Smooth Muscle Insulin-Like Growth Factor-1 Mediates Hypoxia-Induced Pulmonary Hypertension in Neonatal Mice

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Abstract

Insulin-like growth factor (IGF)-1 is a potent mitogen of vascular smooth muscle cells (SMCs), but its role in pulmonary vascular remodeling associated with pulmonary hypertension (PH) is not clear. In an earlier study, we implicated IGF-1 in the pathogenesis of hypoxia-induced PH in neonatal mice. In this study, we hypothesized that hypoxia-induced up-regulation of IGF-1 in vascular smooth muscle is directly responsible for pulmonary vascular remodeling and PH. We studied neonatal and adult mice with smooth muscle-specific deletion of IGF-1 and also used an inhibitor of IGF-1 receptor (IGF-1R), OSI-906, in neonatal mice. We found that, in neonatal mice, SMC-specific deletion of IGF-1 or IGF-1R inhibition with OSI-906 attenuated hypoxia-induced pulmonary vascular remodeling in small arteries, right ventricular hypertrophy, and right ventricular systolic pressure. Pulmonary arterial SMCs from IGF-1-deleted mice or after OSI-906 treatment exhibited reduced proliferative potential. However, in adult mice, smooth muscle-specific deletion of IGF-1 had no effect on hypoxia-induced PH. Our data suggest that vascular smooth muscle-derived IGF-1 plays a critical role in hypoxia-induced PH in neonatal mice but not in

adult mice. We speculate that the IGF-1/IGF-1R axis is important in pathogenesis of PH in the developing lung and may be amenable to therapeutic manipulation in this age group.

Keywords: insulin-like growth factor; pulmonary hypertension; vascular smooth muscle; neonatal; hypoxia

Clinical Relevance

What this study adds to the field: Using conditional gene deletion in mice, this study shows that loss of insulin-like growth factor (IGF)-1 in smooth muscle cells protects against hypoxia-induced pulmonary vascular remodeling, right ventricular hypertrophy (RVH), and pulmonary hypertension (PH) in neonatal mice. Inhibition of IGF-1 receptor (IGF-1R) with OSI-906 diminished hypoxia-induced RVH and pulmonary vascular remodeling in neonatal mice, indicating that targeting the IGF-1/IGF-1R axis may have therapeutic benefits in the treatment of hypoxic PH in that age group.

Pulmonary hypertension (PH) is characterized by a progressive increase in pulmonary arterial pressure accompanied by pulmonary vascular remodeling, leading to right ventricular hypertrophy (RVH) and failure. Proliferation and migration of smooth muscle cells (SMCs) is a characteristic feature of pulmonary vascular remodeling in PH, and dysregulation of many growth factors have been implicated

in this process (1, 2). Several growth factors have been implicated in the vascular remodeling and pathogenesis of pulmonary arterial hypertension, and their definitive functions are still being unraveled (reviewed in Refs. 1, 3, 4). Insulin-like growth factor (IGF)-1 is a member of the insulin/IGF family and plays a crucial role in the growth, differentiation, and postnatal development of many tissues and in

the regulation of overall growth and metabolism of an organism. IGF-1 is a single-chain polypeptide with a high sequence homology to proinsulin and is expressed in most cell types to regulate growth, survival, proliferation, migration, differentiation, adhesion, and apoptosis. Its pleiotropic effects on the vascular system via both endocrine (liver-derived) and paracrine/autocrine mechanisms are

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mediated by cell surface high-affinity tyrosine kinase receptor IGF-1 receptor (IGF-1R) and, to a lesser degree, via the insulin receptor (reviewed in Refs. 5, 6). Through IGF-1R, multiple signaling pathways can be activated including phosphoinositide 3-kinase (PI3K), protein kinase B (PKB, also known as AKT), and extracellular signal-regulated kinase (ERK, also known as mitogen-activated protein kinase [MAPK]). Null mutation of IGF-1 in mice causes intrauterine growth retardation and perinatal lethality with delayed development in brain, bone, muscle, and lung as well as postnatal growth retardation and infertility in surviving mice (7, 8). Mice with a conditional deletion in the liver, the major source of plasma IGF-1, however, have normal postnatal growth and development (9).

Recently, we demonstrated that chronic hypoxia increased IGF-1 expression in lungs of neonatal mice, which could be suppressed by the use of a histone deacetylase inhibitor, apicidin, thus implicating epigenetic mechanisms in the regulation of IGF-1 expression in hypoxia (10). In addition, apicidin was also able to attenuate hypoxia-induced PH in the mice. However, a direct causal relationship between IGF-1 and hypoxia-induced vascular remodeling and RVH was not established.

In this study, we hypothesized that hypoxia-induced up-regulation of IGF-1 in pulmonary vascular SMCs is directly responsible for both vascular remodeling and PH. To test our hypothesis, we deleted *IGF-1* selectively in SMCs of mice and studied neonatal and adult mice in hypoxia. Wild-type (WT) neonatal mice were also treated with an IGF-1R inhibitor, OSI-906, to block IGF-1 signaling in chronic hypoxia. We found that smooth muscle-derived IGF-1 contributes to the development of PH induced by hypoxia in neonatal mice, but not in adult mice, demonstrating a developmental role for IGF-1 in hypoxia-induced PH.

