Hypoxia-Inducible Factor 1α Is a Critical Downstream Mediator for Hypoxia-Induced Mitogenic Factor (FIZZ1/RELMα)—Induced Pulmonary Hypertension

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Objective—Pulmonary hypertension (PH) is characterized by progressive elevation of pulmonary vascular resistance, right ventricular failure, and ultimately death. We have shown that in rodents, hypoxia-induced mitogenic factor (HIMF; also known as FIZZ1 or resistin-like molecule-β) causes PH by initiating lung vascular inflammation. We hypothesized that hypoxia-inducible factor-1 (HIF-1) is a critical downstream signal mediator of HIMF during PH development.

Approach and Results—In this study, we compared the degree of HIMF-induced pulmonary vascular remodeling and PH development in wild-type (HIF-1α+/-) and HIF-1α heterozygous null (HIF-1α−/−) mice. HIMF-induced PH was significantly diminished in HIF-1α−/− mice and was accompanied by a dysregulated vascular endothelial growth factor-A–vascular endothelial growth factor receptor 2 pathway. HIF-1α was critical for bone marrow–derived cell migration and vascular tube formation in response to HIMF. Furthermore, HIMF and its human homolog, resistin-like molecule-β, significantly increased interleukin (IL)-6 in macrophages and lung resident cells through a mechanism dependent on HIF-1α and, at least to some extent, on nuclear factor κB.

Conclusions—Our results suggest that HIF-1α is a critical downstream transcription factor for HIMF-induced pulmonary vascular remodeling and PH development. Importantly, both HIMF and human resistin-like molecule-β significantly increased IL-6 in lung resident cells and increased perivascular accumulation of IL-6–expressing macrophages in the lungs of mice. These data suggest that HIMF can induce HIF-1α, vascular endothelial growth factor-A, and interleukin-6, which are critical mediators of both hypoxic inflammation and PH pathophysiology. (Arterioscler Thromb Vasc Biol. 2016;36:134-144. DOI: 10.1161/ATVBAHA.115.306710.)

Key Words: hypertension, pulmonary ◼ hypoxia-inducible factor 1 ◼ interleukins ◼ macrophages ◼ resistin-like molecule

Pulmonary hypertension (PH) is characterized by remodeling of distal pulmonary arteries and vasoconstriction and in situ thrombosis that lead to enhanced pulmonary vascular resistance and pressure, progressive right-sided heart failure, and ultimately death. In humans, severe pulmonary arterial hypertension is characterized by plexiform lesions that contain phenotypically altered pulmonary smooth muscle cells (SMCs) and endothelial cells (ECs). Despite major advances in diagnosis and treatment of this disease over the past several decades, the underlying mechanisms of PH and its cause are poorly understood. Growing evidence indicates that inflammation plays a key role in triggering and maintaining pulmonary vascular remodeling.

Our group has shown that hypoxia-induced mitogenic factor (HIMF, also known as FIZZ1 or resistin-like molecule [RELM]-α), a member of the resistin family of proteins, is dramatically upregulated in the proliferative phase of a hypoxia-induced PH model. Conversely, inhibition of the HIMF pathway prevents development of hypoxia-induced PH. HIMF has proinflammatory actions and is known to be persistently upregulated in animal lungs in models of allergic inflammation, bleomycin-induced pulmonary fibrosis, and herpes virus-induced pulmonary fibrosis. HIMF exhibits chemotactic actions on bone marrow–derived (BMD) cells, in part, by binding Bruton tyrosine kinase. Interestingly, genetic transfer of HIMF into rodent lungs induces the vascular remodeling and hemodynamic changes of PH in rats and promotes BMD cell recruitment to the remodeled pulmonary vasculature in mice.

HIMF (FIZZ1) is well known as a marker for alternatively activated (M2) macrophages. It has been suggested that this phenotype is positively associated with tissue remodeling and...
vascular growth response in chronic inflammatory conditions, including PH.\textsuperscript{12,13} We also have shown that HIMF expression in the remodeled pulmonary vasculature, but not in airway epithelial cells, positively correlates with increased mean pulmonary arterial pressure in experimental PH.\textsuperscript{14} On the basis of these data, it is reasonable to speculate that macrophage-derived HIMF (FIZZ1) protein plays a critical role in the PH development.

We have recently established a mouse model of PH in which a single systemic injection of recombinant (r)HIMF protein causes early lung inflammation (day 7) and PH development (day 30) that are dependent on the vascular endothelial growth factor (VEGF) and T-helper (Th) type 2 cytokine interleukin (IL)-4 pathways.\textsuperscript{5,4} Using this HIMF injection model, we also have shown that macrophage recruitment to the lung and lung vascular inflammation in response to HIMF is completely suppressed in IL-4 knockout mice.\textsuperscript{5} It has been suggested that HIMF recruits and binds BMD macrophages, dendritic cells, and T cells.\textsuperscript{15,16} Moreover, we have proven that HIMF can activate pulmonary ECs and EC apoptosis to trigger pulmonary vascular inflammation.\textsuperscript{5} Although HIMF is thought to be a shared mediator of both hypoxic and Th2 inflammation,\textsuperscript{14,17,18} the underlying mechanism by which HIMF induces downstream mediators critical for PH development is unknown.

