



Effect of pulsatile stretch on unfolded protein response in a new model of the pulmonary hypertensive vascular wall

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ARTICLE INFO

Keywords:

Pulmonary artery

Pulsatile stretch

ER stress

Unfolded protein response

ABSTRACT

Persistent pulmonary hypertension of the newborn (PPHN) is characterized by hypoxemia and arterial remodeling. Dynamic stretch and recoil of the arterial wall during pulsation (in normal conduit arteries, stretch 20% above diastolic diameter) maintains homeostasis; a static arterial wall is associated with remodeling. PPHN is diagnosed by echocardiography as decreased pulmonary artery wall displacement during systole, causing decreased pulmonary arterial pressure acceleration time in a stiff artery.

We hypothesized that a 'normal' amplitude of pulsatile stretch is protective against ER stress, while the loss of stretch is a trigger for hypoxia-induced stress responses. Using a novel *in vitro* model of pulmonary arterial myocytes subject to repetitive stretch-relaxation cycles within a normoxic or hypoxic environment, we examined the relative impact of hypoxia (pulmonary circuit during unresolved PPHN) and cyclic mechanical stretch (diminished in PPHN) on myocyte homeostasis, specifically on signaling proteins for autophagy and endoplasmic reticulum (ER) stress.

Stretch induced autophagosome abundance under electron microscopy. Hypoxia, in presence or absence of pulsatile stretch, decreased unfolded protein response (UPR) hallmark BIP (GRP78) in contractile phenotype pulmonary arterial myocytes. Inositol requiring enzyme-1 α (IRE1 α) was not activated; but hypoxia induced eif2 α phosphorylation, increasing expression of ATF4 (activating transcription factor-4). This was sensitive to inhibition by autophagy inhibitor bafilomycin A1.

We conclude that in the pulmonary circuit, hypoxia induces one arm of the UPR pathway and causes ER stress. Pulsatile stretch ameliorates the hypoxic UPR response, and while increasing presence of autophagosomes, does not activate canonical autophagy signaling pathways. We propose that simultaneous application of hypoxia and graded levels of cyclic stretch can be used to distinguish myocyte signaling in the deformable pulmonary artery of early PPHN, versus the inflexible late stage PPHN artery.

1. Introduction

Persistent pulmonary hypertension of the newborn (PPHN; WHO PH classification group 1.7 [1]) accounts for 10% of admissions to neonatal ICUs [2,3], and is marked by hypoxemia, pulmonary vasoconstriction and rapid arterial remodeling, and death or bypass oxygenation in 10–20% of patients [4–8]. The initial clinical picture of PPHN is pulmonary arterial vasospasm in context of near-normal arterial histology. Progressive pulmonary arterial remodeling due to smooth muscle proliferation and extracellular matrix deposition impairs arterial distensibility [9], causing a loss of responsiveness to vasodilator therapy [10].

Pulmonary artery histology in late PPHN is characterized by thickened vascular media and adventitia, smooth muscle hyperplasia and extracellular matrix deposition, resulting in an irreversible increase in pulmonary vascular resistance [11,12].

The smooth muscle composition of the hypoxic pulmonary arterial wall is influenced by selective cell cycle re-entry and initiation of apoptotic signaling. We have reported that the effect of hypoxia on pulmonary apoptosis is phenotype-dependent [13]; hypoxia and nitric oxide cumulatively activate apoptosis of contractile pulmonary myocytes, causing a contraction of this subpopulation, while in synthetic myocytes, survival and proliferative signaling is enhanced by hypoxia

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which promotes vascular fibrosis.

Pulmonary hypertension can be diagnosed echocardiographically in hypoxemic neonates by a shortened pulmonary arterial acceleration time, reflecting the rapid rise in systolic pressure in a stiff artery [14,15]. Loss of distensibility, increased elastic modulus and increased stiffness index are detected in pulmonary hypertensive patients by intravascular ultrasound measurements of vascular area change for given pressure change [16]. CT [17] and MRI [18] imaging studies of pulmonary hypertensive adults show moderate increase in diastolic vascular diameter but a marked loss of pulsatile deformation during systole, such that hypertensive pulmonary arteries have increased wall strain but diminished stretch during the cardiac cycle, resulting in loss of capacitance. While in systemic arteries most of the compliance of the system is located in proximal conduit vessels, 80% of the compliance of the pulmonary circuit is located beyond the first intrapulmonary branch; loss of this more distal element of compliance is an early hallmark of pulmonary hypertension, and creates a feed-forward mechanism increasing distal proliferative vasculopathy [19]. Pulsatile flow decreases resistance in the neonatal pulmonary circuit, compared to steady flow [20]. Augmentation of pulsatile deformation of the hypoxic hypertensive pulmonary circuit using a pulsatile catheter acutely restores nitric oxide-dependent vasodilation and decreases resistance [21]; but the mechanism for this effect is not known, and effects of pulsatile stretch on arterial remodeling have not yet been examined.

Cell fate during arterial remodeling is regulated by essential mechanisms including apoptosis, autophagy and endoplasmic reticulum (ER) stress. Hypoxia, oxidative stress, growth factor deprivation or nutrient deficiency can activate apoptosis [22,23], trigger ER stress, and/or activate autophagy [24]. ER stress in smooth muscle cells is directly linked to activation of both autophagy and apoptosis [25]. One element of ER stress is the unfolded protein response (UPR), aggregation of abnormally folded proteins within the ER, which has been reported in severe pulmonary hypertension and is associated with cell death [26]. Activation of UPR pathways opposes differentiation of smooth muscle into a contractile phenotype [27], thus potentiating remodeling. Inhibition of UPR prevents development of hypoxia-induced pulmonary hypertension, making this a potential therapeutic target [28]. Specific triggers for activation of the UPR cascade are unknown. One the other hand UPR, autophagy, and apoptosis are tightly interconnected. UPR is involved in regulation of Bcl2 family proteins via JNK phosphorylation and expression of CHOP via PERK and ATF4 activation [25,29–32]. UPR is also connected to autophagy through the regulation of autophagy flux and ATG proteins expression through IRE1, and PERK arm of the UPR [33–35]. Therefore, targeting linking components of these pathways could be a potential gateway to controlling disease related to these mechanisms.

