Hyperoxia Synergizes with Mutant Bone Morphogenic Protein Receptor 2 to Cause Metabolic Stress, Oxidant Injury, and Pulmonary Hypertension

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Pulmonary arterial hypertension (PAH) has been associated with a number of different but interrelated pathogenic mechanisms. Metabolic and oxidative stresses have been shown to play important pathogenic roles in a variety of model systems. However, many of these relationships remain at the level of association. We sought to establish a direct role for metabolic stress and oxidant injury in the pathogenesis of PAH. Mice that universally express a disease-causing mutation in bone morphogenic protein receptor 2 (Bmpr2) were exposed to room air or to brief daily hyperoxia (95% oxygen for 3 h) for 6 weeks, and were compared with wild-type animals undergoing identical exposures. In both murine tissues and cultured endothelial cells, the expression of mutant Bmpr2 was sufficient to cause oxidant injury that was particularly pronounced in mitochondrial membranes. With the enhancement of mitochondrial generation of reactive oxygen species by hyperoxia, oxidant injury was substantially enhanced in mitochondrial membranes, even in tissues distant from the lung. Hyperoxia, despite its vasodilatory actions in the pulmonary circulation, significantly worsened the PAH phenotype (elevated right ventricular systolic pressure, decreased cardiac output, and increased pulmonary vascular occlusion) in Bmpr2 mutant animals. These experiments demonstrate that oxidant injury and metabolic stress contribute directly to disease development, and provide further evidence for PAH as a systemic disease with life-limiting cardiopulmonary manifestations.

Keywords: pulmonary hypertension; Bmpr2; oxidative stress; mitochondria; hyperoxia

Pulmonary arterial hypertension (PAH) is a progressive, fatal disease of the pulmonary vasculature that results in vessel occlusion and dropout, increasing pulmonary vascular resistance, and ultimately, death from right ventricular failure (1, 2). The muscularization of small vessels in the pulmonary circulation, and a dysregulated balance between smooth muscle and endothelial cell proliferation and apoptosis, are thought to comprise the central cellular mechanisms of pathogenesis (3). What drives these processes at the molecular level continues to be a focus of active investigation, because the molecular etiologies of PAH

CLINICAL RELEVANCE

This study demonstrates that oxidative injury and metabolic dysfunction play a direct pathogenic role in bone morphogenic protein receptor 2-associated pulmonary arterial hypertension (PAH). Further, this study provides evidence that PAH is truly a systemic disease manifesting in multiple organ systems, but with the life-limiting manifestations occurring in the lungs and heart. These conceptual contributions, together with an enhanced understanding of potentially relevant “second hits,” will hopefully permit the investigation and development of novel targeted therapies for this devastating disease.

are incompletely understood. As a result, no curative therapies for PAH have yet been approved for use in humans.

Mitochondrial dysfunction has been shown to occur in monocrotaline-induced PAH, in Fawn hooded rats that spontaneously develop PAH, and in humans with idiopathic PAH (IPAH) or heritable PAH (HPAH) (4–7). Oxidative stress has been associated with PAH in a monocrotaline model, in a Bmpr2 mutant mouse model of PAH, and in patients with IPAH and HPAH (8–10). Few published studies have directly demonstrated that the enhancement of oxidative stress exacerbates the development of pulmonary hypertension, or that interventions to impair mitochondrial function will worsen the PAH phenotype in a susceptible individual. Some studies have shown that improving mitochondrial function (e.g., by restoring normal glucose oxidation with dichloroacetate) ameliorates PAH (11, 12), and that the reduction of reactive oxygen species production may be beneficial in some models of PAH (13–15). However, effective translations into human therapies still require an improved understanding of the specifics of molecular pathophysiology.

The contribution of systemic metabolic stress to the development of PAH is also an area of active investigation. Insulin resistance and impaired signaling of the peroxisome proliferator activating receptor- γ have been shown to drive the development of experimental pulmonary hypertension (16–19). In patients with PAH, previously unrecognized systemic glucose intolerance has been demonstrated that cannot be explained by body mass index (BMI) alone (20). However, it remains unclear whether systemic metabolic dysfunction reflects a systemic nature of PAH, is a contributing factor to the development of PAH, is a consequence of disease or of therapy, or is an epiphenomenon.

