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The Role of Nogo and the Mitochondria–Endoplasmic Reticulum Unit in Pulmonary Hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is caused by excessive proliferation of vascular cells, which occlude the lumen of pulmonary arteries (PAs) and lead to right ventricular failure. The cause of the vascular remodeling in PAH remains unknown, and the prognosis of PAH remains poor. Abnormal mitochondria in PAH PA smooth muscle cells (SMCs) suppress mitochondria-dependent apoptosis and contribute to the vascular remodeling. We hypothesized that early endoplasmic reticulum (ER) stress, which is associated with clinical triggers of PAH including hypoxia, bone morphogenetic protein receptor II mutations, and HIV/herpes simplex virus infections, explains the mitochondrial abnormalities and has a causal role in PAH. We showed in SMCs from mice that Nogo-B, a regulator of ER structure, was induced by hypoxia in SMCs of

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SUPPLEMENTARY MATERIAL

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Fig. S1. Nogo-B levels are increased in human PAH pulmonary arteries.

Fig. S2. Nogo-B levels are increased in the serum of PAH patients.

Fig. S3. Hypoxia induced ER stress in normal human PASMCs to levels similar to PAH PASMCs.

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Fig. S8. Hypoxia increases the distance between the endoplasmic reticulum and the mitochondria in *Nogo*^{+/+} PASMCs.

Fig. S9. Nogo-B is localized in the endoplasmic reticulum and plasma membrane.

Fig. S10. Hypoxia does not change phosphatidylserine synthesis in *Nogo*^{+/+} and *Nogo*^{-/-} PASMCs.

Fig. S11. *Nogo*^{+/+} PASMCs exposed to hypoxia have decreased mitochondrial calcium.

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Fig. S13. Hypoxia decreases K⁺ current in *Nogo*^{+/+} PASMCs.

Fig. S14. Phenylephrine increases intracellular calcium levels in *Nogo*^{+/+}, *Nogo*^{+/-}, and *Nogo*^{-/-} PASMCs.

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the PAs but not the systemic vasculature through activation of the ER stress-sensitive transcription factor ATF6. Nogo-B induction increased the distance between the ER and mitochondria and decreased ER-to-mitochondria phospholipid transfer and intramitochondrial calcium. In addition, we noted inhibition of calcium-sensitive mitochondrial enzymes, increased mitochondrial membrane potential, decreased mitochondrial reactive oxygen species, and decreased mitochondria-dependent apoptosis. Lack of Nogo-B in PASMCs from *Nogo-A/B*^{-/-} mice prevented these hypoxia-induced changes in vitro and in vivo, resulting in complete resistance to PAH. Nogo-B in the serum and PAs of PAH patients was also increased. Therefore, triggers of PAH may induce Nogo-B, which disrupts the ER-mitochondria unit and suppresses apoptosis. This could rescue PASMCs from death during ER stress but enable the development of PAH through overproliferation. The disruption of the ER-mitochondria unit may be relevant to other diseases in which Nogo is implicated, such as cancer and neurodegeneration.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a disease of the pulmonary circulation caused by an excessive proliferation of vascular cells that eventually obliterate the lumen in resistance pulmonary arteries (PAs) but spare systemic vessels (1). Although its idiopathic and familial forms are rare, PAH is associated with several more common diseases like collagen vascular diseases, congenital heart disease, and AIDS, among others (1). The median survival of untreated patients is less than 3 years after the time of diagnosis (1). Existing therapeutic agents were all originally developed as vasodilators for systemic vascular diseases, not as antiproliferative or proapoptotic therapies designed to reverse established pulmonary vascular remodeling. Thus, it is not surprising that, although they improve some symptoms, these drugs do not reverse the disease or prolong survival (2, 3).

Several environmental and genetic triggers for PAH have been proposed, including alveolar hypoxia, loss-of-function mutations of bone morphogenetic protein receptor II (BMPRII), and viral infections with herpes simplex virus (HSV) or HIV (4). Diverse signaling abnormalities accompany the disease (4). As in cancer, a glycolytic metabolic phenotype appears to be a common downstream feature of several genetic and signaling abnormalities in PAH (5). For example, in cancer, loss of p53 or activation of AKT or c-myc results in suppression of mitochondria-based glucose oxidation (GO), with a resultant shift to cytoplasm-based glycolysis (6). This shift is associated with a resistance to mitochondria-dependent apoptosis and provides a potential explanation for the Warburg effect in cancer, in which there is a shift in the energy production from GO toward glycolysis, even in the absence of hypoxia (7). There is a similar metabolic shift in PAH, and modulators or genetic interventions that reverse the suppression of GO reverse the disease in animal models of PAH, as they do in cancer (7–10). In both cancer and PAH, however, the primary cause of this metabolic switch remains unknown (5, 11).

Endoplasmic reticulum (ER) stress is a fundamental cellular response that may accompany several triggers of PAH. For example, hypoxia (4), viral infections (12), the unfolded protein response caused by BMPRII mutations [which occur in 75% of familial and less than 20% of sporadic PAH cases (1)] and inflammation (4), and Notch induction (13) are all associated with some degree of ER stress (14–17). In addition, abnormal ER structure, suggestive of ER stress, was shown in electron microscopy studies of PAH tissues more than 30 years ago (18), but its potential role in PAH pathogenesis remains unknown. The lung and its resistance PAs, which lie adjacent to the alveoli and represent one of the body's major contact points with the environment, are highly susceptible to stress signals such as hypoxia, viruses or pollutants. The resistance PAs [normally exposed to a PO_2 (partial pressure of oxygen) of more than 100 mmHg] are exposed to much more oxidative stress

than systemic resistance arteries (PO_2 of about 50 mmHg) (19, 20), suggesting that the regulation of and threshold for ER stress may be unique in the pulmonary vasculature. We hypothesized that ER stress may participate in pulmonary circulation-specific metabolic and mitochondrial remodeling in PAH.

Nogo is a member of the reticulon family of proteins and is critical for regulating the tubular structure of the ER (21). Nogo exists in three isoforms—A, B, and C—with Nogo-A expressed in the central nervous system, Nogo-B expressed ubiquitously, and Nogo-C expressed in both neurons and skeletal muscle (22–24). Nogo-B was decreased in mice femoral arteries after injury and inhibits smooth muscle cell (SMC) migration but promotes endothelial cell migration (25). Mice lacking Nogo-A/B develop exaggerated neointimal remodeling, which can be rescued with adenoviral-mediated gene transfer of Nogo-B (25). Furthermore, Nogo-A was increased in hypoxic areas of the brain in rat models of cerebral ischemia by an unknown mechanism (26). Nogo-A is thought to promote neuronal survival by suppressing apoptosis during sustained ER stress, but its intracellular mechanisms remain unknown (24). Animals lacking both Nogo-A and Nogo-B have a relatively normal phenotype, suggesting compensation and functional overlap among Nogo isoforms (25,27). Because Nogo is implicated in vascular remodeling and in tissue response to hypoxia, we speculated that Nogo-B might play a role in PAH. Although Nogo-A is absent from blood vessels and the lung, Nogo-B is present in both (25, 27).

