Oxidation of PKG\(\alpha\) mediates an endogenous adaptation to pulmonary hypertension

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Chronic hypoxia causes pulmonary hypertension (PH), vascular remodeling, right ventricular (RV) hypertrophy, and cardiac failure. Protein kinase G \(\alpha\) (PKG\(\alpha\)) is susceptible to oxidation, forming an interprotein disulfide homodimer associated with kinase targeting involved in vasodilation. Here we report increased disulfide PKG\(\alpha\) in pulmonary arteries from mice with hypoxic PH or lungs from patients with pulmonary arterial hypertensive hypoxia. This oxidation is likely caused by oxidants derived from NADPH oxidase-4, superoxide dismutase 3, and cystathionine \(\gamma\)-lyase, enzymes that were concomitantly increased in these samples. Indeed, products that may arise from these enzymes, including hydrogen peroxide, glutathione disulfide, and protein-bound persulfides, were increased in the plasma of hypoxic mice. Furthermore, low-molecular-weight hydroperoxides, which can serve as “superreductants” were attenuated in hypoxic tissues, consistent with systemic oxidative stress and the oxidation of PKG\(\alpha\) observed. Inhibiting cystathionine \(\gamma\)-lyase resulted in decreased hypoxia-induced disulfide PKG\(\alpha\) and more severe PH phenotype in wild-type mice, but not in Cys425er PKG\(\alpha\) knock-in (KI) mice that are resistant to oxidation. In addition, KI mice also developed potentiated PH during hypoxia alone. Thus, oxidation of PKG\(\alpha\) is an adaptive mechanism that limits PH, a concept further supported by polysulfide treatment abrogating hypoxia-induced RV hypertrophy in wild-type, but not in the KI mice. Unbiased transcriptomic analysis of hypoxic lungs before structural remodeling identified up-regulation of endothelial-to-mesenchymal transition pathways in the KI compared with wild-type mice. Thus, disulfide PKG\(\alpha\) is an intrinsic adaptive mechanism that attenuates PH progression not only by promoting vasodilation but also by limiting maladaptive growth and fibrosis signaling.

Significance

This study demonstrates that oxidation of protein kinase G \(\alpha\) (PKG\(\alpha\)) to its disulfide-activated state occurs in pulmonary arteries during chronic hypoxia, and that this is a protective event that limits progression of pulmonary hypertension by at least two mechanisms. Firstly, it induces pulmonary vasodilation that counters and offsets maladaptive vasoconstriction during chronic hypoxia, and, secondly, disulfide PKG\(\alpha\) is protective by preventing maladaptive growth and fibrosis signaling. Consistent with oxidation of PKG\(\alpha\) being protective, administration of polysulfides to mice during hypoxia, which increased the abundance of the disulfide form of the kinase, was therapeutic and limited disease progression.

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for most diseases. This has led to antioxidant supplements being advocated as a panacea, as they have for PH (13, 23), but, in general, they have failed in large-scale clinical trials and often have proven harmful when administered to humans with various diseases (24, 25). This may be because antioxidants prevent intrinsic cellular responses (25, 26), for example by neutralizing ROS species that may otherwise react with redox sensor proteins, such as PKGIα, to initiate adaptive signaling.

The role of redox-regulated PKGIα in controlling the tone of systemic vessels has been widely reported (8–10), whereas the importance of these events in the pulmonary circulation is less well defined. In the present study, the redox state of pulmonary PKGIα and its potential role in the pathogenesis of hypoxia-induced PH was investigated. The novelty of this study is that disulfide PKGIα accumulates during chronic hypoxia and that this serves an endogenous, adaptive redox mechanism by promoting vasodilation that limits PH and the associated adverse pulmonary arterial and right heart remodeling that otherwise ensues. Furthermore, disulfide PKGIα accumulation during chronic hypoxia was associated, likely causatively, with a loss of low-molecular-weight hydopersulfides that can serve as “superreductants” that otherwise maintain the kinase in the reduced state. Pharmacological agents that induce disulfide PKGIα have therapeutic potential in PH, another original finding of this work.