The 2 human homologs of HIMF are RELM\textbeta{} and resistin (hRETN). RELM proteins are 105 to 114 amino acids in length, and their C-terminal signature sequence contains 10 cysteines that are highly conserved and constitute nearly half of the molecule.\textsuperscript{19} RELM\textbeta{} is upregulated in lungs of patients with scleroderma-associated PH, strongly suggesting an causal role of resistin family proteins in PH.\textsuperscript{20} On the contrary, hRETN is expressed by myeloid cells, especially macrophages, and its expression pattern shows a greater similarity to that of murine HIMF (RELMo) than to that of murine resistin.\textsuperscript{21} We have recently shown that, like HIMF, hRETN induces endothelial activation, apoptosis, and release of growth factors for SMCs, suggesting that it has a critical role in EC–SMC cross talk under inflammatory conditions.\textsuperscript{5}

In the present study, we dissected the mechanism by which HIMF induces mediators that contribute to the PH development. Accumulating evidence suggests that hypoxia-inducible factor-1 (HIF-1) is a critical transcription factor in both hypoxic inflammation and Th2 immune activation in the lung.\textsuperscript{22,23} Because we have shown previously that HIMF upregulates angiogenic/proinflammatory mediators in the lung that are downstream of HIF-1, such as VEGF, we hypothesized that HIF-1 is a critical downstream mediator of HIMF during pulmonary vascular remodeling and development of PH. In this context, we investigated whether (1) HIF-1 mediates HIMF-induced pulmonary vascular remodeling and PH development in vivo; (2) whether HIF-1 mediates HIMF-induced EC apoptosis; (3) whether HIMF mediates BMD cell migration/recruitment in response to HIMF; (4) whether HIF-1 is critical to inducing proinflammatory mediators in macrophages and lung resident cells; and finally (5) whether the human homolog of HIMF mediates HIF-1 activation as a downstream signaling pathway in human lung resident cells.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**HIMF Increases Expression of HIF-1α, but Not HIF-2α, in the Lung**

We and others have shown that HIMF is the critical shared mediator for hypoxia and Th2 inflammation in the lung.\textsuperscript{14,17,18} Both hypoxia and inflammation also stimulate the transcription factor HIF-1.\textsuperscript{23} Therefore, we examined whether HIF-1 is activated in HIMF-induced pulmonary vascular remodeling and PH development. In HIMF-injected mice,\textsuperscript{5} we observed that HIF-1α expression in lung epithelial cells and macrophages was increased during the PH development phase (day 30, Figure 1A). We also analyzed HIF protein expression in response to HIMF injection in HIF-1α\textsuperscript{−/−} and HIF-1α\textsuperscript{+/−} mice. Interestingly, HIMF treatment increased HIF-1α protein expression, but not HIF-2α expression, in lung tissue of HIF-1α\textsuperscript{−/−} mice (Figure 1B). However, this HIMF-induced HIF-1α expression was abolished in HIF-1α\textsuperscript{+/−} mice. Next, we examined the expression of VEGF-A and its receptor (VEGFR) in the lung because we have shown previously that VEGF-A is increased and VEGFR2 dysregulated in this animal model, suggesting that HIMF-induced lung inflammation occurs through a VEGFR2-dependent mechanism.\textsuperscript{3} Indeed, HIMF caused significant VEGF-A induction and decreased VEGFR2 expression in HIF-1α\textsuperscript{−/−} mice; however, these responses were abolished in HIF-1α\textsuperscript{+/−} mice (Figure 1B). Interestingly, HIMF treatment did not change VEGFR1 expression in either genotype. These data suggest that HIMF stimulates HIF-1–VEGF-A–VEGFR2 signaling in the lung.

**HIMF Causes HIF-1α–Dependent Pulmonary Vascular Remodeling and PH Development**

To determine the importance of HIMF as a downstream signaling mediator of HIMF in the development of PH, we induced PH by injecting HIMF into HIF-1α\textsuperscript{−/−} and HIF-1α\textsuperscript{+/−} mice as previously described.\textsuperscript{5} At 30 days after HIMF injection (PH development phase), medial thickening was evident in small pulmonary vessels in HIF-1α\textsuperscript{−/−} mice but not in HIF-1α\textsuperscript{+/−} mice (Figure 2A). Similarly, HIMF injection significantly increased airway thickness in HIF-1α\textsuperscript{+/−} mice, but...
this phenomenon was markedly diminished in HIF-1α<sup>−/−</sup> mice (Figure 2B). Furthermore, HIMF-injected HIF-1α<sup>−/−</sup> mice exhibited significant right heart hypertrophy and had higher right heart pressure than did vehicle-injected HIF-1α<sup>−/−</sup> mice (Figure 2C). These hemodynamic changes were absent in HIF-1α<sup>−/−</sup> mice although no baseline differences were apparent between genotypes. Finally, we compared HIMF-induced pulmonary vascular remodeling in each genotype. The 2
genotypes exhibited no baseline differences; however, HIMF injection caused a significant decrease in the number of non-muscularized vessels and increases in the number of partially muscularized and fully muscularized vessels in HIF-1α+/+ mice. This HIMF-induced pulmonary vascular remodeling was significantly diminished in HIF-1α+/− mice (Figure 2D). These data suggest that HIMF causes pulmonary vascular remodeling and development of PH by a HIF-1–dependent mechanism in this animal model.

HIMF Recruits BMD Macrophages, and HIMF-Mediated In Vivo Vascular Formation Is Significantly Diminished in HIF-1α−/− Mice

Vasculogenesis describes the process of de novo blood vessel formation from vascular precursor cells. It has been suggested that BMD cells, especially BMD macrophages, play a central role in postnatal vessel growth as a source of proangiogenic paracrine factors.24 We have previously shown that HIMF can induce vascular tube formation in vivo and vascular growth response in the lung.3 We have also shown significant macrophage recruitment to the lungs of HIMF-treated mice.4 BMD macrophages are known to be recruited at the initiation stage of vascular formation during neovascularization of subcutaneous Matrigel plugs.25 In this context, we examined whether HIF-1 mediates HIMF-induced vascular tube formation in vivo using HIF-1α+/+ and HIF-1α−/− mice. HIMF caused strong vascular tube formation in the Matrigel plugs of HIF-1α+/+ mice (Figure 3A). HIMF-induced vascular tube formation is usually observed in the presence of red blood cells in Matrigel, as shown in Figure 3A. Notably, HIMF treatment caused massive red blood cells recruitment in the Matrigel plugs of HIF-1α+/− mice; however, we observed significantly fewer of the nucleated cells that can form vascular tubes in HIF-1α+/− mice than in HIF-1α+/+ controls (Figure 3B). Because BMD macrophages are thought to be recruited to the area of neovascularization to form vascular tubes and stabilize vascular networks by releasing the factors necessary for vascular growth response,25 we analyzed whether HIMF recruits BMD macrophages to the area of vascular formation using a BM transplant technique in which lethally irradiated mice were rescued with BM transplanted from green fluorescent protein (GFP)-positive transgenic mice, as we have previously reported (Figure 3C).10 We were able to identify BMD cells using anti-GFP antibody. GFP-positive BMD cells in the newly formed vascular tube colocalized with macrophage marker F4/80 and CD11b (Figure 3D and 3E). To determine whether the baseline number of macrophages differed in each genotype, we also compared the ability of BMD cells to differentiate into a macrophage phenotype in response to macrophage colony-stimulating factor (also known as colony-stimulating factor-1). Macrophage colony-stimulating factor is a cytokine required for the differentiation of monocyte lineage cells to macrophages and promotes the formation of high-density vessel networks in pathological angiogenesis.26

