ protein, respectively, in normoxia + BTZ vs. normoxia control.
BTZ inhibited hypoxia-elevated basal \([Ca^{2+}]\), and SOCE in cultured rat distal PASMCs. PASMCs were cultured and divided into four treatment groups: 1) normoxia, 2) normoxia + BTZ, 3) hypoxia, and 4) hypoxia + BTZ. Basal \([Ca^{2+}]\) in PASMCs exposed to hypoxia increased to 1.44 ± 0.01 compared with 1.21 ± 0.06 in PASMCs exposed to normoxia (n = 4, P < 0.01). In addition, BTZ (10 nM, 60 h) treatment effectively eliminated the hypoxic elevation of basal \([Ca^{2+}]\), and SOCE. As shown in Fig. 7, A and B, compared with the normoxia control group, hypoxic exposure significantly increased basal \([Ca^{2+}]\) and SOCE to 1.44 ± 0.01 and 0.85 ± 0.01, respectively. BTZ significantly abolished hypoxia-elevated basal \([Ca^{2+}]\), and SOCE to 1.22 ± 0.02 and 0.66 ± 0.01 in hypoxic, but not normoxic, PASMCs (Fig. 7, A, C, D, and E).

BTZ inhibited PH features in the MCT-PH rat model. Since our results strongly suggest a therapeutic potential of BTZ in the CHPH rat model, we introduced another well-used MCT-PH model to confirm the protective role of BTZ in PH pathogenesis. As previously described, rats were injected with MCT (50 mg/kg sc) to induce severe PH. As seen in Fig. 8, A and B, RVSP increased to 53.32 ± 6.79 mmHg in MCT controls (P < 0.01, n = 6), which was significantly higher than 22.87 ± 2.40 mmHg in saline controls. Moreover, BTZ treatment (1.3 mg/m² sc, twice a week) markedly attenuated MCT-elevated RVSP without altering RVSP in saline control rats (P < 0.01, n = 6). In addition, the RV hypertrophy index and RV/BW (g/kg) showed a similar pattern in all four groups of rats (Fig. 8, C and D; P < 0.01, n = 6). In parallel, lung histochemistry data also demonstrated that MCT-induced dramatic thickening in the wall of the distal PA compared with saline control rats, while BTZ markedly alleviated the MCT-induced PA wall thickening (Fig. 8, E–J; P < 0.01, n = 6).

BTZ inhibited MCT-upregulated TRPC1 and TRPC6 expression in distal PAs isolated from MCT-PH rats. As BTZ could markedly affect the characteristics of MCT-PH rats, we further investigated the potential role of BTZ in the abnormally elevated TRPC-SOCE signaling axis in MCT-PH. MCT increased the mRNA expression of TRPC1 and TRPC6 in distal PAs by 83% and 53%, respectively (Fig. 9, A and B). Similarly, MCT also induced 74% and 147% increases in TRPC1 and TRPC6 protein expression (Fig. 9, C–E). However, treatment with BTZ inhibited these MCT-induced increases in TRPC1 and TRPC6 expression at the mRNA and protein levels. In addition, TRPC1 and TRPC6 expression was not significantly affected in saline controls (Fig. 9; n = 6, P > 0.05).

BTZ normalized MCT-upregulated HIF-1α and BMP4 expression and MCT-downregulated PPARγ expression in rat distal PAs. MCT injection increased HIF-1α and BMP4 mRNA expression by 152% and 99%, respectively (Fig. 10, A and B) but decreased PPARγ mRNA expression by 67% (Fig. 10C). At the protein level, MCT injection caused 88% and 84% increases in HIF-1α and BMP4 expression, respectively, and a 63% reduction of PPARγ (Fig. 10, D–G). Treatment with BTZ normalized HIF-1α, BMP4, and PPARγ expression at the mRNA and protein levels without affecting the saline control rats (Fig. 10; n = 6, P > 0.05).

DISCUSSION

BTZ is the first clinically used proteasome inhibitor approved for the treatment of multiple myeloma (1, 4, 11, 13, 19, 32, 51). Recently, researchers have indicated that BTZ alleviates experimental PH (10, 15, 50, 54). In CHPH and MCT-PH animal models, BTZ could ameliorate endothelial function by upregulating eNOS, phosphorylated eNOS, and NO expression.
In this study we have provided mechanistic evidence that BTZ inhibits the elevation of SOCE and Ca\(^{2+}\) entry via SOCCs, ROCCs, and VDCCs, contributing greatly to hypoxia-induced Ca\(^{2+}\) homeostasis, especially SOCE in PASMCs. It is well accepted that elevated Ca\(^{2+}\) promotes cell contraction and proliferation of PASMCs (15). In flow-induced PA hypertension models, BTZ inhibited contraction and proliferation of PASMCs (9, 17), which play a crucial role in PH development. Our previous publications have shown elevation of Ca\(^{2+}\) in PASMCs isolated from CHPH rats (25, 31, 35, 39). Thus we sought to investigate whether BTZ exerts its protective role by targeting the intracellular Ca\(^{2+}\) homeostasis, especially SOCE in PASMCs.