The close physical proximity of the ER and mitochondria networks in cells allows for the efficient exchange of mediators between the two organelles (28). Calcium from the ER, the largest Ca^{2+} store in the cell, can enter the mitochondria and activate critical Ca^{2+} -dependent mitochondrial enzymes (29, 30) such as pyruvate dehydrogenase (PDH), an enzyme that regulates the influx of pyruvate into the mitochondria and promotes GO. The entry of Ca^{2+} also promotes mitochondrial depolarization and apoptosis because the opening of the mitochondrial transition pore, through which proapoptotic mediators flow to initiate mitochondria-dependent apoptosis, is voltage-gated and promoted by depolarization (31). Because of the mitochondrial Ca^{2+} uniporter's low affinity for Ca^{2+} , its influx depends on Ca^{2+} microdomains in the ER, which allow Ca^{2+} to enter mitochondria down a concentration gradient (32). Such gradients are facilitated by the close proximity of the Ca^{2+} -rich ER to the mitochondria networks (29, 30). Proximity also allows adenosine triphosphate (ATP) from the mitochondria to efficiently reach the ER, its largest consumer. In addition, the two organelles are linked by tethers (28,33) and specialized contact points (mitochondria-associated membranes), which facilitate exchange of phospholipids (34). Thus, these structures form a functional, interacting mitochondria-ER unit. Both suppressed PDH activity and hyperpolarized mitochondria in PASMCs are associated with the pathogenesis of PAH (5, 9, 10). Thus, a potential disruption of the mitochondria-ER unit may explain these abnormalities. Because Nogo regulates the structure of the ER, we hypothesized that induction of Nogo-B disrupts the mitochondria-ER unit. Here, we test the effects of hypoxia and ER stress on PASMC Nogo-B induction, on the mitochondria-ER unit, and on the pathogenesis of PAH.

RESULTS

Nogo-B expression is increased in human PAH

We examined the levels of Nogo-B in the PAs of age- and sex-matched patients with ($n = 5$) and without ($n = 3$) PAH who underwent transplant surgery at our center. Using immunohistochemistry with multiphoton confocal microscopy, we found that Nogo-B levels were increased in the vascular walls of patients with PAH compared to those from control patients (Fig. 1A and fig. S1).

To explore Nogo as a potential biomarker in PAH, we measured serum Nogo-A/B levels in PAH patients ($n = 41$), in age- and sex-matched healthy subjects ($n = 18$), and in patients with secondary pulmonary hypertension as a result of thromboembolic disease ($n = 6$), a condition in which pulmonary hypertension is caused by a mechanical obstruction (embolized thrombus) and not by primary abnormalities intrinsic to the PAs. Nogo-A/B was increased in the serum of PAH patients compared to the control groups (Fig. 1B and fig. S2). Nogo-A/B serum concentrations were highest in patients with the most advanced disease [World Health Organization (WHO) functional classes III and IV] (fig. S2).

We then measured Nogo-B mRNA and protein in primary cultures of normal and PAH human PSMCs, isolated from intrapulmonary resistance PAs ($<300 \mu\text{m}$) immediately after lung tissue extraction in the operating room. Both Nogo-B protein and mRNA levels were up-regulated in PAH PSMCs in vitro (Fig. 1, C and D).

Because Nogo-B levels are decreased in a mouse model of systemic vascular remodeling after injury (25), we also measured Nogo-B in carotid artery SMCs (CASMCs) isolated from two transplant donors (Fig. 1D). To test for differences in the regulation of Nogo-B between the pulmonary and systemic circulations, we exposed normal human PSMCs and CASMCs to two inducers of ER stress, hypoxia and thapsigargin (14, 35). Nogo-B expression was induced in physiologic hypoxia [$PO_2 \sim 40 \text{ mmHg}$ (normal value, 120 mmHg)], whereas the pH and PCO_2 (partial pressure of CO_2) remain normal in normal PSMCs at levels similar to those in the PAH PSMCs, but was not induced in CASMCs. Nogo-B expression was not further increased in hypoxic PAH PSMCs compared to normoxic PAH PSMCs (Fig. 1D). To prove that ER stress was indeed occurring in hypoxic and PAH PSMCs, we studied GRP-78, an ER stress marker (36). Similar to Nogo-B, GRP-78 was increased in PAH and hypoxic normal PSMCs compared to normal PSMCs. Hypoxia did not further increase GRP-78 levels in PAH PSMCs (fig. S3). Thapsigargin induced Nogo-B in both PSMCs and CASMCs (Fig. 1D).

There are three major ER stress sensors: ATF6, PERK, and IRE1 (14). Of these, the transcription factor ATF6 is associated with a prosurvival response, is redox-sensitive (35, 37) (and thus is perhaps more responsive to the reducing signals of hypoxia than the other two ER stress sensors), and increases Nogo expression (36). The protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), which inhibits the cleavage and activation of ATF6 in the Golgi apparatus (38), and an ATF6 small interfering RNA (siRNA) (but not its scrambled RNA control) prevented the Nogo-B induction in PSMCs subject to ER stress and normalized Nogo-B expression in PAH PSMCs, but not in CASMCs (Fig. 1D).

These results were confirmed by immunocytochemistry (Fig. 1E). Nuclear ATF6 was increased in hypoxic and PAH PSMCs, indicative of increased activity; in the same cells, we observed increased Nogo-B protein levels. In addition, compared to normoxic PAH PSMCs, ATF6 activation was not further increased by hypoxia (fig. S3). ATF6 inhibition with AEBSF decreased nuclear ATF6 and Nogo-B in hypoxia (Fig. 1E). In contrast, hypoxia did not increase nuclear ATF6 or Nogo-B levels in CASMC (Fig. 1E). To confirm the specificity of ATF6 activation in PSMCs, we used a dual-luciferase reporter and showed that in identically treated human PSMCs and CASMCs, ATF6 activity was increased by hypoxia only in the PSMCs (fig. S4).

Effect of Nogo-B in PAH is gene dose-dependent

To study the hemodynamic impact and selectivity of the induction of Nogo-B in the pulmonary circulation, we studied mice with either homozygous ($-/-$) or heterozygous ($+/-$) deletion of Nogo-A/B isoforms. Because Nogo-A does not exist in the lungs, Nogo-B is the

likely form of Nogo in the lungs of these mice (27). These mice have a normal phenotype under basal conditions (25, 27), but we hypothesized that lack of Nogo-B would make *Nogo*^{-/-} mice resistant to 3 weeks of chronic normobaric hypoxia-induced PAH (CH-PAH). As in human PAH, Nogo-B levels were increased in the PAs of both CH-wild-type littermates (*Nogo*^{+/+}) and CH-heterozygote (*Nogo*^{+/-}) mice compared to their normoxic controls (Fig. 2A). Immunoblots (Fig. 2B) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (fig. S5) showed a gene dose-dependent increase in Nogo-B protein and mRNA among *Nogo*^{-/-}, *Nogo*^{+/-}, and *Nogo*^{+/+} in CH-PAH PAs.

The CH-*Nogo*^{-/-} mice had normal mean pulmonary artery pressure (mPAP) measured invasively (Fig. 2C), no right ventricular hypertrophy (RVH) (Fig. 2C), and normal pulmonary artery acceleration time (PAAT), measured with echocardiography (fig. S6A). As expected, the CH-*Nogo*^{+/+} mice developed PAH, with increased mPAP, significant RVH (Fig. 2C), and decreased PAAT (fig. S6A). The CH-*Nogo*^{+/-} mice developed PAH to a lesser extent than the CH-*Nogo*^{+/+} mice, suggesting a Nogo-B gene dose-dependent effect on PAH pathogenesis (Fig. 2C). To study the functional effects of PAH in vivo, we exercised all three groups of mice on a treadmill. CH-*Nogo*^{+/+} and CH-*Nogo*^{+/-}, but not CH-*Nogo*^{-/-}, mice had decreased functional capacity compared to normoxic controls (Fig. 2C). All groups had similar systemic blood pressure and heart rate (Fig. 2C and fig. S6B).