As an initial exploration of the relative contributions of oxygen tension and pulsatility to homeostatic balance, we devised a unique *in vitro* model of pulmonary hypertensive smooth muscle, combining hypoxia with cyclic mechanical stretch to simulate the vascular wall environment. Since pulsatile stretch occurs normally in pulmonary arterioles, in this rubric hypoxia in presence of stretch represents pulmonary hypertension without arterial remodeling, while hypoxia in absence of stretch represents pulmonary hypertension after onset of remodeling and loss of pulsatile deformation. Using this model, we examined activation of ER stress and autophagy pathways. We hypothesized that hypoxia would activate ER stress, but that pulsatile stretch would prevent this activation.

2. Methods

2.1. Cell culture

Human pulmonary artery smooth muscle cells (hPASMC) obtained from ATCC (PCS-100-023) were cultured as per instructions, plated on BioFlex collagen-coated 6-well flexible culture dishes, and once

confluent, allocated to four groups: [i] **Normoxic** (control group; 21% O₂, 5% CO₂, balance N₂); [ii] **Normoxic Flexed**, ie growth with pulsatile stretch (cyclic stretch to 10% above resting length, frequency 1 Hz); [iii] **Hypoxic** (late PPHN group; 10% O₂); and [iv] **Hypoxic Flexed**, ie growth with pulsatile stretch (early PPHN group; 10% O₂; cyclic stretch). Myocytes in mechanical stretch groups were placed on the Flexcell FX-3000 Tension Unit (Flexcell International Corporation, Burlington, NC) with cylindrical loading posts and intermittent vacuum to apply a uniform equibiaxial stretch to the collagen matrix simulating the deformation of the pulsatile vascular wall, for 72 h in a dedicated hypoxic or normoxic incubator. PCO₂ and PO₂ were maintained within 1% tolerance. Resting (static) cells were grown in equivalent conditions on collagen matrix plates with no stretch applied.

2.2. Antibodies and reagents

Antibodies against the following proteins were used: smooth muscle type α -actin from Thermo Scientific; BIP (immunoglobulin heavy chain-binding protein, or GRP78), IRE1 α (inositol-requiring protein-1 α), beclin-1 and ATG12 (autophagy related protein) from Cell Signalling; phosphorylated eIF2 α (eukaryotic translation initiation factor 2), ATF4 (activating transcription factor 4) from Abcam; sm-myosin heavy chain, desmin, vimentin, cleaved LC3 β (light chain 3B) and β -actin from Sigma Aldrich. Secondary antibodies were anti-mouse (BD Pharmingen) or anti-rabbit (Sigma Aldrich); enhanced chemiluminescence from Amersham.

2.3. Electron microscopy

After 72 h environmental exposure with or without cyclic stretch, cells were lysed for protein analyses, or collected for transmission electron microscopy (TEM) as per previously published protocol [29]. TEM was performed on ultra-thin sections (100 nm on 200 mesh grids); sections were stained with uranyl acetate and counterstained with lead citrate. Autophagy induction was evaluated based on autophagosome and autophagolysosome formation, while autophagy inhibition was evaluated based on visualization of accumulation of autophagosomes in TEM images.

2.4. Protein content by western blot

Whole cell lysates from myocytes in all treatment groups were clarified by brief centrifugation, and protein concentration determined by Bradford assay. Lysates were separated by SDS-PAGE with appropriate molecular weight markers, transferred to nitrocellulose membranes blocked with 3% non-fat milk in Tris buffered saline with 0.1% Tween (TBS-T), and probed overnight with primary antibodies as follows: smooth muscle phenotype was surveyed by Western blot for protein abundance of smooth muscle type α -actin, sm-myosin heavy chain and intermediate filament proteins desmin and vimentin; UPR intermediates BIP (GRP78), IRE1 α ; ER stress pathway intermediates phosphorylated eIF2 α and ATF4; and autophagy pathway intermediates beclin-1, ATG12 and cleaved LC3 β . Blots were washed with TBS-T and probed with secondary antibodies (anti-mouse or anti-rabbit, as appropriate), then protein bands visualized with enhanced chemiluminescence and normalized to β -actin. Optical density was quantified under non-saturating conditions with automatic background subtraction using a digital imaging densitometer.

2.5. Statistical analysis

Data were analyzed by ANOVA, or ANOVA for repeated measures, with Tukey correction for multiple comparisons, using Prism 8.0 software (GraphPad); $p < 0.05$ was considered significant.