Hyperoxia is known to cause oxidative stress by increasing superoxide production from mitochondria, and in a whole organism hyperoxia has been shown to affect the pulmonary vasculature (21–24). However, short periods of hyperoxia are generally

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regarded as noninjurious. Moreover, oxygen is one of the most potent acute pulmonary vasodilators known, and thus should not directly cause an increase in pulmonary artery pressure. Indeed, exposure of animals to low inspired oxygen concentrations is one of the most widely used and reliable methods for producing experimental pulmonary hypertension. Hyperoxia is also capable of functioning as a metabolic stressor, by causing mitochondrial dysfunction through the oxidative modification of mitochondrial membranes, as well as by shifting the metabolic program of exposed cells toward a reliance on glycolytic metabolism (the so-called Warburg effect, which is thought to underlie the aberrant proliferation and apoptosis resistance observed in malignant cells, as well as in PAH) (4, 6, 21–24, 37, 47, 48).

We have focused our investigations on the murine model of Bmpr2-associated PAH. Mutations in Bmpr2 have been shown to be associated with the majority of cases of heritable PAH, and impaired Bmpr2 signaling has also been implicated in IPAH. The doxycycline-inducible expression in mice of mutant Bmpr2 alleles associated with PAH in humans has been shown to recapitulate the PAH phenotype in mice. Our laboratory has shown that the expression of these same mutant Bmpr2 alleles is sufficient to cause oxidative injury and metabolic dysregulation (characterized by insulin resistance, a shift toward glycolysis, and alterations of the normal anaplerotic mechanisms in the Krebs cycle) *in vitro* and *in vivo* (9, 26, 30, 46). We hypothesized that oxidative and metabolic stresses play a direct role in the development of Bmpr2-mediated PAH. We reasoned that if these processes are pathogenic, then the application of a mixed metabolic stressor/mitochondrial pro-oxidant stimulus in the pulmonary vasculature would enhance the development of PAH in a susceptible animal. We used brief daily hyperoxia (> 95% inspired oxygen for 3 h daily for 6 wk) to enhance mitochondrial

free radical flux. We show that the global expression of a Bmpr2 mutation is sufficient to cause metabolic stress and mitochondrial oxidant injury, even in sites distant from the lungs themselves. The enhancement of these stressors with hyperoxia significantly worsens the PAH phenotype. These experiments have been previously presented in partial form as an abstract (25).

MATERIALS AND METHODS

For a more complete discussion of our methods, please refer to the online supplement.

Generation of Bmpr2 Mutant Mice

FVB/N mice globally expressing the Bmpr2^{R899X} mutation were produced as described elsewhere (26). All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

Cell Culture

Pulmonary microvascular endothelial cells (PMVECs) were isolated from the lungs of Rosa-26 control and Bmpr2^{R899X} mutant mice, each crossed with the Immortomouse strain, as described elsewhere (26). Cells were grown at 37°C in 300 ng/ml doxycycline for at least 72 hours before the assay, and all experiments were performed at approximately 80% confluence.

For experiments performed in A7r5 cells, cells were maintained as previously described (9).

Hyperoxia Exposure

Rosa-26 and Bmpr2^{R899X} mutant mice were started on 1 g/kg doxycycline chow at 8 weeks of age, and were randomly assigned to room-air

Figure 1. Oxidant injury and O₂ flux in murine pulmonary microvascular endothelial cells (PMVECs). F₂-isoprostanes (IsoP) and isofurans (IsoF) were quantified in Rosa26 only (wild-type; WT) or Bmpr2^{R899X} mutation-expressing (R899X) cultured murine PMVECs (A), and the ratio of isofurans to isoprostanes was calculated as one measure of oxygen metabolism (B). The means $\pm$ SEMs are shown for 8–11 independent replicates, with *P* values determined by one-way ANOVA and Student *t* test. (C) Whole-cell respirometry data. The means $\pm$ SEMs are shown for duplicate measurements from three biological replicates.