As in human cells, *Nogo*^{+/+} PSMCs isolated from mice resistance PAs showed increased nuclear ATF6 and Nogo-B in response to hypoxia in an AEBSF-sensitive manner (Fig. 3A). Nuclear ATF6 was significantly increased in hypoxic *Nogo*^{-/-} PSMCs, suggesting that hypoxia induced ER stress in these cells as well despite the absence of Nogo (Fig. 3A). In contrast, *Nogo*^{+/+} CASMCs did not exhibit any changes in nuclear ATF6 or Nogo-B levels in response to hypoxia (Fig. 3A).

Both *Nogo*^{+/+} and *Nogo*^{-/-} PSMCs, but not CASMCs, showed a similar increase in GRP-78 in response to hypoxia (Fig. 3B and fig. S7). To study hypoxic stress in systemic arteries similar in size to resistance PAs, we also studied renal artery SMCs (RASMCs) isolated from *Nogo*^{+/+} resistance renal arteries (<300 μm). As in CASMCs, hypoxia did not increase GRP-78 or Nogo-B mRNA expression in RASMCs (Fig. 3B). This suggests that the resistance pulmonary circulation may have a lower threshold for hypoxia-induced ER stress, specifically ER stress signaling driven by ATF6, indicating a pulmonary circulation-specific induction of Nogo-B in response to hypoxia.

Nogo-B disrupts the mitochondria-ER unit

To study the integrity of the mitochondria-ER unit in hypoxia, we used both structural and functional criteria. Using electron microscopy, we found that the minimal distance between the mitochondria and ER was increased by hypoxia in PSMCs from *Nogo*^{+/+}, but not *Nogo*^{-/-}, mice compared to normoxic controls (Fig. 3C and fig. S8). Using immunogold labeling, we found that most of the Nogo-B was present in the ER (fig. S9A), although small amounts were also located within the plasma membrane (fig. S9B). Thus, the up-regulation of Nogo-B under hypoxia-induced ER stress, by altering ER structure, may be disrupting the mitochondria-ER unit; the absence of Nogo-B could allow for the persistence of a structurally intact unit.

To determine whether these structural changes caused a functional disruption of the mitochondria-ER unit, we used a phospholipid biosynthesis assay. Phosphatidylserine (PtdSer) is a phospholipid that is synthesized in the ER by PtdSer synthase (PSS) and is subsequently transferred to the outer aspect of the mitochondrial inner membrane where it is decarboxylated to phosphatidylethanolamine (PtdEtn) by PtdSer decarboxylase (PSD) (Fig. 3D). Because this reaction does not occur elsewhere in the cell, PtdSer decarboxylation is an

index of mitochondria-ER unit integrity (34). PASMCs were incubated with [^3H]serine, and [^3H]PtdEtn was measured. Although no significant differences were seen in any of the groups in the baseline synthesis of PtdSer (fig. S10), [^3H]PtdEtn production was lower in CH-*Nogo*^{+/+}, but not in CH-*Nogo*^{-/-}, PASMCs compared to normoxic controls (Fig. 3D). Less [^3H]PtdEtn was also synthesized in human PAH PASMCs compared to normal PASMCs (Fig. 3D).

An immediate consequence of the mitochondria-ER unit disruption is a decrease in intramitochondrial calcium ($[\text{Ca}^{2+}]_{\text{m}}$) (29, 30). We measured $[\text{Ca}^{2+}]_{\text{m}}$ with two separate techniques. In the first, we used the cameleon plasmid for mitochondrial FRET (fluorescence resonance energy transfer)- Ca^{2+} and confocal imaging, as previously described (39)., *Nogo*^{+/+}, but not *Nogo*^{-/-}, PASMCs exposed to hypoxia showed a decreased yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP) signal, indicating a decrease in $[\text{Ca}^{2+}]_{\text{m}}$ (Fig. 4A). These results were replicated by using Rhod-2AM, a Ca^{2+} fluorophore with specificity for the mitochondria, as shown by its colocalization with the mitochondria marker MitoGreen (fig. S11).

A decrease in $[\text{Ca}^{2+}]_{\text{m}}$ would decrease critical calcium-dependent mitochondrial enzyme activity [that is, PDH and isocitrate dehydrogenase (IDH)] (29, 32). We directly measured PASMC PDH activity and showed that hypoxia caused a significant decrease in *Nogo*^{+/+}, but not in *Nogo*^{-/-}, PDH activity (Fig. 4B). We then measured α -ketoglutarate (α -KG), a product of IDH and an important mediator of hypoxia-inducible factor (HIF) signaling (40). Hypoxia decreased α -KG levels in *Nogo*^{+/+}, but not *Nogo*^{-/-}, PASMCs (Fig. 4C). The decrease in PDH and IDH activity would suppress GO and overall mitochondrial respiration, a well-known effect of hypoxia on intact cells. Indeed, respiration in the *Nogo*^{+/+} PASMCs previously exposed to hypoxia was much less than in their normoxic controls (Fig. 4D). There was no detectable difference in respiration between normoxic and previously exposed hypoxic *Nogo*^{-/-} PASMCs (Fig. 4D). These data suggest that the induction of Nogo-B causes the metabolic/mitochondrial remodeling and downstream signaling effects previously described in animal and human tissue models of PAH, including CH-PAH (as well as in cancer) (7–10).

Nogo-deficient PASMCs are resistant to the hypoxia-induced changes in mitochondria–NFAT–HIF–Kv channel axis

We have previously described mitochondrial hyperpolarization and decreased mitochondria-derived reactive oxygen species (mROS) in proliferative, antiapoptotic PAH PASMCs (9, 10, 41, 42). These changes are critical for PAH because pharmacologic (dichloroacetate) or molecular reversal of the decrease in PDH activity normalizes this mitochondrial remodeling and reverses PAH in several rodent models (9, 10). However, the initial signals that cause this mitochondrial remodeling and how the many diverse triggers of PAH all relate to this remain unknown. We measured mitochondrial potential ($\Delta\Psi_{\text{m}}$) and mROS in normoxic and hypoxic PASMCs. Although hypoxia, as expected, increased $\Delta\Psi_{\text{m}}$ and decreased mROS in *Nogo*^{+/+} PASMCs, these effects were absent in the *Nogo*^{-/-} PASMCs (Fig. 5A and fig. S12). Furthermore, *Nogo*^{+/-} PASMCs had levels of $\Delta\Psi_{\text{m}}$ and mROS between those of *Nogo*^{-/-} and *Nogo*^{+/+} PASMCs, in keeping with the *Nogo*-B gene dose-dependent effect that we described in the severity of mice CH-PAH (Fig. 2C).^{-/-}

In human tissues and rodent PAH models, there is both decreased opening and down-regulation of redox-sensitive voltage-gated K^+ channels, such as Kv1.5 (10, 41–43). Using whole-cell patch clamping, we showed that the 4-aminopyridine (4-AP)-sensitive K^+ current (that is, Kv current) is suppressed in the hypoxic *Nogo*^{+/+}, but not *Nogo*^{-/-}, PASMCs (Fig. 5B and fig. S13). Inhibition of Kv channels causes plasma membrane depolarization and opening of the voltage-gated Ca^{2+} channels, with a resulting increase in

PASMC intracellular calcium concentration ($[Ca^{2+}]_i$), normally the basis of hypoxic pulmonary vasoconstriction (44). Although *Nogo*^{+/+}, *Nogo*^{+/-}, and *Nogo*^{-/-} PASMCs all had similar levels of $[Ca^{2+}]_i$ at baseline, hypoxia increased $[Ca^{2+}]_i$ in *Nogo*^{+/+}, but not *Nogo*^{-/-}, PASMCs. Hypoxic *Nogo*^{+/-} PASMCs had $[Ca^{2+}]_i$ levels between *Nogo*^{+/+} and *Nogo*^{-/-} PASMCs, in keeping with the gene dose-dependent effects of hypoxia on mROS production (Fig. 5C and fig. S14). These differences in $[Ca^{2+}]_i$ regulation among the three cell types were intrinsic to a mechanism involving the mitochondria [mitochondria are important oxygen sensors that initiate the hypoxic pulmonary vasoconstriction signaling in normal PASMC (44)] because phenylephrine, which causes direct release of Ca^{2+} from the ER, caused a similar increase in $[Ca^{2+}]_i$ (Fig. 5C and fig. S14). These results suggest that the ER remains functionally normal in normoxic cells lacking Nogo-B, consistent with the overall normal cardiovascular phenotype of *Nogo*^{-/-} mice.