BTZ inhibits the elevation of SOCE and Ca\(^{2+}\) entry via SOCCs, ROCCs, and VDCCs, contributing greatly to hypoxia-induced Ca\(^{2+}\) homeostasis, especially SOCE in PASMCs. It is well accepted that Ca\(^{2+}\) promotes cell contraction and proliferation of PASMCs (15). In flow-induced PA hypertension models, BTZ inhibited contraction and proliferation of PASMCs (9, 17), which play a crucial role in PH development. Our previous publications have shown elevation of Ca\(^{2+}\) in PASMCs isolated from CHPH rats (25, 31, 35, 39). Thus we sought to investigate whether BTZ exerts its protective role by targeting the intracellular Ca\(^{2+}\) homeostasis, especially SOCE in PASMCs.
determined the inhibition of BTZ in hypoxia-elevated SOCE in freshly isolated rat distal PASMCs from CHPH rats. To better understand the molecular mechanism of BTZ in the intracellular Ca\(^{2+}\) regulation of PASMCs, we introduced FBS-cultured rat distal PASMCs, which mimic the in vitro hypoxic exposure model. According to our previous studies (43, 46), in vitro cultured PASMCs displayed increases in basal Ca\(^{2+}\) concentration and SOCE in response to prolonged hypoxic exposure (4% O\(_2\), 60 h) quite similar to PASMCs freshly isolated from CHPH rats and, thereby, prove to be a satisfying in vitro model to study the deeper mechanisms. Consistent with this finding, BTZ treatment also suppressed the hypoxia-induced elevation of SOCE in cultured rat distal PASMCs. To exclude the possible influence of Ca\(^{2+}\) influx via VDCCs, the VDCC-specific inhibitor nifedipine was included in the perfusion buffer during the measurement of SOCE.

![Fig. 8. Effect of BTZ on pulmonary hypertension characteristics in monocrotaline (MCT)-induced pulmonary hypertension (MCT-PH) rat model.](image)

![Fig. 9. Effect of BTZ on TRPC1 and TRPC6 expression in distal PAs isolated from MCT-PH rats.](image)
Fig. 10. Effect of BTZ on HIF-1α, BMP4, and PPARγ expression in distal PAs isolated from MCT-PH rats. A–C: mRNA levels of HIF-1α, BMP4, and PPARγ relative to 18S determined by RT-quantitative PCR. D: Western blot of protein levels of HIF-1α, BMP4, and PPARγ. E–G: mean intensities of HIF-1α, BMP4, and PPARγ bands relative to β-actin. Values are means ± SE; n = 6 in each group. #P < 0.05, ##P < 0.01 vs. respective saline control. *P < 0.05, **P < 0.1 vs. respective MCT control. P = 0.81, P = 0.94, and P = 0.91 for HIF-1α, BMP4, and PPARγ mRNA, respectively, and P = 0.94, P = 0.24, and P = 0.98 for HIF-1α, BMP4, and PPARγ protein, respectively, in saline + BTZ vs. saline control.
zonic acid was used to induce Ca\(^{2+}\) release from the cytoplasmic Ca\(^{2+}\) store, we found that BTZ specifically normalized the hypoxia-elevated [Ca\(^{2+}\)], and SOCE without disturbing the normoxia-exposed controls. The exact molecular components of SOCC have not been identified, but it has been generally accepted that TRPC proteins are SOCCs (30). We previously revealed that, among the seven TRPC proteins (TRPC1-7), TRPC1, TRPC4, and TRPC6 were predominantly expressed in distal PAs and PASMCs (44). Hypoxia selectively upregulated TRPC1 and TRPC6 expression at the mRNA and protein levels (46). In addition, knockdown of TRPC1 and TRPC6 blocked the hypoxia-induced SOCE and [Ca\(^{2+}\)], elevation of PASMCs (21), suggesting that TRPC1 and TRPC6 act as SOCC subunits that are responsible for increased SOCE under hypoxia. Therefore, we further investigated the effect of BTZ on TRPC1 and TRPC6 expression. As expected, BTZ significantly suppressed the increase in mRNA and protein levels of TRPC1 and TRPC6 in distal PAs from hypoxic rats.

