An Abnormal Mitochondrial–Hypoxia Inducible Factor-1α–Kv Channel Pathway Disrupts Oxygen Sensing and Triggers Pulmonary Arterial Hypertension in Fawn Hooded Rats

Similarities to Human Pulmonary Arterial Hypertension

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Background—The cause of pulmonary arterial hypertension (PAH) was investigated in humans and fawn hooded rats (FHR), a spontaneously pulmonary hypertensive strain.

Methods and Results—Serial Doppler echocardiograms and cardiac catheterizations were performed in FHR and FHR/BN1, a consomic control that is genetically identical except for introgression of chromosome 1. PAH began after 20 weeks of age, causing death by ≈60 weeks. FHR/BN1 did not develop PAH. FHR pulmonary arterial smooth muscle cells (PASMCs) had a rarified reticulum of hyperpolarized mitochondria with reduced expression of electron transport chain components and superoxide dismutase-2. These mitochondrial abnormalities preceded PAH and persisted in culture. Depressed mitochondrial reactive oxygen species (ROS) production caused normoxic activation of hypoxia inducible factor (HIF-1α), which then inhibited expression of oxygen-sensitive, voltage-gated K^+ channels (eg, Kv1.5). Disruption of this mitochondrial-HIF-Kv pathway impaired oxygen sensing (reducing hypoxic pulmonary vasoconstriction, causing polycythemia), analogous to the pathophysiology of chronically hypoxic Sprague-Dawley rats. Restoring ROS (exogenous H_2O_2) or blocking HIF-1α activation (dominant-negative HIF-1α) restored Kv1.5 expression/function. Dichloroacetate, a mitochondrial pyruvate dehydrogenase kinase inhibitor, corrected the mitochondrial-HIF-Kv pathway in FHR-PAH and human PAH PASMCs. Oral dichloroacetate regressed FHR-PAH and polycythemia, increasing survival. Chromosome 1 genes that were dysregulated in FHRs and relevant to the mitochondria-HIF-Kv pathway included HIF-3α (an HIF-1α repressor), mitochondrial cytochrome c oxidase, and superoxide dismutase-2. Like FHRs, human PAH-PASMCs had dysmorphic, hyperpolarized mitochondria; normoxic HIF-1α activation; and reduced expression/activity of HIF-3α, cytochrome c oxidase, and superoxide dismutase-2.

Conclusions—FHRs have a chromosome 1 abnormality that disrupts a mitochondria-ROS-HIF-Kv pathway, leading to PAH. Similar abnormalities occur in idiopathic human PAH. This study reveals an intersection between oxygen-sensing mechanisms and PAH. The mitochondria-ROS-HIF-Kv pathway offers new targets for PAH therapy. **(*Circulation*. 2006;113:2630-2641.)**

Key Words free radicals ■ hypoxia ■ ion channels ■ mitochondrial membranes

Pulmonary arterial hypertension (PAH) is a disease of small pulmonary arteries characterized by intimal hyperplasia, medial hypertrophy, a thickened adventitia, and endothelial proliferative plexiform lesions. PAH can occur in rare idiopathic and familial forms, but it is most commonly associated with connective tissue diseases, anorexigen use, HIV, or congenital heart disease. PAH typically appears in the third to fifth decade and has high mortality rates (≈50% at 5 years).[1] The endothelium is dysfunctional in PAH. An early proapoptotic endothelial insult may promote PAH by

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damaging normal endothelium, thereby selecting apoptosis-resistant clones that ultimately form plexiform lesions.[2] In the media, impaired apoptosis and excessive proliferation of pulmonary arterial smooth muscle cells (PASMCs) result from decreased expression of voltage-gated potassium channels (Kv 1.5[3,4]), de novo expression of the apoptosis inhibitor survivin,[2,5] and increased expression of the serotonin transporter.[6] In the adventitia, disordered matrix remodeling[7] and transition of fibroblasts into myofibroblasts[8] may contribute to pathological remodeling. The discovery of bone morphogenetic receptor-2 mutations in >50% of familial PAH patients raised hope that the cause for PAH had been revealed. However, it is now clear that bone morphogenetic receptor-2 mutations, which enhance proliferation of PASMCs,[9] occur in only 10% of nonfamilial PAH, and mutation carriers have only a 20% lifetime risk of developing PAH.[10] Thus, bone morphogenetic receptor-2 mutations are neither necessary nor sufficient to cause many cases of idiopathic PAH.[10]

Clinical Perspective p 2641

We chose to study PAH in fawn hooded rats (FHRs), a strain in which PAH occurs spontaneously.[11] FHRs are somewhat an equivalent of Hermansky-Pudlak syndrome, an autosomal recessive disease caused by defective biogenesis of multiple cytoplasmic organelles and granules and characterized by oculocutaneous albinism, pulmonary fibrosis, and a platelet storage pool disorder.[12] As in humans, FHR-PAH is associated with increased endothelin levels,[13] enhanced serotonin-induced vasoconstriction, a platelet storage pool deficiency, and excessive PASMC proliferation.[9] Our study was facilitated by the availability of consomic FHR control rats in which a chromosome 1 was introgressed from Brown-Norway rats.[14] These FHR-BN1 rats lack platelet and vascular abnormalities[15] and, in preliminary data, were free of PAH, suggesting that FHR-PAH is initiated by abnormal chromosome 1 genes. Another clue to the origin of FHR-PAH is the known "hypoxia sensitivity" of FHR, meaning that they develop PAH and alveolar simplification in response to mild hypoxia.[11,16] Although FHR-PAH is reduced by supplemental oxygen,[16,17] it occurs despite normal Pa_{O_2},[11] suggesting a defect in the pulmonary vascular oxygen sensor.[18]

The pulmonary circulation has a unique redox-based mitochondrial oxygen sensor.[19] During normoxic respiration, unpaired electron transport in mitochondrial complexes I and III results in a small production of superoxide radicals, produced in proportion to alveolar Pa_{O_2}.[20] Superoxide, a potentially toxic reactive oxygen species (ROS), is converted by superoxide dismutase-2 (SOD-2), an enzyme found only in mitochondria, to a diffusible second messenger, hydrogen peroxide (H_2O_2). H_2O_2 regulates activity of redox-sensitive transcription factors (eg, hypoxia-inducible factor [HIF-1α][21]) and the activation and expression of Kv channels (eg, Kv1.5).[22] Within seconds of hypoxia (Pa_{O_2}, 40 to 70 mm Hg), decreased PASMC mitochondrial ROS production inhibits oxygen-sensitive Kv channels, causing membrane depolarization, activation of voltage-gated L-type calcium channels, and calcium influx, thereby initiating hypoxic pulmonary vasoconstriction.[20] When atmospheric hypoxia is maintained chronically, hypoxic pulmonary vasoconstriction is depressed as a result of both alterations of the mitochondrial sensor[3] and decreased expression of oxygen-sensitive Kv channels (eg, Kv1.5, Kv2.1[3,22,23]). Human and experimental PAH-PASMCs are deficient in Kv1.5; consequently, they have depolarized membrane potentials that activate voltage-gated L-type calcium channels.[3,24] The resulting increase in cytosolic calcium boosts cell proliferation, whereas elevated K^+ concentrations inhibit proapoptotic caspases, suppressing apoptosis.[2,5,25,26]

Mitochondria are further implicated in PAH because hyperpolarization of PASMC mitochondrial membrane potential ($\Delta\Psi m$) occurs in experimental PAH.[5,26,27] These acquired mitochondrial abnormalities appear to be pathogenetically relevant because returning $\Delta\Psi m$ to depolarized potentials through the use of dichloroacetate restores Kv expression, enhances apoptosis, and reverses PAH.[26,27] Dichloroacetate, a prototypic mitochondrial pyruvate dehydrogenase kinase inhibitor, promotes glucose oxidation, increases mitochondrial NADH, and favors increased mitochondrial electron flux, thereby restoring ROS production.