Nuclear factor of activated T cells (NFATc2), which decreases Kv1.5 expression and is activated in PAH PASMCs by a glycolysis-driven inhibition of glycogen synthase kinase-3 β (GSK-3 β) and an increase in $[Ca^{2+}]_i$ (9, 42), was activated in CH-*Nogo*^{+/+} PASMCs both in vitro and in vivo (fig. S15). Accordingly, in cells in which NFATc2 was activated, there was a down-regulation of Kv1.5. In contrast, NFATc2 was not activated and Kv1.5 was not down-regulated in *Nogo*^{-/-} PASMCs, in vitro or in vivo (fig. S15). Similarly, HIF-1 α , which is also activated in PAH and decreases Kv1.5 expression (41), was activated in CH-*Nogo*^{+/+} PASMCs, in a manner sensitive to the ATF6 inhibitor AEBSF. In CH-*Nogo*^{-/-} PASMCs, there was no significant HIF-1 α activation (Fig. 5D). This is in keeping with the sustained mROS and α -KG production, both regulators of HIF-1 α stabilization, in CH-*Nogo*^{-/-} PASMCs. This suggests that a mitochondrial signal is critical for the activation of HIF-1 α in these conditions, whereas the direct effects of hypoxia on hydroxylation and HIF-1 α stability may not be enough to activate HIF-1 α in these cells.

Our previous work has provided evidence that the above-described changes in $\Delta\Psi_m$ and the associated suppression of mitochondria-dependent apoptosis, as well as its secondary effects on transcription factors like NFATc2 and HIF, critical kinases like GSK-3 β , $[Ca^{2+}]_i$, and Kv channels, largely explain the antiapoptotic and pro-proliferative environment in the PA wall in PAH (9). Accordingly, among the *Nogo*^{+/+}, *Nogo*^{+/-}, and *Nogo*^{-/-} PASMCs, both proliferation and apoptosis resistance correlated positively with Nogo-B protein levels (Fig. 6, A to C, and fig. S16). Once again, there was a gene dose-dependent effect both on the expression of proliferating cell nuclear antigen (PCNA) under hypoxia and on the percent TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling)-positive cells under pro-apoptotic, starvation conditions (0.1% serum) in vitro (Fig. 6, A and B, and fig. S16). This explains the lack of PA remodeling (% media thickness), a hallmark of PAH in the *Nogo*^{-/-} mice in vivo (Fig. 6D and fig. S17).

Exogenous expression of Nogo-B disrupts the mitochondria-ER unit and induces a PAH phenotype in normal PASMC, mimicking hypoxia

To study the direct effects of Nogo-B, the only isoform present in the lungs, on the mitochondria-ER unit and determine whether exogenous Nogo-B can mimic its endogenous induction and the effects of hypoxia, we infected normoxic PASMCs with an adenovirus carrying the *Nogo-B* gene. As a control, we used an adenovirus that carried the green fluorescent protein (*GFP*) gene at the same multiplicity of infection. Infection with the *Nogo-B*, but not the *GFP* virus, increased Nogo-B protein levels, caused mitochondrial hyperpolarization, decreased mROS, and decreased Kv1.5 protein levels (fig. S18). This suggests that the hypoxia-induced changes in mitochondria and Kv channels are downstream of Nogo-B induction.

DISCUSSION

We report that Nogo-B expression is increased in PAH in humans and in a mouse model of PAH and is critical for the development of this deadly proliferative vascular disease. In addition to the well-established role of Nogo isoforms in receptor-mediated signaling, particularly in axonal regeneration in the central nervous system, we propose that Nogo-B has direct intracellular effects on the mitochondria-ER unit. We determined that there was a Nogo-B gene dose-dependent effect for the severity of CH-PAH in mice *in vivo*. The higher the expression of Nogo-B, the more severe the hemodynamic and molecular changes in the PAH exhibited by this mouse model. The absence of Nogo-B in mice caused complete resistance to CH-PAH, whereas the overall cardiovascular phenotype remained normal. Administration of exogenous Nogo-B mimics hypoxia and induces a PAH cellular phenotype in PSMCs. These findings suggest that Nogo-B may be a useful therapeutic target for PAH. In addition, we have identified a pathway that not only explains several key features of PAH but also offers insight into the role of Nogo during ER stress in general. There is emerging evidence that Nogo is expressed during ER stress as part of a rescue response that contributes to cell survival; the absence of Nogo (or inadequate expression) during sustained ER stress may allow or facilitate apoptosis (24). The intracellular mechanism underlying these observations remained unknown (24), but our data now provide a direct link among Nogo, ER stress, and mitochondrial signaling.

Mitofusin 2 (Mfn2) has been proposed to function as a tether, linking the ER with the mitochondria and contributing to an intact mitochondria-ER unit (45). The absence of Mfn2 *in vitro* leads to disruption of the mitochondria-ER unit, decreased $[Ca^{2+}]_m$, mitochondrial hyperpolarization, as well as enlarged ER and generalized ER dysfunction (45). The absence of Mfn2 is embryonically lethal in mice, limiting the exploration of its role in health and disease *in vivo*. In contrast, the absence of Nogo results in an essentially normal phenotype under control conditions, which allowed us to explore its role in the mitochondria-ER unit in a clinically relevant disease model *in vivo*.

We now show that Nogo-B is critical for the function of the mitochondria-ER unit in the pulmonary circulation, but the absence of Nogo-A/B is not lethal in mice and does not result in a generalized ER dysfunction under normal conditions. However, the absence of Nogo-B may increase the susceptibility of PSMCs to apoptosis under sustained stress conditions like hypoxia or growth factor withdrawal. The presence of Nogo-B at adequate levels during ER stress may allow for restructuring of the ER and disruption of the mitochondria-ER unit, which leads to mitochondrial hyperpolarization, closure of the mitochondrial transition pore, and suppression of apoptosis. The decrease in $[Ca^{2+}]_m$ suppresses mitochondrial function and oxidative phosphorylation (see mechanism in Fig. 6E). Although the cells can eventually compensate by increasing glucose uptake and generating ATP through cytoplasmic glycolysis, the short-term effects of this mitochondrial suppression are important for at least two reasons. First, this mitochondrial hyperpolarization is associated with a potentially beneficial decrease in mROS, because oxidative stress exacerbates ER stress. Second, the initial decrease in ATP production might decrease activity of sarcoplasmic reticulum/ER calcium-adenosine triphosphatases and thus limit Ca^{2+} overloading of the ER, another feature of ER stress. Nevertheless, the rescue of PSMCs from apoptosis during ER stress, which might be beneficial in other tissues or disease states, may come at the expense of a proliferative vascular remodeling and the eventual development of PAH.