Materials and Methods

Mice

All mice were cared for in accordance with the University of Illinois at Chicago (Chicago, IL) animal care policy following National Institutes of Health (Bethesda, MD) guidelines. Mouse experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. C57BL/6 (WT) mice were

purchased from Charles River Laboratories (Cambridge, MA). The generation of smooth muscle-specific IGF-1 knockout (SM-IGF1KO) mice and hypoxia exposure protocols are described in the supplemental MATERIALS AND METHODS.

Neonatal Mice

WT, SM-IGF1KO, and IGF-1 flox pups with their dams were randomized and placed into 11% hypoxia following the protocol described in the supplemental MATERIALS AND METHODS, within 24 hours of birth for 2 weeks. Some mice were left to recover in room air for an additional 4 weeks after hypoxia or normoxia for right ventricular systolic pressure (RVSP) measurements.

Adult Mice

The 6-week-old IGF-1 flox and SM-IGF1KO mice were placed into 10% hypoxia for 2 weeks following the protocol for hypoxia exposure described in the supplemental MATERIALS AND METHODS.

OSI-906 Administration to Neonatal Mice

WT pups were exposed to hypoxia (11% oxygen) or normoxia as described previously here. Starting at 5 days after birth, pups in hypoxia or normoxia were randomized to receive vehicle (50% DMSO in physiological saline, 5 ml/kg body weight, intraperitoneally) or the IGF-1R inhibitor, OSI-906 (linestinib, 10 mg/kg body weight in vehicle, intraperitoneally; Selleck Chemicals, Houston, TX) on alternate days until 2 weeks of age.

Measurement of IGF-1 mRNA

RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed using Superscript III (Invitrogen) and oligo (dT)₁₂₋₁₈ primers, and real-time quantitative PCR was set up containing Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The sequences for IGF-1 exon 4-specific primers used are shown in Table 1, and β -actin was used as an endogenous control.

Western Blot Analysis for Phosphorylated AKT/AKT and α -Smooth Muscle Actin in Lung and Cell Lysates

Primary antibodies for the following proteins were used: phosphorylated AKT (pAKT) (Ser473), pan AKT, pERK, ERK1/2 (Cell Signaling Technology, Beverly, MA), and α -smooth muscle actin (α -SMA; Sigma-Aldrich, St. Louis, MO) with β -actin (Santa Cruz Biotechnology, Dallas, TX) as loading control. Band intensities were quantified with NIH ImageJ (National Institutes of Health).

Cell Proliferation Assay

A bromodeoxyuridine (BrdU)-based cell proliferation kit was used with pulmonary arterial SMCs (PASMCs) from WT, flox, and SM-IGF1KO mice, as described in the supplemental MATERIALS AND METHODS.

Statistical Analysis

Statistical analysis of the data was performed using a single-factor ANOVA and standard two-sample Student's *t* test assuming

Table 1. Primer Sequences for Quantitative PCR and Genotyping

Gene (Primer ID)	Species	Sequence	F or R	Assay
Cre (primer A)	Mouse	GCGGTCTGGCAGTAAAACTATC	F	Genotyping
Cre (primer B)	Mouse	GTGAAACAGCATGTGCTGCTCACTT	R	Genotyping
IGF-1 (WT, P2)	Mouse	GGCAATGGAAATCCTATGTCT	F	Genotyping
IGF-1 (flox, P3)	Mouse	AAACCACACTGCTCGACATTG	F	Genotyping
IGF-1 (common, P1)	Mouse	CACTAAGGAGTCTGTATTTGGACC	R	Genotyping
β -actin	Mouse	AAATCGTGCGTGACATCAAAGA	F	q-PCR
β -actin	Mouse	GGCCATCTCCTGCTCGAA	R	q-PCR
IGF-1 (exon 4 specific)	Mouse	GCTATGGCTCCAGCATTC	F	q-PCR
IGF-1 (exon 4 specific)	Mouse	CTTGGGCATGTCAGTGTG	R	q-PCR
α -SMA	Mouse	TGTGCTGGACTCTGGAGATG	F	q-PCR
α -SMA	Mouse	GAAGGAATAGCCACGCTCAG	R	q-PCR

Definition of abbreviations: α -SMA, α -smooth muscle actin; F, forward; PCR, polymerase chain reaction; q-PCR, quantitative PCR; R, reverse; WT, wild type.

unequal variances of the two data sets. Statistical significance was determined using a two-tailed distribution assumption and was set at a 5% level ($P < 0.05$). Data are presented as means ($\pm$ SE).

Results

Postnatal Profile of Lung IGF-1 mRNA Levels in Mice

To determine the pattern of IGF-1 expression in lungs postnatally, we measured IGF-1 mRNA levels in lungs of neonatal mice at birth (<24 h), 1 week, 2 weeks, and 6 weeks after birth (Figure 1). IGF-1 levels were significantly increased (~ 1.9 -fold) in 1-week-old mouse lungs compared with levels at birth and returned to basal levels by 2 weeks of age, remaining low thereafter until 6 weeks of age, implying a role for IGF-1 in early lung growth and development (Figure 1A).