We hypothesized that FHRs have an inherited mitochondrial dysfunction that creates a low-ROS environment, which constitutes an inappropriate "hypoxic signal" and disrupts oxygen sensing. This activates the hypoxic transcriptome with consequences similar to those elicited by chronic atmospheric hypoxia (eg, polycythemia and suppressed hypoxic pulmonary vasoconstriction).[27] This hypothesis also was tested in small arteries and PASMCs obtained from humans with PAH.

Methods

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

In Vivo Experiments

The Animal Policy and Welfare Committee approved the procedures and protocols. Rats were obtained from local colonies and Charles Rivers Laboratories. The natural history of FHR-PAH in normoxia and chronic hypoxia (0.5 atm for 4 weeks[27]) was established by comparison with 2 age-matched control groups of male rats (Sprague-Dawley and FHR/BN1 consomic rats, n=8 per group). Pulmonary artery acceleration time, a validated measure of mean pulmonary arterial pressure in rodents,[5,26] cardiac output, total pulmonary resistance, and right ventricular hypertrophy were serially assessed in anesthetized rats with 2D and Doppler echocardiography.[26] At 40 weeks, invasive catheterization of the carotid artery and pulmonary artery was performed in anesthetized, closed-chest rats (Fi_{O_2}=0.4) with 1.4F Millar catheters (Millar Instruments, Houston, Tex)[22] (n=5 to 12 per group). Cardiac output was measured with a validated thermodilution technique.[26] Hemodynamics and gene and protein expression in resistance pulmonary arteries were studied early (12 weeks), just before PAH (20 weeks), and after establishment of PAH (40 weeks). In PAH regression studies, FHRs with proven PAH were randomized to receive 7 weeks of dichloroacetate at a dose used in humans with mitochondrial disease[26] (0.75 g/L drinking water) or plain water.

Right ventricle hypertrophy was assessed postmortem as the weight ratio of the right ventricle to the left ventricle plus septum.

Medial Pulmonary Artery Thickness

Medial thickness of small (<100 μmol/L) arteries was expressed as a percent of vessel external diameter.[22]

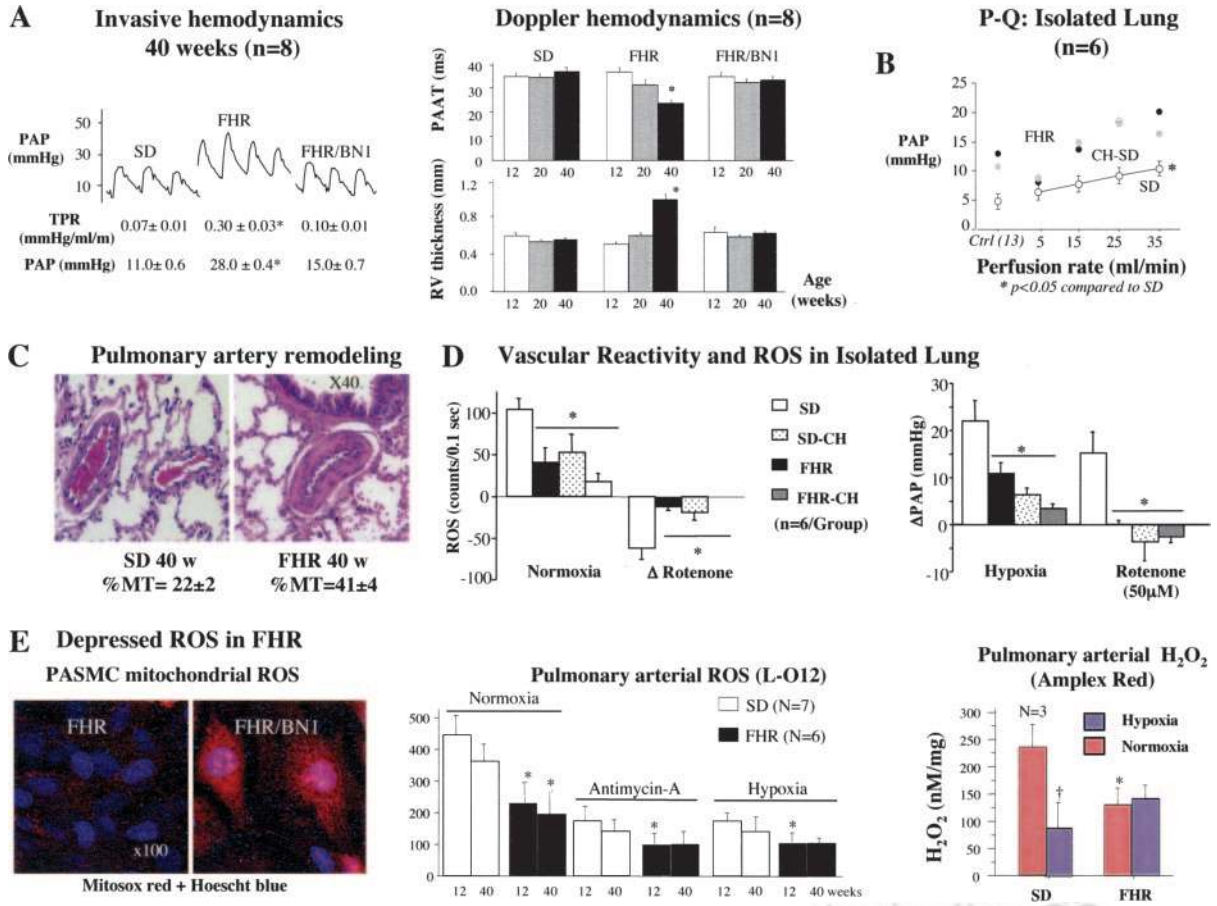

Figure 1. Early loss of ROS precedes spontaneous PAH in FHRs. A, Invasive measurements of pulmonary artery pressure (n=8) confirm PAH in 40-week-old FHRs. Serial assessment of pulmonary artery acceleration time and right ventricle (RV) thickness demonstrates the natural history of PAH. Intergroup differences are absent before 20 weeks of age. B, A left-shifted pressure-flow relationship and cardiac catheterization confirm PAH in FHRs and chronically hypoxic Sprague-Dawley rats. C, Lung histology reveals that 40-week-old FHRs have increased medial hypertrophy of small pulmonary arteries vs 40-week-old Sprague-Dawley rats (*$P<0.05$). D, Hypoxic and rotenone vasoconstriction and lung ROS levels (luminol) are similarly impaired in normoxic FHRs and chronically hypoxic Sprague-Dawley rats vs normoxic Sprague-Dawley rats (*$P<0.05$). E, Normoxic ROS and H_2O_2 production in FHR PASMCs and isolated resistance pulmonary arteries is decreased and sensitivity to the electron transport chain inhibitor antimycin A and hypoxia is attenuated vs Sprague-Dawley rats (*$P<0.05$).

Electrophysiology

PASMCs were enzymatically dispersed from freshly isolated resistance pulmonary arteries (300- to 500-μm diameter). Current density was measured in the whole-cell, voltage-clamp configuration as described.[22]

Isolated Lung Perfusion

ROS and pulmonary vascular resistance were measured simultaneously in isolated, perfused rat lungs. See the online Data Supplement for details.[28]

ROS Production

To avoid the limitations of luminal (redox cycling and lack of ROS specificity) and to directly assess arterial ROS production, resistance pulmonary arteries were assayed with L-012, a superoxide-sensitive, chemiluminescence enhancer that is not subject to redox cycling[29] (100 μmol/L for 30 minutes at 37°C) (Wako Chemicals, Richmond, Va). Pulmonary artery H_2O_2 production was measured by Amplex Red (Molecular Probes).[20] PASMC mitochondrial superoxide production was measured in primary cultured cells with Mitosox (Molecular Probes, Eugene, Ore), a cell-permeable ethidium bromide derivative that is selectively targeted to the mitochondria, where superoxide-mediated oxidation causes red fluorescence (excitation/emission, 510/580 nm).

SOD activity was measured in cultured cells (passages 1 through 5) in triplicate with a colorimetric assay following the manufacturer's instructions (Dojindo, Gaithersburg, Md). To measure SOD2 activity, Cu/Zn-SOD activity was blocked with 1 mmol/L potassium cyanide.

