Attenuating Endoplasmic Reticulum Stress as a Novel Therapeutic Strategy in Pulmonary Hypertension

Peter Dromparis, BSc; Roxane Paulin, PhD; Trevor H. Stenson, PhD; Alois Haromy, MSc; Gopinath Sutendra, PhD; Evangelos D. Michelakis, MD

Background—Evidence suggestive of endoplasmic reticulum (ER) stress in the pulmonary arteries of patients with pulmonary arterial hypertension has been described for decades but has never been therapeutically targeted. ER stress is a feature of many conditions associated with pulmonary arterial hypertension like hypoxia, inflammation, or loss-of-function mutations. ER stress signaling in the pulmonary circulation involves the activation of activating transcription factor 6, which, via induction of the reticulin protein Nogo, can lead to the disruption of the functional ER-mitochondria unit and the increasingly recognized cancer-like metabolic shift in pulmonary arterial hypertension that promotes proliferation and apoptosis resistance in the pulmonary artery wall. We hypothesized that chemical chaperones known to suppress ER stress signaling, like 4-phenylbutyrate (PBA) or tauroursodeoxycholic acid, will inhibit the disruption of the ER-mitochondria unit and prevent/reverse pulmonary arterial hypertension.

Methods and Results—PBA in the drinking water both prevented and reversed chronic hypoxia–induced pulmonary hypertension in mice, decreasing pulmonary vascular resistance, pulmonary artery remodeling, and right ventricular hypertrophy and improving functional capacity without affecting systemic hemodynamics. These results were replicated in the monocrotaline rat model. PBA and tauroursodeoxycholic acid improved ER stress indexes in vivo and in vitro, decreased activating transcription factor 6 activation (cleavage, nuclear localization, luciferase, and downstream target expression), and inhibited the hypoxia-induced decrease in mitochondrial calcium and mitochondrial function. In addition, these chemical chaperones suppressed proliferation and induced apoptosis in pulmonary artery smooth muscle cells in vitro and in vivo.

Conclusions—Attenuating ER stress with clinically used chemical chaperones may be a novel therapeutic strategy in pulmonary hypertension with high translational potential. (Circulation. 2013;127:115-125.)

Key Words: endoplasmic reticulum stress ■ hypertension, pulmonary ■ metabolism ■ mitochondria ■ vascular diseases

Pulmonary arterial hypertension (PAH) is a pulmonary-selective vascular remodeling disease in which cells within the vessel wall, including pulmonary artery smooth muscle cells (PASMCs), are characterized by a proproliferative and antiapoptotic diathesis. Pulmonary arterial remodeling occludes the vessel lumen that leads to right ventricular failure and premature death, with the median survival of untreated patients limited to 3 years. However, even in those receiving standard therapies, prognosis remains poor.

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Although the pathology is restricted to the pulmonary vasculature, sparing the systemic vessels, all approved PAH therapies were originally developed as systemic vasodilators. Moreover, in contrast to the earlier belief that vasoconstriction plays a central role in PAH pathogenesis, it is now accepted that PAH is a result of proliferative remodeling with vasoconstriction playing a limited role.

An additional challenge is that, despite the recent groupings of several conditions that share similar lung histology to idiopathic PAH under a “PAH umbrella,” the pathogenesis of PAH is multifactorial, suggesting that therapies that target 1 molecular abnormality in 1 form of PAH may not be as effective in other forms of the disease. For example, PAH is associated with inflammatory conditions like scleroderma, viral infections with HIV or herpes simplex virus, hypoxia, or loss-of-function mutations in the bone morphogenetic protein receptor 2 (BMPRII). In PAH, like in cancer, many different molecular abnormalities can be active in a patient. Thus, an ideal PAH therapy should target common features of all these diverse biological processes in a manner that remains relatively selective to the pulmonary vasculature.
circulation and is effective in reversing pulmonary vascular remodeling.\(^7\)

An intriguing common feature of many known PAH-triggering or facilitating processes is endoplasmic reticulum (ER) stress. For example, both viral infections and hypoxia are well-known causes of ER stress.\(^8\) More recently, the loss-of-function mutations in BMPRII and the resultant protein trafficking dysfunction have been shown to induce ER stress as well.\(^9\) Despite the emerging evidence for protein trafficking dysregulation\(^10\) and the fact that ER abnormalities compatible with ER stress (eg, dysmorphic and swollen ER) were clearly described in the classic description of PAH pathology by Smith and Heath\(^11\) 30 years ago, ER stress has not been considered a therapeutic target.