Figure 2. Oxidant injury and metabolic stress in A7r5 cells as a function of Bmpr2 mutation and glucose. IsoP (A) and IsoF (B) were quantified in A7r5 cells expressing either wild-type (WT) or mutant (Mut) Bmpr2. In addition, cells were grown in either high glucose (HG, 450 mg/dl) or low glucose (LG, 100 mg/dl). (C) The IsoF/IsoP ratio is shown as a whole-cell measure of oxygen availability. The means $\pm$ SEMs are shown for 3–4 biological replicates. * $P < 0.008$, versus all other groups according to one-way ANOVA. All P values were determined by one-way ANOVA, with confirmatory individual Student t tests. N.S., not significant.

or hyperoxia treatment. The room-air groups were maintained in standard rodent housing. The hyperoxia-treated animals were placed in a temperature-controlled and humidity-controlled chamber equilibrated to greater than 95% O_2 , with continuous fresh gas flow for 3 hours daily for 6 weeks. Animals remained in their cages with free access to food and water. For the remaining 21 hours of each day, these animals were kept in standard rodent housing in room air.

Mitochondrial Isolation

Fresh liver tissue was homogenized using a Dounce glass-on-glass homogenizer (Sigma-Aldrich, St. Louis, MO), in a buffer consisting of 200 mM sucrose, 5 mM HEPES, and 1 mM EGTA. Sequential centrifugation was used to obtain a mitochondrial isolate that consisted of both the heavy and light mitochondrial fractions, with minimal microsomal contamination (27). Samples were either used immediately for analysis or aliquoted and stored at -80°C .

Respirometry

Freshly isolated liver mitochondria were assayed at 1 mg mitochondrial protein/ml MiR06 respiration buffer in an O2K Oxygraph (Oroboros, Innsbruck, Austria). Unstimulated oxygen uptake was determined at 37°C . State 3 respiration was determined by the addition of ADP (2 mM), and State 4 respiration was determined after the exhaustion of ADP in the presence of succinate (10 mM), glutamate (10 mM), and malate (2 mM). For whole-cell respirometry, cells were counted and assayed in complete medium in an O2K Oxygraph. Unstimulated oxygen flux was measured and normalized to cell number.

Lipid Peroxidation Measurements

F_2 -isoprostanes (IsoPs) and isofurans (IsoFs) were quantified in whole-cell lysates, isolated mitochondria, and whole-lung homogenates, as

previously described (28, 29). Calculated amounts for each product were normalized to protein content as measured by bicinchoninic acid assay (Thermo-Fisher Scientific, Rockford, IL), or to tissue wet weight.

ATP Assay

The ATP content of liver tissue was determined with a colorimetric assay, according to the manufacturer's instructions (BioVision, Mountain View, CA). Tissue was weighed, homogenized, deproteinized using perchloric acid, and assayed for ATP content.

Whole Animal Physiology

Echocardiography was performed on anesthetized mice at least 24 hours after the final hyperoxic exposure. Right ventricular systolic pressure (RVSP) was measured as described elsewhere (26, 30, 31).

Immunohistochemistry

Paraffin-embedded sections of lung from Rosa26 and Bmpr2^{R899X} mice were stained with an FITC-conjugated antibody against α -smooth muscle actin, and counterstained using 4'6-diamidino-2-phenylindole. Only actin-positive vessels were assessed for occlusion. All scoring was performed in a blinded fashion.

RESULTS

Expression of Mutant Bmpr2 Causes Oxidant Injury without Changing Instantaneous Oxygen Metabolism

We previously reported that the increased production of reactive oxygen species (ROS; specifically, superoxide and hydrogen peroxide)

Figure 3. Oxidant injury and metabolic stress in liver mitochondria from Bmpr2 mutant mice. IsoP and IsoF were quantified in liver mitochondrial membranes from Rosa26 only (wild-type [WT]) and Bmpr2^{R899X}-expressing (R899X) mice (A) ($n = 3-4$). Whole-cell respirometry was performed on isolated liver mitochondria from WT and R899X animals, and State 3 and State 4 respiration was quantified (B) ($n = 4-6$). (C) The IsoF/IsoP ratio was calculated as a separate measure of oxygen availability and metabolism ($n = 3-4$). The means $\pm$ SEMs are shown. *P* values were determined by one-way ANOVA or Student *t* test.