Immunoblotting

Immunoblotting was performed on 25 μg of protein pooled from resistance pulmonary arteries (n≥4 per group) as described.[27] Expression was normalized to smooth muscle actin.

SEM of Lung Vascular Casts

To measure lung vascularity, Mercox casts were created, coated with gold sputter, and imaged with SEM as described.[30]

Human PAH and Control Tissues

The Health Research Ethics Board at the University of Alberta approved this protocol. Cultures of resistance PASMCs from an idiopathic PAH (iPAH) patient (passages 1 through 5) and a lung donor were obtained from surgical specimens at the time of transplantation. Formaldehyde-fixed, paraffin-embedded lung sections were obtained from 8 PAH and 7 control lobectomy patients (Data Supplement Figure I).

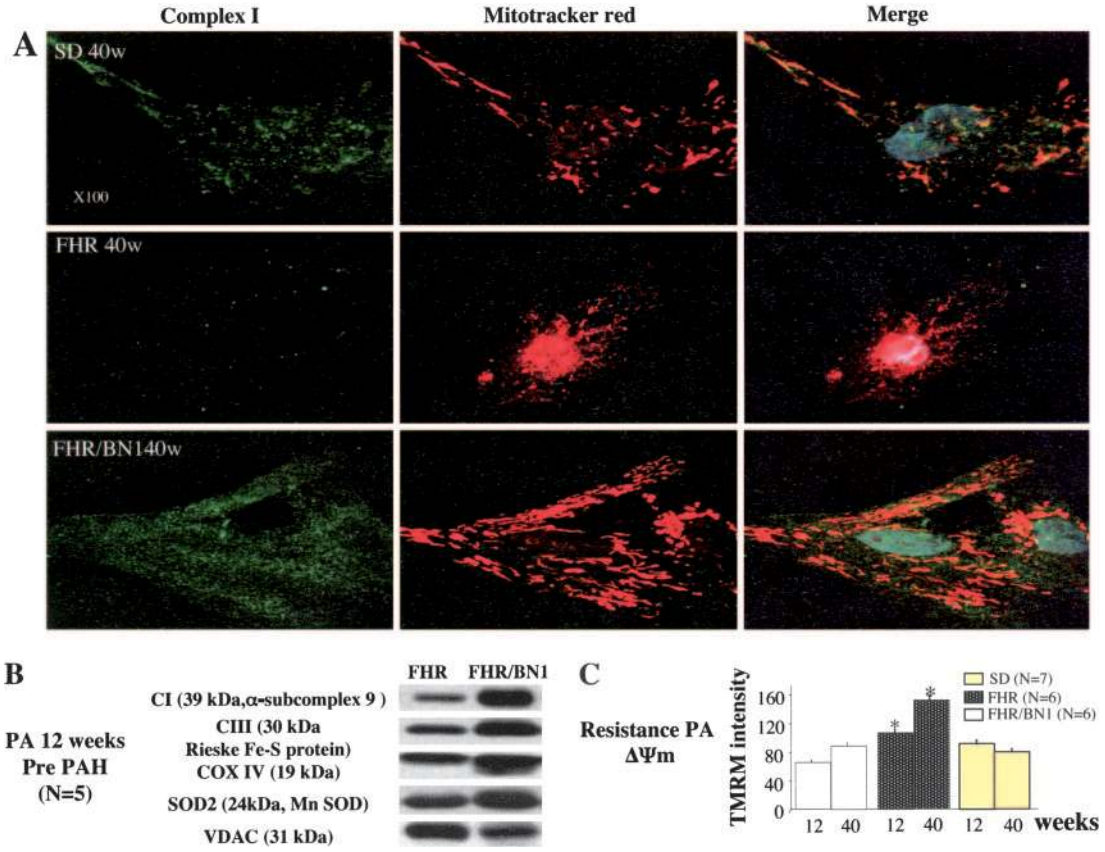

Figure 2. FHRs have abnormal mitochondria before PAH. A, FHR PASMCs express less electron transport chain complex I and have fewer mitochondria arrayed in a less organized reticulum than FHR/BN1 PASMCs. B, Expression of electron transport chain complexes and SOD2 is decreased in FHR pulmonary arteries before PAH. Conversely, voltage-dependent anion channel expression is increased in FHRs, suggesting a preserved or increased number of mitochondria in FHR PASMCs. C, ΔΨm is hyperpolarized in freshly isolated FHR resistance pulmonary arteries before PAH (12-weeks) vs FHR/BN1 and Sprague-Dawley rats (*$P<0.05$).

Immunofluorescence

Human lung sections were processed using microwave antigen retrieval in citrate buffer. Details are given in the online Data Supplement.

Quantitative RT-PCR

RNA was extracted from cells or tissues, and mRNA was measured with TaqMan probes and RT-PCR reagents using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, Calif) as described.[31]

In Silico Analysis of Hypoxic Response Element on Kv1.5

Analysis of the 5′ untranslated region of Kv1.5 (GenBank accession number NM002234) for putative hypoxia response elements (5′. . .(R)CGTG. . .3)′ was performed with DNA strider 1.2 (R signifies a purine [A or G]).

Cytosolic Calcium

Cultured PASMCs (passages 1 through 5) were loaded with Fluo-4-AM (1 μmol/L in DMSO/pluronic for 1 hour at 37°C), placed in medium containing 1 mmol/L probenecid to inhibit probe leakage, and then incubated at 37°C for 30 minutes. Cells were excited at 488 nm (emission, 505 to 530 nm).

ΔΨm was measured in fresh tissues and PASMC cultures (passages 1 through 5) by loading with the potentiometric dye tetramethylrhodamine methyl-ester perchlorate (TMRM, 20 nmol/L; excitation, 543 nm; emission, 565 to 615 nm; Molecular Probes). Red emission increases with mitochondrial hyperpolarization.[5,26]

HIF-1α Dominant-Negative Virus (Adv-HIF-1α DN)

The HIF-1α dominant-negative cDNA is a deletion mutant lacking a DNA-binding domain, transactivation domains, and an oxygen-dependent degradation domain.[32] See the online Data Supplement for details.

Cell Cultures

A description of cell culture techniques is provided in the online Data Supplement.

Statistical Analysis

Values are expressed as mean±SEM. Intergroup differences were assessed by a simple ANOVA with post hoc analysis using Fisher's probable least-significant-difference test. For variables measured serially, a 2-way ANOVA was used, and testing for a group-by-time interaction was performed. Each group had ≥5 animal (unless otherwise specified), and the animal (not the number of vessels or cells) was the unit of analysis. Kaplan-Meier analysis was used to estimate survival probabilities; log-rank testing was used to evaluate equality of survival curves. A value of $P<0.05$ was considered statistically significant.

Results

Hemodynamics

FHRs have normal pulmonary artery pressure until 20 weeks, but by 40 weeks, despite normal systemic blood pressure, cardiac output, and Pa_{O_2} (Data Supplement Table I), they