Very recently, we published evidence that ER stress in the pulmonary circulation leads to the activation of the ER stress sensor activating transcription factor 6 (ATF6), causing upregulation of neurite outgrowth inhibitor (Nogo), a member of the reticulin family of proteins that regulate ER shape.\(^12\) Nogo induction causes disruption of a functional ER-mitochondrial unit, resulting in decreased mitochondrial calcium and inhibition of several key calcium-sensitive mitochondrial enzymes. We and others have described the resulting metabolic abnormalities as critical in PAH pathogenesis.\(^13\) The mitochondrial suppression in PAH, much like in cancer, leads to a switch to a glycolytic phenotype that promotes proliferation and suppresses apoptosis.\(^7,15\) Perhaps because of previously described mitochondrial differences between the pulmonary and systemic circulations,\(^17\) this metabolic abnormality appears to be restricted to the pulmonary circulation in both animals and patients with PAH. Indeed, mice lacking Nogo are phenotypically normal but completely resistant to metabolic and vascular remodeling and hypoxia-induced PAH.\(^12\) This work provided strong evidence of the role of Nogo and ER stress in PAH but did not offer a therapeutic tool. Although anti-Nogo therapies are currently in development,\(^18\) we hypothesized that inhibition of ER stress-induced ATF6 activation, the original signal for Nogo induction, may offer a therapeutic strategy potentially applicable to diverse PAH pathologies. The fact that we had shown that ATF6 is selectively activated in the pulmonary circulation, and not systemic vessels despite systemic hypoxia, provided some evidence that such a therapy may be relatively pulmonary selective.\(^12\)

ER stress can be attenuated with small-molecule chemical chaperones like the fatty acid derivative chemical chaperone 4-phenylbutyric acid (PBA) and the bile acid derivative tauroursodeoxycholic acid (TUDCA). Chemical chaperones mimic native chaperones, promoting folding, preventing aggregation, and restoring trafficking of misfolded proteins.\(^19\)–\(^21\) They have demonstrated antiproliferative and proapoptotic effects in cancer,\(^22\)–\(^24\) which shares a similar metabolic and mitochondrial remodeling with PAH-PASMCs.\(^7,15\) Moreover, these therapies are orally administered and are currently used clinically.\(^19\)

Here, we show that these small molecules limit ER stress-induced mitochondrial suppression, preventing and reversing vascular remodeling, using classic models of pulmonary hypertension in both mice (hypoxia) and rats (monocrotaline [MCT]) and in vitro mechanistic studies.

**Methods**

**Animals**

All experiments were performed with approval by the University of Alberta committee on animal policy and welfare. Male C57BL/6 mice and Sprague Dawley rats were purchased from Charles River. The mice were randomized to normobaric hypoxia (10% O\(_2\) ) or room air, and rats were randomized to injection of saline or MCT (60 mg/kg) as previously described.\(^15\) Animals were further randomized to receive PBA in either a prevention (PBA starting the day of pulmonary hypertension [PHT] induction and continuing for 4 weeks) or a reversal (on the third week of PHT induction and continuing for 2 weeks) protocol in their drinking water. By measuring water consumption and animal weights, we determined that mice and rats received an average dose of \(\approx 500\) mg \(\cdot\) kg\(^{-1}\) \cdot\) d\(^{-1}\).

**Echocardiography and Hemodynamics**

Cardiac output was assessed on isoflurane-anesthetized animals by echocardiography using the Vevo770 imaging system with the 707B (30 MHz) and 716 (15 MHz) probes for mice and rats, respectively. The cardiac output (CO) was calculated after determining the left ventricular outflow tract diameter (LVOT), aortic velocity-time integral (AoVTI), and heart rate (HR) with the following formula:

\[
CO = 7.85 \times \text{LVOT}^2 \times \text{AoVTI} \times \text{HR} / 1000
\]

Pulmonary artery acceleration time was measured by echocardiography as previously described.\(^12,15,25\) Total pulmonary resistance was calculated by the ratio of mean pulmonary arterial pressure to CO. Mean pulmonary artery pressures were assessed in closed-chest animals with Millar catheters (Millar Instruments Inc, Houston, TX) as previously described.\(^12,15,25\) Under inhaled isoflurane anesthesia, the right jugular vein was cannulated, and the catheter was advanced into the pulmonary arteries. Pressures from the right atrium, right ventricle, and pulmonary arteries were recorded continuously, and mean pulmonary artery pressure was calculated electronically (Power Laboratory, with Chart software 5.4, ADInstruments).

**Treadmill Test**

Animals were placed on a calibrated, motor-driven treadmill (Treadmill Simplex II, Columbus Instruments) and run once a week (3 times total) on an undemanding protocol to allow familiarization with the test. Afterward, animals from each therapy group were run until failure with the following protocol: 3 minutes at 10 m/min, 3 minutes at 12 m/min, 20 minutes at 14 m/min, 20 minutes at 16 m/min, and 18 m/min until failure. Failure was defined as >5 consecutive seconds on the shocker grid, and the test was terminated.

**Blood Pressure**

Blood pressure was measured with an iiTC blood pressure apparatus (Life Sciences, Woodland Hills, CA) for mice and rats. Animals were restrained in warming chambers (34°C), and a tail cuff was placed at the base of the tail. Systolic and diastolic blood pressures for each animal were obtained in triplicate and averaged.