Figure 2. Hypoxia-induced mitogenic factor (HIMF) injection causes hypoxia-inducible factor (HIF)-1α-dependent pulmonary vascular remodeling and pulmonary hypertension. A and B, Hematoxylin and eosin–stained small pulmonary vessels (A; scale bar, 100 μm) and pulmonary airway epithelium (B; scale bar, 200 μm) from HIF-1α−/− and HIF-1α+/+ mice 30 d after intravenous injection with HIMF or vehicle. C, Hemodynamics and right ventricular hypertrophy were analyzed 30 d post injection. Left, n=4 to 5 per group; right, n=4 to 7 per group. Data are shown as mean±SEM. D, Comparison of HIMF-induced pulmonary vascular remodeling in HIF-1α+/+ and HIF-1α−/− mice. Bar graphs show the percentage of small pulmonary arteries in HIF-1α−/− and HIF-1α−/− mice that were nonmuscular (NM), partially muscular (PM), or fully muscular (FM). *P<0.05, **P<0.01, ***P<0.001 vs HIF-1α+/+ + HIMF. At least 500 vessels were counted in each group (3–5 animals per group). RVSP indicates right ventricular end systolic pressure; and RV/(LV+S), right ventricular weight/(left ventricular+septal weight). CONT indicates control.
Figure 3. Hypoxia-inducible factor (HIF-1)–dependent bone marrow cell recruitment and vascular formation in response to hypoxia-induced mitogenic factor (HIMF). A and B, HIMF–induced vascular formation in Matrigel plugs from HIF-1α+/+ and HIF-1α+/− mice. A, Representative hematoxylin and eosin images of vascular formation in Matrigel plugs after HIMF (50 nmol/L) administration to HIF-1α+/+ and HIF-1α+/− mice (×40 magnification). B, Bar graph represents mean value of nucleated cell areas in the vascular formation from 5 to 10 images per mouse (n=3–4 mice per group). C, Representative hematoxylin and eosin image of vascular formation in a Matrigel plug from a bone marrow–transplanted mouse injected with HIMF (×400 magnification). D and E, Colocalization of bone marrow–derived (BMD) cells (GFP-positive, green) with macrophage markers F4/80 (D, red, ×630 magnification) or CD11b (E, red, ×1000 magnification). (Continued)
Our results showed that BMD cells from HIF-1α +/- mice and HIF-1α +/- controls differentiated to macrophages (CD11b positive) in response to macrophage colony-stimulating factor to a similar degree (Figure 3F). Thus, the pathophysiological differences in response to HIMF are not based on a difference in baseline numbers of macrophages in each genotype. These results suggest that HIMF recruits macrophages to the area of vascular formation and that this phenomenon is mediated by HIF-1 as a downstream transcription factor of HIMF.

We have previously reported that HIMF and its human homolog hRETN stimulate EC activation and apoptosis in pulmonary microvascular ECs (PMVECs). Furthermore, these HIMF- or hRETN-stimulated ECs produce growth factors and chemokines that enhance perivascular immune cell recruitment and SMC growth response. Here, we characterized EC-derived human SMC growth factors in response to hRETN and found that serpin E1 (also known as plasminogen activator inhibitor-1), tissue inhibitor of metalloproteinase-1, and urokinase plasminogen activator were released from ECs in response to hRETN. These molecules are known to promote SMC growth response (Figure I in the online-only Data Supplement).

We also have previously reported that HIMF recruits BMD cells to the area of remodeled pulmonary vasculature. In a cell culture system, we have shown that HIMF directly causes migration of myeloid cells through the action of Bruton tyrosine kinase, a functional binding partner of HIMF. To investigate whether HIF-1 mediates HIMF-induced BMD cell migration, we isolated BMD cells from HIF-1α +/- and HIF-1α +/- mice and analyzed HIMF-induced BMD migration as previously described, using HIMF protein (20 nmol/L) alone or conditioned medium from HIMF-1α +/- PMVECs treated with or without HIMF (20 nmol/L, 24 hours). BMD cells from HIF-1α +/- mice migrated toward HIMF as expected. To our surprise, HIMF-1α +/- rHIMF-treated PMVEC conditioned medium caused a 3.87-fold increase in BMD migration when compared with untreated control groups (Figure 3G). However, BMD cells from HIF-1α +/- mice did not migrate in response to either rHIMF or HIMF-treated PMVEC conditioned medium. These results suggest that HIMF can stimulate PMVECs to produce chemokines that cause BMD cell migration. The results also show that HIMF-1α activity in BMD cells is critical to HIMF-induced myeloid cell migration.

**HIMF Increases IL-6 Production in Macrophages In Vitro and In Lung Organ Slices Ex Vivo in a HIF-1α–Dependent Manner**

Macrophage accumulation is not only a hallmark of PH but also a critical component of pulmonary vascular remodeling associated with PH. Recent studies have shown that macrophage activation is critical in experimental PH. Macrophages are also known to be responsible for producing a large spectrum of chemokines and proinflammatory and vascular growth factors. In this context, we performed angiogenesis cytokine array analysis using culture medium from BMD macrophages and lung organ slices from each genotype treated with or without HIMF. We found that HIMF significantly induced IL-6 protein expression in BMD macrophages (Figure 4A) and in lung resident cells (Figure 4B). However, this effect was completely suppressed in cells from HIF-1α +/- mice (Figure 4A and 4B).