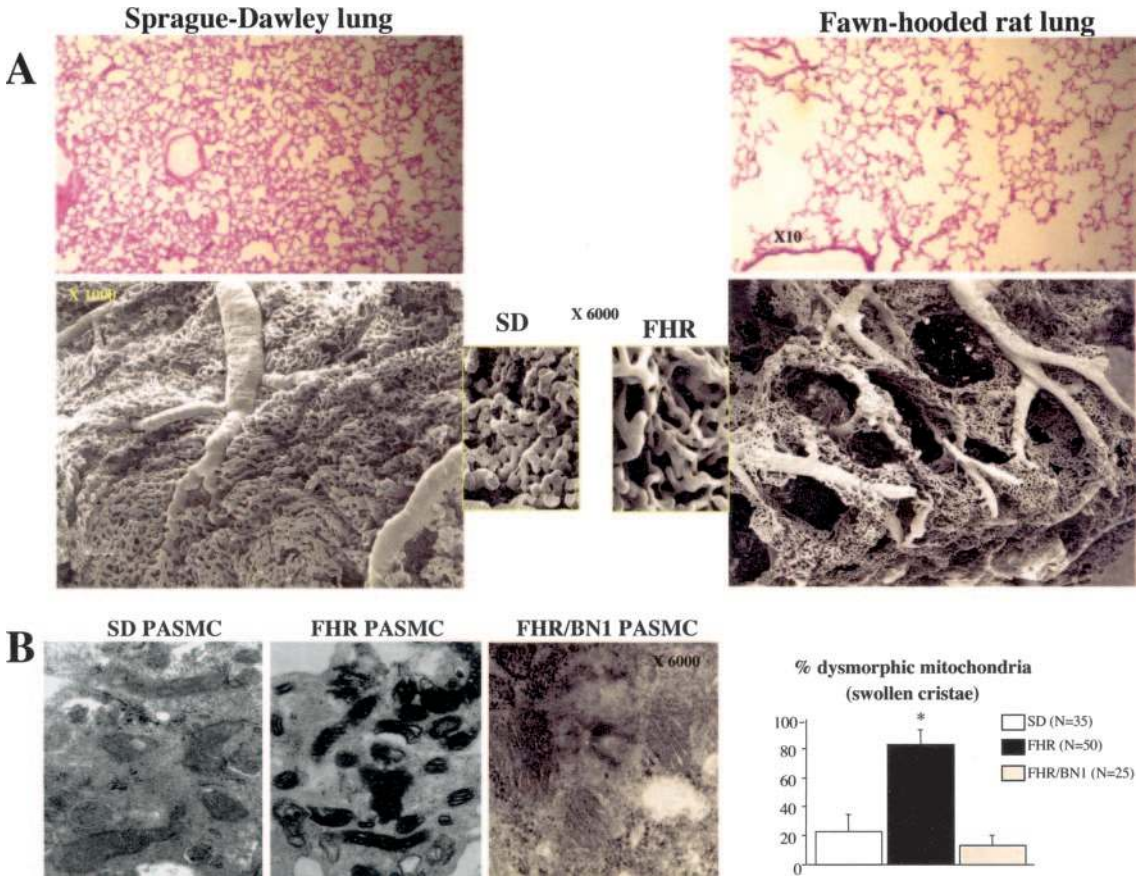

Figure 3. FHRs have alveolar simplification and a dysmorphic mitochondria. A, Light microscopy of lung sections and SEM of Mercox casts reveal alveolar simplification (large air sacs, decreased septation) and a reduced lung capillary network in 40-week-old FHRs. B, Transmission electron microscopy reveals that FHR PASMCs have small, dense, dysmorphic mitochondria vs FHR/BN1 and Sprague-Dawley rats (*$P<0.05$).

manifest PAH and right ventricular hypertrophy. Age-matched FHR/BN1 rats did not develop PAH (Figure 1A). Normoxic FHRs have a leftward shift in their pulmonary vascular pressure–flow relationship, similar to Sprague-Dawley rats exposed to chronic hypoxia (Figure 1B), and similarly have medial hypertrophy of resistance pulmonary arteries (Figure 1C).

Simultaneous measurement of pulmonary artery pressure and ROS production in isolated lungs of normoxic FHRs and chronically hypoxic Sprague-Dawley rats showed a parallel suppression of constriction (to hypoxia and rotenone) and ROS production (Figure 1D). Moreover, both groups lost the hypoxia- and rotenone-sensitive components of total ROS production. The loss of superoxide (total and hypoxia-sensitive components) was not the result of PAH because it also was evident in cultured FHR PASMCs and isolated resistance pulmonary arteries from normotensive FHRs (12 weeks) (Figure 1E). H_2O_2 production also was reduced in FHR pulmonary arteries, and hypoxia failed to inhibit H_2O_2 production (Figure 1E).

FHR Have Dysmorphic, Hyperpolarized PASMC Mitochondria

The normal filamentous mitochondrial reticulum of the PASMCs was disrupted and rarefied in FHR (fewer mitochondria per unit area; Figure 2A). Expression of electron transport chain complexes I and III and COX4 was decreased in FHRs before PAH; conversely, mitochondrial voltage-dependent anion channel was increased, suggesting that there was not a generalized loss of mitochondria (Figure 2B). Expression of SOD2, an intramitochondrial antioxidant enzyme encoded on chromosome 1, was decreased in FHR PASMCs (Figure 2B), likely explaining their low H_2O_2 production (Figure 1E). Loss of electron transport chain complexes (particularly complex I) and mitochondrial hyperpolarization were evident before PAH and may have contributed to the low ROS production (Figure 2C). These abnormalities persist in culture, consistent with a genetic basis for FHR-PAH (Figure 3B and 4).

FHRs Have Alveolar Simplification

FHRs have alveolar simplification and decreased capillary density (Figure 3A). The PASMC mitochondria in FHR PASMCs are small, dense, and dysmorphic (Figure 3B). Mitochondrial hyperpolarization is evident in other organs of young normotensive FHRs (Data Supplement Figure I).

Recapitulation of the FHR Mitochondrial Abnormalities in Human PAH PASMCs

Small pulmonary arteries from PAH patients have reduced expression of mitochondrial electron transport chain complex

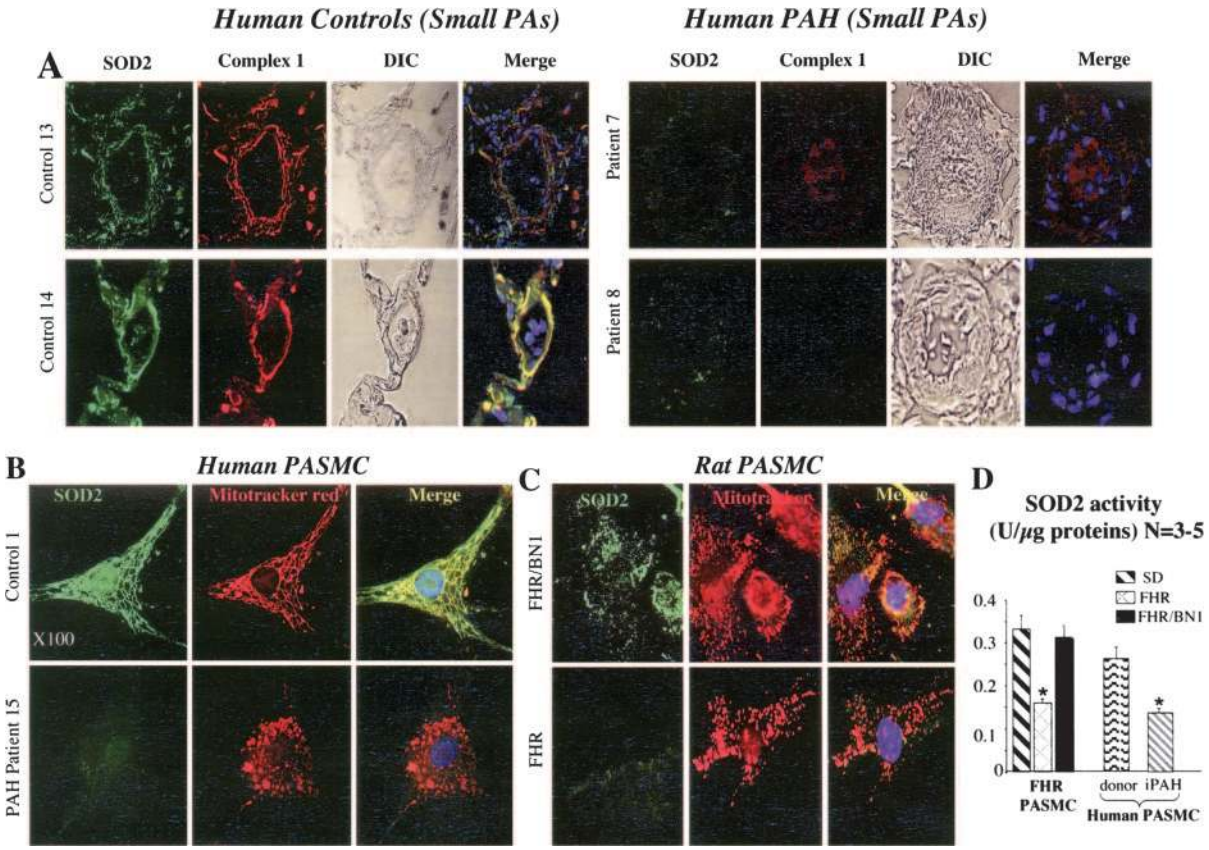

Figure 4. A structural and functional mitochondrial defect in human PAH PASMCs. A, Representative images reflecting findings in 7 controls and 8 PAH patients. SOD2 (green) and complex I (red) expression is decreased in PAH patients vs control subjects. B, C, Cultured PASMCs from both humans (B) and rats (C) show that human PAH and rat FHR PASMCs have a rarefied, disrupted network of dysmorphic mitochondria with diminished localization of SOD2 to the mitochondria. D, SOD2 activity is significantly decreased in FHR and human PAH PASMCs vs control rats and patients, respectively (*$P<0.05$).