Mitochondrial hyperpolarization in PAH has been described in several animal models and human tissues (9, 10, 41). The important role of mitochondria in PAH is shown by the fact that mitochondrial targeting with dichloroacetate (a small molecule that activates PDH and

thus reverses the glycolytic phenotype in PAH, promoting mitochondria-dependent apoptosis) reverses established PAH in several models (9, 10). Dichloroacetate, by the same mechanism, can reverse cancer growth in animals (7) as well as in humans (8), conditions also characterized by mitochondrial hyperpolarization, activation of NFATc2 and HIF-1 α , and down-regulation of Kv1.5 (7, 8). Disruption of the mitochondria-ER unit during sustained stress may contribute to the prosurvival/antiapoptotic metabolic changes reported in cancer or PAH. This response would lead to a suppression of mitochondrial respiration and a switch toward the antiapoptotic glycolytic environment (4, 6); in that sense, the absence of Nogo-B mimics dichloroacetate treatment.

One of the initial events during ER stress, regardless of the specific cause, is the activation of ATF6, which increases Nogo expression (36), supporting the notion that Nogo-B induction is an early event during ER stress. ER stress can be caused by many diverse conditions that lead to PAH in addition to hypoxia, including the unfolded protein response of mutated BMPRII in familial PAH (16), or viral infection of PASMCs with HIV or HSV (12, 15). Recently, overexpression of Notch3 has been implicated in both the pathogenesis of PAH (13) and ER stress (17). Sustained ER stress in systemic vascular SMCs induces the mitochondria-dependent apoptotic pathway (46), in keeping with the fact that Nogo-B appears to be down-regulated in systemic vascular injury (25). The fact that ATF6 activation may occur only in pulmonary, not systemic, vasculature explains the selective induction of Nogo-B in the pulmonary circulation. This finding strengthens the argument that Nogo-B therapeutic targeting may achieve relative selectivity for the pulmonary circulation in PAH.

The chronic hypoxia model of PAH, although widely used, does not recapitulate all the features of human PAH. For example, although animals exposed to chronic hypoxia develop several features of the pulmonary vascular pathology of human PAH, they do not develop the plexiform lesions that are frequently seen in some forms of PAH. These are proliferative lung vascular lesions, mainly consisting of endothelial cells, but their origin and exact role in the pathogenesis of PAH remain incompletely understood. Nevertheless, the hypoxia-exposed mice that we studied are directly relevant to the common form of pulmonary hypertension that patients with chronic obstructive pulmonary disease or residents of high altitudes develop, because their pulmonary circulation is exposed to hypoxia (a form of pulmonary hypertension that is not associated with plexiform lesions). Furthermore, the relevance of our work to clinical PAH is strengthened by our corroborating results from tissues, blood, and cell lines of PAH patients.

We focused on PASMC in our findings reported here and did not study endothelial cells, which are also implicated in PAH (4). Although most investigators agree that PA endothelial cell apoptosis is one of the earliest events in the pathogenesis of PAH, less is known about endothelial cell biology as the disease progresses; many suspect that there is a switch toward a proliferative and apoptosis-resistant phenotype (4). We observed that Nogo-B is expressed in the PA endothelia of patients with established PAH but not in the PAs from normal control subjects (Fig. 1A). Although we did not study the effects of Nogo-B on the biology of these endothelial cells, it is possible that, as in the PASMC, increased Nogo-B marks a proliferative endothelial cell with suppressed mitochondrial function and a switch toward a glycolytic phenotype. Indeed, it has recently been shown that human PA endothelial cells from PAH patients exhibit a glycolytic phenotype both in vitro and in vivo (47).

More work is also needed to identify the cellular source(s) of circulating Nogo-A/B and better define its role as a potential biomarker in larger PAH cohorts. In our small cohort, circulating Nogo-A/B levels failed to separate normal controls from patients with early, compensated PAH (WHO functional classes I and II) but could identify advanced and

decompensated PAH (WHO functional classes III and IV) from early compensated PAH (fig. S2).

In summary, Nogo-B targeting represents a potential selective therapeutic strategy for PAH. The link between Nogo-B and mitochondria signaling during ER stress may be relevant to other diseases in which Nogo isoforms are implicated, such as cancer, diabetes, and neurodegenerative diseases (24).

MATERIALS AND METHODS

Breeding of *Nogo-A/B*^{-/-} mice

Nogo-A/B-deficient mice (missing both Nogo-A and Nogo-B isoforms) were backcrossed for seven generations to a C57BL/6 background and used to generate *Nogo-A/B*-deficient, heterozygote, and littermate control mice (25, 27).

Blood pressure and heart rate

The CODA2 (Kent Scientific Corp.) mousetail cuff system was used to measure systemic blood pressure and heart rate in nonanesthetized mice. Briefly, mice were restrained in a chamber and an occlusion cuff was placed proximally, followed by a volume pressure cuff placed distally on a mousetail. The volume-pressure recording was used to obtain the mouse blood pressure and heart rate.

Chronic hypoxic mouse model

All experiments were conducted with the approval of the University of Alberta Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines. Eight-week-old *Nogo*^{+/+}, *Nogo*^{+/-}, and *Nogo*^{-/-} mice were placed into hypoxic chambers (Reming Bioinstruments; 10% O₂ and 90% N₂) for 3 weeks, as described (9).

Electrophysiology

With standard whole-cell patch-clamping techniques, cells were voltage-clamped at a holding potential of -70 mV and currents were evoked by 200-ms test pulses from -70 to +70 mV with 20-mV steps, filtered at 1 kHz, and sampled at 2 to 4 kHz, with or without 4-AP (5 mM, Sigma-Aldrich), as described (9, 10).

Right heart catheterization

Mice weighing 25 to 30 g were anesthetized with ketamine (60 mg/kg, intraperitoneally) and xylazine (20 mg/kg, intraperitoneally) and placed in a supine position on a heated table. The right jugular vein was cannulated, and right heart catheterization was performed. A sheath was used through which a high-fidelity Millar catheter was advanced (microtip, 1.4F, Millar Instruments Inc.). The sheath was 0.2 mm in inner diameter and 0.58 mm in outer diameter with a specially curved tip to facilitate passage through the right heart and main PA. Pressures in the right atrium, right ventricle, and PA were recorded continuously, and mean PA pressure was calculated electronically (Power Lab, with Chart software 5.4, ADInstruments) (9, 10).

Electron microscopy

Hitachi H-7000 transmission electron microscopy was used for immunogold (BD Biosciences) conjugated to Nogo (N-18) and nonconjugated images. To estimate the mean distance between the ER and the mitochondria, we measured the minimum distance between the mitochondrial outer membrane and the nearest ER membrane as described (48). Electron microscopy images of *Nogo*^{+/+}, CH-*Nogo*^{+/+}, *Nogo*^{-/-}, and CH-*Nogo*^{-/-} were loaded in the

analysis software ImageJ. Images were then magnified until the outer mitochondrial membrane and the ER membrane were clearly visible. Using the ImageJ software, we drew a line, marking the shortest distance between the outer mitochondrial membrane and the ER by an experimenter blinded to the study. Mean data represent a minimum of 10 images per group with four to five mitochondria analyzed per image.

Echocardiography

PA acceleration time (using pulsed wave Doppler) was measured in the parasternal short axis view with the VeVo 770 imaging system with the 707B probe (30 MHz), as described (9, 42).