**Medial Wall Thickness**

The percent medial wall thickness was determined as previously described.\(^12,15,25\) Briefly, 5-μm-thick lung sections were stained with hematoxylin and eosin. Vessels >50 μm were identified and measured at the 2 ends of the shortest external diameter of the distal PAs, and the average was taken ([2× wall thickness/external diameter] \(\times\)100).
PBA Prevents and Reverses Pulmonary Hypertension in Mice and Rats

To determine whether chemical chaperones could be a potential PHT therapy, we studied mice exposed to 4 weeks of chronic normobaric hypoxia (CH-PHT) treated with PBA in a prevention (PBA starting the day of CH exposure and continuing for 4 weeks) or reversal protocol (PBA starting on the third week after onset of CH and continuing for 2 weeks). Mice treated with PBA in either protocol had lower mean pulmonary artery pressure (35.8 ± 1.3 versus 21.8 ± 0.9 and 19.0 ± 0.7 mm Hg in PBA reversal and prevention treatments, respectively) and total pulmonary resistance (0.71 ± 0.08 versus 0.45 ± 0.07 and 0.33 ± 0.08 mm Hg • min⁻¹ • mL⁻¹ in PBA reversal and prevention protocols, respectively) compared with untreated CH-PHT controls (Figure 1A). Pulmonary artery acceleration time measured echocardiographically, which is inversely related to mean pulmonary artery pressure, was also improved with PBA treatment (Figure IA in the online-only Data Supplement). The decreased afterload resulted in lower right ventricular hypertrophy and improved functional capacity assessed by a treadmill test (Figure 1A). These parameters were not affected in normal normoxic mice treated with PBA. Systolic and diastolic blood pressures were not affected by hypoxia or PBA treatment (Figure IB in the online-only Data Supplement). In a second, more severe and inflammatory model of PHT, 27 rats were injected with MCT (MCT-PHT) and treated in similar prevention (PBA starting the day of MCT injection and continuing for 4 weeks) and reversal (PBA starting on the third week after MCT injection and continuing for 2 weeks) protocols. Similar to the CH-PHT mice, the PBA-treated MCT-PHT rats had decreased mean pulmonary artery pressure (50.2 ± 5.1 versus 33.7 ± 4.3 and 31.3 ± 4.7 mm Hg in PBA reversal and prevention protocols, respectively), total pulmonary resistance (0.71 ± 0.08 versus 0.45 ± 0.07 and 0.33 ± 0.08 mm Hg • min⁻¹ • mL⁻¹ in PBA reversal and prevention protocols, respectively; Figure 1B), and right ventricular hypertrophy (Figure II in the online-only Data Supplement).
PBA Prevents and Reverses Pulmonary Vascular Remodeling in PHT

Consistent with the improved hemodynamics, PBA-treated CH-PHT and MCT-PHT animals had reduced medial wall thickening of the resistance PAs (50- to 100-μm diameter) and reduced muscularization (in >50-μm-diameter PAs) in both the reversal and prevention protocols (Figure 2A and Figure III in the online-only Data Supplement). Resistance vessels in vehicle-treated PHT animals from both models had increased expression of the proliferation marker Ki67 in SMA-positive cells, which was reduced by PBA in both treatment protocols (Figure 2B). Furthermore, PBA induced apoptosis in resistance PA SMA-positive cells by ≈3.6- and 4.5-fold in the CH-PHT and MCT-PHT animals, respectively (Figure 2B). Proliferation and apoptosis indexes were not affected in von Willebrand Factor–positive cells (PA endothelial cell marker; Figure IV in the online-only Data Supplement). No changes in any histology-assessed parameters were observed in the lungs of PBA-treated healthy animals (Figure 2 and Figure III in the online-only Data Supplement).

PBA Reduces Markers of ER Stress in the Pulmonary Vasculature in PHT

Upon ER stress, ATF6 is cleaved in the ER and translocates to the nucleus,28 where it functions as a transcription factor. To determine whether the beneficial effects observed in vivo can be associated with decreased cleavage of ATF6, we assessed nuclear ATF6 expression in resistance pulmonary arteries. Vehicle-treated CH-PHT mice had elevated nuclear ATF6 levels in SMA-positive PA cells compared with vehicle-treated normoxic mice (Figure 3A), suggesting ATF6 cleavage in the diseased PA. PBA reduced nuclear ATF6 expression in both the prevention and reversal protocols (Figure 3A), which corresponded to decreased expression of the ATF6 target gene, glucose-regulated protein 78 (GRP78) 29 (Figure V in the online-only Data Supplement). To more appropriately quantify the reduction of ATF6 cleavage, we performed immunoblots on isolated PAs (more than the fourth division). In keeping with the immunofluorescence, CH-PHT PAs had increased levels of cleaved (ie, active form) ATF6 (≈60 kDa) and increased expression of its target products GRP78 and Nogo (Figure 3B), supporting ATF6 activation in the PAs in vivo. This was blocked by PBA in both the prevention and reversal protocols. In contrast to ATF6, the proapoptotic ER stress pathway was not upregulated by hypoxia or PBA treatment, as assessed by C/EBP homologous protein (CHOP) expression (Figure VI in the online-only Data Supplement). Similar effects on the ATF6 axis were observed in whole-lung samples in both CH-PHT (Figure VII in the online-only Data Supplement) and MCT-PHT (Figure VIII in the online-only Data Supplement).
Chemical Chaperones Attenuate Hypoxia-Induced ATF6 Activation In Vitro