I and are deficient in SOD2 expression/activity (patient details are given in Data Supplement Table II), similar to what occurs in FHR pulmonary arteries and PASMCs (Figure 2). Mitochondria in normal human and rat PASMCs form an intricate, filamentous network, in which SOD2 and Mitotracker Red are tightly colocalized. Conversely, in FHRPAH and human PAH, PASMC colocalization of SOD2 and Mitotracker Red is lost, and the mitochondrial reticulum is disrupted (Figure 4B and 4C). The decrease in SOD2 expression in both human iPAH patients and FHR is associated with a reduction in SOD2 activity (Figure 4D). Thus, before PAH, FHR PASMCs have defective mitochondrial structure/function, which creates a low-ROS environment (Figure 1E).

Normoxic HIF-1α Activation Decreases PASMC Kv Channel Expression

HIF activation and Kv channel suppression are seen in FHR only after 20 weeks, in apparent response to the earlier change in the mitochondrial redox environment. HIF-1α activation was increased at 40 weeks in FHRs compared with FHR-BN1 rats (Figure 5A). Over this period, there was a concomitant decrease in pathways that normally repress HIF activation, including the HIF-destabilizing enzyme proline dehydroxylase-1 (HPH-1) and the HIF-1α repressor HIF-3α (Figure 5B). HIF-3α, formerly called inhibitory PAS domain protein,[33] is encoded on chromosome 1 and was decreased in human PAH (Figure 5C). More impressive than the change in total HIF-1α expression (data not shown) was increased HIF-1α nuclear translocation, a marker of HIF activation.[34] Normoxic HIF activation was almost universal in both cultured FHR (Figure 5A) and human small pulmonary arteries from PAH patients (Figure 5C). As a positive control for PASMC HIF activation, Sprague-Dawley rat PASMCs were exposed to hypoxia (5% O_2 during 48 hours). This created the same nuclear translocation of HIF-1α seen in normoxic FHR PASMCs (Figure 5D). In FHR PASMCs, HIF activation and Kv1.5 downregulation were reversed by hyperoxia (95% O_2 for 48 hours), consistent with a left shift in the oxygen sensing of FHRs (Figure 5D). These data suggest that HIF-1α activation accounts for the downregulation of Kv1.5. This is definitively proved by the finding that inhibition of HIF-1 with a HIF-α dominant-negative adenovirus restores Kv1.5 expression in FHR PASMCs (Figure 6D). To establish whether normoxic HIF activation was a consequence of a low-ROS environment, exogenous H_2O_2 was administered to FHR PASMCs. Restoring ROS reversed the nuclear translocation of HIF-1α and increased Kv1.5 expression (Figure 5E).

As FHR-PAH evolved (weeks 20 to 40), there was a concordant decrease in PASMC K^+ current density (Figure 6A) and expression of oxygen-sensitive Kv channels (Kv1.5

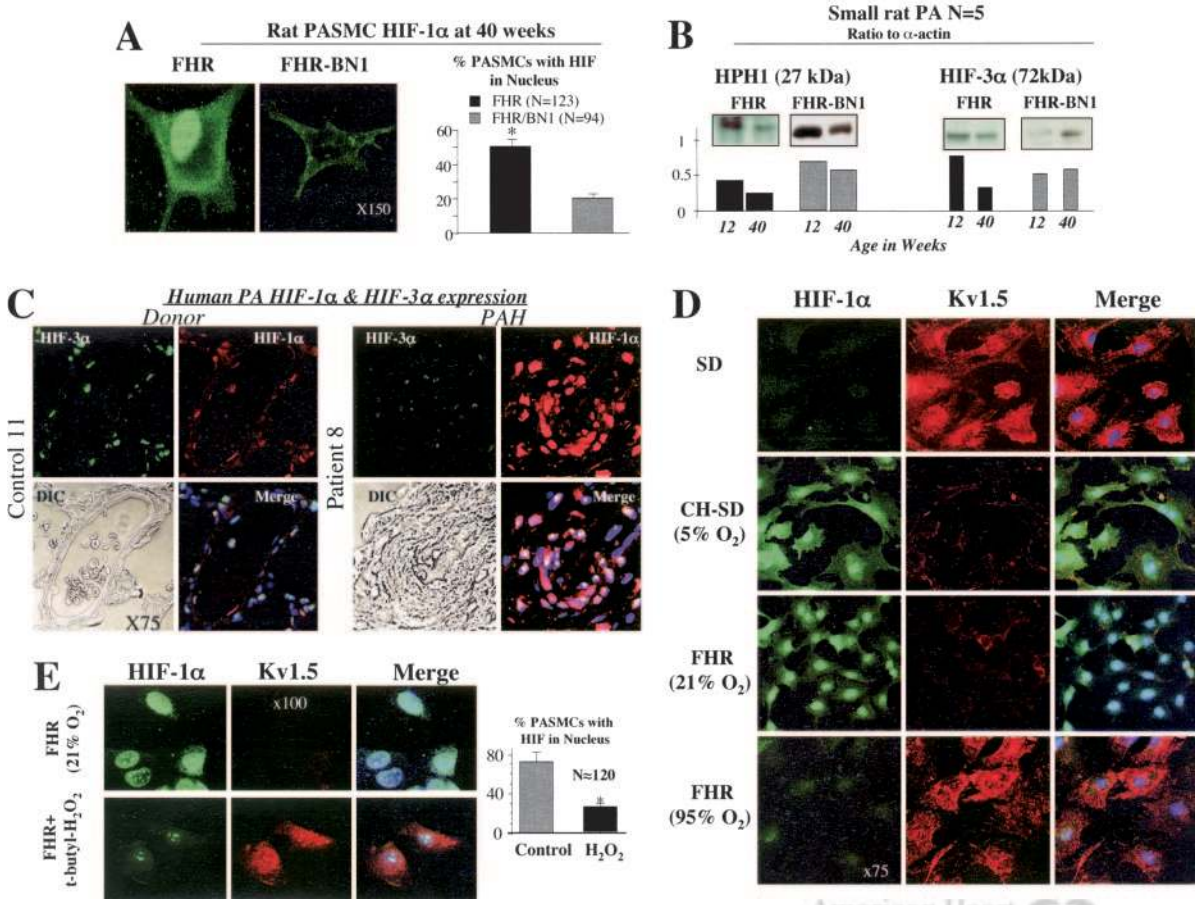

Figure 5. Normoxic HIF-1α activation in FHR and human PAH. A, HIF-1α activation in FHR is confirmed by its translocation to the nucleus. B, There is loss of HIF repression in FHR pulmonary arteries at 40 weeks evidenced by decreased expression of HPH-1 and HIF-3α. C, Decreased HIF-3α expression (green) and increased nuclear HIF-1α (red) in human PAH (image representative of findings in 4 PAH and 4 control patients). D, Sprague-Dawley PASMCs were exposed to chronic hypoxia for 48 hours to create a positive control for HIF-1α activation. The resulting nuclear translocation of HIF-1α and accompanying Kv1.5 downregulation were recapitulated in normoxic FHR PASMCs. Hyperoxia reversed the FHRs' abnormality, consistent with a left shift in their oxygen sensor. E, t-butyl H_2O_2 (100 μmol/L for 48 hours) inhibits HIF-1α activation and restores Kv1.5 expression in FHR PASMCs.

and Kv3.1b) (Figure 6B). Loss of K^+ channel function/expression caused FHR PASMC depolarization and increased cytosolic calcium (Figure 6C). To assess the putative contribution of normoxic HIF activation to Kv channel downregulation, an HIF-1α dominant-negative construct, delivered via a replication deficient adenovirus, was administered to FHR PASMCs. Inhibiting HIF-1α restored Kv1.5 expression, increased PASMC Kv current (that portion sensitive to 4-aminopyridine, a Kv channel blocker), and repolarized membrane potential. This indicates that HIF-1α activation is the major cause of Kv downregulation (Figure 6D).