**Human RELMβ Increases HIF-1α and IL-6 Expression Through a Nuclear Factor-κB–Mediated Mechanism in Human Primary Lung Fibroblasts**

In our previous study, we showed that RELMβ expression was elevated in the remodeling vasculature of patients with PH,
HIMF Increases IL-6 Production in Perivascular Macrophages and SMC in a HIF-1α–Dependent Manner In Vivo

Involvement of IL-6 signaling has been strongly implicated in clinical and experimental PH. We further examined IL-6 induction in response to HIMF in the remodeled pulmonary vasculature in vivo. HIMF caused massive IL-6 expression in the remodeled vascular media (α-SMA–positive cells) and in infiltrating inflammatory cells of HIF-1α+/− mice but not in those of HIF-1α−/− mice (Figure 5A). Next, we analyzed whether those IL-6–expressing inflammatory cells in the remodeled small pulmonary vessels are macrophages by using the marker for macrophage F4/80. We observed that IL-6–expressing macrophages (F4/80+ cells) are recruited to the media of remodeling small pulmonary vessels in HIMF-treated HIF-1α+/− mice but not in HIF-1α−/− mice (Figure 5B). Notably, in addition to those IL-6–expressing macrophages, we observed the recruitment of IL-6–expressing α-SMA–positive cells that were distinct from resident SMC in the remodeling vasculature (Figure 5D and 5E). These data suggest that HIMF stimulates the recruitment of IL-6–expressing macrophages and α-SMA–positive cells to the pulmonary vessels via a HIF-1α–dependent mechanism.

Discussion

We have shown previously that HIMF induces proinflammatory and proangiogenic cytokines (eg, VEGF) that are critical to hypoxic inflammation and known to lie downstream in the signaling pathway of transcription factor HIF-1. Thus, we hypothesized that HIF-1 is a critical downstream signal mediator of HIMF-induced pulmonary vascular remodeling and development of PH. In this study, we showed that HIMF can induce expression of HIF-1α, but not of HIF-2α, in the lung. Moreover, HIF-1α was required for HIMF-induced vascular remodeling, vascular tube formation, BM cell recruitment, and IL-6 induction in macrophages and lung resident cells. Importantly, HIMF significantly increased lung VEGF-A expression and downregulated VEGFR2 in a HIF-1α–dependent manner. VEGFR2 dysfunction is a hallmark of the severe pathophysiology observed in human and experimental PH. In addition, increased HIF-1 and VEGF expression are observed in plexiform lesions in patients with severe PH. Our results are in accordance with human conditions and suggest that the mechanism of HIMF-induced PH is mediated, at least in part, by HIF-1–VEGF–A–VEGFR2 signaling in the lung.

We have shown previously that in mice, HIMF causes PH by initiating pulmonary EC apoptosis and mediating lung vascular inflammation in a Th2 cytokine IL-4–dependent mechanism. Indeed, HIMF/RELMα was upregulated in the lungs of animals exposed to either ovalbumin immunization or chronic hypoxia, suggesting that it is a common mediator in both hypoxic PH and Th2 lung inflammation. Several investigators have reported that Th2-skewed stimulation causes severe pulmonary vascular muscularization, indicating that pulmonary vascular remodeling is under the control of the immune system.39 However, a model of PH development and right heart failure after Th2 antigen challenge alone has not been reported. Mizuno et al determined factors that are critical to PH development by using a rat model of PH created with a combination of VEGFR2 tyrosine kinase inhibitor Sugen 5416 and Th2 immune system activation (Su-ovalbumin). In that study, the authors found that the lung expression of HIF-1α, VEGF, and IL-6 was significantly increased after treatment with both ovalbumin immunization alone and Su-ovalbumin. Notably, IL-6 protein expression in the lung was positively correlated with the mean pulmonary artery pressure, suggesting that the increased HIF-1α and IL-6 in lung may be a critical component of the pathogenesis of PH as a consequence of Th2 inflammation.

In our present study, we showed that HIMF treatment induced IL-6 expression in cultured BM macrophages, ex vivo lung organ slices, and in vivo pulmonary vasculature by a HIF-1α–dependent mechanism. IL-6 is elevated in several lung diseases and has been suggested to contribute to the pathogenesis of asthma and chronic obstructive pulmonary disease, as well as PH. IL-6 is an important regulator of effector CD4 T-cell differentiation; it promotes IL-4 production during Th2 differentiation and inhibits Th1 differentiation. IL-6 also is known to promote Th17 cell differentiation in a manner dependent on TGF-β. It is produced by inflammatory cells such as macrophages but primarily by lung resident cells (eg, epithelial cells, ECs, SMCs, and fibroblasts) in response to a variety of stimuli such as allergens, viruses, and hypoxia. We used an ex vivo lung organ culture system that allows epithelial, interstitial, and microvascular cells to interact in the alveolar wall without systemic circulatory influences. Our finding that IL-6 expression in response to HIMF was more robust in lung resident cells than in macrophages in a circulation-free system is consistent with the observation that lung resident cells are the primary source of IL-6 production. Notably, we did not observe a significant IL-6 increase in pulmonary vascular SMCs in response to HIMF or hRETN in vitro (data not shown). However, in vivo, we observed that IL-6–expressing macrophages and α-SMA–positive cells were recruited to the remodeling vasculature of HIF-1α+/− mice but not of HIF-1α−/− mice. These results suggest that HIMF can induce IL-6 in the lung resident cells and recruit circulating IL-6–expressing cells (such as macrophages and α-SMA–positive cells) to the remodeling vasculature through a HIF-1α–dependent mechanism. It also has been suggested that lung resident cells, especially epithelial cells