We then exposed cultured murine PASMCs to 48 hours of physiological mild hypoxia (normoxia: $pO_2 = 122.1 \pm 0.6$ mm Hg, $pH = 7.38 \pm 0.02$; hypoxia: $pO_2 = 41.9 \pm 0.7$ mm Hg, $pH = 7.39 \pm 0.01$) to mimic our in vivo CH-PHT conditions. Similar to our in vivo findings, hypoxia caused an increase in nuclear translocation of ATF6, suggesting that hypoxia increases ATF6 cleavage in vitro (Figure 4A). Double staining with antibodies against both ATF6 and GRP78 showed that in the same cells in which ATF6 was activated, GRP78 expression was increased. Both PBA and a structurally distinct chemical chaperone, TUDCA, blocked hypoxia-induced increases in ATF6 nuclear localization and GRP78 expression (Figure IX in the online-only Data Supplement). Similar to the PAs in vivo, hypoxia did not affect ATF6 cleavage in the resistance PA SMA$^+$ cells in normoxic mice. PBA did not affect ATF6 cleavage in the resistance PA SMA$^+$ cells in normoxic mice. In contrast, thapsigargin, an inducer of severe and non-physiological ER stress, substantially upregulated both GRP78 and CHOP in PASMCs (Figure IX in the online-only Data Supplement).

Chemical Chaperones Inhibit the Decrease in Mitochondrial Calcium and Function in Hypoxic PASMCs

We have previously shown that in PASMCs, hypoxia-induced ATF6 activation triggers an increase in Nogo expression, which disrupts the ER-mitochondrial unit. Spatial disruption of this unit dissociates mitochondria from ER-associated calcium microdomains, reducing intramitochondrial calcium. Hypoxia predictably reduced the levels of mitochondrial calcium as determined by fluorescence resonance energy transfer imaging (Figure 5A). Both PBA and TUDCA inhibited the reduction in mitochondrial calcium despite ongoing hypoxia. Ruthenium red, a mitochondrial calcium uniporter inhibitor, reduced the fluorescence resonance energy transfer signal (low fluorescent protein/cyan fluorescent protein ratio), confirming the sensitivity of this technique to measure mitochondrial calcium in our model (Figure 5A). These results were also replicated with rhodamine-2AM, a mitochondria-specific, calcium-sensitive dye (Figure X in the online-only Data Supplement).

**Figure 3.** 4-Phenylbutyrate (PBA) reduces endoplasmic reticulum stress in vivo. A, PBA in prevention and reversal protocols reduces hypoxia-induced activating transcription factor 6 (ATF6) cleavage in the resistance pulmonary artery (PA) smooth muscle actin (SMA$^+$)–positive cells (50- to 100-μm-diameter vessels) as assessed by nuclear ATF6 levels with immunofluorescence and confocal microscopy. Top, low magnification showing SMA (green), ATF6 (red), and nuclei (blue). Bottom, high magnification of boxed region showing ATF6 (red) and nuclei (blue; n = 10 vessels per mouse, 3–4 mice per group; $^*$P < 0.05 vs normoxia vehicle; $^+$P < 0.05 vs hypoxic vehicle). B, PBA in prevention and reversal protocols reduces ATF6 cleavage and GRP78 and Nogo protein expression in isolated PAs of chronic normobaric hypoxia–pulmonary hypertension (CH-PHT) mice (mean data obtained from blot of n = 5 mice per group; $^*$P < 0.05 vs normoxia vehicle; $^+$P < 0.05 vs hypoxic vehicle).
In keeping with increased mitochondrial calcium, PBA and TUDCA inhibited the hypoxia-induced decrease in the calcium-sensitive mitochondrial enzyme PDH (Figure 5B). PBA and TUDCA also increased the activity of another calcium-dependent mitochondrial enzyme, isocitrate dehydrogenase, as assessed by its product, α-ketoglutarate (0.60 ± 0.1 versus 0.80 ± 0.04 and 0.75 ± 0.04 μg per well for PBA- and TUDCA-treated hypoxic PASMCs, respectively; *P < 0.05). Both PDH and isocitrate dehydrogenase produce NADH, which feeds into the electron transport chain, proportionally generating mitochondrial reactive oxygen species (mROS). In keeping with restored activity of these enzymes, PBA and TUDCA inhibited the hypoxia-induced decrease in mROS (Figure 6A). Finally, because calcium is positively charged, it also influences the mitochondrial membrane potential (∆Ψm). Consistent with decreased mitochondrial calcium and as previously described, hypoxic PASMCs had increased ∆Ψm, which was blocked by both PBA and TUDCA (Figure 6A). Taken together, these results suggest that PBA and TUDCA prevent ER stress–induced mitochondrial suppression, a cellular hallmark of PAH.