Chronic Hypoxia Induces IGF-1 mRNA Expression in Lungs of Neonatal Mice, but Not Adult Mice

Chronic hypoxia exposure of neonatal mice significantly increased lung IGF-1 levels after 1 and 2 weeks of exposure (Figure 1), suggesting that a further increase of IGF-1 levels in hypoxia is associated with

pathogenesis of hypoxia-induced PH in neonatal mice (Figure 1A). IGF-1 levels in neonatal mouse lungs after 4 weeks of recovery in room air after hypoxia exposure were no different than levels in lungs from room air controls at 6 weeks of age, indicating that the chronic hypoxia-induced up-regulation of *IGF-1* expression is transient or reversible in room air. In contrast, IGF-1 levels in lungs were not up-regulated in adult mice exposed to chronic hypoxia for 2 weeks (Figure 1B).

SMC-Specific Deletion of IGF-1 in Mice

Recently, we implicated IGF-1 and its epigenetic regulation in the pathogenesis of hypoxia-induced PH in neonatal mice (10). To test if smooth muscle-specific IGF-1 had a causative role in this, we generated mice with a conditional deletion of IGF-1 in SMCs (SM-IGF1KO mice) using the Cre-Lox recombinant technology. Mice homozygous for IGF-1 in which exon 4 was flanked with LoxP sites (floxed) were bred with mice expressing Cre-recombinase directed by the mouse transgelin (smooth muscle protein-22 α [SM22 α]) promoter (Figures 2A and 2B) (11, 12). The resultant SM22 α -Cre-mediated deletion of exon 4 was examined by analysis of *IGF-1* expression in the thoracic aorta of mutant

mice and showed marked reduction of IGF-1 transcripts in the medial smooth muscle layer (Figure 2C; see also Figure E1 in the online supplement). PASMCs isolated from SM-IGF1KO mice showed a significant (88%) reduction in IGF-1 mRNA levels compared with cells from floxed mice (Figure 2D). RNA isolated from lungs of 6-week-old adult SM-IGF1KO mice exhibited significantly reduced IGF-1 levels compared with floxed controls (Figure 2E). Together, these data indicate that *IGF-1* expression was successfully diminished in an SMC-specific manner in the lungs of SM-IGF1KO mice. Lung-to-body weight ratios were similar in neonatal SM-IGF1KO mice and floxed controls (Figure 2F and Figure E2). In addition, unlike homozygous null mutations of IGF-1, SM-IGF1KO mice were viable, appeared normal, and were obtained in normal Mendelian ratio without loss of fertility (7, 8).

IGF-1 Deficiency in SMCs Is Protective against Hypoxia-Induced RVH and Increased RVSP in Neonatal Mice, but Not in Adult Mice

After 2 weeks of hypoxia, IGF-1 floxed neonatal mice exhibited RVH, as indicated by an elevated ratio of right ventricular weight to left ventricular + septum weight (RV/[LV + S]) compared with control mice in normoxia (Figure 3A). However, SM-IGF1KO

Figure 1. Hypoxia increases insulin-like growth factor (IGF)-1 mRNA expression in neonatal mouse lungs. (A) Lung IGF-1 mRNA expression is up-regulated during chronic hypoxia in wild-type (WT) neonatal mice. IGF-1 expression levels were examined in lungs by quantitative PCR (q-PCR); β -actin was used as an endogenous control. Data are expressed as fold changes compared to at birth in room air ($n = 4$ –5 per group; $*P < 0.05$ compared to at birth, $^{\#}P < 0.05$ compared with normoxia group at the same time point). (B) A 2-week exposure to chronic hypoxia does not affect lung IGF-1 levels in adult mice. Data expressed as fold changes compared with normoxia group ($n = 5$) and are presented as means ($\pm$ SE).

Figure 2. Selective deletion of IGF-1 in smooth muscle of mice. (A) Schematic representation of the floxed mouse IGF-1 allele depicting exon 4 deletion mediated by smooth muscle protein-22 α -Cre along with the location of genotyping primers. (B) Presence of only IGF-1 flox and Cre bands indicates a homozygous knockout. (C) IGF-1 expression levels quantified by q-PCR with exon 4-specific primers are significantly reduced in thoracic aorta from adult smooth muscle-specific IGF-1 knockout (SM-IGF1KO) mice compared with floxed controls. Data were normalized with β -actin levels and results expressed as fold changes compared with floxed group ($n = 3$; $*P < 0.05$). (D) Reduced IGF-1 mRNA levels in pulmonary arterial smooth muscle cells (PASMCs) from SM-IGF1KO mice compared with floxed mice. Data normalized with β -actin as control. Results expressed as fold changes compared with floxed group ($n = 4$; $*P < 0.05$ compared with floxed controls). (E) Quantification of IGF-1 mRNA levels by q-PCR in lungs from adult floxed and SM-IGF1KO mice show significantly reduced levels in SM-IGF1KO mice. Data normalized to β -actin levels and expressed as fold changes compared with floxed mice ($n = 5$; $*P < 0.05$ compared with floxed controls). (F) Lung-to-body weight ratios were determined in 2-week-old SM-IGF1KO mice and floxed controls, and were found to be similar ($n = 5-6$). Data are presented as means ($\pm$ SE).