particularly those with scleroderma-associated PH. hRELMβ is also known to increase fibroblast proliferation and differentiation that results in deposition of extracellular matrix proteins. Fibroblast differentiation is a major component of vascular remodeling in PH. Furthermore, some RELM proteins in humans and mice mediate nuclear factor-κB (NF-κB) signaling in multiple cell types. In this context, we examined whether hRELM mediates HIF-1 and NF-κB signaling in human primary lung fibroblast (HLF). We observed a statistically significant increase in HIF-1α protein expression in HLF that was significantly suppressed by the inhibition of IκB–NF-κB (IKK), a critical upstream mediator of NF-κB activation (Figure 4C). Furthermore, hRELM significantly increased IL-6 production in HLF, but this increase was significantly attenuated in the presence of IKK-β inhibitor (Figure 4D). These data suggest that the IKK-β–NF-κB–HIF-1α axis is required for hRELMβ–induced production of proinflammatory cytokine IL-6 in HLF.
and SMCs, contribute to the Th2/Th17 type of immune response by secreting cytokines that can differentiate native T cells to Th2 and Th17 cells. We have published previously that HIMF amplifies Th2 inflammation, as evidenced by increased IL-4 in the lung. Furthermore, we have shown that IL-17 expression in the lung is significantly suppressed in global HIMF/RELMα knockout mice when compared with wild-type mice during allergic inflammation. It is reasonable to speculate that an increase in IL-6 in lung resident cells in response to HIMF can induce a sequential Th2/Th17 response. In accordance with our results, a recent study by Jang et al. showed that hRETN preferentially binds myeloid cells such as monocytes and macrophages in the lung during parasitic infections and promotes proinflammatory cytokine expression. They also reported that increased circulating hRETN expression correlated positively with IL-6, chemokine (C-C motif) ligand 2, and tumor necrosis factor-α in human patients infected with soil-transmitted helminths or filarial nematodes. Because these cytokines, particularly IL-6, are PH markers in the context of inflammation and immunity, their findings support our current observation that RELM proteins can induce proinflammatory cytokines in humans during inflammation.

The mechanism by which hypoxia induces HIMF has been investigated, at least in part, by Vergadi et al. They reported that HIMF/FIZZ1 was induced in alveolar macrophages that were transiently recruited to the lung after 4 days of hypoxia and that inhibition of HIMF/FIZZ1-expressing M2 macrophages ameliorated hypoxic PH. It has been previously suggested that HIMF/FIZZ1/RELMα is induced by Th2 cytokines IL-4 and IL-13 via the STAT6 pathway in both macrophages and lung epithelial cell during allergic inflammation; however, we have

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**Figure 5.** Hypoxia-induced mitogenic factor (HIMF) induces interleukin (IL)-6 expression in macrophages and smooth muscle cells in vivo. A, HIMF treatment increased IL-6 expression in the small pulmonary vessels of HIF-1α+/+ mice but not in those of HIF-1α+/- mice. HIMF increased α-smooth muscle actin–positive cells (α-SMA+, red, identifying vascular media) and IL-6 expression (green) in the remodeled pulmonary vasculature. IL-6 expression colocalized with α-SMA+ cells and inflammatory cells that infiltrated into the vasculature. These increases were strongly suppressed in HIF-1α+/- mice. B, IL-6–expressing macrophages (F4/80+, far red) were recruited to the remodeling small vessels in HIMF-treated HIF-1α+/- mice but not in HIF-1α+/- mice. C–E, Magnified merged images of IL-6–expressing macrophages (*) and α-SMA+ cells (#) are shown. Arrows indicate resident α-SMA+ cells without IL-6 expression (scale bars, 100 μm).
published that hypoxia-induced HIMF expression in the lung occurred through a pathway independent of IL-4 or STAT6, suggesting that this protein can be induced by other pathways, such as hypoxia signaling. In support of this observation, recent work by Colegio et al. showed that tumor-derived lactic acid can lead to HIMF/FIZZ1 expression in tumor-associated macrophages by a HIF-1α-dependent mechanism. This finding suggests that HIF-1α may be an upstream regulator of HIMF/FIZZ1 expression in macrophages under certain conditions. The data also suggest that HIMF is induced by alveolar macrophages in response to hypoxia, and that increased perivascular HIMF/FIZZ1 can further activate lung resident cells and BMD macrophages to contribute directly to pulmonary vascular remodeling. In our study, hypoxia stimulation did not cause HIMF induction in naïve BMD macrophages in vitro (data not shown). A recent study by El Kasmi et al. showed that adventitial fibroblasts derived from hypertensive pulmonary arteries produce IL-6, which activates naïve BM macrophages to a pro-PH phenotype that is distinct from classical M1 or M2 macrophage phenotypes. Interestingly, that study showed that paracrine IL-6 activates STAT3, HIF-1α, and C/EBPβ (CCAAT/enhancer binding protein beta) in macrophages, and that these transcription factors are necessary for macrophage activation and polarization. It is possible that HIMF/FIZZ1 is produced by alveolar macrophages in response to hypoxia-produced cytokines in the pulmonary vasculature, and thus IL-6 induced by HIMF may stimulate additional macrophage activation to a pro-PH phenotype and promote a proinflammatory feed-forward mechanism in the remodeling vasculature. In accordance with our observations, recent data showed that IL-6, which is increased in hypoxia-induced PH, also stimulates the increase of HIMF/FIZZ1-expressing M2 macrophages, supporting a positive feed-forward mechanism between IL-6 and HIMF/FIZZ1-expressing M2 macrophages in the development of PH.