Chemical Chaperones Induce Apoptosis and Normalize Proliferation in Hypoxic PASMCs

Mitochondria-driven apoptosis is mediated largely by the release of proapoptotic factors through the mitochondrial transition pore. Because both mROS and ∆Ψm depolarization facilitate the opening of the voltage- and redox-sensitive mitochondrial transition pore, we explored the effects of chemical chaperones on PASMC apoptosis under hypoxic conditions. PBA and TUDCA increased apoptosis measured by the percentage of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive PASMCs by ≈3.93- and ≈3.05-fold, respectively (Figure 6B). Furthermore, PBA and TUDCA both reduced proliferation measured by the percentage of PASMCs expressing Ki67, in keeping with the effects of these chaperones in vivo (Figure 6B).

Discussion

We show that the small-molecule chemical chaperone PBA prevents and reverses PAH in 2 standard rodent models, CHPHT in mice and MCT-PHT in rats. In vitro, PBA and TUDCA inhibit the ATF6 ER stress pathway in PASMCs exposed to
physiological hypoxia. The downstream effects of this inhibition include decreased expression of Nogo, increased mitochondrial calcium levels, restoration of the activity of critical metabolic enzymes, decreased $\Delta \Psi_m$, and increased mROS, mimicking the genetic deletion of Nogo $^{12}$ (Figure 7). These agents reduced proliferation and induced apoptosis, in vivo and in vitro, reversing and preventing the pulmonary vascular remodeling of PHT.

Hypoxia, viruses, or loss-of-function mutations can cause ER stress and can activate the unfolded protein response. $^8$ There are 3 arms of the unfolded protein response: inositol-requiring enzyme-1, protein kinase ER-like kinase (PERK), and ATF6. $^{33}$ Although the PERK pathway is generally pro-apoptotic, activating transcription factors like CHOP, the ATF6 response is typically associated with antiapoptotic and pro-survival signaling. $^{34-36}$ The prosurvival response is designed to transiently suppress apoptosis to allow cellular repair and normalization of protein trafficking during stress. We had previously postulated that a very fast and efficient way to achieve this would be to inactivate mitochondria, major inducers of apoptosis. $^{12}$ Because mitochondria are dependent on ER for calcium supply, disruption of the strategically arranged “mitochondria-ER unit” by reshaping the ER on Nogo induction can achieve this goal. Indeed, PASMCs from Nogo$^{-/-}$ mice fail to show ER-mitochondrial disruption, any tested indexes of mitochondrial suppression, or a proliferative/antiapoptotic phenotype on hypoxic exposure, and Nogo$^{-/-}$ and Nogo$^{+/+}$ mice are resistant to CH-PHT in a gene-dose-dependent manner. Moreover, adenoviral delivery of Nogo results in hyperpolarized $\Delta \Psi_m$ and decreased mROS. $^{12}$ Thus, although in the short term this ER stress–induced mitochondrial suppression can protect against apoptosis, the signaling changes that follow downstream promote a state of proliferation and apoptosis resistance that, if sustained, may lead to vascular remodeling and PAH.

The relative contribution of the proapoptosis versus prosurvival ER stress pathway is complex and incompletely understood but may depend on the degree and duration of ER stress, as well as being cell-type specific. $^{33}$ In keeping with this, exposure of PASMCs to a severe and nonphysiological ER stress stimulus, thapsigargin, caused greater ATF6 activation (Figure 4B) and GRP78 upregulation (Figure VIII in the online-only Data Supplement) but also activated the pro-apoptotic ER stress response (Figure VIII in the online-only Data Supplement), in contrast to hypoxia. Under the mild stress of physiological hypoxia (ie, $pO_2 \approx 40$ mm Hg), the ATF6 activation may be favored in the pulmonary circulation, which is generally under more oxidized conditions (as a result of exposure to much higher oxygen levels and baseline mROS levels compared with systemic vessels). $^{12,17}$ The reason is that, compared with the other ER stress sensors, ATF6 is redox sensitive and has a lower activation threshold under a shift toward more reduced conditions (hypoxia, decreased mROS). $^{28}$ Thus, the relatively mild ER stress caused by hypoxia (CH-PHT) or inflammation (MCT-PHT), coupled with an overall loss of oxidative signals resulting from mitochondrial suppression, may promote ATF6 activation over the other ER stress pathways in the pulmonary circulation.

The reversal of ER stress–induced ATF6 signaling with chemical chaperones could be a potentially attractive therapeutic strategy because it satisfies many of the current
therapeutic challenges in PAH: It inhibits a pathway potentially common to several, but perhaps not all, PAH pathologies; it is effective in decreasing proliferation and inducing apoptosis; and it is relatively specific to the pulmonary circulation. Obviously, such a strategy may logically have short-term adverse effects because it may remove the beneficial survival response designed to protect against a serious stress. Although we did not observe any gross adverse effects in the treated healthy animals, the possibility remains that if these animals were exposed to another stressor, they might be less able to defend against the cellular stress acutely. On the other hand, Nogo–/– mice have a normal development and phenotype.12 Furthermore, there is ample published experience with these types of agents in humans.