Results and Discussion

Disulfide PKGIα Level Is Elevated in Pulmonary Tissues from Hypoxic Mice and Lungs from Pulmonary Hypertensive Patients. Mouse models of hypoxic PH reproduce the pulmonary vessel constriction and muscularization (27, 28), observed in humans in Group 3 of the World Health Organization PH classification system (3, 29). For this reason, we established and validated this PH model in C57BL/6 mice by subjecting them to chronic hypoxia (10% O2) for 28 d (SI Appendix, Fig. S1 A–D). Subsequently, wild-type (WT) alone, or WT together with “redox-dead” Cys4Ser PKGIα knock-in (KI) mice that cannot form PKGIα disulfide dimer (8, 10) were compared in their responses to 3, 14, or 28 d of hypoxia; 3 or 14 d of hypoxia increased the amount of total, as well as disulfide, PKGIα in WT pulmonary vessels compared with those maintained in normoxic room air (Fig. 1 A and B). Endothelially elevated disulfide PKGIα levels, as well as a trend toward increased total PKGIα, were evident in whole lungs of mice subjected to hypoxia for 28 d (Fig. 1C), or those from human pulmonary arterial hypertension (PAH) patients (Fig. 1D). This elevation in total PKGI expression was as observed by others (16), and was considered an adaptive process. However, it was unclear whether the increase in disulfide PKGIα contributes to pulmonary adaptation to hypoxia. PKGIα oxidation was not altered in the RV and was slightly reduced in the left ventricle and septum of hypoxic WT mice (SI Appendix, Fig. S2A). As anticipated, disulfide PKGIα was not evident in tissues from the KI mice regardless of the experimental intervention (Fig. 1 B and C). Although total PKGIα was up-regulated in pulmonary vessels (Fig. 1 A and B) and lungs (Fig. 1C) of the KI mice in response to hypoxia, as occurred in WT.

Next, we went on to investigate the molecular basis for pulmonary PKGIα oxidation during chronic hypoxia. Our attention turned to the ROS that might mediate oxidation of PKGIα, and their potential enzymatic sources. Previous studies showed that hydrogen peroxide (H2O2) (8, 30), persulfides (9), or nitric oxide-related species (10) induced oxidation of PKGIα. In addition, expression of the H2O2-producing superoxide dismutase (SOD) enzyme was altered in hypoxic PH (12, 31). The H2O2-generating enzyme nonphagocytic NADPH oxidase-4 (Nox4), that can also increase cystathionine γ-lyase (CSE) (32), was upregulated in pulmonary vascular cells and pulmonary vessels during chronic hypoxia in vitro (33) and in vivo (20). For these reasons, expression of SOD, Nox4, and CSE enzymes was compared in normoxic or hypoxic tissues. Hypoxia increased Nox4, SOD3, and CSE protein expression comparably in WT or KI pulmonary arteries (Fig. 1 A and B) and lungs (Fig. 1C). SOD1 or SOD2 expression was not significantly altered in the lungs of mice subjected to hypoxia (SI Appendix, Fig. S2B), although the latter was previously observed to decrease in PH lungs (34, 35). To ensure accurate measurement of Nox4 protein expression, the custom-made Nox4 antibodies were validated by using Nox4-knockout lung tissue (SI Appendix, Fig. S2C).

Up-regulation of oxidant-producing proteins may reflect an adaptive role for oxidants whereby they lower pulmonary blood pressure by disulfide PKGIα-dependent vasodilation. Indeed, increased SOD3 expression is consistent with an established role for this enzyme in protecting the lung from hypoxia-induced PH (36–38). Consistent with such a protective role for the dismutase, SOD3 depletion from smooth muscle cells potentiated the severity of phenotypic responses to PH in mice (39), whereas its overexpression in lung attenuated PH-induced arterial remodeling and pressure (37). Thus, the increased SOD3 observed herein was likely an adaptive response that limits dysfunction during PH, with the rational likelihood that the increased Nox4 that also generates H2O2 is likewise beneficial. In line with this, Fawn-Hooded rats that produce less H2O2 develop spontaneous PH, compared with Sprague Dawley controls. However, treatment of Fawn-Hooded rats with an SOD2 mimetic, which is anticipated to enhance H2O2 derived from superoxide, abrogated the PH phenotype (27), consistent with a crucial role for oxidants in the regulation of pulmonary vasotone.