Dichloroacetate Improves FHR Survival

Oral dichloroacetate therapy reduced established FHR-PAH and improved survival (Figure 7A through 7C). In vivo, dichloroacetate depolarized PASMC $\Delta\Psi$m; in vitro, FHR PASMC dichloroacetate (48 hour) restored Kv1.5 expression (Figure 7D and 7E, respectively). Likewise, in PASMC culture, dichloroacetate rapidly reversed the "hypoxic" phenotype of FHR, increasing SOD activity, eliminating nuclear HIF-1α translocation, and restoring Kv1.5 mRNA and protein expression (Figure 7F through 7H). Because dichloroacetate is a prototypic inhibitor of the mitochondrial enzyme pyruvate dehydrogenase kinase,[35] these data are consistent with the primacy of the mitochondria in FHR-PAH. In human PAH and FHR-PAH PASMCs, dichloroacetate improved mitochondrial function, depolarizing the abnormally hyperpolarized $\Delta\Psi$m and increasing SOD activity by 32% (Figure 7). Rotenone partially inhibited dichloroacetate-induced $\Delta\Psi$m depolarization, consistent with a key rule for complex I (Data Supplement Figure II).

Discussion

We report here for the first time that PAH can result from disruption of a mitochondrial pathway that is normally used in oxygen sensing. In FHRs, mitochondrial dysfunction resulting from a genetic abnormality on chromosome 1 is the first detectable abnormality (Figure 8). The FHR hyperpolarized, dysmorphic mitochondria are deficient in components of several electron transport complexes, particularly complex I and SOD2. The net result of these abnormalities is reduced total ROS production and inability to vary ROS production in proportion to

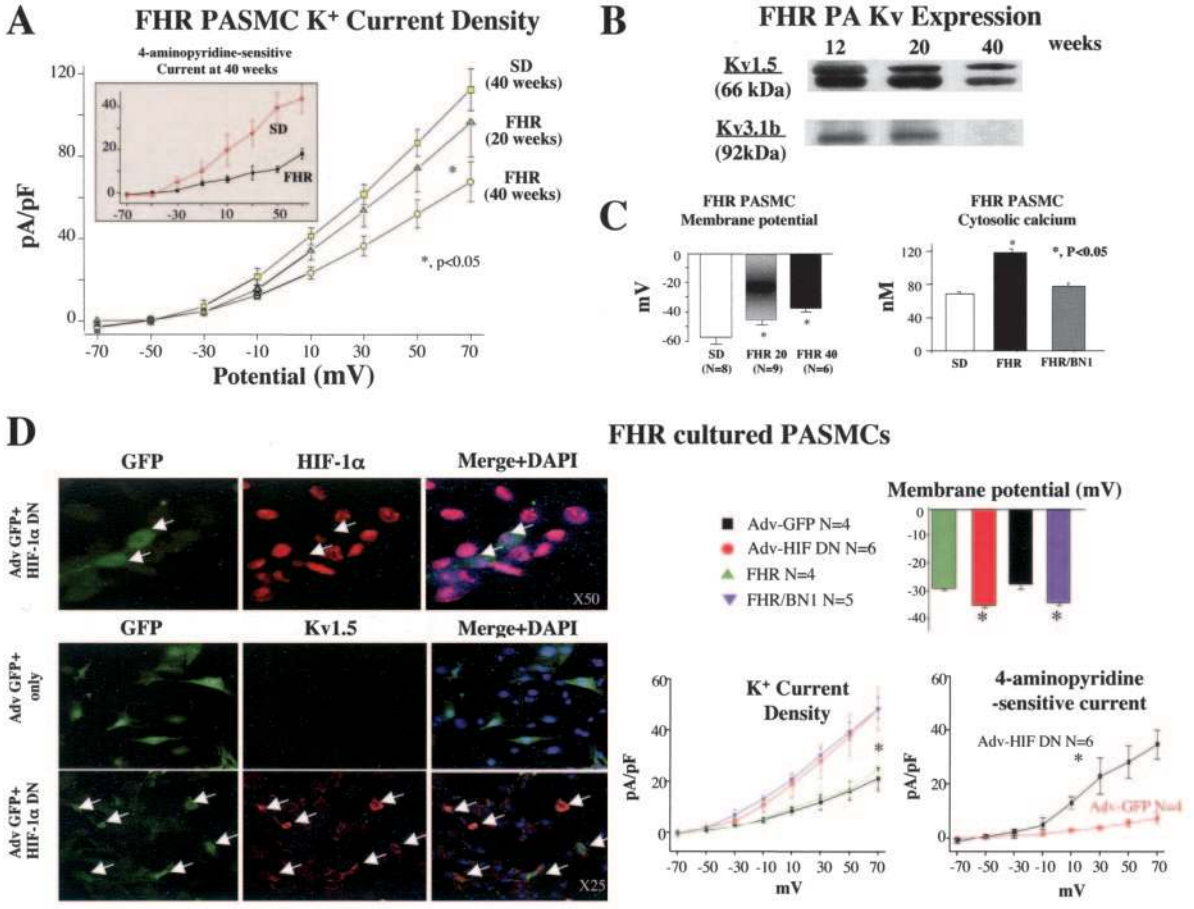

Figure 6. HIF-1α activation decreases K^+ current density and depolarizes FHR PASMCs, increasing cytosolic calcium. A, B, Mean current-voltage relationship in freshly isolated FHR PASMCs (*$P<0.05$). As PAH develops, FHR PASMCs progressively lose total and 4-aminopyridine–sensitive Kv current (inset) (*$P<0.05$) in parallel with a decrease in the expression of Kv1.5 and Kv3.1b. C, This depolarizes membrane potential and increases cytosolic calcium vs FHR/BN1 and Sprague-Dawley rats (*$P<0.05$). D, Direct inhibition of HIF-1α using a dominant-negative construct with a GFP reporter restores K^+ current density by increasing 4-aminopyridine–sensitive current vs GFP alone (*$P<0.05$). This restores the membrane potential of cultured FHR PASMCs to values similar to FHR/BN1 PASMCs. The "GFP-only" vector had no effect.

P_{O_2} (Figures 1 and 2). Loss of these ROS second messengers creates a "hypoxia-like" redox milieu, reminiscent of that seen in chronically hypoxic Sprague-Dawley rats, even to the extent of suppressing acute hypoxic pulmonary vasoconstriction and eliciting mild polycythemia (Data Supplement Table I). The low-ROS state of the FHRs activates the master transcription factor HIF-1α. We hypothesize that HIF-1α activation could inhibit Kv1.5 expression through the binding of HIF-1α to a putative hypoxic response element (ACGTG) that we found at position -1208 to 1203 within the 5′-untranslated region of Kv1.5 (Figure 8). This element is similar in sequence and gene proximity to hypoxic response elements for other HIF-regulated genes.[36] Humans with PAH have a similar mitochondrial abnormality, and their PASMC mitochondria are deficient in electron transport chain complex I and SOD2 (Figures 2 and 4). As in FHRs, this results in normoxic HIF activation.