Chemical chaperones are currently being explored therapeutically in a variety of ER stress–associated conditions.19 In humans, they have been investigated in patients with cystic fibrosis,33,34 [beta]-thalassemia,39 spinal muscular atrophy,40 ornithine transcarbamylase deficiency,41 primary biliary cirrhosis,42,43 and cancer.44 Dosing in clinical trials, ranging up to 600 mg ⋅ kg⁻¹ ⋅ d⁻¹ in children,41 is consistent with the ≈500-mg ⋅ kg⁻¹ ⋅ d⁻¹ dose in our studies. At these doses, PBA is well tolerated with minimal toxicities.37–40,42 In our in vitro studies, our PBA dose (2 mmol/L) is also comparable to the low-millimolar PBA plasma concentrations reported in treated humans.37,44 In addition to these clinical research trials, both PBA and TUDCA are approved by the Food and Drug Administration for urea cycle and cholestatic liver disorders, respectively.19 The extensive clinical investigations with these agents make a rapid translation to PAH patients possible.

BMPRII receptor mutations occur in 75% of sporadic and 20% of familial PAH cases,1 and restoration of BMPRII signaling remains an intense area of PAH research. These loss-of-function mutations often result in protein misfolding, aggregation, abnormal trafficking, and ER stress. PBA, at a dose similar to the dose used here, restores BMPRII trafficking in HeLa cells transfected with a human disease–causing BMPRII mutant (mutated at a cysteine residue, thus affecting sulfide bonding/protein folding, causing ER accumulation).9 PBA enhanced plasma membrane transport and Smad signaling in the mutant-expressing cells. The ability of PBA to restore BMPRII signaling resulting from mutation-dependent trafficking deficits further supports the use of these agents as a PAH therapy.

Our study has limitations. Although it is believed that the structural features of PBA are conducive to chaperone
activity. PBA also has histone deacetylase (HDAC) inhibitor properties. Indeed, HDAC inhibitors have demonstrated antiproliferative and proapoptotic characteristics in cancer and thus may contribute to the beneficial effects reported herein. However, PBA 20 mmol/L, a 10-fold-higher dose than in our in vitro studies, inhibits HDAC activity by only ≈25%, suggesting that PBA has relatively weak HDAC inhibitor activity. Moreover, a second chemical chaperone, TUDCA, which, to the best of our knowledge lacks HDAC inhibitor activity, had effects similar to PBA (Figures 5 and 6), further suggesting that the beneficial effects are HDAC inhibitor independent.

In this study, we focus on the PASMCs and do not address potential direct effects of these agents on PA endothelial cells, which contribute to plexiform lesions and vascular remodeling in PAH patients. Nevertheless, in our in vivo models, the proliferation and apoptosis indicators were not localized in PA endothelial cells, suggesting that (at least at the late stage of disease in which we studied these lungs) the effects of PA endothelial cells do not contribute significantly to the beneficial effects of these drugs. Recent work has suggested that human PAH–PA endothelial cells are proliferative and share a very similar mitochondrial suppression with PAH-PASMCs; thus we speculate that a similar mechanism could be involved, but more studies are needed. In addition, our studies did not directly address the potential effects of these drugs on the right ventricle. However, PBA-treated animals had improved cardiac output and performance on the treadmill test, consistent with improved cardiac function.

Although ER stress has not been definitively linked with all known triggers of PAH, this work, along with our recently published work, strengthens the evolving metabolic theory of PAH and links 2 fundamental cellular processes, ER stress and mitochondrial biology, potentially opening new avenues for therapies. The increase in PDH activity with PBA is also compatible with the beneficial proapoptotic effects of dichloroacetate, a PDH activator that prevents and reverses several PAH models. Like dichloroacetate, PBA promotes mitochondria-dependent apoptosis (marked by ΔΨm depolarization and an increase in mROS) without activating the ER stress–dependent apoptotic pathway, as shown by the fact that PBA does not increase CHOP levels (Figures VI and IX in the online-only Data Supplement). It is intriguing that these chemical chaperones are also considered for the treatment of diabetes mellitus, while evidence for a generalized insulin-resistant–like abnormality in PAH is accumulating. Finally, it is satisfying to eventually attempt to translate the important observations made by Smith and Heath in their classic description of PAH pathology over 30 years ago.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Pulmonary arterial hypertension (PAH) carries poor prognosis despite aggressive medical interventions. The reason for the failure of current therapies is 2-fold. First, most approved therapies (endothelin antagonists, phosphodiesterase type V inhibitors, and prostacyclin analogs) are vasodilators that were originally developed for systemic vascular diseases, not PAH, and are limited by systemic side effects. Second, the elevated pulmonary pressures in PAH are more a result of vascular remodeling than of vasoconstriction, particularly at the time of clinical presentation. Thus, agents that reverse this vascular remodeling and are specific to the pulmonary circulation are desperately needed. In addition, an efficacious therapy must address the many diverse triggers of PAH, both acquired and genetic, and because multiple triggers often occur within the same patient, identification of a “bottleneck” target is important. Many PAH-associated conditions are linked to endoplasmic reticulum stress, and although early pathology reports described dysmorphic endoplasmic reticulum in PAH plexiform lesions, the involvement of endoplasmic reticulum stress in PAH was only recently proposed. Here, we show that the orally administered generic chemical chaperone 4-phenylbutyrate (PBA) reduces endoplasmic reticulum stress, preventing/reversing pulmonary vascular remodeling and lowering pulmonary pressures in 2 animal models of PAH without apparent effects on the systemic vasculature. PBA and tauroursodeoxycholic acid (a chemical chaperone with a function similar to PBA) also normalized the cellular “PAH phenotype” in pulmonary artery smooth muscle cells in vitro. Our work supports targeting endoplasmic reticulum stress as an effective and selective strategy for the treatment of PAH. Because both PBA and tauroursodeoxycholic acid are used clinically, mostly under research protocols, translation to human PAH is possible.
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SUPPLEMENTAL MATERIAL