Nox4 up-regulation during PH has been observed multiply at both the messenger RNA (mRNA) and protein levels (4, 20, 21, 33, 40), but little is known about the downstream targets of the H2O2 generated by this enzyme (21). A large body of literature describes detrimental effects of Nox4 in PH (21, 22, 41, 42). For example, inhibition of Nox4 by VCCS88646, VCC202273, or GKT136901 reduced PASM C proliferation in vitro, and vascular remodeling together with RV hypertrophy in monocrotaline-treated rats (22), consistent with a causative role for Nox4 in PH. However, the specificity and isoform selectivity for many of these inhibitors has been questioned (43), and thus the protection observed may result from inhibition of other superoxide generating Nox isoforms, rather than Nox4 alone. Indeed, a recent report demonstrated that constitutive or inducible Nox4 knock-out mice develop a similar PH phenotype to WT when subjected to chronic hypoxia for 21 d (44). Therefore, Nox4 up-regulation may serve as a protective rather than a detrimental mechanism. Nox4-mediated cysteine oxidation of Kγ1.5 channels leads to the inhibition of the channel activity, sustained depolarization, and pulmonary vasoconstriction observed during the pathogenesis of hypoxia-induced PH (21). In contrast, another group showed that Kγ1.5 oxidation induces activation of these channels, which couples to vasodilation (rather than vasoconstriction) in response to H2O2 (45). Oxidants also induce disulfide PKGIα (8, 10, 30), which significantly mediates vasodilation by H2O2, including via phosphoactivation of large-conductance potassium channels (46). It is likely that chronic hypoxia results in oxidation of multiple other proteins, but studying the “redox-dead” PKGI KI allowed a specific role for oxidation of this kinase in adaptation to PH to be defined (7, 47).

The expression of CSE enzyme was also increased during hypoxia. CSE is known for its generation of the vasorelaxant H2S (48), while this enzyme may also directly generate cysteine persulfides from cystine (49). The term “persulfide” is defined here as all molecular species containing more than one sulfur atom in each low-molecular-weight and protein/peptidyl thiol moiety. H2S can additionally be oxidized to the persulfides by the product of SOD3 and Nox4, namely H2O2 (9, 50), or directly by Cu/Zn SOD (51). These persulfide species cause vasodilation...
and blood pressure lowering by promoting disulfide PKGI\(\alpha\) (9). Thus, the increased expression of SOD3, Nox4, and CSE would provide an integrated mechanism that limits dysfunction during PH by generating oxidant species that couple to vasodilation by increasing disulfide PKGI\(\alpha\). Such vasodilation would reduce pulmonary and RV pressure to limit the progressive adverse remodeling, consistent with the disease-limiting effects of therapies that enhance cGMP-dependent activation of PKGI (52–54). Therefore, protein oxidation, as observed in idiopathic PAH (55), may provide beneficial, adaptive mechanisms, as opposed to the solely deleterious role it has historically been associated with. It is notable that the increased disulfide PKGI\(\alpha\), as well as the Nox4 and CSE enzymes that likely contribute to oxidation of the kinase, are observed in samples from humans with PAH (Fig. 1D).

**Alteration of Reactive Oxygen and Sulfur Species Metabolome During Chronic Hypoxia.** To further explore the molecular nature of the species that mediate PKGI\(\alpha\) oxidation, reactive oxygen and sulfur metabolites were analyzed in lungs or plasma of mice subjected to chronic hypoxia for 3 and 14 d. Hypoxia increased pulmonary disulfide PKGI\(\alpha\) levels (Fig. 2A), substantiating the observations shown in Fig. 1. A one-step fluorescence-based Amplex Red assay was employed to assess \(\text{H}_2\text{O}_2\) abundance in these samples, as it is a product of both Nox4 and SOD3. We observed a time-dependent increase of \(\text{H}_2\text{O}_2\) in lungs of mice and PAH patients (Fig. 2D).