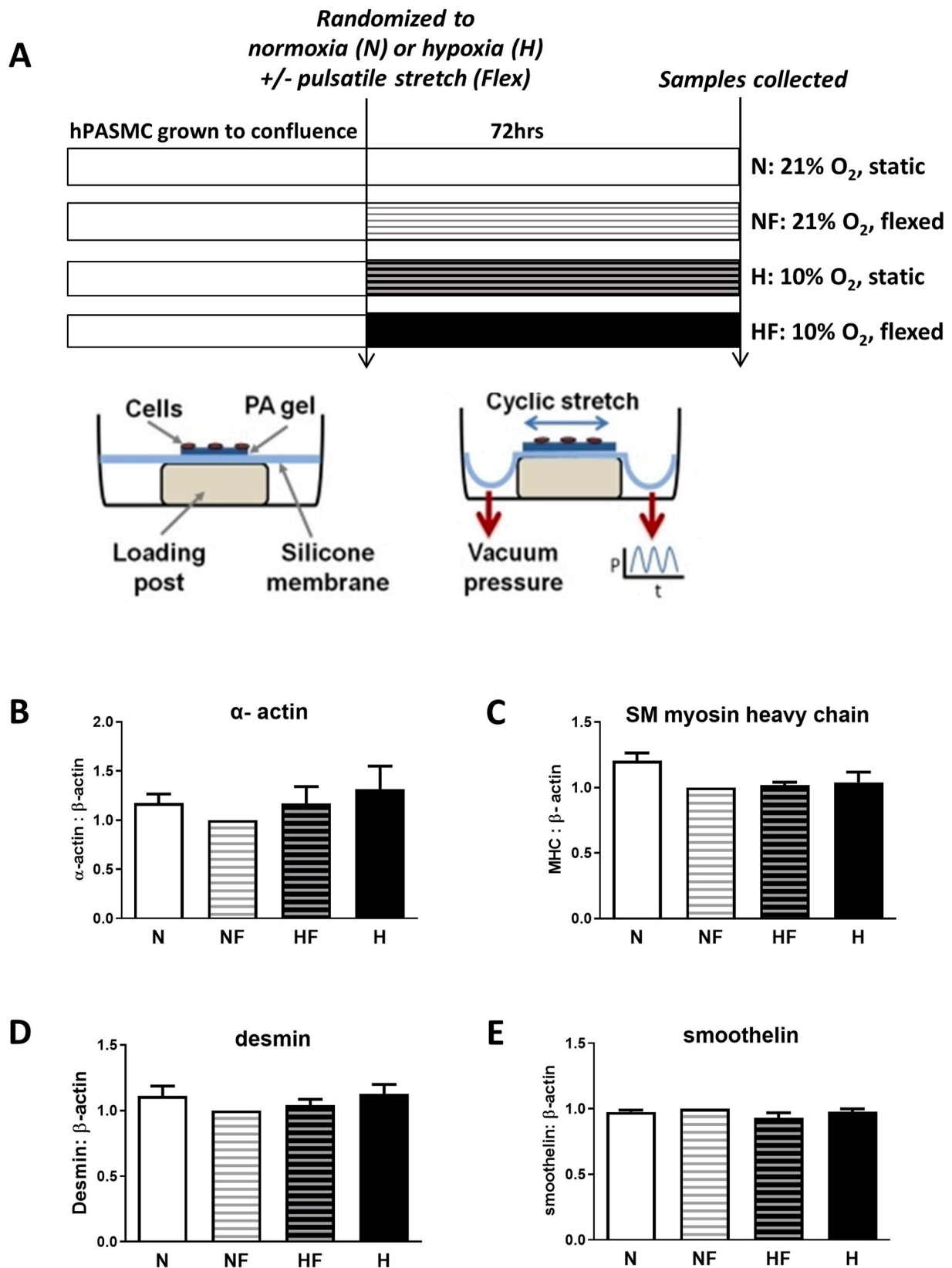


Fig. 1. A) Schematic of cell culture model. Serum-fed human pulmonary arterial smooth muscle cells (PASMCMs) in confluent culture under routine culture conditions were randomized to static growth, or to cyclic pulsatile stretch to 10% above resting length, 60 times per minute to model systolic arterial deformation, for 72 h in a normoxic (21% O₂) or hypoxic (10% O₂) incubator with CO₂ maintained constant at 5%. Immunoblotting of cell lysates for **phenotypic marker proteins** showed no difference in abundance of **B) α-smooth muscle-actin**, **C) smooth muscle myosin heavy chain**, **D) desmin** or **E) vimentin** ($p = ns$ for all comparisons).

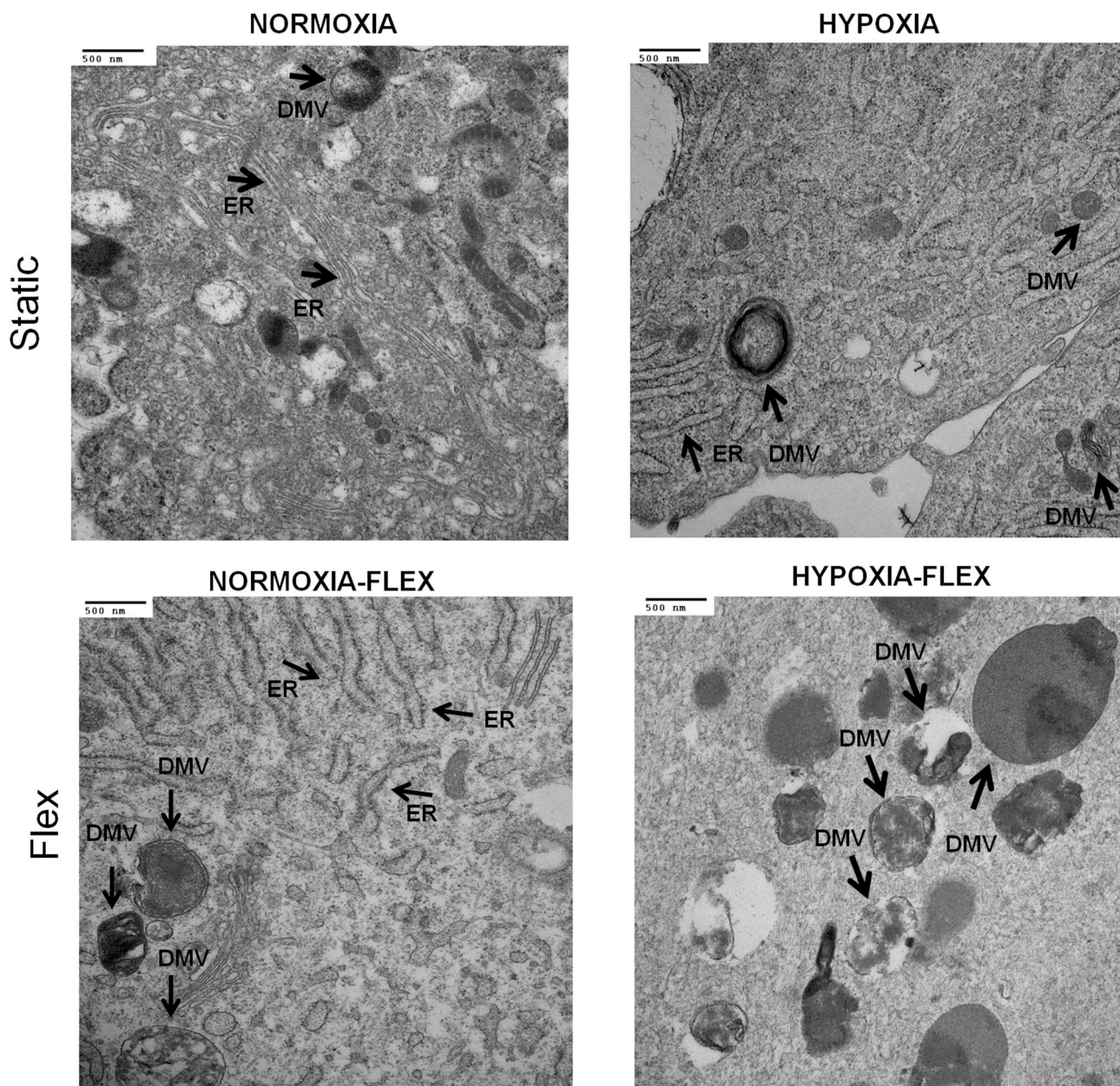


Fig. 2. Hypoxia and cyclic stretch alter ER appearance and incidence of double membrane vacuoles in pulmonary artery myocytes. PASMCS grown in normoxia (N; 21% O₂, 5% CO₂ for 72hrs), normoxia flexed (NF; 21% O₂, 5% CO₂ with cyclical stretch for 72hrs), hypoxia (H; 10% O₂, 5% CO₂ for 72hrs), or hypoxia flexed conditions (HF; 10% O₂, 5% CO₂ with cyclical stretch for 72hrs) were fixed and processed for transmission electron microscopy (TEM). All flexed cells had an increased number of double membrane vacuoles (DMVs). Hypoxic myocytes had visible alterations in endoplasmic reticulum (ER) structure, while in hypoxic flexed myocytes, these changes in ER structure coexisted with an accumulation of intracellular DMVs. Magnification is 25,000x in all images.