Figure 3. Neonatal SM-IGF1KO mice exhibit diminished right ventricular hypertrophy (RVH) and right ventricular pressures in response to chronic hypoxia, unlike adult mice. (A) Attenuated hypoxia-induced RVH in neonatal SM-IGF1KO mice. Newborn SM-IGF1KO and floxed mouse pups were exposed to hypoxia (11% oxygen) or normoxia for 2 weeks. The weight ratio of right ventricle to left ventricle + septum (RV/[LV + S]) was obtained as a measure of RVH ($n = 5-8$; $*P < 0.05$ compared with the floxed-normoxia group, $^{\#}P < 0.05$ compared with floxed-hypoxia group). Schema is shown above. H, hypoxia; N, normoxia. (B) Diminished right ventricular systolic pressure (RVSP) in hypoxia-recovered SM-IGF1KO mice ($n = 6-8$; $*P < 0.0005$ compared with floxed-normoxia group, $^{\#}P < 0.05$ compared with floxed-hypoxia group). (C) Hypoxia-induced RVH is similar in adult SM-IGF1KO mice and floxed controls. Chronic hypoxia increased the RV/(LV + S) in adult SM-IGF1KO and floxed mice to a similar degree ($n = 6-8$; $*P < 0.05$ compared with floxed-normoxia). (D) RVSP measured in adult SM-IGF1KO and floxed mice in chronic hypoxia are similar and significantly increased compared with normoxic groups ($n = 6-8$; $*P < 0.05$ compared with floxed-normoxia). Data are presented as means ($\pm$ SE).

neonatal mice had a significant attenuation in hypoxia-induced RVH compared with floxed controls (Figure 3A). After 4 weeks of recovery in room air, both IGF-1 floxed and SM-IGF1KO neonatal mice exposed to hypoxia (6 wk old) had increased RVSP (Figure 3B); however,

RVSP in SM-IGF1KO mice was significantly lower compared with IGF-1 floxed mice exposed to hypoxia.

In contrast, adult IGF-1 floxed and SM-IGF1KO mice had similar RVH and RVSP in response to chronic hypoxia (Figures 3C and 3D).

IGF-1 Deficiency in SMCs Is Protective against Hypoxia-Induced Pulmonary Vascular Remodeling in Small Pulmonary Arteries of Neonatal Mice, but Not in Adult Mice

Morphometric evaluation of the pulmonary vessel wall thickness in lung cross-sections

after chronic hypoxia showed that neonatal SM-IGF1KO mice exposed to chronic hypoxia exhibited a significantly blunted increase in medial wall thickness of vessels less than 50 μm in diameter compared with IGF-1 floxed mice (Figures 4A and 4B), indicating that selective *IGF-1* deletion attenuates hypoxia-induced remodeling of small vessels in neonatal mice. However, conditional *IGF-1* deletion in neonatal mice resulted in an increase in wall thickness of vessels 50–100 μm in diameter compared with floxed controls in room air with no further increase in wall thickness after hypoxia exposure (Figure 4B).

In adult mice exposed to chronic hypoxia, medial wall thickness of small pulmonary vessels was significantly increased in floxed mice (Figure 4C), consistent with vessel remodeling. In contrast to neonatal mice, wall thickness was not significantly different between adult floxed and SM-IGF1KO mice in hypoxia, indicating no benefits of gene deletion in small vessel remodeling. Both groups of adult mice also had increased remodeling in 50- to 100- μm -diameter vessels in hypoxia.

Mean Linear Intercept in Lungs of Mice Exposed to Chronic Hypoxia Is Not Affected by Smooth Muscle-Specific Deletion of IGF-1

Mean linear intercept (MLI) was used to assess alveolar enlargement as a measure of impaired lung development in chronic hypoxia. In neonatal mice, MLI was significantly greater in both hypoxia groups compared with normoxia groups, and there were no differences between the hypoxia groups (Figure 4D and Figure E3). In adult lungs, chronic hypoxia did not increase MLI (Figure 4E). Interestingly, MLI was higher in neonatal SM-IGF1KO mice compared with floxed controls in room air (Figure 4D), possibly indicating a mild inhibition in alveolar development. However, the alveolarization was similar in adults, with no differences observed between 6-week-old SM-IGF1KO mice and floxed controls in normoxia or hypoxia (Figure 4E).

Up-regulation of IGF-1 Levels in Lungs Are Induced by Hypoxia in Neonatal, but Not Adult Mice

Neonatal floxed mice exposed to 2 weeks of chronic hypoxia had a significant increase

in lung IGF-1 mRNA compared with floxed mice in normoxia, as noted previously here with neonatal WT mice (Figure E4A, Figure 1A). However, there were no differences observed in IGF-1 levels between SM-IGF1KO and floxed mice in normoxia or hypoxia (Figure E4A).

Adult SM-IGF1KO mouse lungs had reduced IGF-1 levels, consistent with endogenous deletion (Figure E4B). However, IGF-1 was not significantly increased in lungs of adult floxed or SM-IGF1KO mice exposed to chronic hypoxia, suggesting that IGF-1 signaling may not play a major role in the pathogenesis of hypoxia-induced PH in the adult. In addition, the right ventricular expression of IGF-1 mRNA was similar in hypoxia and normoxia in neonatal mice (Figure E4C).