**Fig. 1.** Disulfide PKGI\(\alpha\) is increased in pulmonary arteries and lungs of mice subjected to chronic hypoxia and in lungs of PAH patients. (A) Disulfide PKGI\(\alpha\), total PKGI, CSE, Nox4, and SOD3 protein expression in vessels of WT mice subjected to either normoxia or chronic hypoxia for 3 d. (B) Disulfide PKGI\(\alpha\), total PKGI, CSE, Nox4, and SOD3 protein expression in vessels of WT or Cys42Ser PKGI\(\alpha\) KI mice subjected to either normoxia or chronic hypoxia for 14 d. (C) Disulfide PKGI\(\alpha\), total PKGI, CSE, Nox4, and SOD3 protein expression in lungs of WT or Cys42Ser PKGI\(\alpha\) KI mice subjected to either normoxia or chronic hypoxia for 28 d. (D) Disulfide PKGI\(\alpha\), total PKGI, CSE, and Nox4 protein expression in lungs of PAH patients. *\(P < 0.05\), **\(P < 0.01\) versus normoxia or respective WT; \(n = 6\) to 8 per group; monomer, PKGI\(\alpha\) monomer; dimer, PKGI\(\alpha\) dimer; Nox4, NADPH oxidase 4; SOD, extracellular SOD; WT, WT mice; PKG \(\alpha\) KI, “redox-dead” Cys42Ser PKGI\(\alpha\) KI mice. In some cases, the aspect ratio of the original immunoblots was altered to enable a concise multipanel figure with a consistent presentation style; the original uncropped representative images of these immunoblots are also available in SI Appendix, Figs. S8 and S9.
subjected to hypoxia, whereas plasma H$_2$O$_2$ was only elevated after 3 d (Fig. 2A). This was anticipated and rationalized by the concomitantly increased Nox4 and SOD3 expression in pulmonary arteries after 3 d or 14 d of hypoxia (Fig. 1A and B). The ROS-generating activity of Nox4 is regulated primarily by its expression (56–58); in addition, both mRNA and protein levels of Nox4 can be up-regulated via hypoxia-inducible factor 1α (33). Therefore, it is likely that H$_2$O$_2$ elevation in hypoxic lungs is, at least partially, a result of increased protein abundance and activity of this oxidase. Overall, the elevated amounts of H$_2$O$_2$ observed in hypoxic tissues are consistent with increased expression and activity of both Nox4 and SOD3, but other ROS-producing enzymes may also contribute.

Fig. 2. Reactive oxygen and sulfur species level in lung and plasma of mice subjected to chronic hypoxia. (A) Representative disulfide PKGIα increase in lungs of WT mice subjected to either normoxia or chronic hypoxia for 3 and 14 d. (B) Low-molecular-weight persulfides and oxidized forms of sulfur metabolism in lung and plasma of WT mice subjected to either normoxia or chronic hypoxia for 3 and 14 d. Thiol and hydroperoxysulfide-containing compound were alkylated with tyrosine and a hydroxysulfenyl-containing derivative, β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM), and their HPE-IAM adducts were quantified by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). (C) Protein persulfidation in lung and plasma of WT mice subjected to either normoxia or chronic hypoxia for 3 and 14 d. Protein-bound hydroperoxysulfide were alkylated with HPE-IAM, and pronase digest samples were quantitatively analyzed by LC-ESI-MS/MS. *P < 0.05, **P < 0.01 versus normoxia; n = 6 to 8 per group. In some cases, the aspect ratio of the original immunoblots was altered to enable a concise multipanel figure with a consistent presentation style; the original uncropped representative images of these immunoblots are also available in SI Appendix, Fig. S10.
Next, the sulfur metabolites were analyzed by mass spectrometry, as before (49, 59). Hypoxia induced a time-dependent reduction in plasma homocysteine (Fig. 2A), which may reflect the increased expression and activity of pulmonary CSE, consuming this as a substrate. Another H₂S producing enzyme, namely cystathionine β-synthase, can also metabolize homocysteine (60); however, we could not detect this protein in the lung. Reactive sulfur intermediates, such as low-molecular-weight persulfides (RS₃H and RSS₃R, n > 1), as well as total protein persulfidation were assessed (Fig. 2 B and C and SI Appendix, Fig. S3). These sulfur intermediates have inherent chemical properties that confer reactivity to different biological targets (49, 59), and are anticipated to alter the thiol redox state of proteins, including PKGIα. Cysteine (CysSSH) and glutathione persulfides (GSSH) were time-dependently decreased in both lungs and plasma, although the change in GSSH levels failed to reach statistical significance in plasma (Fig. 2B). This is notable, as these hydroperoxsulfide species are superior reductants that are capable of rapid neutralizing reactions with ROS, as well as the reduction of disulfide-containing molecules (49, 61), including those on PKGI. Other sulfur derivatives such as HS⁻, HSS⁻, and thiosulfates (HS₂O₃⁻) were also decreased in the plasma and lung in hypoxia (SI Appendix, Fig. S3). Reduction of such reducing equivalents will favor accumulation of oxidized products, and is consistent with the increased disulfide PKGIα present in the pulmonary system during hypoxia. It was notable that lung cysteine was decreased in a time-dependent manner during hypoxia, while its plasma level was hardly detectable at 14 d of hypoxia, after a transient increase at 3 d (Fig. 2B). Cysteine is an alternative substrate for CSE that can be utilized by this enzyme specifically during oxidative stress (49). Therefore, these data further corroborate an increase in systemic CSE activity, and are consistent with increased oxidative stress and higher amounts of disulfide PKGIα during hypoxia.