3. Results

3.1. Phenotypic characterization after hypoxia with or without pulsatile stretch

As described in Fig. 1a, human pulmonary artery myocytes were grown on culture surfaces of collagen adherent to silicone membrane until confluent, and then randomized *in situ* to static or flexed growth for 72 h in dedicated hypoxic or normoxic incubators. Cyclic stretch was applied by vacuum, causing deformation of the silicone membrane around a loading post, and resulting in circumferential deformation of the culture surface. Cells remained serum-fed during this study, and were thus not subject to phenotypic selection pressure. The amplitude of stretch applied in both the flexed growth groups did not itself result in

alteration in smooth muscle phenotype, as measured by abundance of smooth muscle marker proteins α -actin (Fig. 1b), myosin heavy chain (Fig. 1c), and differentiated intermediate filament markers desmin (Fig. 1c) or smoothelin (Fig. 1d) in myocyte lysates (histograms shown as band density ratio to β -actin; $p = ns$).

3.2. Double membrane vesicle appearance by TEM

We examined the morphology of ER and the abundance of double-membrane vesicles (DMV) in flexed and non-flexed cells grown in normoxic or hypoxic conditions, to differentiate the effects of mechanical stretch versus oxygen environment on ER stress (Fig. 2). Mechanical stretch increased the appearance of cytoplasmic DMV, regardless of oxygen environment. Stretch-induced structural changes in the ER

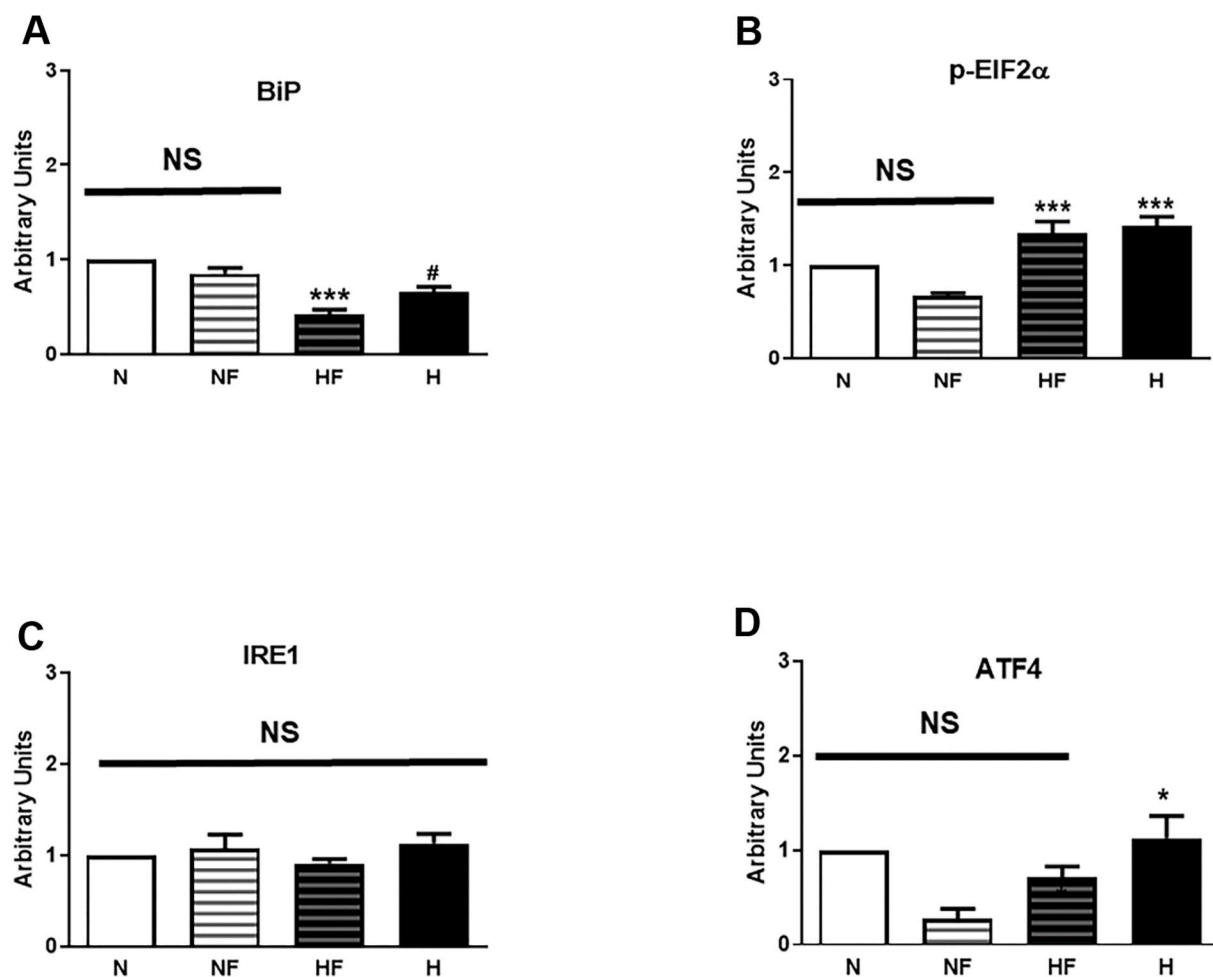


Fig. 3. Effects of hypoxia and pulsatile stretch on unfolded protein response elements. PASCs grown in normoxia (N; 21% O₂, 5% CO₂ for 72hrs), normoxia flexed (NF; 21% O₂, 5% CO₂ with cyclical stretch for 72hrs), hypoxia (H; 10% O₂, 5% CO₂ for 72hrs), or hypoxia flexed conditions (HF; 10% O₂, 5% CO₂ with cyclical stretch for 72hrs) were lysed per described protocol, protein contents quantified, and equivalent samples prepared for immunoblotting of unfolded protein response (UPR) intermediates. **A)** BiP abundance is significantly decreased in all hypoxic groups compared to normoxic static or normoxic flexed cells ($^{#}p < 0.05$). **B)** Abundance of phosphorylated eIF2 α significantly increased in both hypoxic and hypoxic flexed groups compared to normoxic controls ($^{***}p < 0.001$). **C)** Neither oxygen limitation nor mechanical stretch exposures changed IRE1 α abundance. **D)** ATF4 abundance trended higher in hypoxic flexed cells compared to normoxic flexed cells, but a significant increase in ATF4 occurred only in static hypoxic cells compared to controls ($^{*}p < 0.05$).

including widening of the tubular structure, and an increased number of ribosomes, both observed during normoxic growth. In contrast, during hypoxic conditions, the application of stretch decreased the tubular distance and also decreased ribosomal structures in the ER.