Cell Culture
PASMCs from five C57BL/6 mice were freshly isolated from third generation pulmonary arteries, with an enzymatic cocktail containing papain (1mg/ml), dithiothreitol (0.5mg/ml), collagenase (0.6 mg/ml), and bovine serum albumin (0.6 mg/ml) (Sigma-Aldrich, St. Louis, MO) and pooled as previously described 1,2. PASMCs were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Gibco, Invitrogen, Burlington, ON) and placed in a humidified incubator set at 37°C in either normoxic conditions (21% O₂; pO₂~120mmHg) or hypoxic conditions (4% O₂; pO₂~40mmHg) with 4-phenylbutyric acid (2mM; Sigma-Aldrich, St. Louis, MO), tauroursodeoxycholic acid (1mM; Calbiochem, San Diego, CA) and thapsigargin (400nM; Sigma-Aldrich, St. Louis, MO) for 48 hours. For apoptosis/proliferation experiments, cells were exposed to normoxia/hypoxia for 48 hours prior to drug treatment. Cells of passage ≤6 were used.

Confocal Microscopy
Immunofluorescence imaging was performed on a Ziess LSM 510 confocal microscope (Carl Zeiss, Toronto, ON) as previously described 1-3. ApopTag apoptosis detection kit (Serotologicals, Norcross, GA) and Ki67 antibody (1:100, Abcam, Cambridge, MA) were used as previously described 1. ATF6 and GRP78 (1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), CHOP (1:100, Cell Signaling, Danvers, MA), tetramethylrhodamine methyl ester (TMRM 10nM; Invitrogen, Burlington, ON), MitoSOX (5µM; Invitrogen, Burlington, ON), and Rhodamine 2AM (5µM; Invitrogen, Burlington, ON) were used as previously described 1-3. Fluorescein isothiocyanate (FITC 1:1000; Invitrogen, Burlington, ON), tetramethylrhodamine isothiocyanate (TRITC 1:50 Dako, CA), Far Red (1:1000; Invitrogen, Burlington, ON) and Zenon kit (Invitrogen, Burlington, ON) secondary antibodies were used as previously described 1-3.

PDH Activity
PDH activity was measured with a commercially available MitoProfile Dipstick assay kit (Mitosciences, Eugene, OR) as previously described. Briefly, 50µL of protein (1mg/ml) was incubated with a dipstick containing the PDH complex antibody in a 96 well plate, followed by activity buffer. A flat top scanner was used to measure the band intensity.

**Alpha-ketoglutarate assay**

αKG levels were measured with a commercially available spectrophotometric αKG assay kit (BioVision, Milpitas, CA), as previously described. PASMCs were grown, harvested, lysed, and a protein concentration was obtained to normalize between wells. Optical density at 570nm was measured after the kit-based reaction occurred.

**Immunoblot**

Tissues were collected and immunoblotting was performed as previously described. The films were digitized and quantified with 1D Image Analysis Software (Kodak, Rochester, NY). Expression was normalized to α-actin to correct for loading differences. Antibodies: ATF6 (1:1000; Imgenex, San Diego, CA), GRP78 (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), Nogo (1:10000; previously described and characterized), CHOP (1:1000, Cell Signaling, Danvers, MA) and α-actin (1:400; Abcam, Cambridge, MA).

**ATF6 luciferase**

PASMCs (20,000 cells/well) were seeded into a 96 well plate and allowed to adhere. Cells were transfected with the ATF6 dual luciferase plasmid (SABiosciences, Mississauga, ON) using a Xfect transfection reagent (Clontech, Mountain View, CA) under normoxic conditions. After 24 hours, media was replaced with treatment media and cells were placed in normoxia or hypoxia. ATF6 activity was assessed with the dual-luciferase reporter assay system (Promega, Madison, WI). After 48 hours, cells were lysed by a freeze-thaw cycle in passive lysis buffer (provided in kit) and luminescence was measured with an illuminometer. ATF6 activity was assessed by ATF6-driven firefly luminescence normalized to a constitutively-driven Renilla luminescence to standardize the transfection as previously described.
FRET analysis