We have previously shown that HIMF triggers pulmonary EC activation and apoptosis by activating caspase-3 and stress-activated kinases such as JNK (c-Jun N-terminal kinases) and p38 MAPK (mitogen-activated protein kinases). To determine whether HIF-1α regulates HIMF-induced EC apoptosis and activation, we compared HIMF-mediated caspase-3 and stress-activated kinase activation in PMVECs from HIF-1α+/− and HIF-1α−/− mice. However, PMVECs from the 2 genotypes showed similar degrees of caspase-3 and stress-activated kinase activation (data not shown), suggesting that HIF-1α does not mediate this phenomenon. We have shown previously that conditioned media from hRETN-treated PMVECs causes significant SMC proliferation and have identified endothelin-1 as one of the growth factors released from ECs in response to hRETN. Endothelin-1 is known to induce a pulmonary SMC–specific increase in HIF-1α level by upregulating HIF-1α synthesis and downregulating HIF prolyl hydroxylase 2–mediated degradation. In our study, we identified additional human SMC growth factors that are released from ECs in response to hRETN, including serpin E1, tissue inhibitor of metalloproteinase-, and urokinase plasminogen activator. Similar to endothelin-1, serpin E1 promotes HIF-1α–dependent SMC growth response. hRETN is considered to be an important modulator of chronic inflammatory diseases such as obesity and atherosclerosis. Therefore, we speculate that in humans, pulmonary SMC HIF-1α expression is increased by pulmonary SMC growth factors from hRETN-induced PMVECs as a result of EC–SMC cross talk during inflammation and vascular remodeling.

Another human homolog of HIMF, hRELMβ, has been reported to be increased in lung epithelial cells, fibroblasts, alveolar macrophages, and desquamated alveolar cells of patients with scleroderma-associated PH and idiopathic pulmonary fibrosis, suggesting a profibrotic role in the lung. Both HIMF/FIZZ1 and hRELMβ have been suggested to cause differentiation of fibroblasts into myofibroblasts. These activated fibroblasts modulate vascular function and remodeling either directly or indirectly through secretion of a variety of cytokines, chemokines, growth factors, and matrix proteins. HIF-1α is known to promote extracellular matrix remodeling under hypoxic conditions in fibroblasts. Previous studies have shown that RELM proteins mediate NF-κB signaling in multiple cell types. In the present study, we show that hRELMβ significantly increases HIF-1α expression and IL-6 production and that these effects are significantly suppressed by inhibition of IKK-β, a critical upstream mediator of HIF-1α required for HIF-1α protein accumulation. These data suggest that hRELMβ induces the production of proinflammatory cytokine IL-6 via the IKK-β–NF-κB–HIF-1α axis in HLF. Interestingly, we did not observe HIF-1α induction by hRETN in HLF (data not shown), indicating separate and distinct roles of these proteins in humans. Thus, RELMβ may play a more direct role than hRETN in matrix protein deposition during tissue and vascular remodeling by activating local fibroblasts.

In this study, HIF-1α−/− mice exhibited significantly less pulmonary vascular remodeling and PH development when compared with HIF-1α+/− mice in response to HIMF injection. In addition, a previous study showed that digoxin, a HIF-1 inhibitor, could prevent and reverse hypoxia-induced pulmonary vascular remodeling, right ventricle hypertrophy, and PH. HIF-1α has been reported to have a cell type–specific role in cardiovascular diseases such as cardiac hypertrophy. For example, HIF-1α in ECs likely plays a protective role, whereas HIF-1α in cardiomyocytes plays a pathological role in cardiac hypertrophy and dysfunction. Additional studies are needed to determine the cell-specific role of HIF-1α in HIMF- and hRETN-mediated right ventricular hypertrophy and failure.

In conclusion, we have shown that HIF-1α is a critical downstream mediator for HIMF-induced pulmonary vascular remodeling and development of PH. Our data show that HIMF-induced PH is mediated, at least in part, by HIF-1α–VEGF-A–VEGFR2 signaling in the lung, and that HIMF acts by increasing the expression of IL-6 in both inflammatory and lung resident cells. HIMF also displayed a HIF-1α–dependent chemotactic effect on BMD cells and on BM macrophages. Because we observed HIMF-induced HIF-1α–dependent IL-6 expression in macrophages and lung resident cells, future study is needed to identify which HIF-1α–expressing cell type induces proinflammatory and immune-activating mediators in response to HIMF and its human homologs. Our results suggest that blocking the RELM protein and its downstream signaling pathway may
be an effective immunosuppressive therapy for the treatment of PH in humans.

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Disclosures

None.

References

Pulmonary hypertension (PH) is characterized by elevated pulmonary artery pressure that leads to progressive right-sided heart failure and ultimately death. The exact mechanisms that underlie PH development are unknown, but growing evidence indicates that inflammation plays a key role in triggering and maintaining pulmonary vascular remodeling. We have shown that in a mouse model of PH, hypoxia-induced interleukin-6 serum concentrations in severe primary pulmonary hypertension.

Adult pulmonary endothelial cell activation and mediating lung vascular inflammation. In a key role in triggering and maintaining pulmonary vascular remodeling. We have shown that in a mouse model of PH, hypoxia-induced interleukin-6 serum concentrations in severe primary pulmonary hypertension.


Hypoxia-Inducible Factor 1α Is a Critical Downstream Mediator for Hypoxia-Induced Mitogenic Factor (FIZZ1/RELM α)—Induced Pulmonary Hypertension
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MATERIALS AND METHODS

HIMF-induced pulmonary hypertension mouse study
The Hif1a<sup>tm1jhu</sup> mouse strain was generated by homologous recombination to replace exon 2 of the Hif1a gene with a neo<sup>R</sup> expression cassette in the J1 line of embryonic stem cells (derived from 129/Sv mice), followed by injection of the genetically modified cells into blastocysts that were isolated from C57BL/6 mice. The strain has been maintained by heterozygous (HIF-1α<sup>+/−</sup>) × wild-type (HIF-1α<sup>+/+</sup>) mating for the last 15 years, and the mice segregate the Hif1a<sup>tm1jhu</sup> allele on an outbred 129 × B6 genetic background. HIF-1α<sup>+/−</sup> and HIF-1α<sup>−/−</sup> male littermate mice (8–12 weeks old) were used for in vivo experiments, and male mice at age 4–6 weeks were used for in vitro cell isolation. Housing and procedures involving experimental animals were approved by the Animal Care and Use Committee of the Johns Hopkins University. rHIMF protein was produced in T-REx 293 cells as previously described. We injected rHIMF (200 ng/animal in 100 µL saline) intravenously as reported previously. FLAG protein (200 ng/animal in 100 µL saline, Sigma-Aldrich, St. Louis, MO) was used as a negative control (rHIMF is FLAG-tagged at its C-terminus). Endotoxin level of rHIMF protein was < 0.1 ng/µg. Lung tissue was collected at 30 days after the injection (pulmonary hypertension development phase), as we described previously.