3.3. Effects of hypoxia and pulsatile stretch on UPR pathway intermediates

We then examined the effects of mechanical stretch and environmental hypoxia on stress-sensing ER transducers mediating canonical UPR pathways. In this and following figures, data are present in the following order: normoxic static culture (control), normoxic flexed culture (to model a normal, dynamically stretched vascular wall), hypoxic flexed culture (to model an early pulmonary hypertensive vascular wall), and hypoxic static culture (to model a non-pulsatile, late pulmonary hypertensive vascular wall). Hypoxia, and in particular hypoxia plus flex, decreased free BiP abundance (Fig. 3a). The arms of UPR found upstream to BiP include IRE1, which is not altered in this preparation (Fig. 3c); and activation of PERK, which phosphorylates eIF2 α . Phosphorylation of eIF2 α was increased by hypoxia (Fig. 3b). Its downstream target ATF4 was increased only in hypoxic static culture (Fig. 3d).

3.4. Effects of hypoxia and pulsatile stretch on autophagy pathway intermediates

Key intermediates in autophagy pathways were examined next. Activation of autophagy can be discerned as beclin-dependent or independent. Abundance of beclin was unchanged in all treatment conditions (Fig. 4a). The association of ATG5 with ATG12 is required for elongation of the phagophore; this was not significantly altered by hypoxia or mechanical stretch (Fig. 4b). Finally the ratio of LC3 β -II to LC3 β -I was studied, as an indicator of autophagosome formation; this was also unchanged in all treatment groups (Fig. 4c).

3.5. Effects of autophagic flux inhibition on UPR signal versus autophagy signal

The final series of histograms show the direction and degree of change from untreated state, following treatment of myocytes in all environmental and mechanical stretch groups with bafilomycin-A1, an inhibitor of autophagic flux. We observed that inhibition of autophagy by bafilomycin alters signal in PERK-mediated UPR pathways, especially in hypoxic static culture. While free BiP was unaltered (Fig. 5a), phosphorylation of EIF2 α was significantly decreased in both hypoxia groups

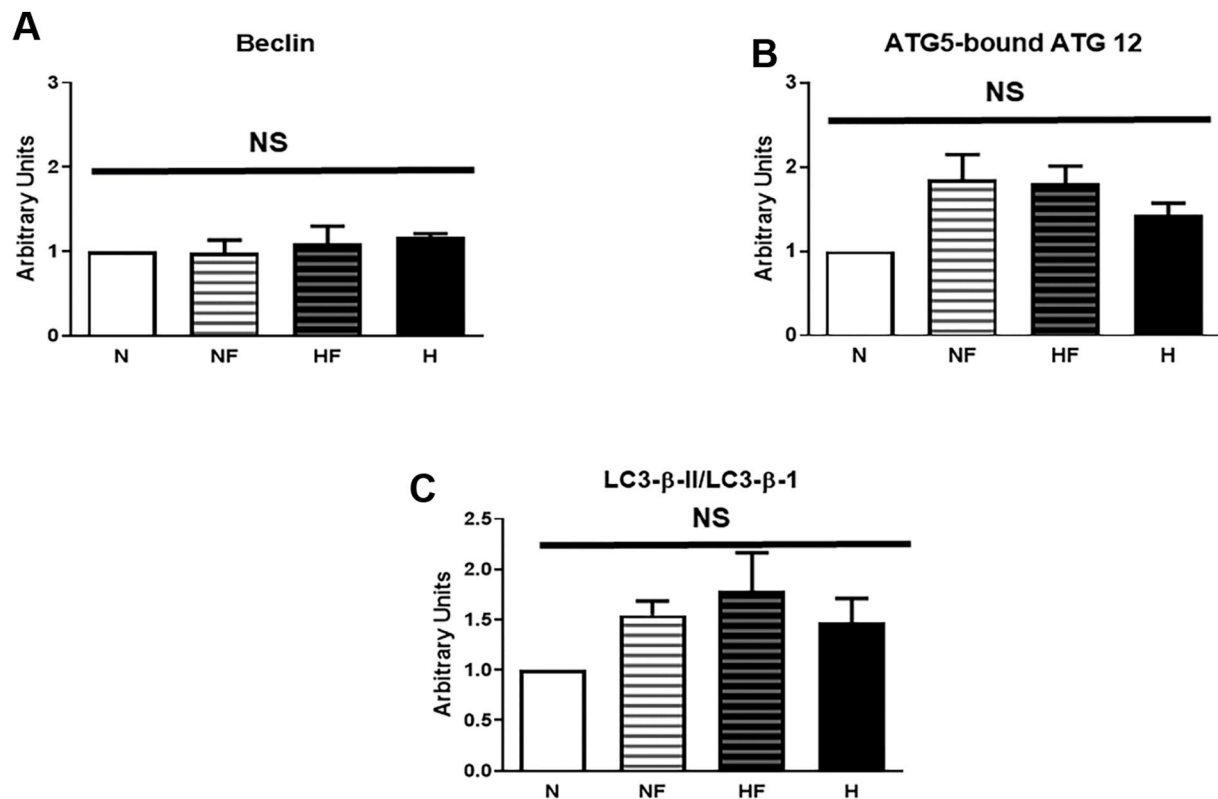


Fig. 4. Autophagy pathway intermediates after hypoxia or pulsatile stretch. PASCs grown in normoxia (N; 21% O₂, 5% CO₂ for 72hrs), normoxia flexed (NF; 21% O₂, 5% CO₂ with cyclical stretch for 72hrs), hypoxia (H; 10% O₂, 5% CO₂ for 72hrs), or hypoxia flexed conditions (HF; 10% O₂, 5% CO₂ with cyclical stretch for 72hrs) were lysed per described protocol, for immunoblotting of key autophagy pathway proteins A) beclin-1, B) conjugated ATG5-12, C) LC3β-II/LC3β-I ratio. No significant differences were seen between groups (p = ns).