PASMCs were plated on glass dishes and transfected with the 4mtD3CPV cameleon plasmid using a Xfect transfection reagent (Clontech, Mountain View, CA) under normoxic conditions. After 24 hours, media was replaced with treatment media and placed in normoxia or hypoxia. After 48 hours, media was diluted with 4% paraformaldehyde in a 1:1 ratio and cells were fixed under treatment conditions for 60 minutes. The mitochondrial calcium uniporter (MCU) inhibitor ruthenium red (10µM, Calbiochem, San Diego, CA) was incubated in a separate dish for 3 hours prior to fixation to ensure sensitivity. Cells were washed with distilled water and mounted on a slide using Prolong Gold (Invitrogen, Burlington, ON) and imaged 48 hours later on a Zeiss LSM 510 confocal microscope. Excitation occurred at 458 nm and the emission filters were 480 to 520 nm for cyan (when Ca\(^{2+}\) is not bound) and 535 to 590 nm for FRET (yellow when Ca\(^{2+}\) is bound). The ratio of yellow/cyan intensities was used to standardize the rate of infection for each cell, as previously described\(^2\).
Supplement Figure 1. A. Chronic hypoxic mice treated with PBA have lower pulmonary artery acceleration time (PAAT) compared to vehicle treated hypoxic mice (n=9-15 mice/group; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxic vehicle using one-way ANOVA with Fisher’s least difference post-hoc. Red lines represent mean values). B. Systolic or diastolic systemic blood pressure was not significantly different between any of the groups (p = 0.12 and 0.51 for systolic and diastolic blood pressures, respectively, using one-way ANOVA).
**Supplement Figure 2.** Monocrotaline rats treated with PBA (300-500mg/kg/day) had lower RV/LV+Septum (n=5-12 rats/group; *p≤0.05, vs. sham vehicle, +p≤0.05 vs. monocrotaline vehicle using Kruskal-Wallace with Mann-Whitney U test to compare inter-group differences. Red lines represent the median value). Each point represents one animal.
Supplement Figure 3. PBA in prevention and reversal protocols reduces muscularization of capillary vessels (<50 μm diameter) in both the chronic hypoxic (left) and monocrotaline (right) models of PAH (n~20 vessels/animal, 5 animals *p≤0.05, vs. healthy + vehicle, +p≤0.05 vs. PHT + vehicle).
Supplement Figure 4. Proliferating markers (proliferating cell nuclear antigen; PCNA; red) colocalize with SMA-positive (purple, left), but not vWF-positive (purple, right), cells in PHT distal PAs. PBA does not induce apoptosis (TUNEL, green, right) in vWF-positive cells in either the prevention or reversal protocols.
Supplement Figure 5. PBA in prevention and reversal protocols reduces expression of GRP78 in the resistance SMA+ PASMCs (50-100 μm diameter vessels) as assessed by immunofluorescence and confocal microscopy on whole lung tissue. Top: low magnification showing SMA (green), GRP78 (red) and nuclei (blue); bottom: high magnification of boxed region showing GRP78 (red) and nuclei (blue) (n~10 vessels/mouse 4-5 animals/group; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxic vehicle). PBA did not effect expression resistance SMA+ PASMCs from normoxic mice.
Supplement Figure 6. CHOP expression is decreased in the PAs of CH-PHT animals. Treatment with PBA does not significantly effect CHOP expression. (*p<0.05 vs. Vehicle using Kruskall-Wallace test with Mann-Whitney U test).
**Supplement Figure 7.** PBA in prevention and reversal protocols reduces hypoxia-induced ATF6 cleavage (top) and expression of downstream target GRP78 (bottom) as assessed by immunoblot on whole lung tissue. Actin was used as a loading control (each lane represents one animal; p<0.05 using Kruskall-Wallace test with Mann-Whitney U test to compare each treatment to vehicle).
**Supplement Figure 8.** A. Immunoblot showing increased GRP78 expression in MCT-PHT compared to healthy controls (left) and reduced expression of GRP78 in whole lung tissue in monocrotaline rats treated with PBA in prevention and reversal protocols (right). B. Immunoblot showing increased Nogo expression in MCT-PHT compared to healthy controls (left) and reduced expression Nogo in whole lung tissue in monocrotaline rats treated with PBA in prevention and reversal protocols. Actin was used as a loading control (each lane represents one animal; p<0.05 using Kruskall-Wallace test with Mann-Whitney U test to compare healthy to MCT-PHT and each treatment to vehicle).
**Supplement Figure 9.** Hypoxia activates GRP78, but not nuclear (activated) CHOP, in PASMCs assessed by immunofluorescence and confocal microscopy. PBA and TUDCA reduce GRP78 in hypoxic PASMCs without affecting CHOP activation (n=50 cells/group; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia vehicle using one-way ANOVA with Tukey’s post hoc analysis).
Supplemental Figure 10. PBA (2mM) and TUDCA (1mM) maintain mitochondrial calcium in hypoxia assessed by Rhodamine 2AM and confocal microscopy (n~50 cells/group/experiment, n=4 experiments; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia vehicle using one-way ANOVA with Tukey’s post hoc analysis).