IGF-1 Deletion in SMCs Diminishes Hypoxia-Induced Activation of AKT and Decreases Expression of α -SMA in the Lungs of Neonatal Mice

IGF-1 and its receptors activate the downstream signaling of the serine/threonine-specific kinase AKT. Therefore, lung extracts from neonatal mice exposed to chronic hypoxia or normoxia were probed for phosphorylation of AKT. As shown in Figure 5A, floxed mouse lungs had increased pAKT levels compared with SM-IGF1KO mice in hypoxia, suggesting that IGF-1 deletion in SMCs attenuates hypoxia-induced activation of AKT in neonatal mice. p44/42 MAPK (ERK1/2) activation was not observed in hypoxia, and ERK1/2 remained unchanged in floxed and SM-IGF1KO normoxic and hypoxic neonatal lungs.

Smooth muscle contractile protein α -SMA immunoreactivity was increased in the lungs of floxed mice compared with SM-IGF1KO mice exposed to hypoxia (Figure 5A). Hypoxia also increased α -SMA mRNA expression significantly in lungs of neonatal floxed mice, but not in SM-IGF1KO lungs, which was consistent with vessel remodeling in neonatal mice in hypoxia (Figure 5B).

IGF-1 Deletion in SMCs Reduces Hypoxia-Induced Proliferation of PSMCs

To examine whether IGF-1 deletion in SMCs affects their proliferative behavior, PSMCs isolated as before from SM-IGF1KO and floxed mice were exposed to hypoxia or normoxia and analyzed using

a BrdU-based assay (13). As shown in Figures 6A and 6B, PSMCs from SM-IGF1KO mice exhibited decreased proliferation in both normoxia and hypoxia compared with floxed controls. PSMCs isolated from SM-IGF1KO also showed significantly lower basal pAKT levels compared with cells from floxed mice in normoxia and in acute hypoxia (Figures 6C and 6D). Concurrently, in acute hypoxia, SM-IGF1KO cells had increased pERK levels compared with floxed PSMCs (Figures 6C and 6D).

IGF-1R Inhibition Attenuates Hypoxia-Induced RVH and Pulmonary Vessel Remodeling in Neonatal Mice

We used OSI-906, a dual inhibitor of IGF-1R/insulin receptor tyrosine kinases, to disrupt IGF-1 signaling in neonatal WT mice exposed to hypoxia (14). In vehicle-treated mice, chronic hypoxia increased RVH, but this was significantly attenuated by OSI-906 treatment (Figure 7A). The body weights of neonatal mice receiving OSI-906 treatment in room air were reduced when compared with vehicle-treated controls, and both groups had reduced body weights in chronic hypoxia (Figure E5); however, the lung weight:body weight ratios were similar. In addition, OSI-906 treatment significantly reduced remodeling in vessels of 50–100 μm in diameter and prevented a significant increase in medial thickening of vessels less than 50 μm in diameter (Figure 7B). It was observed that the chronic drug treatment also led to a significant increase in medial wall thickness of vessels 50–100 μm in diameter in room air. In addition, OSI-906 treatment in room air reduced the density of vessels that were less than 50 μm in diameter in the lungs of 2-week-old neonatal mice compared with vehicle-treated controls (Figure E6). MLI was slightly, but significantly, increased in the lungs of OSI-906-treated neonatal mice compared with the vehicle-treated group in room air (Figure E7); however, both groups showed increased MLI in hypoxia.

OSI-906 Treatment Inhibits PSMC Proliferation

To determine if IGF-1R inhibition with OSI-906 affects proliferation, mouse PSMCs were starved overnight, treated with OSI-906 or DMSO, and exposed to hypoxia or normoxia for 48 hours. Although hypoxia increased the proliferation of

Figure 4. Neonatal SM-IGF1KO mice exhibit reduced pulmonary vascular remodeling in small arterioles after chronic hypoxia exposure. Paraffin-embedded histological sections from inflation-fixed lungs were stained with hematoxylin and eosin (H&E). (A) Representative photomicrographs of lung sections from normoxic or hypoxic neonatal SM-IGF1KO and floxed mice are shown. Green arrowheads in the images indicate arterioles in lung sections (400 $\times$ magnification); A, airway; scale bars, 50 μ m. (B) Morphometric assessment of medial wall thickening in lung vessels of neonatal SM-IGF1KO mice and floxed controls in normoxia and hypoxia. Medial wall thickening in vessels smaller than 50 μ m from SM-IGF1KO was reduced compared with floxed mice in hypoxia ($n = 3-4$ per group; * $P < 0.05$ compared with flox-normoxia group, # $P < 0.05$ compared with flox-hypoxia group). H, hypoxia; N, normoxia. (C) Pulmonary vascular remodeling of small vessels from adult SM-IGF1KO and floxed mice in hypoxia is similar. Morphometric

mouse PASMCs, OSI-906 treatment potently inhibited proliferation in both hypoxia and in normoxia (Figure 7C). pAKT levels were decreased in cell lysates from mouse PASMCs treated with OSI-906 in normoxia and hypoxia (Figure 7D). No increase in pAKT:AKT ratio levels were observed in PASMCs exposed to hypoxia compared with normoxia. pERK/ERK levels remained unaffected for the time of treatment exposure.

Overall, these data demonstrate a beneficial effect of downstream IGF-1R inhibition in hypoxia-induced RVH and vascular remodeling.