Thus, the abundance of some reactive sulfur species is attenuated in hypoxia-induced PH. Interestingly, a decrease in reactive persulfide species accompanied by increased CSE expression was recently reported in the lungs of patients with chronic obstructive pulmonary disease (62). Such lower amounts of hydroperoxsulfides may appear counterintuitive to increased CSE activity (61). A likely explanation of this is that the sulfur species produced by CSE are eliminated by H₂O₂ or other endogenous electrophiles accumulating in the hypoxic tissue. Indeed, oxidized glutathione was increased in plasma (Fig. 2B), while no increase in plasma H₂O₂ was observed after 14 d of hypoxic exposure (Fig. 2A), further supporting this suggestion. Furthermore, protein-bound persulfides (CySSH/CysSH) were increased in plasma after 14 d of hypoxia (Fig. 2C). Enhanced protein persulfidation in plasma may represent a compensatory induction of persulfide biosynthesis in response to the sulfide consumption during hypoxia. Although the exact underlying mechanisms still remain unclear, these observations are consistent with systemic oxidative stress, including within the pulmonary system.

**Effect of CSE Inhibition and Polysulfide Donors on Hypoxia-Induced PH.** To experimentally test the importance of CSE in limiting PH during chronic hypoxia, C57BL/6 mice were subjected to chronic hypoxia for 14 d with or without t-propargylglycine (t-PPG)—a pharmacological inhibitor of this enzyme. 14 d of hypoxia, albeit not when t-PPG was also present, increased pulmonary disulfide PKGIα levels compared with normoxia (Fig. 3A). Moreover, t-PPG treatment of mice subjected to hypoxia increased RV pressure and hypertrophy compared with vehicle-treated hypoxic mice (Fig. 3A). CSE expression was decreased in WT or KI mice by administering small interfering RNA (siRNA) to them in vivo. This silencing approach, which was initially validated in mouse PASMCs (SI Appendix, Fig. S4A), decreased lung CSE protein by nearly 50%, with a trend toward decreased disulfide PKGIα in the same samples (Fig. 3B). As with inhibition of CSE by t-PPG, siRNA-induced knockdown of this enzyme increased RV pressure and hypertrophy in WT mice compared with vehicle-treated controls subjected to normoxia or hypoxia (Fig. 3B). There was no effect of CSE siRNA on RV systolic pressure (RVSP) or RV hypertrophic remodeling in the KI mice (SI Appendix, Fig. S4B). These observations are consistent with the causal role of CSE-derived persulfide species that facilitate disulfide PKGIα and limit PH and the consequent dysfunction.

**Redox-Dead Cys42Ser PKGIα KI Mice Develop More Severe Hypoxia-Induced PH Phenotype.** The role of disulfide PKGIα as an adaptive mechanism during hypoxic PH was examined in more depth by comparing disease progression in WT versus KI mice. KI mice showed potentiated increases in RV hypertrophy and pressure (Fig. 4 A and B) compared with WT during hypoxia. This potentiated dysfunction in the KIs that cannot form the targeting and activating disulfide PKGIα during hypoxia was further evidenced by an exacerbated decline in pulmonary vascular blood flow indexes, together with a higher pulmonary vascular resistance (PVR) (Fig. 4C). Cardiac function decline was moderate in both genotypes (SI Appendix, Fig. S5).