is a consequence of the expression of mutant Bmpr2 in cell culture. To confirm that these ROS participate in actual oxidant injury with the covalent modification of macromolecules, we quantified two different lipid peroxidation products, F₂-IsoPs and IsoFs, in cultured murine PMVECs from Rosa26 and Bmpr2^{R899X} mice (Figure 1A). In mutant endothelial cells, both lipid peroxidation products were increased compared with wild-type cells, confirming that oxidant injury was occurring.

By examining the IsoF-to-IsoP ratio, a time-averaged index of the ambient oxygen concentration during oxidant injury can be obtained, because IsoF formation is favored over IsoP formation at increased oxygen concentrations (28, 32–35). In cultured PMVECs, the ratio of IsoFs to IsoPs was unchanged between wild-type and Bmpr2 mutant cells (Figure 1B), suggesting similar ambient oxygen concentrations and relatively intact mitochondrial oxygen consumption. This was confirmed with whole-cell, high-resolution respirometry, showing that instantaneous oxygen

metabolism was not different between wild-type and mutant PMVECs (Figure 1C).

To ensure that these results were not specific to cell type or to the particular Bmpr2 mutation, and to explore the interplay further between metabolic stress and oxidant injury, we quantified IsoPs and IsoFs in a vascular smooth muscle cell line (A7r5 cells), stably transfected with either wild-type or mutant Bmpr2 (Figure E1 in the online supplement). This cell line not only offered the advantage of representing a vascular smooth muscle phenotype and a different disease-causing Bmpr2 mutation (details are provided in the online supplement), but the A7r5 cell type tolerates manipulation of culture conditions well, allowing us to manipulate the ambient glucose concentrations to examine the effects on oxidant injury and oxygen metabolism. At baseline, the expression of a mutant Bmpr2 in A7r5 cells is sufficient to cause oxidant injury, as measured by both IsoPs and IsoFs (Figure E2). Under basal conditions, A7r5 cells are grown in a high-glucose medium,

Figure 4. Time-averaged indices of systemic metabolic stress in Bmpr2 mutants. Expression of the Bmpr2^{R899X} mutation (R899X) resulted in decreased serum glucose (A) and decreased body weight at sacrifice (Sac; B), compared with Rosa26 alone (wild-type; WT). The means $\pm$ SEMs are shown. *P* values were determined by Student *t* test.

with a glucose concentration that would correspond to a serum glucose concentration of 450 mg/dl in a human, resulting in hyperglycemic stress. We found that when grown in medium containing 1 gm/L glucose (corresponding to a human serum glucose of 100 mg/dl and a glucose concentration identical to that of the normal endothelial cell medium used in the experiments in Figure 1), IsoP and IsoF formation is markedly attenuated in both wild-type and Bmpr2 mutant cells, although a trend toward increased oxidant injury remains in the mutant cells (Figures 2A and 2B). Moreover, the IsoF/IsoP ratio normalized with relief of hyperglycemic stress (Figure 2C), confirming that the Bmpr2 mutation alone does not substantially perturb oxygen metabolism under basal conditions in cultured cells.

Bmpr2 Mutations *In Vivo* Cause Systemic Mitochondrial Oxidant Injury and Systemic Metabolic Dysfunction

We next sought evidence for systemic oxidant injury and metabolic dysfunction *in vivo* in the murine Bmpr2 mutant model of