Treadmill test

A graded submaximal exercise tolerance test was performed on a calibrated, motor-driven treadmill in a Plexiglas cage (Treadmill Simplex II, Columbus Instruments). Incremental increases in treadmill belt speed were made as described: 1-min warm-up at 3 m/min, then 1 min at 4 m/min, 2 min at 5 m/min, 2 min at 6 m/min, 6 min at 7 m/min, and finally 8 m/min until the mouse exhibited signs of exhaustion. Exhaustion was defined as the mouse spending >50% of the time or >10 consecutive seconds on the shock grid as described (9).

Cell culture

PASMCs, CASMCs, and RASMCs were freshly isolated from PAs, carotid arteries, and renal arteries of mice and humans with an enzymatic cocktail containing papain (1 mg/ml), dithiothreitol (0.5 mg/ml), collagenase (0.6 mg/ml), and bovine serum albumin (0.6 mg/ml) (Sigma-Aldrich). Human PASMCs were established in accordance with University of Alberta ethics approval from resistance PAs isolated immediately after extraction at transplant surgery. Cultured cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Gibco, Invitrogen) and placed in an incubator set at 37°C in either normoxic conditions (gas-perfused incubator with 5.2% CO₂, 21% O₂, and balanced N₂) or hypoxic conditions (gas-perfused incubator with 5.2% CO₂, 4% O₂, and balanced N₂). Thapsigargin (600 nM, Sigma Aldrich) and AEBSF (250 µM; Sigma Aldrich) were prepared as aqueous solutions and added to the medium of confluent cells.

Confocal microscopy

Imaging was performed with a Zeiss LSM 510 confocal microscope (Carl Zeiss). ApopTag apoptosis detection kit (TUNEL Serologicals) and the PCNA antibody (DAKO) were used as previously described (9, 10). Nogo (N-18) (Santa Cruz Biotechnology), ATF6 (Santa Cruz Biotechnology), HIF-1α (Abcam), GRP-78 (Santa Cruz Biotechnology), NFATc2 (Novus Biologicals), Kv1.5 (Santa Cruz Biotechnology), tetramethylrhodamine methyl ester (TMRM) (10 nM, Molecular Probes), and MitoSOX (5 µM, Molecular Probes) were used as previously described (9, 10). FLUO-3 (10 µM), TMRM, MitoSOX, FLUO-3, and Rhod-2AM (5 µM) were from Molecular Probes, Invitrogen. Fluorescein isothiocyanate (FITC)- and tetramethylrhodamine isothiocyanate (TRITC)-conjugated (DAKO) secondary antibodies were used in immunofluorescence.

Medial wall thickness

Percent medial wall thickness was measured at the two ends of the shortest external diameter of the distal PAs, and the average was taken ($[2 \times \text{wall thickness}/\text{external diameter}] \times 100$).

Adenoviral infection

PASMCs were grown on poly-L-lysine (Sigma-Aldrich)-coated glass coverslips and infected at a multiplicity of infection of 100 in serum-free DMEM (Gibco, Invitrogen) for 6 hours, allowing an infection rate of ~80% as previously described (49). Medium was then exchanged for DMEM containing 10% FBS.

Mitochondrial calcium imaging

Rhod-2AM (Gibco, Invitrogen) was used to measure mitochondrial calcium as indicated in the commercially available protocol. The cameleon plasmid for mitochondrial FRET-Ca²⁺ and imaging was used as previously described (39). Briefly, PASMCs were plated on confocal dishes and the plasmid was introduced by electroporation. For the dual-emission ratio imaging of Ca²⁺ in the cameleon plasmid-treated cells, we used a Zeiss LSM 510 confocal microscope in which the excitation was set at 458 nm and the emission filters used were 480 to 520 nm for cyan (when Ca²⁺ is not bound) and 535 to 590 nm for FRET (yellow when Ca²⁺ is bound). The ratio of FRET/cyan was used to standardize the rate of infection for each cell.

ATF6 luciferase assay

ATF6 dual-luciferase reporter kit (SA Biosciences) was used to assess the ATF6 activity in human PASMCs and CASCs as described in the commercially available protocol. Briefly, PASMCs (20,000 cells per well) were plated on a 96-well plate containing the transfection agent (SureFect; SA Biosciences) and the ATF6 Cignal reporter (which contained a control *Renilla* reporter as well). After 16 hours of transfection, the transfection media were replaced with regular PASC growth media and exposed to normoxia or hypoxia for 48 hours. ATF6 activity was assessed with the Promega dual-luciferase reporter assay system (SA Biosciences). Briefly, cells were lysed with the passive lysis buffer (provided in the kit) before luminescence was detected with an illuminometer. ATF6 activity was standardized to the transfection of each cell by normalizing data to *Renilla* luminescence.

Immunoblotting

Tissues were collected and immunoblotting was performed as previously described (9, 10) [25 µg of protein in pooled sample (four different animals) per lane]. The films were digitized and quantified with 1D Image Analysis Software (Kodak). Expression was normalized to α-actin to correct for loading differences. The Nogo 1761 antibody detecting Nogo-B has been described and characterized (50).

α-KG measurements

α-KG levels were measured as described in a commercially available spectrophotometric α-Ketoglutarate Assay Kit (BioVision). PASMCs cells were grown to confluency in a T75 flask. Cells were then harvested and lysed, and protein concentration was adjusted to equal levels between groups. α-KG levels were measured by measuring optical density at 570 nm after the kit-based reaction was completed as previously described (8).

PDH activity

PDH activity was measured with the MitoProfile Dipstick Assay Kit (MitoSciences). Protein was collected after cells were homogenized. Protein (50 µl of 1 µg/ml) was placed in a 96-well dish and incubated with the dipstick containing the PDH complex antibody and then incubated in activity buffer. PDH activity was measured by intensity of band with a flat top scanner as previously described (8).

qRT-PCR

Samples were added to a microwell plate, along with TaqMan probes and reagents. qRT-PCR was performed with the ABI Prism 7700 Sequence Detector (Applied Biosystems), and 18S RNA was used as a housekeeping gene (Applied Biosystems). Identical amounts of RNA were added in each group (~100 ng of RNA per group), as previously described (10).

Phospholipid biosynthesis and functional assessment of mitochondria-ER contacts

PASMCs in 60-mm culture dishes were incubated at 37°C for 7 to 12 hours with 15 μ Ci of [3-³H]serine (0.4 mM). The reaction was terminated by the addition of 1 ml of chloroform/methanol (2:1, v/v) on ice, and ³H-labeled phospholipids were extracted. PtdSer and PtdEtn were isolated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, v/v) after addition of carrier phospholipids. The phospholipids were identified according to standards with iodine vapor, and radioactivity was measured, as described (34).

Whole-cell respiration

PASMCs were harvested (5×10^6 cells/ml) in Hanks' balanced salt solution (HBSS) supplemented with 10 mM glucose and placed in a sealed glass chamber (Warner Instruments). Cells were kept at a constant temperature of 37°C and continuously stirred. Real-time oxygen consumption was measured by an oxygen electrode (Warner Instruments) connected to a Strathkelvin 782 oxygen meter. Rate of oxygen consumption was calculated with the oxygen meter's software (version 4.0, Strathkelvin Instruments Ltd.).

siRNA studies

Human donor and idiopathic PAH PASMCs were grown to 80% confluence in six-well culture dishes. The transfection agent siPORTamine (Ambion siRNA Transfection II kit 1631) was preincubated at room temperature for 10 min at a ratio of 1:12 in OptiMEM1 culture medium (Gibco, Invitrogen) before being mixed with 50 nM ATF6 siRNA (Ambion) or scrambled RNA (Ambion). The culture media were aspirated from the cells, and the transfection agent–RNA complex mixture was allowed to spread over the monolayer of cells. Plates were incubated at 37°C for 48 hours.