OSI-906 Treatment Reduces AKT Activation in the Lungs of Neonatal Mice Exposed to Chronic Hypoxia

To understand the effect of IGF-1R inhibition on downstream AKT activation in lungs of neonatal mice exposed to hypoxia or normoxia, lung lysates from OSI-906-treated mice exposed to hypoxia or normoxia were probed for pAKT. Hypoxia increased pAKT levels compared with normoxic vehicle group (Figure E8). OSI-906 administration considerably increased AKT activation, both in normoxic and hypoxic lungs compared with the vehicle-treated groups. However, pAKT levels were diminished in OSI-906-treated hypoxic lungs compared with normoxic lungs.

Discussion

In this study, we present new evidence that augmented IGF-1 signaling in vascular SMCs plays a detrimental role in the pathogenesis of chronic hypoxia-induced PH. Interestingly, this effect was observed only in a neonatal, and not adult, model of PH. We generated mice with a conditional deletion of *IGF-1* in SMCs and exposed both neonatal and adult mice to hypoxia to evaluate PH phenotypes. When exposure to hypoxia was in the immediate neonatal period, knockout mice had reduced RVH, pulmonary vascular remodeling, and RVSP compared with control mice. α -SMA, a

specific marker of vascular remodeling, was also reduced in lungs of SM-IGF1KO mice. On the other hand, adult SM-IGF1KO mice exhibited no differences in RVH, vascular remodeling, or RVSP compared with floxed controls in hypoxia. Interestingly, chronic hypoxia also significantly increased IGF-1 transcripts in neonatal lungs, and not in adult lungs. Therefore, we conclude that the role of IGF-1 in hypoxia-induced PH in mice is specific to the postnatal developmental stage of the lung. Independently, we also demonstrated that inhibition of the IGF-1/IGF-1R/insulin receptor axis with OSI-906 reduces hypoxia-induced RVH and vascular remodeling in neonatal mice. In addition, PASMCs from the IGF-1-deleted mice or from WT mice after OSI-906 treatment exhibited reduced proliferative capacity. Taken together, our data demonstrate the importance of vascular smooth muscle-specific IGF-1 signaling in the pathogenesis of hypoxia-induced PH in neonatal mice and point to a potential therapeutic benefit of targeting the IGF-1/IGF-1R axis early in the treatment of pulmonary vascular disease in this age group.

Earlier, we had shown that IGF-1 was up-regulated in neonatal hypoxia involving an epigenetic mechanism, and that the increased *IGF-1* expression was associated with the development of RVH and pulmonary vascular remodeling in hypoxia-induced PH (10). In this study, we used a conditional gene deletion approach in mice to provide evidence that dysregulation of the vascular smooth muscle IGF-1 signaling in hypoxia can contribute to the pathogenesis of PH, but only in the neonatal stage. The rationale for our study stems from the observations that chronic hypoxia increases pulmonary IGF-1 expression in neonatal lungs and that IGF-1 is a critical growth factor involved in lung development that is expressed by SMCs and can induce SMC hyperplasia. Because null mutations of IGF-1 or IGF-1R in mice are lethal and can impair distal lung morphogenesis but conditional liver-specific deletion of IGF-1 results in normal

growth patterns confirming paracrine/autocrine functions of IGF-1 (15), we used the SMC-specific IGF-1 knockout mouse to test if IGF-1 signaling participates in the pathophysiology of PH in the developing lung.

IGF-1/IGF-1R has been shown to be required for normal lung development, pulmonary vascularization, and proper alveolarization (8, 15, 16). In our study, we observed that lung IGF-1 levels in mice increased significantly after birth before returning to baseline at around 2 weeks of age, coinciding with the period of postnatal alveolarization in mice and consistent with an important function of IGF-1 in lung development after birth (Figure 1A). In addition, we show that chronic hypoxia further elevates IGF-1 levels in the neonatal period. Absence of IGF-1 results in prenatal lung hypoplasia and abnormal vascularization in mice (17, 18). In direct contrast with total gene knockout, SMC-specific deletion of IGF-1 did not affect viability or body weights of neonatal mice at 2 weeks of age (8). Lung-to-body weight ratios in SM-IGF1KO mice were similar to those of control mice, indicating an absence of pulmonary hypoplasia with compensated postnatal lung development, presumably maintained through paracrine/endocrine IGF-1 mechanisms. It is noteworthy that total lung IGF-1 levels were not significantly diminished in neonatal SM-IGF1KO mice, suggesting similar compensatory mechanisms contributing to maintain IGF-1 levels. Although incomplete Cre-mediated gene deletion can be envisaged in neonatal SM-IGF1KO mice, the protective phenotype exhibited in chronic hypoxia indicates successful deletion. Absence of an IGF-1 increase in lungs of adult mice in hypoxia suggested that IGF-1 plays a role in the pathogenesis of PH only in the neonatal stage, and not in adults.

