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

Running title: Dromparis et al.; ER Stress as a Therapeutic Target in Pulmonary Hypertension

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Abstract:

Background—Evidence suggestive of endoplasmic reticulum (ER) stress in the pulmonary arteries (PA) of patients with pulmonary arterial hypertension (PAH) has been described for decades but has never been therapeutically targeted. ER stress is a feature of many conditions associated with PAH like hypoxia, inflammation or loss-of-function mutations. ER stress signaling in the pulmonary circulation involves the activation of ATF-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 PAH that promotes proliferation and apoptosis resistance in the PA wall. We hypothesized that chemical chaperones known to suppress ER stress signaling, like 4-phenylbutyrate (PBA) or tauroursodeoxycholic acid (TUDCA), will inhibit the disruption of the ER-mitochondrial unit and prevent/reverse PAH.

Methods and Results—PBA in the drinking water both prevented and reversed chronic hypoxia induced pulmonary hypertension in mice, decreasing pulmonary vascular resistance, PA remodeling, right ventricular hypertrophy and improving functional capacity without affecting systemic hemodynamics. These results were replicated in the monocrotaline rat model. PBA and TUDCA improved ER stress indices in vivo and in vitro, decreased ATF6 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 PA 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.

Key words: metabolism; mitochondria; pulmonary hypertension; vascular disease; endoplasmic reticulum stress
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 pro-proliferative and anti-apoptotic diathesis. Pulmonary arterial remodeling occludes the vessel lumen which leads to Right Ventricular (RV) failure and premature death, with the median survival of untreated patients limited to 3 years\(^1\). However, even in those receiving standard therapies, prognosis remains poor\(^2\).

Although the pathology is restricted to the pulmonary vasculature, sparing the systemic vessels, all approved PAH therapies were originally developed as systemic vasodilators\(^3\). 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\(^4\).

An additional challenge is that, despite the recent groupings of several conditions that share similar lung histology to idiopathic PAH (iPAH) under a ‘PAH umbrella’, the pathogenesis of PAH is multifactorial, suggesting that therapies that target one molecular abnormality in one 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 HSV, hypoxia, or loss-of-function mutations in the bone morphogenetic protein receptor 2 (BMPR2)\(^5\)-\(^6\). 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 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. More recently, the loss-of-function mutations in BMPR2 and the resultant protein trafficking dysfunction have been shown to induce ER stress as well. Despite the emerging evidence for protein trafficking dysregulation and the fact that ER abnormalities compatible with ER stress (for example dysmorphic and swollen ER) have been clearly described in the classic description of PAH pathology by Dr. Heath 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. 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. The mitochondrial suppression in PAH, much like in cancer, leads to a switch towards a glycolytic phenotype that promotes proliferation and suppresses apoptosis. Perhaps because of previously described mitochondrial differences between the pulmonary and systemic circulations, 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 the metabolic and vascular remodeling and hypoxia-induced PAH. 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, 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\textsuperscript{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\textsuperscript{19-21}. They have demonstrated anti-proliferative and pro-apoptotic effects in cancer\textsuperscript{22-24}, which shares a similar metabolic and mitochondrial remodeling with PAH-PASMCs\textsuperscript{7,15}. Moreover, these therapies are orally administered and are currently used clinically\textsuperscript{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) as well as 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 and Sprague Dawley rats were purchased from Charles River. The mice were randomized to normobaric hypoxia (10\% O\textsubscript{2}) or room air, and rats were randomized to injection of saline or monocrotaline (60mg/kg) as previously described\textsuperscript{15,25}. Animals were further randomized to receive PBA in either a prevention (PBA starting the day of pulmonary hypertension (PHT) induction, onward for 4 weeks) or a reversal (on the third week of PHT induction, onward 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
~500mg/kg/day.

**Echocardiography and Hemodynamics**

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

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

Pulmonary Artery Acceleration Time (PAAT) was measured by echocardiography as previously
described\textsuperscript{12, 15, 25}. Total pulmonary resistance was calculated by the mPAP/CO ratio. Mean
pulmonary artery pressures were assessed in closed-chest animals with Millar catheters (Millar
Instruments Inc., Huston, TX) as previously described\textsuperscript{12, 15, 25}. Under inhaled isoflurane
anesthesia, the right jugular vein was cannulated and the catheter advanced into the pulmonary
arteries. Pressures from the right atrium, right ventricle and pulmonary arteries were recorded
continuously and mean PAP was calculated electronically (Power Lab, 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 a non-demanding protocol to allow
familiarization of the test. Afterwards animals from each therapy group were run until failure
with the following protocol: 3 min at 10m/min, 3 min at 12m/min, 20 min at 14m/min, 20 min at
16m/min, and 18m/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 using 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, 5µm thick lung sections were stained using hematoxylin and eosin (H&E) stain. Vessels >50µm were identified and measured at the two ends of the shortest external diameter of the distal PAs, and the average was taken ([2 x wall thickness/external diameter] x 100).