(Fig. 5f), and ATF4 abundance decreased in hypoxic static culture (Fig. 5g), while IRE1 abundance was increased (Fig. 5b). No changes were seen in autophagy pathway intermediates beclin (Fig. 5c), LC3 ratio (Fig. 5d) or ATG5-bound ATG12 (Fig. 5e), following the bafilomycin treatment of all groups.

4. Discussion

The arterial wall normally exists in a state of dynamic tension, stretching in systole and relaxing in diastole. Coupling of right ventricle to pulmonary artery follows the Windkessel model; during systole, the bolus of blood entering the pulmonary artery causes acute distention due to arterial compliance, and reduces ventricular afterload. During diastole, elastic recoil of conduit arteries displaces blood distally into small vessels, ensuring continuous pulmonary flow throughout the cardiac cycle [36]. Wall tension is associated with remodeling; dynamic stretch of the artery wall has the opposite effect. Normal arteries stretch and recoil during pulsation, thus smooth muscle cells undergo cyclic mechanical strain during the cardiac cycle, critical for maintaining homeostatic signaling. Pulsatile stretch can alter myocyte phenotype commitment [37], induces generation of reactive oxygen species [38], increases mobilization of calcium [39], initiates ER stress [40] and induces apoptosis in vascular myocytes [41]. On the other hand the loss of pulsatile stretch, as studied in context of non-pulsatile ventricular assist devices, reveals acute and chronic vascular effects of non-pulsatile flow including increased vascular stiffness due to smooth muscle proliferation increasing wall thickness, increased collagen and diminished elastin content, smooth muscle atrophy and loss of vasodilator responsiveness [42]. Both the concentric outward remodeling of large conduit pulmonary arteries and the concentric inward narrowing of small conduit and resistance arteries observed in PPHN are sufficient to decrease

compliance and increase impedance [43]. Physiological pulsatile stretch in a normal conduit artery is estimated by echocardiographic methods at 10% above initial diameter, at a frequency equivalent to the heart rate [44]. Modeling the hypertensive arterial wall by application of supra-physiological levels of cyclic stretch to myocytes does not fit well with the known decrease in systolic deformation reported in imaging studies of pulmonary hypertensives [17,18]. Hence in this study, we used 10% stretch at 1 Hz (denoted the flexed groups) to model normal arterial pulsation; however, a static normoxic culture remains the control condition, as the accepted literature standard.

The degree of cellular hypoxia achieved in this study is determined by incubator oxygen tension, by diffusion of oxygen through non-hemoglobin-containing media to the cell growth plane, and by density and metabolic activity of cultured cells [45]. Agitation of media due to cyclic stretch will increase oxygen diffusion to some extent, as will media changes. We measured steady state media oxygen content just under 5 mg/ml at the cell growth interface for pulmonary arterial myocytes grown in a 21% O₂ incubator, and approximately 2.5 mg/ml for myocytes cultured in 10% O₂ [46]. These media oxygen concentrations are comparable to those of arterial tissues in normal or pulmonary hypertensive conditions [47]. This level of pulmonary arterial hypoxia induces catalase [48] but inhibits mitochondrial superoxide dismutase, increasing mitochondrial ROS [49].

Both hypoxia and stretch increase intercellular connectivity and induce mitochondrial ROS generation, triggering contractile pathway activity and adaptive remodeling [50]; but stretch also triggers nitric oxide synthesis [51], mediating smooth muscle relaxation. Myocytes adapt to pulsatile mechanical loading by maintaining a target mechanobiological equilibrium in which mechanical stiffness remains proportional to wall stress; this is modulated by rapid changes in cell stiffness (cytoskeletal remodeling) as well as in tissue stiffness (matrix