PAH. We initially analyzed tissues from 14-week-old mice (Bmpr2^{R899X} transgenic and Rosa26 control mice) that had received doxycycline-containing chow for 6 weeks. The majority of these mice demonstrated normal RVSP (only 20% of the transgenic mice developed elevated RVSP, as will be described). To assess for a more generalized, systemic oxidant injury downstream from a Bmpr2 mutation in a tissue distant from the site of the major disease phenotype that also ties in to metabolic stress, we quantified lipid peroxidation products (IsoPs and IsoFs) in the membranes of liver mitochondria. As with whole-cell analysis of cultured lung endothelial cells and smooth muscle cells, the expression of a Bmpr2 mutation caused substantial oxidant injury to the membranes of liver mitochondria, compared with wild-type liver mitochondrial membranes (Figure 3A). Because normal mitochondrial function depends at least in part on the integrity of the mitochondrial membrane, we next assessed oxygen metabolism in control and Bmpr2 mutant liver mitochondria to see whether the substantial oxidant injury to the mitochondrial membranes in the mutant animals exerted an observable deleterious effect. According to high-resolution respirometry, no significant differences were evident between control and Bmpr2 mutant liver mitochondria for either State 4 (resting, ADP-limited oxygen consumption in the presence of glutamate and malate) or State 3 (maximally respiring, ADP-stimulated oxygen consumption) respiration (Figure 3B). However, examination of the IsoF/IsoP ratio in liver mitochondrial membranes suggested that mitochondrial oxygen metabolism was markedly different in the Bmpr2 mutants, as evidenced by a substantially lower IsoF/IsoP ratio in the mitochondrial membranes compared with control membranes (Figure 3C). This implies less available molecular oxygen in the mutant mitochondria. Total ATP content in the livers of wild-type and Bmpr2 mutant mice was quantified and found to be grossly preserved in the mutant animals compared with wild-type animals (Figure E3). Along with the observed changes in oxygen availability, a slight but statistically significant decrease in serum glucose levels was observed in the Bmpr2 mutant animals compared with control animals (Figure 4A), accompanied by a significant decrease in total body weight in the Bmpr2 mutant animals compared with control animals that was not associated with increased mortality or obvious differences in food intake, activity, or overall health of the animals (Figure 4B). We have previously undertaken quantitative analyses of food intake and activity in wild-type and Bmpr2^{R899X} mice, and detected no differences between the two groups (30), although these parameters were not directly measured in the present study.

Brief Daily Hyperoxia Produces Oxidant Injury and Metabolic Changes Similar to Bmpr2 Mutation

With the finding that Bmpr2 mutations were sufficient to cause systemic oxidative and metabolic dysfunction, we wanted to assess the effects of additional oxidative and metabolic stress in their capacity as a “second hit.” The stimulus we chose involved daily exposure to 3 hours of greater than 95% oxygen for 6 weeks. Upon the exposure of wild-type mice to daily hyperoxia, we found that oxidized lipids in liver mitochondrial membranes increased substantially, compared with room-air control membranes (Figures 5A and 5B). Surprisingly, this was accompanied by a decrease in the IsoF/IsoP ratio down to what is seen in the Bmpr2 mutant mitochondria in room air, suggesting that overall ambient oxygen concentrations in liver tissue had decreased despite an increase in whole-body oxygen exposure and an increase in oxidant injury (Figure 5C). Along with this, wild-type animals exposed to hyperoxia showed a decrease in body weight

Figure 5. Brief daily hyperoxia causes significant oxidant injury and metabolic stress. Rosa26 only (wild-type; WT) or Bmpr2^{R899X} expressing (R899X) mice were either maintained in room air (RA) or treated with 3 hours of daily hyperoxia (Hyperox). Isoprostanes (A) and isofurans (B) were quantified in liver mitochondrial membranes ($n = 3-5$). (C) The IsoF/IsoP ratio was calculated as a measure of oxygen availability and metabolism ($n = 3-5$). Body weights were determined on a weekly basis for all animals in the experiment (D), and serum glucose was measured at the time of death (E) ($n = 8-18$). The means $\pm$ SEMs are shown in A–D. The means are shown in E, with whiskers indicating 5th to 95th percentiles. For A, $*P < 0.02$, $**P < 0.0003$, and $\#P < 0.03$, compared with WT RA. For B, $*P < 0.04$ and $**P < 0.02$, compared with WT RA. For E, $*P < 0.05$, versus WT RA. All P values were determined by one-way ANOVA or Student t test.