**Muscularization**

Lung sections (5µm) were stained for smooth muscle actin and von Willebrand Factor (endothelial cell maker). Vessels (<50µm) were classified as fully (100%), partially, or non-muscularized (0%), based on the percentage of vWF surrounded by SMA in each vessel.

**Cell culture and in vitro experiments**

PASMC isolation, culture, confocal microscopy, PDH activity, αKG levels, immunoblot, dual luciferase reporter assays, and FRET imaging were performed as previously described\(^{12, 15, 25, 26}\). See supplement for detailed methods.

**Statistics**

All values are expressed as mean±SEM unless otherwise stated. For in vitro analysis, differences between groups were assessed by either student’s t-test or one-way analysis of variance (ANOVA) using Tukey’s post hoc analysis as a appropriate. Normality of our in vivo data was
assessed by the Shapiro-Wilk normality test. Inter-group differences for in vivo experiments were assessed by either ANOVA using Fisher’s least significant differences post hoc analysis or Kruskal-Wallis with a Mann-Whitney U test as appropriate. Vessels from the same animal were assumed to be independent for purposes of analysis. All analyses were performed using SPSS 19 (IBM Corp., Armonk, NY). Significance was defined as p<0.05.

Results

PBA prevents and reverses pulmonary hypertension in mice and rats

To determine if 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 onward for 4 weeks) or reversal protocol (PBA starting on the third week after onset of CH and for 2 weeks onward). Mice treated with PBA in either protocol had lower mean pulmonary artery pressure (mPAP; 35.8±1.3 mmHg vs. 21.8±0.9 and 19.0±0.7 mmHg in PBA reversal and prevention treatments, respectively) and total pulmonary resistance (TPR; 2.56±0.16 vs. 1.13±0.92 and 0.92±0.04 mmHg.min.ml⁻¹ in PBA reversal and prevention protocols, respectively) compared to untreated CH-PHT controls (Figure 1A). Pulmonary artery acceleration time (PAAT) measured echocardiographically, which is inversely related to mPAP, was also improved with PBA treatment (Supplement Figure 1A). The decreased afterload resulted in lower RV hypertrophy (RVH) 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 pressure was not affected by hypoxia or PBA treatment (Supplement Figure 1B). In a second, more severe and inflammatory model of PHT 27, rats injected with monocrotaline (MCT-PHT) and treated in similar prevention (PBA starting the day...
of MCT injection, for 4 weeks onward) and reversal protocols (PBA starting on the third week after MCT injection, for 2 weeks onward). Similar to the CH-PHT mice, the PBA treated MCT-PHT rats had decreased mPAP (50.2±5.1 mmHg vs. 33.7±4.3 and 31.3±4.7 mmHg in PBA reversal and prevention protocols, respectively), TPR (0.71±0.08 vs. 0.45±0.07 and 0.33±0.08 mmHg.min.ml⁻¹ in PBA reversal and prevention protocols, respectively) (Figure 1B) and RVH (Supplement Figure 2).

**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 (50-100μm diameter) and reduced muscularization (in <50μm diameter PAs) in both the reversal and prevention protocols (Figure 2A, Supplement Figure 3). Resistance vessels in vehicle-treated PHT animals from both models had increased expression of the proliferation marker Ki67 in smooth muscle actin-positive cells (SMA), which was reduced by PBA in both treatment protocols (Figure 2B). Furthermore, PBA induced apoptosis in resistance pulmonary artery SMA-positive cells, by ~3.6 and 4.5 fold in the CH-PHT and MCT-PHT animals, respectively (Figure 2B). Proliferation and apoptosis indices were not affected in von Willebrand Factor-positive cells (PA endothelial cell marker) (Supplement Figure 4). No changes in any histology-assessed parameters were observed in the lungs of PBA-treated healthy animals (Figure 2, Supplement Figure 3).