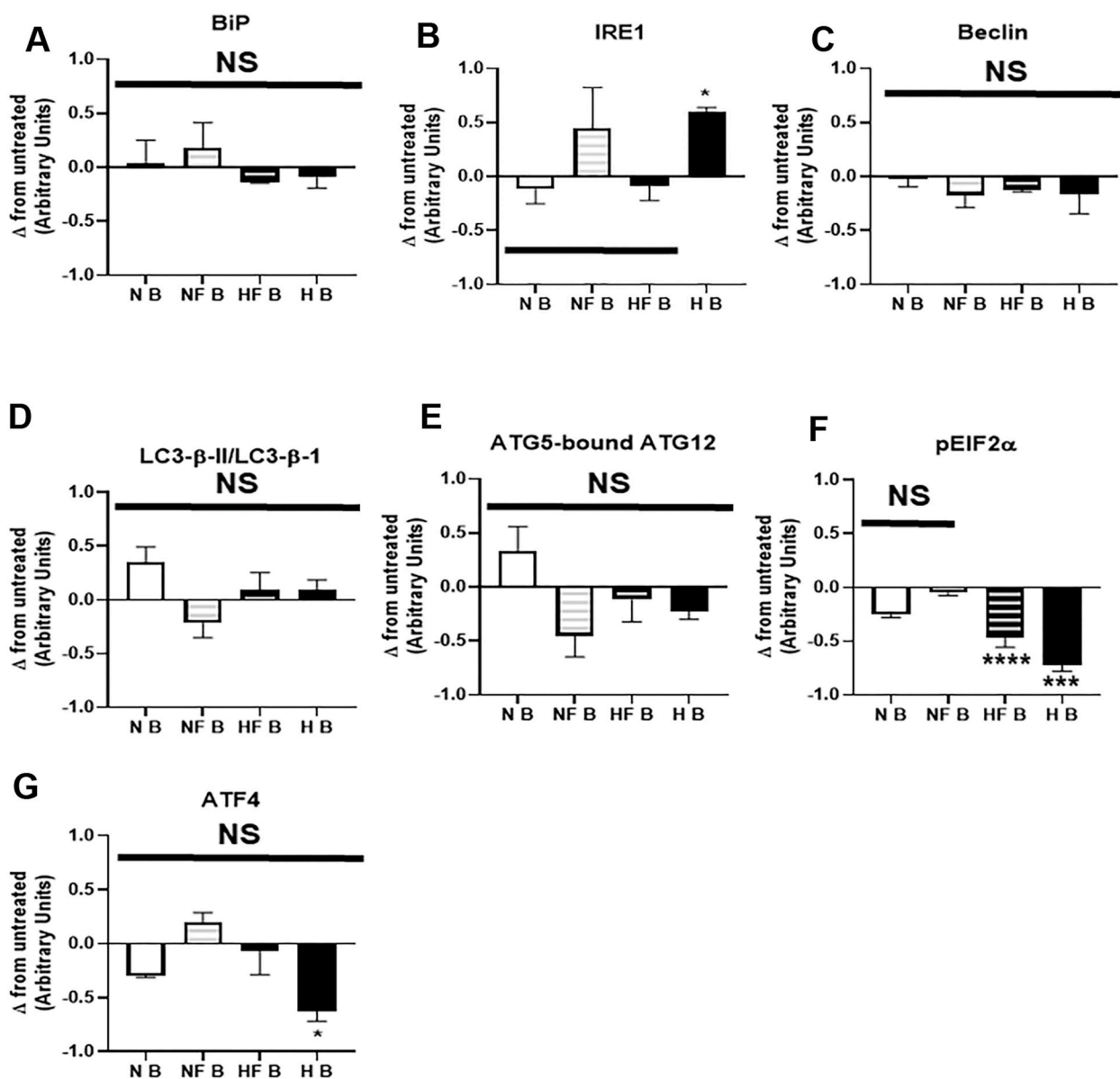


Fig. 5. Effect of Bafilomycin on UPR and autophagy responses to hypoxia or pulsatile stretch. PASCs grown in normoxia (N; 21% O₂, 5% CO₂ for 72hrs), normoxia flexed (NF; 21% O₂, 5% CO₂ with cyclical stretch for 72hrs), hypoxia (H; 10% O₂, 5% CO₂ for 72hrs), or hypoxia flexed conditions (HF; 10% O₂, 5% CO₂ with cyclical stretch for 72hrs) were treated daily with autophagy inhibitor 5 nM bafilomycin-A1 during the 72 h period of exposure to static or stretched conditions, then lysates subject to immunoblot for UPR and autophagy pathway intermediates, depicted here as change in band density from that of bafilomycin-untreated cells in similar conditions. Treatment with bafilomycin-A1 did not alter the expression of BiP (A), Beclin (C), LC3β-II/LC3β-I (D) or ATG5-bound ATG12 (E). Abundance of phosphorylated eIF2α (F) was decreased by bafilomycin in hypoxic myocytes in flexed and static groups. Only hypoxic myocytes in static culture had significantly increased expression of IRE1 (B) and decreased ATF4 (G) when treated with bafilomycin, compared to bafilomycin-untreated myocytes in the identical O₂ and stretch conditions (*p < 0.05; ***p < 0.001; ****p < 0.0001).

deposition) and mural proliferation [52]. Physiological levels of stretch maintain growth arrest in contractile myocytes; models of pulmonary myocyte stretch-induced proliferation and copious ROS generation more reflect lung overdistension by mechanical ventilation, rather than pulsatile deformation [38]. Biomechanical forces regulate phenotype and protein translation through the activation of ER stress-induced UPR, to acutely inhibit protein synthesis or increase degradation, or to prompt the synthesis of protective proteins in response to dynamic changes in pressure stimuli [53]. Hypoxia and unbalanced mitochondrial ROS are also known to activate UPR pathways [54]. The cumulative signal for arterial remodeling is determined by the balance of proliferation with apoptosis and autophagy pathways. In this investigation, one cannot assume that hypoxia, and pulsatile or cyclic mechanical stretch, will have concordant effects.

The ER and lysosomes are known to be involved in cellular responses to environmental stressors through UPR and autophagy mechanisms [55–58], depicted in Fig. 6. Therefore, we first investigated the morphology of ER and abundance of double-membraned vesicles (DMV) by transmission electron microscopy in pulmonary arterial myocytes following exposure to two distinct forms of environmental stress: oxygen tension and mechanical stretch. We found that stretch increased cytoplasmic DMV in both normoxic and hypoxic cell culture. Increased numbers of DVM in cytosol have been cited as a potential marker of autophagy in a number of different models [59–62]. In normoxic conditions, application of pulsatile stretch also induced structural changes in the ER including widening of the tubular structure, and an increased number of ribosomes. However, the same degree of stretch with simultaneous exposure to hypoxia decreased both the tubular distance