Phosphorylation of vasodilator-stimulated phosphoprotein (VASP) was decreased, while myosin light chain (MLC) phosphorylation was increased in lungs (Fig. 4D) and pulmonary arteries (SI Appendix, Fig. S6A) of the KI compared with WT during hypoxia. Lack of VASP phosphorylation in the KIs is consistent with deficient disulfide PKGIα targeting, and was observed previously (10). Phosphorylated MLC status is modulated by MLC phosphatase, the activity of which is phosphoregulated by PKGIα (64, 65). Disulfide PKGIα is anticipated to activate MLC phosphatase which, in turn, dephosphorylates MLC and enables smooth muscle relaxation (64). The Cys42Ser PKGIα KIs are likely deficient in this activity, resulting in higher MLC phosphorylation. This may be consistent with Cys42 oxidation targeting PKGIα, which is plausible given the disulfide occurs in the middle of the N-terminal leucine zipper of the kinase that binds a similar zipper in MLC phosphatase (47, 64). The enhanced phosphorylation of MLC in the KI during hypoxia is in agreement with enhanced vasconstrictory kinase activation (66, 67), with an inability to suitably activate the disulfide PKGIα-dependent activation of MLC phosphatase that enables compensatory vasodilation in WT.

**H₂O₂-dependent Vasodilation Is Impaired in "Redox-Dead" Cys42Ser PKGIα KI Pulmonary Arteries.** Disulfide PKGIα contributes to blood pressure homeostasis, being a component of endothelium-derived hyperpolarizing factor-dependent vasodilation (8, 47). It decreases calcium concentration in vascular smooth muscle cell, and mediates oxidant-induced vasodilation (68). Since PASMCs are abundant in PKGIα and its disulfide-dimerized form, it was rational to test whether this mechanism is preserved in pulmonary vessels. First- or second-order pulmonary arteries from the KIs demonstrated impaired vasodilatory responses to H₂O₂ compared with the WT, despite equal constriction to the pressor.
agonist U-46619 in each genotype (SI Appendix, Fig. S6B). Isolated perfused mouse lung was next employed to test vascular responses in pulmonary resistance vessels. Potentiated pressor agonist-induced constriction and deficient pulmonary vasodilatory responses to $H_2O_2$ were observed in the perfused lungs from the KI mice, compared with the WT (SI Appendix, Fig. S6C), further supporting the crucial vasodilatory role of disulfide PKGIα in pulmonary circulation. This is consistent with a global role for PKGIα oxidation in vasodilation (8, 11).

Fig. 3. Effect of CSE inhibition and polysulfides in hypoxia-induced PH. (A) Pulmonary disulfide PKGIα expression, RV pressure, and RV to left ventricle + septum (LV+S) ratio in C57BL/6 mice subjected to either normoxia or chronic hypoxia for 14 d with or without CSE inhibitor L-PPG (50 mg/kg/d). (B) CSE protein expression and disulfide PKGIα level in lungs of mice treated with CSE siRNA (1.3 mg kg$^{-1}$·d$^{-1}$); RV pressure and RV to LV+S ratio in WT mice subjected to either normoxia or chronic hypoxia for 14 d with or without CSE siRNA (1.3 mg kg$^{-1}$·d$^{-1}$). (C) Disulfide PKGIα formation in response to persulfides donor K$_2$Sx treatment in human pulmonary artery smooth muscle cells. (D) RV pressure and RV to LV+septum ratio in C57BL/6 mice subjected to either normoxia or chronic hypoxia for 14 d with or without persulfides donor K$_2$Sx (2 mg kg$^{-1}$·d$^{-1}$); RV to LV+S ratio in WT or KI mice subjected to chronic hypoxia for 14 d with or without K$_2$Sx (2 mg kg$^{-1}$·d$^{-1}$). *P < 0.05, **P < 0.01 versus control; n = 5 to 7 per group. In some cases, the aspect ratio of the original immunoblots was altered to enable a concise multipanel figure with a consistent presentation style; the original uncropped representative images of these immunoblots are also available in SI Appendix, Fig. S10.