(Figure 5D) and a decrease in total serum glucose (Figure 5E), strikingly similar to what was seen in the Bmpr2 mutant mice in room air, although not to the same degree. Wild-type animals treated with hyperoxia also showed hepatic ATP levels, similar to what was seen in the room-air Bmpr2 mutants (Figure E4).

In contrast to the wild-type animals, the Bmpr2 mutant mice exposed to hyperoxia did not show a pronounced enhancement of their already elevated metabolic stress or oxidant injury. Levels of IsoFs and IsoPs in mitochondrial membranes were similar in mutant mice exposed to hyperoxia and their room-air control counterparts (Figures 5A and 5B). Hyperoxia did not perturb the IsoF/IsoP ratio in liver mitochondrial membranes further in mutant animals, compared with those maintained in room air (Figure 5C). Serum glucose levels were also similar between the room-air and hyperoxia-treated mutant mice (Figure 5E). Body weight showed a very slight further reduction in the hyperoxic mutant animals, compared with their room-air counterparts (Figure 5D).

Bmpr2 Mutation and Brief Daily Hyperoxia Act Synergistically and Result in a More Severe PAH Phenotype

To confirm a causative role for oxidant injury and metabolic stress in the development of PAH, and to support the role of a “second hit” in the development of disease, we examined RVSP by invasive hemodynamic measurements, and cardiac output by surface echocardiography, in wild-type and Bmpr2 mutant mice exposed to room air or brief daily hyperoxia. In wild-type mice, hyperoxia exerted no significant effect on RVSP or cardiac output. In Bmpr2 mutant mice, however, brief daily hyperoxia significantly increased RVSP and decreased cardiac output (Figures 6A and 6B). In addition to raising the average RVSP in Bmpr2 mutants, hyperoxia also increased the penetrance of the Bmpr2 mutation, with 41% (7/17) of mutant mice in the hyperoxia group developing PAH, compared with only 20% (3/15) in the room-air group (Figure 6A), although this did not quite reach statistical significance ($P = 0.09$, according to χ^2 analysis). Expression of the Bmpr2 transgene was confirmed by quantitative PCR, and this expression was positively correlated with RVSP ($r = 0.7$, $P < 0.001$) and weakly negatively correlated with cardiac output ($r = -0.4$, $P = 0.08$).

Expression of the Bmpr2 mutation resulted in a qualitative increase in the muscularization of vessels in the lung, as measured by immunohistochemistry for α -smooth muscle actin with a trend (not statistically significant) toward more extensive muscularization in the Bmpr2 mutant mice exposed to hyperoxia (Figures 7A and E5). An examination of lung sections also showed that hyperoxia treatment resulted in increased vascular occlusion in both wild-type and Bmpr2 mutant lungs, although the increase was only statistically significant in the Bmpr2 mutants (Figure 7B). Closer examination showed that vascular occlusion in the Bmpr2 mutants was attributable, at least in part, to an increase in occlusion of the pulmonary microvasculature by cellular aggregates (Figure 7A, inset). These occlusions were not attributable to platelet aggregation or to the presence of acellular thrombi, although a precise characterization of the nature of the occlusive cells has remained elusive.

DISCUSSION

In the present study, we set out to test the hypothesis that oxidant injury and metabolic stress (defined at the molecular and cellular levels as mitochondrial membrane oxidation and altered mitochondrial oxygen consumption, and at the systemic level as changes in body weight and glucose homeostasis) play a direct role in the development of Bmpr2-mediated PAH. We have demonstrated that disease-associated Bmpr2 mutations are sufficient to induce both oxidant injury and metabolic stress *in vitro* (Figures 1 and 2) and *in vivo*, and that these downstream effects are systemic (Figures 3–5). We have shown that oxidant injury and metabolic stress are interrelated both *in vitro* (Figure 2) and *in vivo* (Figures 3–5), and that modulating one affects the other. Finally, we have shown that the application of a combined oxidative and metabolic stressor (i.e., brief daily

Figure 6. Brief daily hyperoxia (Hyperox) increases the penetrance and severity of the pulmonary arterial hypertension (PAH) phenotype in Bmpr2 mutant mice. Rosa26 only (wild-type; WT) or Bmpr2^{R899X} mutant (R899X) mice were maintained in room air (RA) or treated with 3 hours of daily hyperoxia (Hyperox). Right ventricular systolic pressures (RVSP) were measured by invasive hemodynamics (A), and cardiac output was quantified by echocardiography (B). The means $\pm$ SEMs are shown. $*P < 0.03$, versus WT RA. P values were determined by Student t test. N.D., not statistically significantly different.