**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 nucleus²⁸ where it functions as a transcription factor. To determine if 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 to 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)\(^{29}\) (Supplement Figure 5). To more appropriately quantify the reduction of ATF6 cleavage, we performed immunoblots on isolated PAs (>4\(^{th}\) division). In keeping with the immunofluorescence, CH-PHT PAs had increased levels of cleaved (i.e. active form) ATF6 (~60kDa) 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 pro-apoptotic ER stress pathway was not upregulated by hypoxia or PBA treatment, as assessed by CHOP expression (Supplement Figure 6). Similar effects on the ATF6 axis were observed in whole lung samples in both CH-(Supplement Figure 7) and MCT-PHT (Supplement Figure 8).

**Chemical chaperones attenuate hypoxia-induced ATF6 activation in vitro**

We then exposed cultured murine PASMCs to 48 hours of physiologic mild hypoxia (normoxia: \(pO_2=122.1\pm0.6\text{mmHg, pH}=7.38\pm0.02; \) hypoxia: \(pO_2=41.9\pm0.7\text{mmHg, pH}=7.39\pm0.01\)), to mimic our in vivo CH-PHT conditions. Similar to our in vivo findings, hypoxia caused an increase in nuclear translocation of ATF6, suggesting 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 increased. Both PBA, and a structurally distinct chemical chaperone TUDCA, blocked hypoxia-induced increases in ATF6 nuclear localization (Figure 4A) and GRP78 expression (Supplement Figure 9). Similar to the PAs in vivo, hypoxia did not activate the pro-apoptotic CHOP pathway. In contrast, thapsigargin, an inducer of severe and non-physiological ER stress\(^{28}\), substantially upregulated both GRP78 and

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CHOP in PASMCs (Supplement Figure 9).

To better assess ATF6 transcriptional activity, we performed a dual luciferase ATF6 reporter assay. In keeping with our immunofluorescence data, hypoxia caused a ~113% increase in ATF6 luciferase signal compared to normoxia, but not to the same extent as thapsigargin (~216% increase) (Figure 4B). Hypoxia-induced ATF6 activation was blocked by PBA, which had no effects under normoxic conditions (Figure 4B). In addition, PBA reduced mRNA levels of GRP78 and Nogo by ~50%, further supporting effective ATF6 inhibition in hypoxic PASMCs (Figure 4C).

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 unit12. Spatial disruption of this unit dissociates mitochondria from ER-associated calcium microdomains, reducing intra-mitochondrial calcium30. Hypoxia predictably reduced levels of mitochondrial calcium as determined by Forster resonance energy transfer (FRET) 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 FRET signal (YFP/CFP 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 mitochondrial-specific, calcium-sensitive dye (Supplement Figure 10).

In keeping with increased mitochondrial calcium, PBA and TUDCA inhibited the hypoxia-induced decrease in the calcium-sensitive31 mitochondrial enzyme pyruvate dehydrogenase (PDH) (Figure 5B). PBA and TUDCA also increased the activity of another
calcium-dependent mitochondrial enzyme, isocitrate dehydrogenase (IDH)\(^31\) as assessed by its product, alpha-ketoglutarate (0.60±0.1\(\mu\)g/well vs. 0.80±0.04 and 0.75±0.04 \(\mu\)g/well for PBA and TUDCA treated hypoxic PASMCs respectively, p<0.05). Both PDH and IDH produce NADH that 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 (\textbf{Figure 6A}). Finally, since calcium is positively charged, it also influences the mitochondrial membrane potential (\(\Delta \Psi_m\)). Consistent with decreased mitochondrial calcium and as previously described\(^{12,13,25}\), hypoxic PASMCs had increased \(\Delta \Psi_m\), which was blocked by both PBA and TUDCA (\textbf{Figure 6A}). Taken together, these results suggest that PBA and TUDCA prevent ER-stress induced mitochondrial suppression, a cellular hallmark of PAH\(^{12,13,25}\).

\textbf{Chemical chaperones induce apoptosis and normalize proliferation in hypoxic PASMCs}

Mitochondrial driven apoptosis is largely mediated by the release of pro-apoptotic factors through the mitochondrial transition pore (MTP)\(^32\). Since both mROS and \(\Delta \Psi_m\) depolarization facilitate the opening of the voltage- and redox-sensitive MTP, we explored the effects of chemical chaperones on PASMC apoptosis under hypoxic conditions. PBA and TUDCA increased apoptosis measured by the percentage of TUNEL-positive PASMCs by ~3.93 and ~3.05 fold, respectively (\textbf{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 (\textbf{Figure 6B}).