Histology and laboratory testing
Once appropriate hemodynamic measurements were made, mice were euthanized by exsanguination, and the heart and lungs were removed en bloc. For histology, the lung was inflated under constant pressure with 1% low-melting-point agarose in phosphate-buffered saline (PBS) and placed on ice. The inflated lung was then placed in 4% paraformaldehyde and subsequently processed for histology as described previously. The heart was then bisected into the right ventricle (RV) and left ventricle (LV) plus septum (S). Each portion of the heart was weighed, and the RV and LV+S ratio was determined (RV/(LV+S)).

Pulmonary vascular remodeling analysis
We assessed pulmonary vascular remodeling of the mice as previously published. For initial analysis, sections were stained with hematoxylin and eosin. Additional lung sections were dual labeled with antibodies to von Willebrand factor (vWF; Dako, Glostrup, Denmark) and smooth muscle actin (α-SMA; Dako), which stain endothelium and vascular smooth muscle, respectively; sections were counterstained with hematoxylin as we have described. To assess remodeling of the lung arteries and arterioles, an investigator blinded to treatment group examined 100 arteries at random per lung section under 40x objective using an Olympus-BHS microscope (Olympus, Tokyo, Japan) attached to a QImaging Retiga 4000RV digital camera (QImaging, British Columbia, Canada). Small arteries with an internal diameter ≤ 80 µm were then classified as non-muscular, partially muscular, or fully muscular, according to α-SMA staining. Average data reflect results from five lungs per group. Negative control sections for the immunohistochemical experiments received identical treatments but were not exposed to the primary antibody; they showed no specific staining.

In vivo hemodynamics
In vivo right ventricular function was assessed by a pressure-volume catheter as described previously. Briefly, mice were anesthetized with 1%–2% isoflurane, 75–100 mg/kg intraperitoneal (i.p.) urethane, 5–10 mg/kg i.p. etomidate, and 1–2 mg/kg i.p. morphine. Then they underwent tracheostomy and were ventilated with 6–7 µL/g tidal volume at 130 breaths/minute. An incision was made between the seventh and eighth ribs to expose the right ventricular apex, through which we inserted a 1.4-Fr pressure-volume catheter (SPR 839; Millar Instruments Inc., Houston, TX) as described previously.

Bone marrow transplantation and in vivo Matrigel plug vascular formation study
We carried out the bone marrow transplantation and in vivo Matrigel plug vascular formation study as described previously. Briefly, female C57BL/6 mice (6–8 weeks old; Charles River Laboratories, Wilmington, MA) were used as bone marrow transplant recipients, and 4–6-week-old male transgenic enhanced green fluorescent protein (EGFP) mice on a C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME) were used as bone marrow donors. Whole bone marrow was collected from the transgenic EGFP mice, and 2 x 10^6 bone marrow cells were transplanted into lethally irradiated (1,050 cGy) recipient mice through intravenous injection. After the recipients were allowed to recover for 4 weeks, Matrigel (500 µL, BD Biosciences, San Jose, CA) containing vehicle or HIMF (50 nM) was injected subcutaneously into the thigh of mice. In a separate study, Matrigel was also injected into HIF-1α−/+ and HIF-1α+/− male littermates. The Matrigel plugs with adjacent subcutaneous tissue were harvested at 7 days after injection for histological analysis.

**Immunofluorescence and confocal microscopy**

Immunofluorescence staining was carried out as described previously. Briefly, the paraffin sections were blocked with appropriate blocking serum (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature and then treated with anti-α-SMA (Dako), anti-E-cadherin (E-Cad, Cell Signaling Technology), anti-IL-6 (Abcam, Cambridge, UK), anti-F4/80 (AbD Serotec, UK), or anti-HIF-1α (Novus Biologicals, Littleton, CO) antibody. Then the sections were incubated with the appropriate fluorochrome-coupled secondary antibody (Jackson ImmunoResearch, West Grove, PA). Finally, the sections were washed in PBS, mounted with ProLong® Gold antifade reagent with DAPI (Invitrogen), and sealed with a glass coverslip. Negative control sections for the immunohistochemical experiments received identical treatments but were not exposed to the primary antibody. Staining was imaged with a Zeiss 510 Meta or LSM 700 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY) at the Johns Hopkins School of Medicine Microscope Core Facility.

**Ex vivo lung organ culture**

Lung organ culture was carried out as we have described previously. Briefly, mice were euthanized and bled through the abdominal aorta. The lungs were isolated and infused with 1.5% low-melting-point agarose solution in culture medium (37°C). After the trachea was clamped, the trachea, lungs, and heart were cooled to solidify the agarose. Once the lung had cooled, complete transverse serial sections (3–5 mm in thickness, six slices from different lobes/lung) were gently sliced and mounted on Transwell chambers (8-mm pore size; Costar, Cambridge, MA). The slices were cultured in Waymouth’s MB752/1 containing 1% heat-inactivated fetal bovine serum (FBS) and supplemented with insulin, transferrin, hydrocortisone, sodium selenite, 100 U/mL penicillin, and 10 mg/mL streptomycin. Two days after the isolation, the lung slices were stimulated with control vehicle or HIMF (20 nM); 24 hours later, the culture medium was harvested for cytokine analysis.