Figure 7. Vascular muscularization and occlusion are more prominent in *Bmpr2* mutants, and after brief daily hyperoxia. (A) Increased small-artery muscularization, as measured by immunostaining for α -smooth muscle actin (green), is present in *Bmpr2* mutant lungs compared with wild-type murine lungs (top, arrowheads). Brief daily hyperoxia increases vascular muscularization (arrowheads) and occlusion (arrows) in both wild-type and *Bmpr2* mutant lungs (bottom). In the *Bmpr2* mutants, multiple vessels could be found that were occluded with cellular aggregates (bottom right, inset). (B) Occluded vessels were statistically significantly increased only in the *Bmpr2* mutant animals treated with brief daily hyperoxia. Vessels were counted and scored in a blinded fashion. Measurements were derived from 10 high-power fields (HPF) per animal ($n = 3$ in each group). * $P < 0.002$, versus all other groups according to ANOVA with Tukey honestly significant difference *post hoc* analysis. hr, hour.

hyperoxia) will substantially exacerbate the PAH phenotype in a susceptible animal (Figures 6 and 7), providing strong support for the idea that both oxidant injury and metabolic stress play causative roles in the development of disease.

The idea of PAH as a systemic, metabolic disease has been gaining traction for some time, and data in support of this idea are rapidly accumulating (36, 37). Global alterations in insulin signaling have been shown to drive the development of PAH (16, 38), and a mixed group of patients with PAH has been shown to exhibit previously unrecognized systemic glucose intolerance that could not be explained by obesity or increased BMI. Imaging studies using fluorodeoxyglucose uptake and positron emission tomography scans have shown increased glucose metabolism in both the hearts and lungs of PAH patients, compared with control subjects (39, 40). Multiple mitochondrial abnormalities have been shown to contribute to PAH, including abnormalities of specific glycolytic enzymes (e.g., hexokinase-2), mitochondrial fission/fusion, mitochondrial/endoplasmic reticulum dynamics, and mitochondrial ROS production (41–45). In light of these and other studies, our choice to examine mitochondria from tissue not obviously affected by disease (i.e., liver that was clearly not edematous or otherwise affected by profound right heart failure) permitted us to highlight the systemic nature of the metabolic and oxidative stresses that contribute to the development of PAH. We have recently shown that widespread metabolic reprogramming affecting many interconnected pathways

occurs downstream from *Bmpr2* mutations (46), and this study provides supporting evidence that at least some of these metabolic alterations occur *in vivo* in a “complete” disease model.

The use of brief daily hyperoxia as a combined metabolic stressor and pro-oxidant stimulus was also intended to highlight several important aspects of the roles of oxidant injury and metabolic stress in the development of PAH. Pulmonary hypertension is classically caused or exacerbated in animal models using hypoxia as opposed to hyperoxia. Moreover, oxygen is an extremely potent acute pulmonary vasodilator, and brief exposure to supraphysiological inspired oxygen concentrations is generally regarded as safe in most mammals. Hyperoxia has been shown to increase superoxide anion production by mitochondria (21–24). In addition, hyperoxia has been demonstrated to alter the metabolic behavior of cells in culture, shifting the metabolic program to one more heavily reliant on glycolysis and glucose turnover to maintain ATP content (47, 48). Finally, hyperoxia is a systemic stimulus that should nonetheless exert its greatest impact in the lung, and it is a clinically relevant stimulus because supplemental oxygen use is one of the most commonly used therapeutic interventions in virtually every patient care setting. The finding that this particular stimulus can drive both the penetrance and severity of pulmonary vascular disease in susceptible individuals (e.g., mice expressing mutant *Bmpr2*), together with the finding that the same stimulus in wild-type animals produces deleterious changes that are nonetheless