Disulfide PKGIα formation transduces increased abundance of oxidants to vasodilation in the systemic circulation (8, 10), with the same basic events clearly in operation in the pulmonary system. This oxidative activation of PKGIα occurs in the pulmonary tissues of WT mice during chronic hypoxia for 28 d, and the evidence presented thus far indicates this offsets the hypertension in airway blood vessels that occurs at this time. This adaptive mechanism that enhances PKGIα activity to trigger vasodilation lowers RV pressure, thus reducing the associated RV hypertrophic remodeling. It is plausible that interventions that increase disulfide PKGIα may be therapeutic, limiting progression to heart failure in PAH patients.
Sustained pulmonary vasoconstriction is influenced by endothelial release of vasoactive mediators and calcium, as well as by changes in MLC phosphorylation (3). Protein kinase A (PKA) is another cyclic nucleotide-dependent kinase that, like PKGIα, is also redox-regulated by interprotein disulfide bond formation (69). PKA plays a role in opposing vasoconstriction by phosphorylating targets that reduce intracellular calcium concentration and promote relaxation (1, 64). Redox-dead Cys17Ser PKARIα KI mice which cannot be disulfide-activated (70) have increased aortic vascular reactivity to vasopressor phenylephrine and deficient H2O2-dependent relaxation (71). We subjected Cys17Ser PKARIα KI mice to chronic hypoxia, but, in contrast to the Cys42Ser PKGIα KI, found no differences in RV hypertrophy between the genotypes (SI Appendix, Fig. S7). These observations are consistent with a specific adaptive role for disulfide PKGIα in the lung in hypoxic PH scenario.

**Redox-Dead** Cys42Ser PKGIα KI Mice Develop Potentiated Pulmonary EndoMT During Hypoxia-Induced PH. Smooth muscle cell proliferation contributes to excessive muscularization of pulmonary vessels during PH (2, 3). PKGI not only modulates vasotone and the pressure within arteries but also continues to emerge as an important player in cell differentiation, growth, proliferation, and cancer progression (72–74). RV hypertrophic remodeling and pressure were assessed in WT and KI mice subjected to hypoxia for 3 d, a time when pulmonary disulfide PKGIα was elevated (Figs. 1A and 5A). WT mice developed no RV hypertrophic remodeling at this time point, and the RV pressure was similar to that in normoxic mice, meaning that PH was reversible in the WT upon return to normoxia—as occurs, for practical reasons, when RV pressure is measured (Fig. 5A). Interestingly, the PKGI KI mice developed a larger increase in RV pressure and hypertrophy, compared with the WTs, consistent with a more pronounced and sustained pulmonary vasoconstriction—perhaps marking the start of remodeling processes in the KI due to hypoxia. It is likely that the disulfide PKGIα is crucial in the pulmonary circulation even after 3 d of hypoxic exposure, by limiting increases in PAP and RVSP, and this is likely why the KI mice...
that cannot form the disulfide in PKGI develop a larger or more sustained pressure increase.

Three days of hypoxia was therefore considered a logical time point to monitor changes in gene expression, as this is before structural remodeling in the WT had occurred, in an attempt to define additional events that are important in the pathogenesis of hypoxic pulmonary disease. Thus, a transcriptomic screen using an Affymetrix microarray was performed on lungs from WT or KI mice subjected to normoxia or hypoxia for 3 d. Pathway analysis of these mRNA expression abundance data

![Fig. 5. Enhanced pulmonary vascular growth signaling and EndoMT in redox-dead Cys42Ser PKGI KI mice subjected to chronic hypoxia.](image-url)

(A) Pulmonary disulfide PKGαx level in C57BL/6 mice subjected to hypoxia for 1, 3, and 7 d; RVSP and RV to LV+S ratio from WT or PKG KI mice subjected to short-time chronic hypoxia for 3 d. *P < 0.05, **P < 0.001 versus control or WT; n = 6 to 8 per group. (B) Unbiased pathway analysis of processes with the largest number of alterations in gene expression in lungs of WT or KI mice subjected to 3 d of hypoxia compared with normoxic animals, listed in descending order. (C) Representative confocal images in lung sections from WT or KI mice subjected to chronic hypoxia for 28 d, stained simultaneously with nuclear (DAPI, blue), smooth muscle (α-SMA, red), and endothelial (CD31, green) markers. (Scale bar, 50 μm.) (D) Desmin, α-SMA, Twist-1, and phospho-vimentin protein expression in lungs from WT or KI mice subjected to chronic hypoxia for 28 d. *P < 0.05 versus control; n = 6 to 8 per group. ECM, extracellular matrix; TGF, transforming growth factor; BMP, bone morphogenetic protein; JAK/STAT, janus kinase/signal transducer and activator of transcription proteins; MAPK, mitogen-activated protein kinase; α-SMA, α-smooth muscle actin; CD31, cluster of differentiation 31; WT, wild type mice; PKG KI, “redox-dead” Cys42Ser PKGαx KI mice. Unique effect is gene changes which are unique to either the WT or KI group. Common effects are gene changes that are common to both the WT as well as the KI group. Similar effects are gene changes which are neither common nor unique to WT or KI groups. This terminology reflects the definitions in the Metalcore Training Manual (Version 5.0). In some cases, the aspect ratio of the original immunoblots was altered to enable a concise multipanel figure with a consistent presentation style; the original uncropped representative images of these immunoblots are also available in SI Appendix, Fig. S11.)
revealed an up-regulation of progrowh, extracellular matrix remodeling and endothelioal-to-mesenchymal transition (EndoMT) cellular signaling pathways in the KI compared with the WT after 3 d of hypoxia (Fig. 5B). This was notable, as EndoMT recently emerged as an important regulator of pulmonary vascular remodeling in rodent models of PH and human disease (75).