Statistics

All values are expressed as means $\pm$ SEM. Intergroup differences were appropriately assessed by either unpaired Student's *t* test or one-way analysis of variance (ANOVA) with post hoc analysis as indicated (SPSS 19)., *P* < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Nogo-B and ATF6 are increased in pulmonary arteries (PAs), pulmonary arterial smooth muscle cells (PASMCs), and serum from patients with PAH. **(A)** Resistance PAs in the lungs of five patients with idiopathic PAH had increased levels of Nogo-B protein (red) compared to normal PAs from three transplant donors, as shown by immunofluorescence and confocal microscopy ($n = 7$ PAs per patient). $*P < 0.01$, unpaired Student's t test. Results from one patient are shown here; see fig. S1A for the remaining results. Although the antibody detects both Nogo-A and Nogo-B, Nogo-A is not expressed in the lungs (27). Resistance PAs were co-stained with an antibody to smooth muscle actin (SMA; green). PA morphology is shown in the differential interference contrast (DIC) panel. In the merged panel, Nogo-B and SMA colocalization is shown (yellow), along with nuclei stained blue with 4',6-diamidino-2-phenylindole (DAPI). Mean data were calculated for all patients ($n = 5$ PAH, $n = 3$ normal; seven PAs per patient were analyzed) and expressed as arbitrary fluorescence units (AFUs). **(B)** Serum levels of Nogo-A/B in PAH patients are increased compared to normal and secondary pulmonary hypertension (thromboembolic disease) (Sec) patients. Sample sizes are shown in parentheses. $*P < 0.01$ versus normal; $\#P < 0.01$ versus secondary PAH using ANOVA with Fisher's least significant difference (FLSD) post hoc analysis. A previously described ELISA method was used (51). Individual patient values are shown in fig. S2. **(C)** PASMCs isolated from resistance PAs taken from patients with idiopathic PAH have higher levels of Nogo-B protein (45 kD) than do PASMCs from normal PAs as shown by immunoblot ($n = 4$ experiments per group). $*P < 0.01$ using unpaired Student's t test. Although the antibody detects both Nogo-A and Nogo-B isoforms,

Nogo-A is absent from vascular and lung tissues; in addition, Nogo-B (45 kD) is separated from Nogo-A (180 kD) by molecular weight. **(D)** PSMCs from normal donor patients exposed to ER stress inducers, hypoxia, and thapsigargin (Tg) and PSMCs from PAH patients have increased Nogo-B mRNA values compared to normal vehicle-treated PSMCs, as shown by qRT-PCR. Thapsigargin, but not hypoxia, increased Nogo-B mRNA in CASMCs from transplant donors. An ATF6 inhibitor [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)] and ATF6 siRNA decrease Nogo-B mRNA in both hypoxia- and thapsigargin-treated PSMCs and in PAH PSMCs ($n = 3$ experiments per group). $*P < 0.05$ versus normoxia; $^{\dagger}P < 0.05$ versus hypoxia; $^{\#}P < 0.05$ versus thapsigargin; $**P < 0.05$ versus PAH normoxia by ANOVA with FLSD post hoc analysis. **(E)** PSMCs from normal donor patients exposed to hypoxia and PSMCs from PAH patients have increased nuclear ATF6 (red) and Nogo-B (green) levels as shown by immunocytochemistry. ATF6 colocalization with the nuclei, stained with DAPI (blue), is shown in pink in the merged panel. Nuclear ATF6 and Nogo-B were decreased by the ATF6 inhibitor AEBSF in hypoxia-treated normal donor PSMCs and PAH PSMCs. Hypoxia did not increase nuclear ATF6 or Nogo-B levels in CASMCs ($n = 70$ cells per group). $*P < 0.05$ versus normoxia PSMC; $^{\dagger}P < 0.05$ versus hypoxia PSMC; $**P < 0.05$ versus normoxia PAH PSMC using ANOVA with Tukey's post hoc analysis. Mean data are presented in AFUs.

Fig. 2.

In mice, chronic hypoxia-induced PAH is dependent on Nogo-B. (A) Resistance PAs in the lungs of control (*Nogo^{+/+}*) and heterozygote (*Nogo^{+/-}*) littermates exposed to chronic hypoxia have increased levels of Nogo-B protein (red) compared to normoxia-treated controls, as shown by immunofluorescence and confocal microscopy. Nogo-deficient (*Nogo^{-/-}*) mice had no detectable Nogo-B protein in either normoxic or chronic hypoxic PAs. Although the antibody detects both Nogo-A and Nogo-B, Nogo-A is not expressed in the lungs (27). Resistance PAs were co-stained with an antibody to SMA (green). PA morphology is shown in the differential interference contrast (DIC) panel. Nogo-B and SMA

colocalization (yellow) is shown along with the nuclear stain DAPI (blue) in the merged panel. **(B)** Lungs of *Nogo*^{+/+} and *Nogo*^{+/-}, but not *Nogo*^{-/-}, mice exposed to chronic hypoxia had increased levels of Nogo-B protein when compared to those from normoxic mice, as shown by immunoblot ($n = 5$ mice per group). $*P < 0.05$ versus normoxia controls using ANOVA with FLSD post hoc analysis. **(C)** (Top) Representative pressure traces obtained by closed chest, right heart catheterization of anesthetized mice with a Millar catheter advanced through the internal jugular vein into the right atrium (RA), right ventricle (RV), and PA. (Bottom) *Nogo*^{+/+} and *Nogo*^{+/-}, but not *Nogo*^{-/-}, mice exposed to chronic hypoxia showed increased mean PA pressure, RV mass [RV/LV (left ventricle) + septum weight ratio], and decreased functional capacity (distance run on rodent treadmill). Systemic blood pressure was not different among groups ($n = 8$ mice per group). $*P < 0.05$ versus normoxia controls using ANOVA with FLSD post hoc analysis.

Fig. 3.

Hypoxia-induced Nogo-B disrupts the mitochondria-ER unit in mice PASC. (A) *Nogo*^{+/+} PASCs exposed to hypoxia had increased nuclear ATF6 (red) and Nogo-B (green) levels as shown by immunofluorescence. ATF6 colocalization, with the nuclear stain DAPI (blue), is shown in pink in the merged panel. Nuclear ATF6 and Nogo-B were decreased by the ATF6 inhibitor AEBSF in hypoxia-treated *Nogo*^{+/+} PASCs. Hypoxia-treated *Nogo*^{-/-} PASCs had increased nuclear ATF6 levels but no detectable Nogo-B. Hypoxia did not increase nuclear ATF6 and Nogo-B levels in *Nogo*^{+/+} CASCs ($n = 70$ cells per group). * $P < 0.05$ versus normoxia; # $P < 0.05$ versus hypoxia using ANOVA with Tukey's post hoc analysis. Mean data are presented in AFUs. (B) *Nogo*^{+/+} PASCs, but not *Nogo*^{+/+} CASCs or RASCs, exposed to hypoxia had increased Nogo-B and GRP-78 mRNA

values, as shown by qRT-PCR., *Nogo*^{-/-} PSMCs exposed to hypoxia had increased GRP-78 mRNA, whereas no detectable Nogo-B mRNA was observed ($n = 3$ experiments per group). $*P < 0.001$ versus normoxia using unpaired Student's t test. (C) *Nogo*^{+/+}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had structurally disrupted mitochondria-ER units as shown by the increase in the minimal distance between the mitochondria and ER in electron microscopy photomicrographs (arrows) ($n = 40$ contact sites in PSMCs isolated from three mice per group). The distance between the outer mitochondrial membrane and the nearest ER membrane was measured on magnified photomicrographs using ImageJ software. $*P < 0.001$ using unpaired Student's t test. (D) *Nogo*^{+/+}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had functionally disrupted mitochondria-ER units as shown by decreased mitochondrial synthesis of phosphatidylethanolamine (PtdEtn) ($n = 6$ per group). $*P < 0.01$ versus normoxia using unpaired Student's t test. Human PAH PSMCs had decreased mitochondrial synthesis of PtdEtn compared to normal donor PSMCs ($n = 3$ per group). $*P < 0.01$ using unpaired Student's t test. Data presented as $\times 10^{-3}$ disintegrations per minute (dpm) per microgram of protein. PSS, PtdSer synthase; PSD, PtdSer decarboxylase.