\textbf{Discussion}

We show that the small molecule chemical chaperone PBA prevents and reverses PAH in two
standard rodent models, CH-PHT in mice and MCT-PHT in rats. In vitro, PBA and TUDCA inhibit the ATF6 ER-stress pathway in PASMCs exposed to physiologic 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 (UPR)$^8$. There are three arms of the UPR: inositol requiring enzyme-I (IRE1), protein kinase ER-like kinase (PERK) and ATF6$^{33}$. While the PERK pathway is generally pro-apoptotic, activating transcription factors like CHOP, the ATF6 response is typically associated with anti-apoptotic and pro-survival signaling$^{34-36}$. The pro-survival 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 manner to achieve this would be to inactivate mitochondria, major inducers of apoptosis$^{12}$. Since mitochondria are dependent on ER for calcium supply, disruption of the strategically arranged “mitochondria-ER unit” by reshaping the ER upon Nogo induction can achieve this goal. Indeed, PASMCs from Nogo$^{+/}$ mice fail to show ER-mitochondrial disruption, any tested indices of mitochondrial suppression, or a proliferative/anti-apoptotic phenotype upon hypoxic exposure and Nogo$^{+/}$ and +/- 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 from 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 pro-apoptosis versus pro-survival ER stress pathway is complex, incompletely understood and may depend on the degree and duration of ER-stress as well as be cell-type specific. In keeping with this, exposure of PASMCs to a severe and non-physiological ER-stress stimulus, thapsigargin, caused greater ATF6 activation (Figure 4B) and GRP78 upregulation (Supplement Figure 8), but also activated of the pro-apoptotic ER-stress response (Supplement Figure 8), in contrast to hypoxia. Under the mild stress of physiologic hypoxia (i.e. pO2~40mmHg), the ATF6 activation may be favored in the pulmonary circulation, which is generally under more oxidized conditions (due to exposure to much higher oxygen levels and baseline mROS levels, compared to systemic vessels). This is because, compared to 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). 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: a) it inhibits a pathway potentially common to several, but perhaps not all, PAH pathologies; b) it is effective in decreasing proliferation as well as inducing apoptosis; and c) it is relatively specific to the pulmonary circulation. Obviously, such a strategy may logically have short-term adverse effects since 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

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stressor, they might be less able to defend to the cellular stress acutely. On the other hand, Nogo-
/- mice have a normal development and phenotype. 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. In humans, they have been investigated in patients with cystic
fibrosis, β-thalassemia, spinal muscular atrophy, ornithine transcarbamylase deficiency,
primary biliary cirrhosis, and cancer. Dosing in clinical trials, ranging up to 600mg/kg/day
in children, is consistent with the ~500 mg/kg/day dose in our studies. At these doses, PBA is
well tolerated with minimal toxicities. In our in vitro studies, our PBA dose (2mM) is also
comparable to the low millimolar PBA plasma concentrations reported in treated humans.
In addition to these clinical research trials, PBA and TUDCA are both FDA-approved for urea
cycle and cholestatic liver disorders, respectively. 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 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 one 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). 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. While it is believed that the structural features of PBA are
conducive to chaperone activity, PBA also has histone deacetylase inhibitor (HDACi) properties\textsuperscript{19}. Indeed, HDACi’s have demonstrated anti-proliferative and pro-apoptotic characteristics in cancer, and thus may contribute to the beneficial effects reported herein. However, PBA (20mM), a 10-fold higher dose than our in vitro studies, inhibits HDAC activity by only \textasciitilde25\%\textsuperscript{45}, suggesting that PBA has relatively weak HDACi activity. Moreover, a second chemical chaperone, TUDCA, which to the best of our knowledge lacks HDACi activity, had effects similar to PBA (Figures 5 and 6), further suggesting the beneficial effects are HDACi-independent.