Our previous study showed that HIF-1\(\alpha\) upregulated expression of TRPC1 and TRPC6, enhanced SOCE and [Ca\(^{2+}\)], in PASMCs, and accelerated PH (47). BMP4 also contributed to PASMC proliferation via upregulation of TRPC1 and TRPC6, resulting in SOCE promotion and [Ca\(^{2+}\)]\(^{\text{cyt}}\) elevation, which ultimately mediated pulmonary vascular remodeling in CHPH (5, 20). Further studies demonstrated that, by binding functional HIF-1 binding sites located in the promoter region of the BMP4 gene, HIF-1\(\alpha\) accelerated BMP4 transcription and, subsequently, induced upregulation of TRPC1 and TRPC6, leading to an increase in SOCE and basal [Ca\(^{2+}\)]\(^{\text{cyt}}\), in PASMCs and promoting CHPH pathogenesis (42). PPAR\(\gamma\) was confirmed to attenuate CHPH and MCT-PH, potentially by normalizing the abnormal intracellular Ca\(^{2+}\) homeostasis, downregulating expression of TRPC1 and TRPC6 and resulting in inhibition of SOCE and [Ca\(^{2+}\)]\(^{\text{cyt}}\). Under hypoxia, HIF-1\(\alpha\) acts as an upstream regulator of PPAR\(\gamma\), and suppressed PPAR\(\gamma\) expression. Interestingly, PPAR\(\gamma\) demonstrated a negative feedback on HIF-1\(\alpha\) and HIF-1\(\alpha\)-mediated signal pathways (49). Next, we determined the impact of BTZ on HIF-1\(\alpha\), BMP4, and PPAR\(\gamma\) expression. As anticipated, BTZ inhibited CH-upregulated HIF-1\(\alpha\) and BMP4 expression and normalized CH-downregulated PPAR\(\gamma\) expression in distal PAs isolated from CHPH rats.

To determine whether BTZ carries out these functions directly on PASMCs, PASMCs isolated from normoxic rats were cultured in vitro and exposed to normoxia or prolonged hypoxia with or without BTZ. We found that BTZ inhibited hypoxia-triggered PASMC proliferation in a dose-dependent manner (1, 5, 10, and 20 nM). Because 20 nM BTZ caused cell death in hypoxic and normoxic conditions, 10 nM BTZ was adopted for the following in vitro experiments. In parallel with the inhibitory effect of BTZ on PASMCs under prolonged hypoxia, molecular and functional changes, such as decreased HIF-1\(\alpha\) and BMP4 expression, PPAR\(\gamma\) upregulation, reduction of TRPC1 and TRPC6 expression, and lowered SOCE and basal [Ca\(^{2+}\)], suggest that the impact of BTZ on proliferation and basal [Ca\(^{2+}\)], in PASMCs likely resulted from inhibition of upregulated HIF-1\(\alpha\), BMP4, TRPC1, and TRPC6 expression, as well as normalization of the downregulated PPAR\(\gamma\) expression.

In addition to the CHPH model, the MCT-PH model is commonly used to study PH. Consistent with the effect on CHPH, in the MCT-PH model, BTZ inhibited RV pressure, RV hypertrophy, and pulmonary vascular remodeling, which were associated with decreased expression of HIF-1\(\alpha\), BMP4, TRPC1, and TRPC6 and increased expression of PPAR\(\gamma\).

BTZ alleviates PH, at least partly, by regulating expression of HIF-1\(\alpha\), BMP4, PPAR\(\gamma\), TRPC1, and TRPC6. Further studies may be needed to validate whether BTZ alleviated PH by targeting HIF-1\(\alpha\)-BMP4-TRPC1/TRPC6 and HIF-1\(\alpha\)-PPAR\(\gamma\)-TRPC1/TRPC6 pathways. If BTZ has an inhibitory effect on PH via the pathway, HIF-1\(\alpha\) is a key upstream molecule in the context of regulation. BTZ is a kind of proteasome inhibitor and HIF-1\(\alpha\) is degraded by the ubiquitin-proteasome system (36). However, our investigation showed that BTZ decreased HIF-1\(\alpha\) expression, suggesting its impact on HIF-1\(\alpha\) ubiquitination. If BTZ has a suppressive effect on PH via HIF-1\(\alpha\), BMP4, TRPC1, TRPC6, and PPAR\(\gamma\), respectively, it is necessary to determine the impact of BTZ on their transcription and degradation mechanisms. Recently, our study confirmed that BMP4 increased TRPC1 and TRPC6 expression, SOCE, and [Ca\(^{2+}\)], by upregulating NADPH oxidase 4 (NOX4) expression in PASMCs (12). In addition to suppressing TRPC1 and TRPC6, PPAR\(\gamma\) also downregulated caveolin-1 protein and inhibited SOCE and [Ca\(^{2+}\)], in PASMCs (53). Therefore, we assumed that NOX4 and caveolin-1 are involved in the mechanism whereby BTZ alleviates PH.

In summary, this study presents essential data suggesting that the suppressive effects of BTZ on CHPH and MCT-PH occur, at least partly, through its inhibitory actions on HIF-1\(\alpha\), BMP4, TRPC1, and TRPC6 and its stimulatory action on PPAR\(\gamma\), resulting in decreased SOCE and [Ca\(^{2+}\)], in PASMCs. All the results presented in this study demonstrate only a potential correlation between the beneficial effects of BTZ on PH and a suppressed Ca\(^{2+}\) regulatory pathway, especially the TRPC-SOCE signaling axis. We do not know whether the inhibited Ca\(^{2+}\) homeostasis is directly regulated by BTZ or through other indirect pathways. Research will continue to focus on the mechanism by which BTZ suppresses the TRPC-SOCE-[Ca\(^{2+}\)]\(^{\text{cyt}}\) pathway and attenuates the development and progression of PH.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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