Fig. 4. Hypoxia induction of mitochondrial Ca^{2+} , pyruvate dehydrogenase (PDH), and isocitrate dehydrogenase (IDH) depends on Nogo-B. (A) *Nogo*^{+/+}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had decreased mitochondrial Ca^{2+} compared to normoxic controls, as shown by the ratio of bound mitochondrial Ca^{2+} [yellow fluorescent protein (YFP), yellow] to unbound mitochondrial Ca^{2+} [cyan fluorescent protein (CFP) signal, blue] with the fluorescence resonance energy transfer (FRET) and confocal microscopy. Mean data are presented as YFP/CFP signal ratio ($n = 25$ cells per group). * $P < 0.05$ versus normoxia using unpaired Student's t test. (B) *Nogo*^{+/+}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had

decreased PDH activity compared to normoxic controls ($n = 3$ experiments per group). $*P < 0.01$ versus normoxia using unpaired Student's t test. Mean data presented in AFUs. (C) *Nogo*^{+/+}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had decreased α -ketoglutarate (α -KG) levels, a direct index of IDH activity, compared to normoxic controls ($n = 4$ experiments per group). $*P < 0.05$ versus normoxia using unpaired Student's t test. (D) *Nogo*^{+/+}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had decreased whole-cell respiration rates ($n = 5$ experiments per group). $*P < 0.01$ versus normoxia using an independent one-sample t test. Mean data are presented as percent decrease of hypoxia-treated PSMCs to normoxic control.

Fig. 5. Hypoxia disruption of the mitochondria-mROS-Kv channel axis depends on Nogo-B. (A) *Nogo*^{+/+} and *Nogo*^{+/-} PSMCs exposed to hypoxia had hyperpolarized mitochondria as assessed by tetramethylrhodamine methyl ester (TMRM) and decreased mROS production as assessed by MitoSOX compared to normoxic controls. In contrast, *Nogo*^{-/-} PSMCs exposed to hypoxia had similar mitochondrial potential ($\Delta\Psi_m$) and mROS production as normoxic PSMCs ($n = 100$ cells per group). * $P < 0.05$ versus normoxic control using ANOVA with Tukey's post hoc analysis. (B) *Nogo*^{+/+}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had lower outward K^+ current density compared to normoxic controls, measured by whole-cell patch clamping. The K^+ current in normoxic *Nogo*^{+/+} and *Nogo*^{-/-} PSMCs was sensitive to 4-aminopyridine (4-AP) ($n = 8$ to 10 cells per group). * $P < 0.05$ versus normoxia using ANOVA with FLSD post hoc analysis. Mean data are shown as current density (pA/pF) over membrane potential (mV). (C) *Nogo*^{+/+} and *Nogo*^{+/-} PSMCs exposed to hypoxia and phenylephrine had increased intracellular Ca^{2+} , as assessed by

FLUO-3, compared to normoxic controls., *Nogo*^{-/-} PSMCs showed increased intracellular Ca²⁺ to phenylephrine but not hypoxia (*n*= 100 cells per group). **P* < 0.05 versus normoxic control using ANOVA with Tukey's post hoc analysis. (D) *Nogo*^{+/+} PSMCs exposed to hypoxia had increased nuclear HIF-1α (red) and Nogo-B (green) levels, as shown by immunofluorescence. HIF-1α colocalization with the nuclear stain DAPI (blue) is shown in pink in the merged panel. Nuclear HIF-1α and Nogo-B levels were decreased by the ATF6 inhibitor AEBSF in hypoxia-treated *Nogo*^{+/+} PSMCs., *Nogo*^{-/-} PSMCs exposed to hypoxia had similar HIF-1α levels compared to normoxic controls and no detectable Nogo-B levels (*n* = 70 cells per group). **P* < 0.05 versus normoxia; #*P* < 0.05 versus hypoxia using ANOVA with Tukey's post hoc analysis. Mean data are presented in AFUs.

Fig. 6.

Nogo-B levels correlate positively with the degree of proliferation under hypoxia and the degree of resistance to apoptosis under starving conditions. (A) *Nogo*^{+/+} and *Nogo*^{+/-}, but not *Nogo*^{-/-}, PSMCs exposed to hypoxia had increased proliferation (% PCNA-positive cells) compared to normoxic controls using immunofluorescence microscopy ($n = 100$ cells per group). * $P < 0.05$ using ANOVA with Tukey's post hoc analysis. (B) *Nogo*^{-/-}, but not *Nogo*^{+/+} and *Nogo*^{+/-}, PSMCs exposed to 0.1% serum had increased apoptosis (% TUNEL-positive cells) compared to controls treated with 10% serum using immunofluorescence microscopy ($n = 100$ cells per group). * $P < 0.05$ versus controls, using

ANOVA with Tukey's post hoc analysis. (C) *Nogo*^{+/+} and *Nogo*^{+/-}, but not *Nogo*^{-/-}, mice exposed to chronic hypoxia had increased cell proliferation in resistance PAs compared to normoxic controls, as shown by % PCNA-positive cells in resistance PAs, using immunofluorescence microscopy ($n = 100$ cells from 15 PAs per group). $*P < 0.05$ versus normoxic control using ANOVA with Tukey's post hoc analysis. (D) *Nogo*^{+/+} and *Nogo*^{+/-}, but not *Nogo*^{-/-}, mice exposed to chronic hypoxia had increased % media wall thickness in resistance PAs ($< 300 \mu\text{m}$) compared to normoxic controls, as assessed by hematoxylin and eosin staining and light microscopy. These lungs were obtained from the same mice that underwent hemodynamic assessment in Fig. 2 ($n = 40$ PAs per group). $*P < 0.05$ versus normoxic controls, using ANOVA with Tukey's post hoc analysis. (E) A schematic diagram showing how Nogo links ER stress to mitochondrial function. Many PAH-associated triggers, including hypoxia, bone morphogenetic protein receptor II (BMPRII) mutations, viruses, inflammation, or induction of Notch, are known triggers of endoplasmic reticulum (ER) stress. ER stress activates ATF6 in a pulmonary circulation-specific manner, which increases Nogo expression. This in turn disrupts the mitochondria-ER unit, resulting in a suppression of mitochondrial function, explaining the metabolic remodeling and several molecular responses in PAH. IDH, isocitrate dehydrogenase; αKG , α -ketoglutarate; mROS, mitochondrial-reactive oxygen species; MTP, mitochondrial transition pore; NFAT, nuclear factor of activated T cells; PDH, pyruvate dehydrogenase.