**Murine bone marrow-derived (BMD) macrophage culture and cell marker analysis**

Murine BMD macrophages were generated from HIF-1α−/+ and HIF-1α+/− male littermates with a well-established method. Briefly, bone marrow was isolated from femurs and tibias of 4–6-week-old mice. To deplete adherent stromal cells, we cultured the harvested BMD cells in MEMα supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin-100 µg/mL streptomycin, and recombinant murine M-CSF (20 ng/mL, R&D Systems, Minneapolis, MN). We plated nonadherent cells in 6-well plates, performed red blood cell lysis, and then cultured them for 10 days to differentiate BMD macrophages. Macrophage marker expression was determined by flow cytometry. Briefly, cells were incubated with Fc Block (BD Biosciences) and then stained with fluorochrome-conjugated antibodies to surface markers (CD11b, BD Biosciences). Samples were acquired on an LSR II flow cytometer system, and data were analyzed by FACSDiva software (BD Biosciences). For the cytokine analysis, cells were stimulated with vehicle or rhIMF (20 nM) under serum-free conditions for 24 hours, and the medium was analyzed for IL-6 level.

**Murine BMD cell migration**
Murine BMD cells were isolated from HIF-1α+/+ and HIF-1α−/− male littermates, and the cell migration assay was performed as we have described previously. Briefly, BMD cells were detached with trypsin-EDTA, washed in serum-free medium, and then counted. Cells (1x10⁶ cells/0.5 mL) were placed on Transwell membranes and allowed to migrate to the underside for 24 hours at 37°C in the presence or absence of HIMF or conditioned media from pulmonary ECs treated with or without HIMF. The Transwell membranes were stained with Diff-Quick Stain Kit (IMEB Inc., San Marcos, CA). Randomly chosen areas of migrated cells were visualized and photographed with a Nikon Eclipse TE2000-E microscope, and image quantification was carried out with Image J software (National Institutes of Health, Bethesda, MD).

**Human lung fibroblast (HLF) cell culture and HIF-1α expression analysis**

Normal human lung fibroblasts (Lonza, Walkersville, MD) were cultured in Fibroblast Growth Media (FGM-2, Lonza) supplemented with 2% FBS, human fibroblast growth factor-B, insulin, gentamicin, and amphotericin-B (Bulletkit CC-4126; Lonza). Only HLF cells from passages 5-6 were used. For hRELMβ stimulation, HLF cells were serum- and growth factor–starved (FGM-2 media with 0.1% FBS) for 24 hours and then treated with hRELMβ (100 ng/mL, Peprotech, Inc.) or vehicle in the presence or absence of IKK-2 inhibitor (IKK-2 inhibitor IV, 500 nM, EMD Millipore, Billerica, MA). Preliminary studies were performed to optimize both incubation time and recombinant protein concentrations (data not shown). Cell lysates were collected for measurement of HIF1α expression, and medium was collected for cytokine analysis. HIF-1α protein expression in HLF cells after hRELMβ stimulation was analyzed by HIF-1α ELISA (R&D Systems).

**Cytokine analysis**

We measured IL-6 levels in medium from ex vivo mouse lung organ slices, BMD macrophages, and HLF cells using an ELISA for mouse or human IL-6, as appropriate (eBioscience, San Diego, CA).

**Immunoblotting**

Protein samples were prepared with extraction buffer as described previously. Briefly, mouse lung tissue lysates or cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein was transferred onto nitrocellulose membrane. Membranes were incubated with primary antibodies against mouse HIF-1α, HIF-2α (both from Novus Biologicals), VEGF-A, VEGFR1, β-actin (all from Santa Cruz Biotechnology, Dallas, TX), or VEGFR2 (Cell Signaling Technology, Danvers, MA). After membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA), the signal was visualized by ECL Western Blotting Detection substrate kit (GE Healthcare, Buckinghamshire, England). Each band was quantified by Image J software.

**Human cell culture and EC activation**

Human PMVECs (HMVEC-L; Lonza) were cultured in endothelial cell basal medium-2 (VEM-2, Lonza) supplemented with 5% FBS, human epidermal growth factor, human VEGF, human fibroblast growth factor (with heparin), long R3 insulin-like growth factor I, hydrocortisone, ascorbic acid, gentamicin, and amphotericin B (Bulletkit CC-3202; Lonza). PMVECs within passage 5 were used for this study. For EC cell lysate and conditioned medium preparation, human PMVECs grown in 100-mm dishes were serum– and growth factor–starved (VEM-2 media with 1% FBS) for 24 h and then treated with recombinant human RETN (rhRETN, 20 nM, Peprotech Inc.) or vehicle as described previously. Cell lysate was collected after 1 hour of stimulation according to the manufacturer's guidance. In a separate experiment, cells were stimulated by hRETN or vehicle for 24 hours, and cell media was collected and cell viability analysis was performed. Both cell lysates and medium were collected and stored at -80°C for antibody array analysis.
Characterization of PMVEC conditioned media
A Proteome Profiler Antibody Array for Human Angiogenesis (R&D Systems) was used to determine the PSMC proliferative factors that are released from PMVEC in response to hRETN. Array membranes were blocked with blocking buffer provided in the kit for 1 hour at room temperature on a rocking platform. Cell lysates (190 μg protein per sample) or EC media samples (400 μg protein each) were reacted with the blocked membrane overnight at 4 ºC. After the membranes were washed with washing buffer, HRP-conjugated detection antibody was added for signal detection. Each membrane was scanned, and the signal was analyzed by Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA).

Statistical analysis
All data are presented as mean ± SEM. Differences between multiple groups were compared by analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. Two-group analysis was performed by Student’s t-test. A value of $P < 0.05$ was considered statistically significant.
References


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Supplemental Figure 1. Identification of smooth muscle cell (SMC) growth factors released from human pulmonary microvascular endothelial cells (PMVEC) in response to hRETN. Angiogenic factors produced from PMVEC in response to hRETN that are known to cause SMC proliferation in a HIF-1–dependent mechanism. 1, Serpin E1; 2, TIMP-1 (tissue inhibitor of metalloproteinase-1); and 3, uPA (urokinase plasminogen activator) were decreased in cell lysate (A) and released into the cell media (B). Cell-free media control (M CONT) analysis confirms that these factors were not derived from the media itself. Mean pixel density of each factor is shown in graphs on the right. For the results of cell media, each value was normalized by the cell number.