In this study, we focus on the PASMC and do not address potential direct effects of these agents on PA endothelial cells (PAECs), which contribute to plexiform lesions and the vascular remodeling in PAH patients. Nevertheless in our in vivo models, the proliferation and apoptosis indicators were not localized in PAECs suggesting that (at least at the late stage of disease in which we studied these lungs) effects of PAECs do not contribute significantly to the beneficial effects of these drugs. Recent work has suggested that human PAH-PAECs are proliferative and share a very similar mitochondrial suppression with PAH-PASMCs\textsuperscript{16}; thus we speculate that a similar mechanisms could be involved but more studies are needed. In addition, our studies did not directly address 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 recent published work\textsuperscript{12}, strengthens the evolving metabolic theory of PAH\textsuperscript{13,15} and links two 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 pro-apoptotic effects of dichloroacetate, a PDH activator that prevents and reverses several PAH models\textsuperscript{13-15,46}. Like dichloroacetate, PBA promotes mitochondria-dependent apoptosis (marked by ΔΨ\textsubscript{m} depolarization and increase in mROS) without activating the ER stress dependent apoptotic pathway, as shown by the fact that PBA does not increase CHOP levels (Supplement Figures 6 and 9). It is intriguing that these chemical chaperones are also considered for the treatment of diabetes\textsuperscript{47}, while evidence for a generalized insulin-resistant-like abnormality in PAH is accumulating\textsuperscript{48}. Lastly, it is satisfying to eventually attempt to translate important observations made by Dr. Heath in his classic description of PAH pathology more than 30 years ago\textsuperscript{11}.

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**Conflict of Interest Disclosures:** None.

**References:**


**Figure Legends:**

**Figure 1.** PBA prevents and reverses chronic hypoxia- and monocrotaline-induced PHT.  
A. Chronic hypoxic mice treated with PBA had lower mPAP (top left), total pulmonary resistance (bottom left), decreased RV/LV+septum (top right), and improved treadmill running distance (bottom right) compared to vehicle-treated mice (n=9-15 mice/group; *p*<0.05 vs. normoxic vehicle, +*p*<0.05 vs. hypoxic vehicle. Red lines represent mean values). PBA had no effects on normoxic mice.  
B. Monocrotaline rats treated with PBA had lower mPAP (top) and total...
pulmonary resistance (bottom) compared to vehicle-treated rats (n=5-12 rats/group; *p<0.05, vs. sham vehicle, +p<0.05 vs. monocrotaline vehicle. In contrast to the CH-PHT, normality was not confirmed, and therefore, the MCT-PHT data are shown with box plots, with red lines representing median values. Each point represents one animal.

Figure 2. PBA prevents and reverses pulmonary vascular remodeling, attenuates proliferation and induces apoptosis in PAs in animal PHT models. A. PBA in prevention and reversal protocols reduces the percent medial wall thickness in the resistance PA’s (50-100μm diameter) in both the chronic hypoxic (left) and monocrotaline (right) models of PHT. Images show H&E stained resistance PA’s from vehicle and PBA treated animals. (n~20 vessels/animal, 5 animals/group; p<0.05). B. PBA in prevention and reversal protocols reduces cell proliferation (Ki67+ nuclei) in the resistance SMA+ PASMCs (50-100μm diameter vessels). PBA in a reversal protocol induces apoptosis (TUNEL+ nuclei) in the resistance SMA+ PASMCs. Confocal images show resistance PAs from a vehicle and PBA treated PHT-animal stained with both Ki67 (green, top) and TUNEL (green, bottom) positive nuclei (blue) (left) and merged with SMA (red) (right). (n~10 vessels/mouse and n~20 vessels/rat, 5 animals/group; *p<0.05).

Figure 3. PBA reduces ER stress in vivo. A. PBA in prevention and reversal protocols reduces hypoxia-induced ATF6 cleavage in the resistance PA SMA+ cells (50-100μm 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/mouse 3-4 mice/group; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxic vehicle). PBA did not affect
ATF6 cleavage in the resistance PA SMA+ cells in normoxic mice. B. PBA in prevention and reversal protocols reduces ATF6 cleavage and GRP78 and Nogo protein expression in isolated PAs of 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).

Figure 4. PBA reduces ATF6 activation in PASMCs in vitro. A. PBA (2mM) and TUDCA (1mM) block hypoxia-induced ATF6 cleavage assessed by nuclear ATF6 localization (red colocalizing with blue nuclei) without affecting normoxic cells, as assessed by immunofluorescence and confocal microscopy. Cytoplasmic expression of GRP78 (green) was also decreased by PBA or TUDCA in hypoxia, but not normoxia (n=50 cells/experiment, 3 experiments; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia vehicle). B. PBA (2mM) blocks hypoxia-induced ATF6 transcriptional activity assessed by an ATF6-driven dual reporter luciferase assay. Hypoxia induced ATF6 activation to a lesser extent than thapsigargin. PBA has no effect on normoxic PASMCs (n=18 wells/group; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia). C. PBA (2mM) reduces expression of the ATF6 target genes GRP78 (left) and Nogo (right) in hypoxic PASMCs. (n=5, +p<0.05 vs. hypoxia vehicle).