Alfemytrix microarray mRNA analysis was performed on the whole lung: therefore, it was necessary to establish whether the increased growth and EndoMT were evident in pulmonary blood vessels. Increased coexpression of α-smooth muscle actin (α-SMA) and cluster of differentiation 31 (CD31) in lung endothelial cells of the KI mice subjected to hypoxia was prominent compared with that measured in WT (Fig. 5C). Protein expressions of α-SMA and desmin, as well as the EndoMT transcriptional regulator Twist-1 and phosphorylated Vimentin (Fig. 5D), were increased in the lungs of the KI to a greater extent than those of WT following hypoxia. The KI mice subjected to 28 d of hypoxia demonstrated significantly exacerbated pulmonary vascular muscularization compared with WT exposed to the same intervention, as evidenced by a greater accumulation of α-SMA expressing cells in pulmonary vessels (SI Appendix, Fig. S6D). It is plausible that increased disulfide PKGIα during hypoxia may prevent pulmonary vascular muscularization, possibly by impairing EndoMT. Whether this mechanism serves to alleviate pressure and PVR, perhaps independently of the disulfide PKGIα pressure-lowering role, remains to be definitively elucidated.

In summary, disulfide PKGIα accumulates during chronic hypoxia in mouse and man, likely due to the accumulation of H₂O₂, glutathione disulfide, and protein-bound persulfides under these conditions. Depletion of superreducing persulfide species in mouse hypoxic tissues may also contribute to disulfide PKGIα abundance, which serves as an endogenous, adaptive redox signaling mechanism that limits PH to attenuate RV hypertrophy and disease progression. Disulfide PKGIα may also prevent the progression of EndoMT and so limit adverse pulmonary vascular remodeling. Pharmacological interventions that enhance disulfide PKGIα levels, such as polysulfides as demonstrated herein, may provide a novel therapeutic strategy to combat disease resulting from PH.

**Materials and Methods**

**Animals, Induction of Hypoxic Pulmonary Hypertension, and Treatment.** All animal procedures were performed in accordance with the Home Office Guidance and the Operation of the Animals (Scientific Procedures) Act 1986 in the United Kingdom and were approved by the King’s College Animal Welfare and Ethical Review Body. Mice constitutively expressing PKGIα Cys42Ser were produced on a pure C57BL/6 background by Taconic Biosciences as described (8, 76) and bred on-site. Age- and body weight-matched WT or PKGIα Cys42Ser KI male offspring were used in most of the studies. In some experiments, age- and body weight-matched adult C57BL/6 male mice were purchased from Charles River, as highlighted in more detail in Results and Discussion. Animals had ad libitum access to standard chow and water and were kept in specific pathogen-free conditions under a 12-h daytime cycle at 20 °C and 60% humidity before hypoxic exposure. Hypoxic PH was induced by exposing mice to normobaric hypoxia (10% of inspired O₂) in a large ventilated chamber (Bioperex, Ltd) (SI Appendix, Fig. S1). The CO₂ level was monitored continuously with CO₂ meter and soda lime. Fresh cage, water, and food changes were performed once every 7 d to 10 d for all of the animals. Additional materials and procedures can be found in SI Appendix, SI Materials and Methods.

**Study Approval.** All animal procedures were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 in the United Kingdom and were approved by the King’s College Animal Welfare and Ethical Review Body. The protocol of the study using human samples was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (No. 111/08 and S8/15). Informed consent was obtained in written form from each subject.

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