insufficient to cause clinically apparent disease, strongly argues for the causal role of oxidant injury and metabolic stress in PAH. These findings provide support for a “two hit” or “multiple hit” model of PAH development, as has been described for many other complex diseases. Moreover, this study may point the way toward investigations of relevant second hits that could lead to the development of PAH in susceptible individuals, for example, exposure to inhaled pro-oxidants or even oxygen itself, metabolic stresses that may accompany morbid obesity or significant illness or injury (e.g., critical illnesses related to septic shock, acute lung injury, or acute respiratory distress syndrome), or possible pharmacologic exposures that could induce oxidant injury and/or metabolic stress (e.g., some agents used as part of combination antiretroviral therapy for HIV).

The effects of the expression of a Bmpr2 mutation and/or treatment with brief daily hyperoxia on mitochondrial oxygen metabolism, ATP production, serum glucose, and body weight deserve further discussion. One interpretation of these findings contends that the overall effect of the expression of mutant Bmpr2 or of brief daily hyperoxia involves a nonclassical mitochondrial uncoupling. Mitochondrial uncoupling is classically described as oxygen consumption that does not result in ATP production, effectively divorcing electron transport from oxidative phosphorylation. Although instantaneous oxygen consumption is unaffected by the expression of mutant Bmpr2 in whole cells or in isolated mitochondria, we here show that a time-averaged, *in vivo* index of oxygen availability in mitochondria (i.e., the IsoF/IsoP ratio) is significantly reduced by mutant Bmpr2 and by brief daily hyperoxia, without a statistically significant change in steady-state tissue ATP levels. Decreased oxygen concentrations in mitochondria are almost certainly attributable to increased mitochondrial oxygen consumption over time. The alternative explanation would involve a decrease in cellular oxygen supply, an argument that seems particularly unlikely in the setting of brief daily hyperoxia. Increased oxygen consumption without increased ATP synthesis is the definition of uncoupling, and other physiologic changes observed in Bmpr2 mutants and hyperoxia-treated animals (e.g., decreased serum glucose levels and decreased body weight) have also been associated with increased mitochondrial uncoupling in other model systems (49, 50). IsoF/IsoP ratios in cultured cells did not reflect the same perturbations of oxygen metabolism as observed *in vivo*, and this is likely a reflection of the metabolic adaptation that cells undergo as a requirement of growing in culture. However, an important alternative explanation would involve increased overall energy expenditure in the Bmpr2 mutants and hyperoxia-treated animals, resulting in increased ATP turnover and subsequent increased glucose (or other carbon source) utilization to meet the increased metabolic demand and maintain ATP concentrations. To support this alternative explanation, measurements of *in vivo* flux rates for glucose and ATP would be necessary. The mechanism by which brief daily hyperoxia might cause this type of mitochondrial uncoupling (or the mechanism by which hyperoxia might cause an increased overall energy expenditure) remains to be elucidated, although we hypothesize that alterations in mitochondrial membrane structure attributable to lipid peroxidation, as demonstrated in the present study, may play a key role.

The present study demonstrates important pathogenic roles for oxidant injury and metabolic stress in the development of PAH. These results open potential avenues toward novel treatments for PAH. We speculate that interventions against either oxidant injury or metabolic stress may exert beneficial effects on nascent or established pulmonary hypertension. Furthermore, we hypothesize that treating either oxidant injury or metabolic stress alone may exert an impact on the other stressor,

and conceivably alleviate the need to treat both processes. This study leaves open the questions of whether oxidant injury or metabolic stress is the more important of the two processes, and whether there may be tissue specificity for each process. The present study provides a strong impetus for ongoing efforts to define the mechanisms underlying oxidant injury and metabolic reprogramming in PAH. These and future studies will hopefully identify untested therapeutic targets for this debilitating disease.

Author disclosures are available with the text of this article at www.atsjournals.org.

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