The role of IGF-1 in vascular function and pulmonary disease has been investigated previously in several studies. IGF-1 stimulates SMC growth and inhibits apoptosis *in vitro* (19). In PASMCs, IGF-1

Figure 4. (Continued). assessment of medial wall thickness in arterioles smaller than 50 μ m and 50- to 100- μ m-diameter arterioles from H&E-stained lung sections are increased in hypoxia compared with in normoxia ($n = 3$ –5 per group; $*P < 0.05$ compared with flox-normoxia group). (D) Evaluation of alveolarization by mean linear intercept (MLI) measurement in inflation-fixed lung sections stained with H&E was performed as described in the supplemental MATERIALS AND METHODS. The MLI in hypoxia-exposed neonatal SM-IGF1KO and floxed mice was similar ($n = 5$ –8; $*P < 0.05$ compared with flox-normoxia group). (E) In adults, the MLI was unchanged in both chronic hypoxia and between SM-IGF1KO and floxed controls ($n = 5$). Data are presented as means ($\pm$ SE).

Figure 5. Protein kinase B (PKB, also known as AKT) activation in chronic hypoxia is diminished in lungs of neonatal SM-IGF1KO mice. (A) Western blot analysis of lung lysates from neonatal SM-IGF1KO and floxed mice in normoxia and hypoxia probed with phosphorylated AKT (pAKT) (Ser473) and pan AKT showed diminished activation of AKT in SM-IGF1KO mice in hypoxia. pERK and ERK1/2 levels were similar in hypoxia and normoxia. Neonatal floxed mice have increased α -smooth muscle actin (α -SMA) expression in hypoxia, which was diminished in SM-IGF1KO mice with β -actin serving as loading control. A representative blot from three experiments is presented. Panels below show plots of pAKT/AKT and α -SMA/actin proteins quantified from the band intensities (* $P < 0.05$ compared with flox-normoxia, # $P < 0.05$ compared with flox-hypoxia). (B) Diminished α -SMA expression in neonatal SM-IGF1KO mouse lungs exposed to chronic hypoxia. α -SMA mRNA expression was analyzed in lungs of neonatal SM-IGF1KO and floxed mice in normoxia and hypoxia and normalized to β -actin levels. Data are expressed as fold changes compared with flox-normoxia group ($n = 4-5$; * $P < 0.05$ compared with flox-normoxia group). Data are presented as means ($\pm$ SE).

Figure 6. Decreased proliferation of PSMCs from SM-IGF1KO. (A) PSMCs isolated from SM-IGF1KO and floxed mice were starved and exposed to hypoxia or normoxia before bromodeoxyuridine (BrdU) labeling for assay as described in MATERIALS AND METHODS. Data from four experiments are represented as fold change of the normoxic group (* $P < 0.05$ compared with normoxia group, # $P < 0.05$ compared with flox-hypoxia group). (B) Reduced proliferation of isolated PSMCs from SM-IGF1KO in normal conditions. PSMCs from SM-IGF1KO and floxed control mice were assayed with BrdU labeling in normoxia after overnight starvation. Data are represented as fold change compared with the floxed group from four experiments (* $P < 0.05$ compared with floxed group). (C) Diminished activation of AKT in PSMCs from SM-IGF1KO. PSMCs isolated from two floxed (FL) and two SM-IGF1KO mice (KO) were serum starved for 48 hours before being exposed to normoxia or hypoxia for 4 hours. Cell lysates were analyzed by Western blotting and probed for pAKT, AKT, pERK, ERK1/2, and β -actin. Experiments were repeated three times. A representative blot from two mice of each group is shown, and band intensities were quantified in a plot of pAKT:AKT and pERK:ERK ratios (D) from four experiments (* $P < 0.05$ compared with FL-normoxia group). Data are presented as means ($\pm$ SE). FL, floxed.

has been shown to inhibit apoptosis through an increase in p38, MAPK, and inducible nitric oxide synthase expression (20). *In vivo*, transgenic mice with an overexpression of IGF-1 in SMCs develop hyperplasia and increased SMC layer thickness in several organs without a change in plasma IGF-1 levels, implicating a paracrine effect (21). Chronic expression of IGF-1 within the vessel wall is also associated with increased arterial contractility (22–24), and direct stimulation of aortic SMCs with IGF-1 *in vitro* increases the expression of SMC differentiation markers, α -SMA, calponin, and SM22 α (25). In addition, IGF-1 has the ability to stimulate elastin in vascular SMCs (26), and increased IGF-1 expression has been associated with remodeling in sheep models

of neonatal PH (27, 28). IGF-1 has also been found to play a role in chronic lung disease, such as respiratory distress syndrome and bronchopulmonary dysplasia, of premature and newborn infants (see Refs. 29–34). However, the exact role of IGF-1 in pulmonary SMC proliferation, differentiation, and structural remodeling of vessels leading to the pathogenesis of PH sequelae has not been fully established. In our study, we find that the small arteriole remodeling, as a result of chronic hypoxia, is abolished in neonatal mice deficient in SMC-derived IGF-1.

Chronic hypoxia is known to impair pulmonary vascularization, alveolarization, and lung development, and the effects are dependent on the age and degree of lung maturation at the onset of exposure (35, 36).