Figure 5. PBA prevents the hypoxia-induced decrease in PASMC mitochondrial calcium. A. PBA (2mM) and TUDCA (1mM) maintain mitochondrial calcium in hypoxia assessed by the fluorescence ratio of bound mitochondrial Ca^{2+} [yellow fluorescent protein (YFP) signal, yellow] to unbound mitochondrial Ca^{2+} [cyan fluorescent protein (CFP) signal, cyan] measured with fluorescence resonance energy transfer (FRET) imaging and confocal microscopy (n=30 cells/group/experiment, n=3 experiments; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia.
vehicle). **B.** PBA (2mM) and TUDCA (1mM) maintain activity of the mitochondrial, calcium-sensitive enzyme PDH in hypoxia (n=6/group; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia vehicle).

**Figure 6.** PBA prevents the hypoxia-induced decrease in mitochondrial function, induces apoptosis and reduces proliferation in PASMCs. **A.** PBA (2mM) and TUDCA (1mM) maintain mitochondrial-ROS (top) and ΔΨm (bottom) in hypoxic PASMCs as assessed by MitoSOX (red, top) and TMRM (red, bottom), respectively. Nuclei are shown in blue (n~50 cells/group/experiment, n=3 experiments; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia vehicle). **B.** PBA (2mM) and TUDCA (1mM) increase apoptosis as assessed by percent TUNEL-positive nuclei (top) and reduce proliferation as assessed by percent Ki67-positive nuclei (bottom) in hypoxic PASMCs (n~50 fields/group from 3 experiments; *p<0.05 vs. normoxia vehicle, +p<0.05 vs. hypoxia vehicle).

**Figure 7.** Chemical chaperones like PBA and TUDCA reverse pulmonary vascular remodeling by attenuating the ER stress response in PASMCs. PBA and TUDCA block the ER stress response induced by a variety of PAH-associated stimuli including hypoxia, inflammation, viruses and mutations. This results in maintenance of mitochondrial calcium, PDH activity, mitochondrial-derived ROS and ΔΨm, through a mechanism that likely involves inhibition of ATF6 and Nogo upregulation. This inhibits the cancer-like metabolic remodeling in PAH (i.e. suppression of mitochondrial function and a shift toward glycolysis) facilitating apoptosis and inhibiting proliferation. These combined effects contribute to the prevention and reversal of pulmonary vascular remodeling in PAH.
A) Normoxia and Hypoxia treatment with Vehicle, PBA, and TUDCA on ATF6, GRP78, and Merge + DAPI.

B) ATP6 Luciferase levels in Normoxia and Hypoxia with Vehicle, PBA, and Thapsigargin.

C) GRP78 and Nogo (2ΔACT) levels in Hypoxia with Vehicle and PBA.

Nuclear ATF6 (AFU) graph shows significant differences between Vehicle, PBA, and TUDCA in both Normoxia and Hypoxia conditions.
PBA
TUDCA

Hypoxia

ER Stress

Viruses

Inflammation

Mutations

\( \downarrow ATF6 \)

\( \downarrow Nogo \)

\( \uparrow \text{Mitochondrial } Ca^{++} \)

\( \uparrow \text{PDH Activity} \)

\( \downarrow \Delta \Psi m \)

\( \uparrow \text{mROS} \)

Reversal of the glycolytic phenotype

\( \downarrow \text{Proliferation/} \uparrow \text{Apoptosis} \)

\( \downarrow \text{Pulmonary Vascular Remodeling} \)
Attenuating Endoplasmic Reticulum Stress as a Novel Therapeutic Strategy in Pulmonary Hypertension

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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. 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. ApopTag apoptosis detection kit (Sero~tologicals, Norcross, GA) and Ki67 antibody (1:100, Abcam, Cambridge, MA) were used as previously described. 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. 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.

PDH Activity
PDH activity was measured with a commercially available MitoProfile Dipstick assay kit (Mitosciences, Eugene, OR) as previously described $^2$. 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 $^2, 4$. 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 $^1-3$. 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 $^5$), 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 $^2$. 
**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 (50-100μm 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).
Supplement 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).
Supplemental References