PASMC mitochondria form an intricate network,[20] an underappreciated feature, relevant to their role in signaling P_{O_2} to plasmalemmal Kv channels. This network is disrupted in human and FHR-PAH. In FHRs, our natural history study shows that these ultrastructural changes precede hemodynamic perturbations (Figure 2). Thus, mitochondrial dysfunction is an early event in the pathogenesis of PAH, whether it results from a genetic abnormality (FHR) or is acquired (human iPAH). The sequence of pathogenic abnormalities proposed (mitochondrial dysfunction, ROS deficiency, normoxic HIF-1α activation, and finally Kv downregulation) is supported by serial observation of the order in which changes in genomic and proteomic expression occurred. Four additional experiments place mitochondrial dysfunction and decreased ROS earlier in the pathogenesis of PAH than HIF activation or Kv channel downregulation. First, restoring ROS through exogenous administration of H_2O_2 prevents nuclear HIF-1α translocation in FHR PASMCs and restores Kv1.5 expression (Figure 5E). Second, selective HIF-1α inhibition with an HIF-1α dominant construct inhibits HIF translocation and increases Kv1.5 expression (Figure 6D). Third, dichloroacetate, which normalizes mitochondrial function in FHRs, reverses HIF activation and increases Kv1.5 expression, thereby reducing PAH and improving survival (Figure 7). Fourth, human PAH PASMCs manifest a virtually identical mitochondrial pathology (Figures 4 and 5C). Thus,

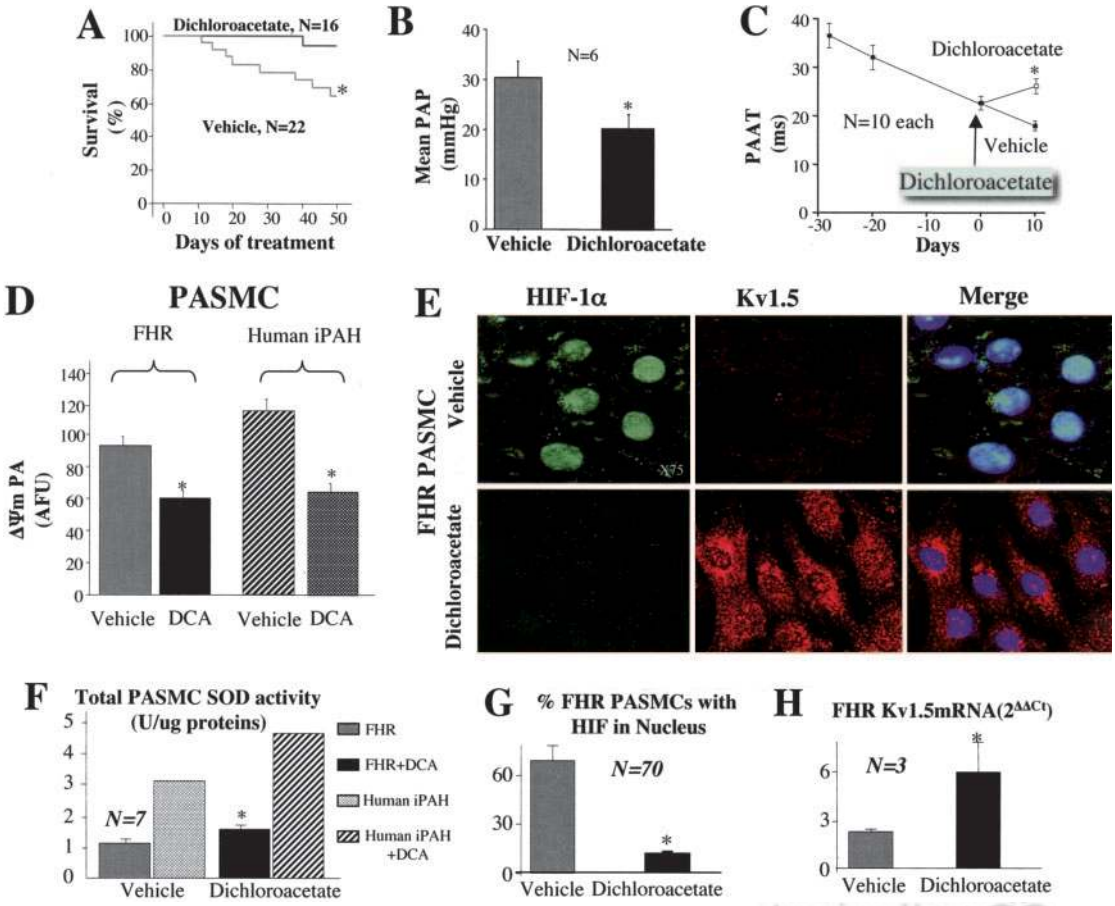

Figure 7. Dichloroacetate corrects ΔΨm, normalizes the HIF-1α-Kv1.5 axis, and reduces PAH. A, B, Dichloroacetate improves survival and causes regression of PAH in treated vs untreated FHR (*$P<0.05$). C, Note the rapid effect of dichloroacetate, with lengthening of pulmonary artery acceleration time within 10 days of initiating therapy in treated vs untreated FHR (*$P<0.05$). D, Dichloroacetate depolarizes ΔΨm in human PAH PASMCs and FHR-PAH PASMCs. E, By inhibiting HIF-1α nuclear translocation, dichloroacetate restores Kv1.5 expression in FHR PASMCs. F, Dichloroacetate increases total pulmonary artery SOD activity in FHR and human PAH (*$P<0.05$). G, H, Dichloroacetate blocks nuclear translocation of HIF-1α and thereby increases Kv1.5 expression vs untreated groups (*$P<0.05$).

in FHR-PAH and probably in human PAH, deficient mitochondrial ROS production is upstream of normoxic HIF-1α activation, which in turn is upstream of decreased Kv1.5 expression. The mitochondrial abnormality in FHRs relates to genes on chromosome 1, explaining the absence of PAH in FHR/BN1.

FHRs have reduced vasoconstriction to acute hypoxia and the mitochondrial complex I inhibitor rotenone (Figure 1D), as occurs in normal rats exposed to chronic hypoxia.[3,37] This reflects impairment of the mitochondrial sensor function (inability to acutely change ROS in response to hypoxia) (Figure 1E) and decreased expression of oxygen-sensitive Kv channels (Figure 6B). Rapid inhibition of Kv channels (eg, Kv1.5) by hypoxia initiates PASMC membrane depolarization, increases cytosolic calcium, and ultimately causes vasoconstriction (Figures 1D, 6C).[18,31,38] A progressive decrease in Kv1.5 (±Kv3.1b) expression underlies the reduced Kv current that occurs as FHR-PAH develops (Figure 6A and 6B). Indeed, impaired K^+ channel function/expression is increasingly recognized as a hallmark of human and experimental PAH.[3,24,39] Decreased Kv expression not only alters tone but also promotes vascular remodeling by increasing cytosolic calcium (promoting constriction/proliferation) and cytosolic K^+ (inhibiting caspase-dependent apoptosis[26,40]). In apoptosis-prone PASMCs, ΔΨm is depolarized and Kv currents are increased[25]; conversely in FHR PASMCs (Figures 2C and 6A) and human PAH, ΔΨm is hyperpolarized and Kv current is decreased, which would be predicted to create an apoptosis-resistant state and to contribute to the vascular remodeling in FHRs. Apoptosis resistance, marked by expression of PASMC survivin, has recently been recognized to contribute to human and experimental PAH.[2,5]

HIF-1α activation contributes to polycythemia, loss of K^+ current, and a form of pulmonary hypertension elicited by chronic hypoxia.[41] In FHRs, the stimulus for HIF-1α activation is not a hypoxia (Po_2 is normal; see the Data Supplement Table I) but rather downregulation of 3 HIF repressor pathways: loss of H_2O_2 (Figure 1), impaired HIF-3α expression,[33,42] and downregulation of HPH-1 (Figure 5), a redox-sensitive prolyl hydroxylase[43] that targets HIF-1α for degradation.[44] A central role for HIF-1α in PASMC K^+ channel regulation was first demonstrated by the finding that HIF-1α haploinsufficiency preserves PASMC K^+ current and reduces pulmonary hypertension in chronically hypoxic rodents.[41] Accordingly, we show an inverse relationship between nuclear HIF-1α translocation and Kv1.5 expression (Figures 5D, 5E, and 6D).