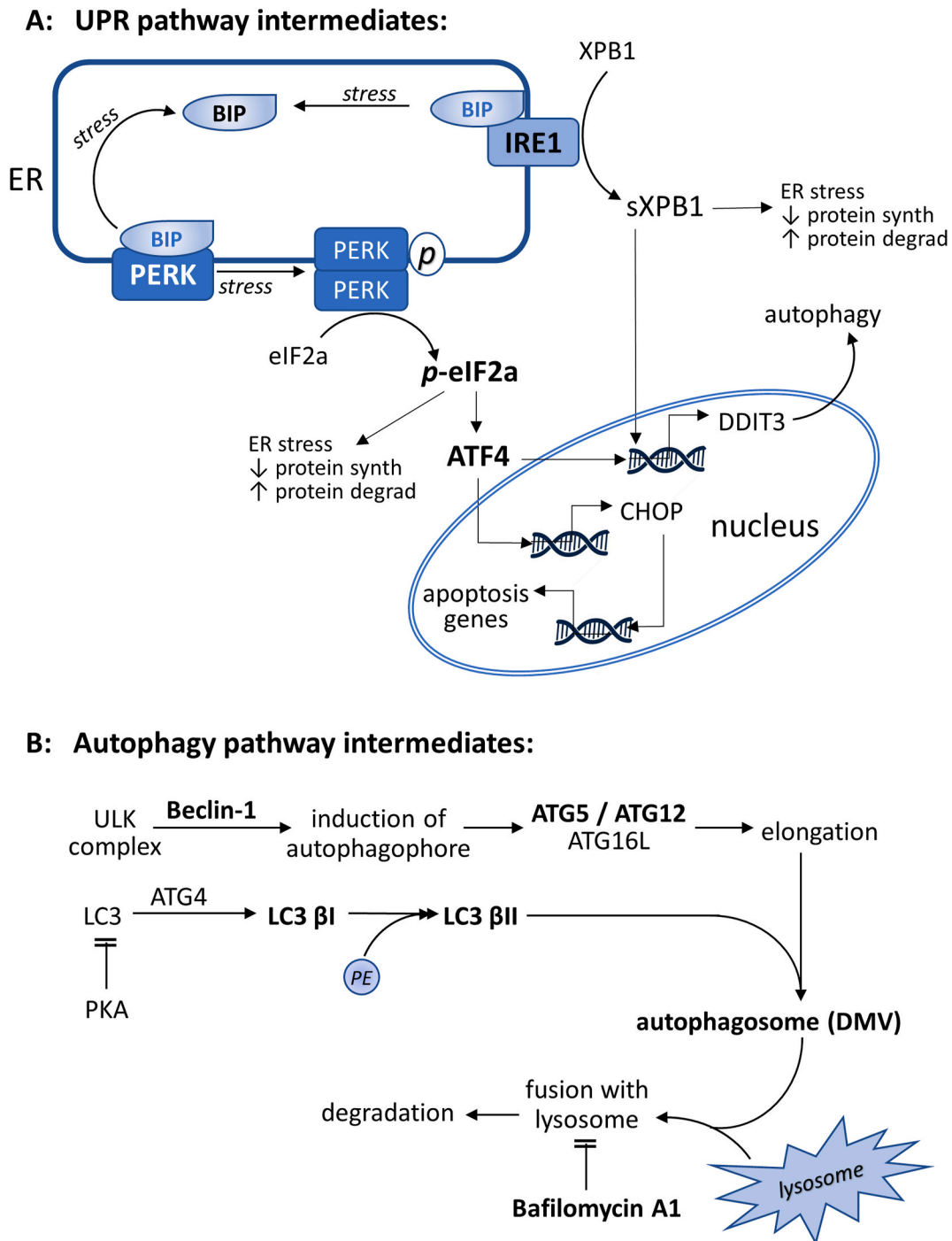


Fig. 6. Unfolded protein response and autophagy pathways. ER stress due to environmental hypoxia or mechanical strain can be transduced through canonical UPR (A) and/or autophagy (B) pathways. Intermediates measured in this study are bolded.

and the appearance of ribosomal structures in the ER.

Given that stretch-induced changes in ER structure were observed to differ in different oxygen tensions, we next examined whether UPR may be elicited by the stretch responses of pulmonary artery myocytes, analysing BIP (GRP78), IRE1 α , eIF2 α phosphorylation and ATF4 expression as markers for UPR [25,63,64]. In normoxic conditions, stretch did not alter BIP expression; but in hypoxic myocytes in static culture, and more so in hypoxic myocytes subject to stretch, BIP expression significantly decreased. IRE1 α expression was unchanged by oxygen tension or pulsatile stretch. Phosphorylation of eIF2 α was low in normoxia, and was markedly increased by hypoxia in both static and

stretched culture. These findings support the initiation of a PERK-mediated ER stress response, as a result of hypoxia. But the downstream intermediate ATF4 is significantly increased only in hypoxic myocytes in static culture, suggesting that stretch may reset this axis to prevent ATF4 induction by phosphorylated eIF2 α , ameliorating UPR.

Following on our TEM finding that pulsatile stretch increased autophagosome formation in both normoxic and hypoxic conditions, we selected beclin-1, ATG5-12 conjugation and LC3 β lipidation [65,66] to investigate the initiation of autophagy in pulmonary artery myocytes. Beclin-1 expression was unchanged in all groups; ATG5-bound ATG12

and LC3 β -II/LC3 β -I ratio trended to increase in stretched versus static growth conditions, but non-significantly. These data do not indicate activation of autophagy by either mechanical strain or hypoxia.

We used bafilomycin-A1, which inhibits the fusion of autophagosomes with lysosomes, to evaluate the mechanism of DVM accumulation during pulsatile stretch. Phosphorylation of LC3 by protein kinase A (PKA) is known to prevent its recruitment to autophagosomes [67]; we have previously shown cAMP and PKA activity are decreased in hypoxic pulmonary artery myocytes [68,69], hence had hypothesized this effect could potentiate autophagy in hypoxic myocytes. We saw no change in upstream autophagy markers beclin-1, LC3 β -II/LC3 β -I or ATG5-bound ATG12 with bafilomycin-A1 treatment, indicating no change in autophagy flux; the increase in DVM observed in hypoxic myocytes subject to pulsatile stretch may in fact be due to decreased autophagosome degradation following lysosome fusion resulting in a persistence of autophagosomes, rather than an increase in new autophagosome formation. Finally, we examined whether bafilomycin-A interference with autophagosome degradation would alter UPR signaling, as it has been reported that autophagy and UPR are interconnected [29,63]. In this model, eif2 α phosphorylation decreased when autophagy was inhibited in hypoxic myocytes, in both static and stretched culture. Hypoxic cells in static culture demonstrated a loss of ATF4 but an increase in IRE upon bafilomycin treatment; these changes were not seen in myocytes subject to pulsatile stretch, where UPR changes were diminished in the first instance by stretch. These data suggest that the PERK-mediated pathway driving UPR in hypoxic myocytes (eif2 α phosphorylation and ATF4 expression) is potentiated by the autophagy process, such that inhibition of the terminal step of autophagy can suppress this part of the UPR signal.

We conclude that in the pulmonary circuit, hypoxia induces one arm of the unfolded protein response and causes ER stress. Pulsatile stretch appears to ameliorate the hypoxic UPR response, and while increasing presence of autophagosomes, does not activate canonical autophagy signaling pathways. The study is exploratory, and limited to UPR pathways; a more detailed study of smooth muscle cell cycle regulation by mechanical forces versus oxygen tension is warranted. The novelty of the physical model used in this study is also a limitation, as comparable literature is scant. Mechanical stretch is known to damp distally in the pulmonary circuit, such that large conduit arteries deform as much as 20% but small conduit arterioles may not deform at all during systole [70]; so our model of smooth muscle hypoxia plus pulsatile stretch is understood to represent forces present in a region of the pulmonary circulation but not its entirety. Untangling the interaction between physiological levels of mechanical stretch and the vascular oxygen environment is important to understand vascular homeostasis and the triggers for initiation of vascular remodeling. We propose that the simultaneous application of hypoxia and graded levels of cyclic stretch can serve to distinguish myocyte signaling in the deformable conduit pulmonary artery of early PPHN, from signaling in the inflexible conduit artery of late stage, remodeled PPHN.

Funding sources

Project supported by grant-in-aid from Heart and Stroke Foundation of Canada, # G-19-0024330.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.101080>.

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