A peroxisome proliferator-activated receptor (PPAR)- γ ligand was able to attenuate TGF- β signaling and improve alveolar development, but not pulmonary vascular remodeling, in neonatal PH (37), and inhibition of CXCR4 improved alveolarization (38). In our study, IGF-1 deletion in SMCs of mice was beneficial in hypoxia-induced pulmonary vascular remodeling and PH in neonatal mice but did not improve lung alveolarization in hypoxia. In addition, a mild, but significant, increase in MLI was observed in 2-week-old SM-IGF1KO mice in room air, indicating possible delayed alveolar development or a consequential shift in lung compliance. It is well known that vascular growth and alveolarization are interrelated during the saccular and alveolar stages of lung

Figure 7. Inhibition of IGF-1/IGF-1 receptor axis with OSI-906 reduces RVH and pulmonary vascular remodeling in neonatal mice exposed to chronic hypoxia. Newborn WT mice exposed to hypoxia or normoxia for 2 weeks after birth were administered OSI-906 or vehicle between Days 5 and 14, as described in MATERIALS AND METHODS. (A) Reduction of hypoxia-induced RVH in OSI-906-treated mice ($n = 5-6$; $*P < 0.05$ compared with normoxia vehicle, $#P < 0.05$ compared with hypoxia vehicle). (B) Pulmonary vascular remodeling of small vessels in hypoxia was diminished after chronic administration of OSI-906 ($n = 3-5$; $*P < 0.05$ compared with normoxia-vehicle, $#P < 0.05$ compared with hypoxia-vehicle). (C) Proliferation of mouse PSMCs is reduced after treatment with OSI-906. Mouse PSMCs were starved overnight before treating with OSI-906 or DMSO and exposure to hypoxia or normoxia. BrdU labeling of cells was performed 16 hours before assay. Experiment was repeated three times, and data are represented as fold change relative to normoxia-vehicle group ($*P < 0.05$ compared with normoxia-DMSO group, $#P < 0.05$ compared with hypoxia-DMSO group). (D) OSI-906 treatment suppresses AKT activation in mouse PSMCs. Mouse PSMCs were serum starved for 24 hours and treated with OSI-906 or DMSO and exposed to hypoxia or normoxia for 4 hours. Cell lysates probed by Western blotting for AKT and ERK activation were normalized to β -actin levels. Experiments were repeated three times, and a representative blot is shown. Band intensities were quantified to depict pAKT: AKT ratios from three to four blots ($*P < 0.05$ compared with DMSO control). Data are presented as means ($\pm$ SE).

development (39). However, the absence of lung hypoplasia in SM-IGF1KO mice suggests that other nonangiogenic mechanisms related to alveolar septation/development may contribute to this (40).

We have previously shown that chronic hypoxia increases pAKT levels in lungs of neonatal mice and that IGF-1 is able to activate AKT in PSMCs (10). Tang and colleagues (41) have recently demonstrated that the absence of AKT1, but not AKT2, diminishes pulmonary vascular remodeling and PH in mice exposed to chronic

hypoxia. To understand the molecular mechanisms affected by IGF-1, we isolated PSMCs from mice and found that basal AKT activation was decreased in SM-IGF1KO mice (Figure 6C). Although basal ERK levels were similar in normoxia, activation of ERK was noted in PSMCs of SM-IGF1KO mice compared with floxed controls in hypoxia. This effect is likely compensatory because lung ERK levels were not affected in normoxic and chronically hypoxic lungs.

In this study, we show that SMC-specific deletion of IGF-1, as well as

inhibition of IGF-1R with OSI-906, attenuated the hypoxia-induced activation of AKT in lungs of neonatal mice and in isolated PSMCs, suggesting that the pathogenesis of neonatal PH is at least partly mediated through the activation of the IGF-1/IGF-1R/AKT pathway.

Treatment with OSI-906, a small-molecule inhibitor of both receptor tyrosine kinases (14), alleviates the RVH and lung vascular remodeling mediated by IGF-1 in neonatal mice exposed to chronic hypoxia. Although administration of OSI-906 increased pAKT levels in both

normoxic and hypoxic neonatal lungs, it diminished pAKT levels in chronic hypoxia compared with normoxia. The reason for the noticeable increase in pAKT levels in vehicle-treated normoxia mice is not clear, and will require further investigation, although possible activation of AKT due to receptor cross-talk initiated by small-molecule inhibitors, through IGF-1R/epidermal growth factor receptor 1/epidermal growth factor 2/platelet-derived growth factor receptor has been described in cancer cell chemoresistance (reviewed in Ref. 42). Our study shows that OSI-906 administration protects against RVH and pulmonary vessel remodeling induced by chronic hypoxia

in neonatal mice, and thus, inhibition of the IGF-1/IGF-1R signaling axis may be an approach in the treatment of hypoxia-induced PH in the developing lung.

In conclusion, our study demonstrates that smooth muscle-derived IGF-1 plays a causal role in the development of neonatal hypoxia-induced PH in mice. We are reporting, for the first time, the use of a conditional knockout mouse model to implicate growth factor IGF-1 in the pathogenesis of neonatal PH. Likely due to the dysregulation of IGF-1 in the neonatal period, a conditional knockout of IGF-1 in SMCs is protective only in neonatal mice exposed to chronic hypoxia, and not in

adult mice. This highlights the complexity of the interactions between normal physiological processes and pathophysiologic stimuli, with particular focus on age (43). In addition to implicating IGF-1 in the development of neonatal PH, we have shown that targeting the IGF-1/IGF-1R pathway using a small-molecule inhibitor can be a possible therapeutic strategy. However, additional investigations must be performed to elucidate the exact mechanisms by which IGF-1 mediates neonatal PH and its involvement in human disease. ■

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

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