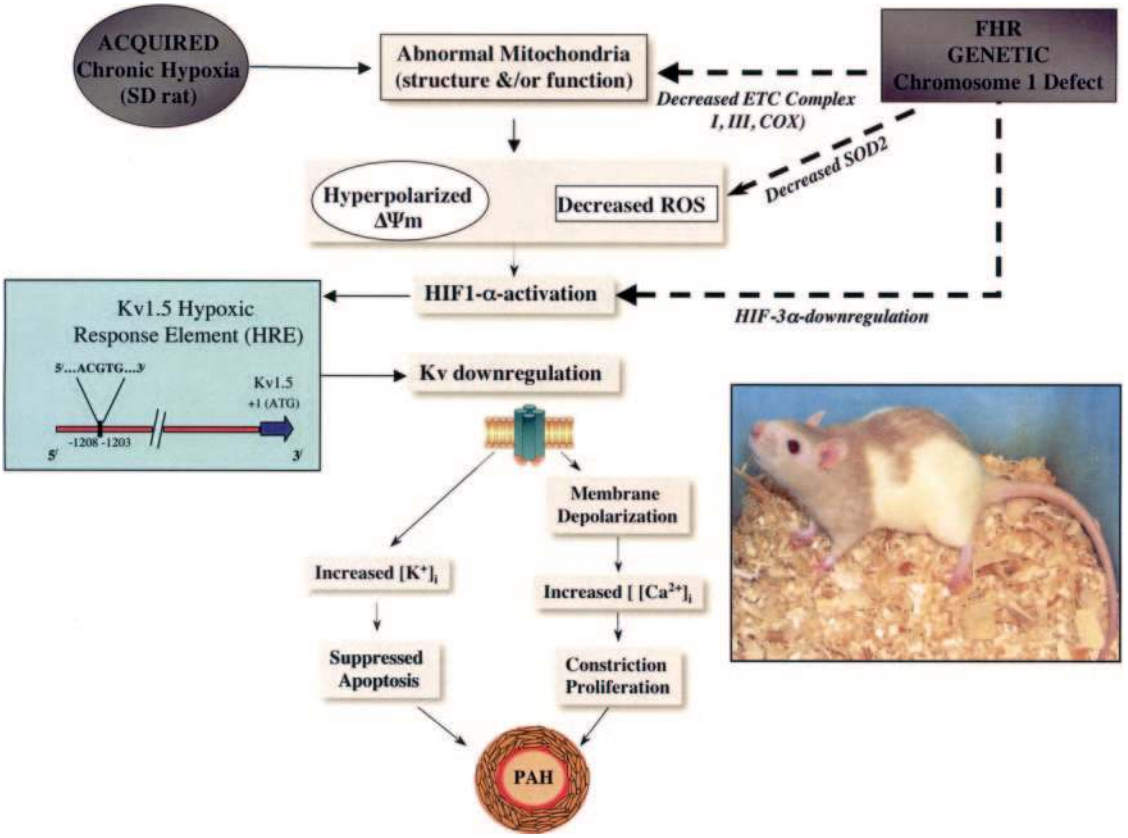

Figure 8. Proposed mitochondria-ROS-HIF-Kv mechanism for PAH. Disorders of the mitochondria-HIF-Kv pathway in FHR and humans lead to PAH.

Our findings extend prior reports of increased HIF-1α expression in PAH plexiform lesions[45] by demonstrating that HIF activation is independent of Po_2, persists in cell culture, and is driven by impaired mitochondrial function/ROS production. Likewise, the observed decrease in SOD2 is consistent with a prior report in PAH patients[46] but clarifies the importance of that observation (interruption of redox signaling). The proposed role of normoxic HIF activation in PAH is analogous to von Hippel-Lindau syndrome, sporadic renal carcinoma,[44] and pheochromocytoma,[47] conditions in which normoxic HIF activation drives a proproliferative, antiapoptotic phenotype. Like the FHR, these patients often have abnormal electron transport chain complex expression/function.[47,48] These similarities offer additional support for the view that PAH is a proliferative, apoptosis-resistant disease with similarities to neoplasia.[5,49]

The consomic rats, which share the same genetic background as FHR apart from chromosome 1, are invaluable in identifying genes that cause the inherited mitochondrial abnormality and PAH in FHRs. They permit a search for candidate PAH genes 1 chromosome at a time. The absence of PAH or mitochondrial disease in FHR/BN1 focused the search for candidate PAH genes on those on chromosome 1 that were dysregulated at 12 weeks, before PAH. DNA microarray analyses of gene expression suggested (data not shown) and immunoblots confirmed (Figure 2B) downregulation of several such genes (*SOD2*, *COX6a2*, *COX8h*) that are relevant to the disordered mitochondria-HIF-Kv pathway. However, expression of components of mitochondrial electron transport chain megacomplexes I and III (although not encoded on chromosome 1) also was decreased early in FHR-PAH. Downregulation of these complexes, even if secondary to an inherited abnormality of SOD2 or COX, likely contributes to the observed $\Delta\Psi$m hyperpolarization and decreased ROS.

If the FHR is a genetic mitochondropathy, why is the disease onset delayed until adulthood, and why is the pathology restricted to the lung despite the widespread hyperpolarization of $\Delta\Psi$m (Data Supplement Figure I)? There is precedent in other mitochondrial diseases. Leigh syndrome, caused by COX deficiency, has protean presentations, ranging from isolated myopathy to multisystem disease, and onset varies from childhood to adulthood.[50] Perhaps overt disease occurs primarily in vessels in which the mitochondria-HIF-Kv pathway is most active in controlling vascular tone and structure. The pulmonary circulation, a prototypic component of the body's specialized oxygen homeostatic system,[18] is preferentially susceptible to mitochondrial dysfunction because the loss of mitochondria-derived ROS, which normally signals Po_2, impairs oxygen sensing and creates a false hypoxic signature that triggers a downstream HIF-Kv remodeling cascade.

Conclusions

We identified a previously unsuspected role for mitochondria in the pathogenesis of human and rodent PAH and demonstrated that the mitochondria can be targeted therapeutically.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The contribution of vasoconstriction to the pathophysiology of pulmonary arterial hypertension (PAH) has been overemphasized, resulting in an excessive focus on vasodilator therapy. Only 20% of patients respond to vasodilator therapy. Recently, we have learned that PAH is due predominantly to vascular obstruction, resulting from excess cell proliferation and impaired apoptosis, with similarities to neoplasia and vascular restenosis. We show that fawn hooded rats, the only animals that spontaneously develop PAH, have a defect in the oxygen-sensing system of the normal pulmonary arterial smooth muscle cell. An inherited disruption of a mitochondria-based redox oxygen sensor (encoded on chromosome 1) creates a false "hypoxic signal" and initiates a cascade that causes PAH to develop with maturation leading to death in adulthood. Before PAH, hyperpolarized, dysmorphic mitochondria have depressed production of reactive oxygen species (ROS). Loss of ROS, which normally serves as signaling molecules, acutely impairs hypoxic pulmonary vasoconstriction and chronically activates the master hypoxia inducible factor (HIF)-1α despite normal P_{O_2}. HIF-1α activation downregulates oxygen-sensitive voltage-gated K^+ channels (Kv1.5), a feature of all experimental and human PAH syndromes. Remarkably, humans with PAH share with fawn hooded rats a disrupted smooth muscle cell mitochondrial network and normoxic HIF-1α activation. Enhancing mitochondrial function using dichloroacetate, a pyruvate dehydrogenase kinase inhibitor previously used in humans, regresses fawn hooded rat PAH and improves survival. This study illustrates a previously unsuspected link between oxygen sensing and PAH. Importantly, we identify the mitochondria-ROS-HIF-Kv pathway as a source of new targets for PAH therapy.