LRP1 Deficiency in Vascular SMC Leads to Pulmonary Arterial Hypertension That Is Reversed by PPARγ Activation

Laurent Calvier, Philippe Boucher, Joachim Herz, Georg Hansmann

Rationale: Arterial remodeling—a hallmark of many cardiovascular pathologies including pulmonary arterial hypertension (PAH)—is regulated by TGFβ1 (transforming growth factor-β1)–TGFβ receptors and the antagonistic, vasoprotective BMPR2 (bone morphogenetic protein receptor 2)–PPARγ (peroxisome proliferator–activated receptor-γ) axis. However, it is unclear which factors drive detrimental TGFβ1 pathways in the hypertensive pulmonary vasculature.

Objective: We hypothesized that LRP1 (low-density lipoprotein receptor–related protein 1) expression is decreased in PAH, leading to enhancement (disinhibition) of TGFβ1 signals and that the PPARγ agonist pioglitazone can restore vascular homeostasis and prevent PAH resulting from LRP1 deletion in vascular smooth muscle cells (SMCs).

Methods and Results: Targeted deletion of LRP1 in vascular SMC (smLRP1−/−) in mice disinhibited TGFβ1–CTGF (connective tissue growth factor) signaling, leading to spontaneous PAH and distal pulmonary arterial muscularization as assessed by closed-chest cardiac catheterization and anti-αSMA staining. Pioglitazone inhibited the canonical TGFβ1–CTGF axis in human pulmonary artery SMC and smLRP1−/− main pulmonary artery (CTGF and NOX4) and reversed PAH in smLRP1−/− mice. TGFβ1 boosted pSmad3 in PASMC from smLRP1−/− mice versus controls. Pioglitazone-activated PPARγ binds to Smad3 in human pulmonary artery SMC (coimmunoprecipitation), thereby blocking its phosphorylation and overriding LRP1 deficiency. Finally, mRNA and protein expression of LRP1 was decreased in pulmonary plexiform lesions of patients with end-stage idiopathic PAH (laser capture microdissection, qPCR, and immunohistochemistry). Downregulation of LRP1 protein was also demonstrated in explanted PASMC from patients with PAH and accompanied by enhanced TGFβ1–pSmad3–CTGF signaling and increased TGFβ1–induced PASMC proliferation that was prevented by pioglitazone.

Conclusions: Here, we identify LRP1 as an inhibitor of TGFβ1–mediated mechanisms that regulate vascular remodeling in mice and clinical PAH and PPARγ as a therapeutic target that controls canonical TGFβ1 pathways. Hence, pharmacologic PPARγ activation represents a promising new therapy for patients with PAH who lack the vasoprotective LRP1 in vascular SMC. (Circ Res. 2019;124:1778-1785. DOI: 10.1161/CIRCRESAHA.119.315088.)

Key Words: humans ■ hypertension, pulmonary ■ muscle, smooth, vascular ■ peroxisome proliferator–activated receptors ■ vascular diseases

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Pulmonary arterial hypertension (PAH) is characterized by remodeling of pulmonary arteries, leading to increased pulmonary vascular resistance, right ventricular (RV) hypertrophy, heart failure, and death. VSMC proliferation, extracellular matrix, and vascular remodeling are hallmarks of PAH. All of these features in VSMC are counteracted by the vascular TGFβ1 Overexpression pathway, which may be reversed by PPARγ agonists.

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Nonstandard Abbreviations and Acronyms

BMPER  bone morphogenetic protein endothelial cell precursor
BMPR2  bone morphogenetic protein receptor 2
CTGF  connective tissue growth factor
HPASMC  human pulmonary artery smooth muscle cell
LDL  low-density lipoprotein
LRP1  low-density lipoprotein receptor–related protein
LV  left ventricle
PAH  pulmonary arterial hypertension
PPARγ  peroxisome proliferator–activated receptorγ
RV  right ventricle
TGFβ1  transforming growth factor beta
TGFBR1  transforming growth factor receptor-β1
TGFBR5  transforming growth factor beta receptor 5
VSMC  vascular smooth muscle cell

Pulmonary arterial hypertension is a proliferative cardiovascular condition regulated by TGFβ1 transforming growth factor-β1–TGFβ1 receptors and the antagonistic, vasoprotective BMPR2 (bone morphogenetic protein receptor 2)–PPARγ (peroxisome proliferator–activated receptor–γ) axis and characterized by lipid-related insulin resistance. LRP1 associates with multiple ligands, including TGFβ1 and its receptor and its downstream target, CTGF (connective tissue growth factor), and inhibits canonical (pSmad3–CTGF) and noncanonical (pStat3–FoxO1) TGFβ1 pathways, glycolysis, and proliferation.

What Is Known?

- Pulmonary arterial hypertension is a proliferative cardiovascular condition regulated by TGFβ1 transforming growth factor-β1–TGFβ1 receptors and the antagonistic, vasoprotective BMPR2 (bone morphogenetic protein receptor 2)–PPARγ (peroxisome proliferator–activated receptor–γ) axis and characterized by lipid-related insulin resistance.
- LRP1 associates with multiple ligands, including TGFβ1 and its receptor and its downstream target, CTGF (connective tissue growth factor), and inhibits canonical (pSmad3–CTGF) and noncanonical (pStat3–FoxO1) TGFβ1 pathways, glycolysis, and proliferation.

What New Information Does This Article Contribute?

- First study demonstrating that LRP1 in VSMC protects from pulmonary arterial hypertension (PAH) in vivo, while LRP1 deficiency leads to PAH.
- Vascular expression of LRP1 is decreased in human PAH.

Nonstandard Abbreviations and Acronyms

BMPER  bone morphogenetic protein endothelial cell precursor
BMPR2  bone morphogenetic protein receptor 2
CTGF  connective tissue growth factor
HPASMC  human pulmonary artery smooth muscle cell
LDL  low-density lipoprotein
LRP1  low-density lipoprotein receptor–related protein
LV  left ventricle
PAH  pulmonary arterial hypertension
PPARγ  peroxisome proliferator–activated receptorγ
RV  right ventricle
TGFβ1  transforming growth factor beta
TGFBR1  transforming growth factor receptor-β1
TGFBR5  transforming growth factor beta receptor 5
VSMC  vascular smooth muscle cell

Pulmonary arterial hypertension (PAH) is characterized by remodeling of pulmonary arteries, leading to increased pulmonary vascular resistance, right ventricular (RV) hypertrophy, heart failure, and death. VSMC proliferation, extracellular matrix, and vascular remodeling are hallmarks of PAH. All of these features in VSMC are counteracted by the vasoprotective BMPR2 (bone morphogenetic protein receptor 2) and its downstream effector PPARγ (peroxisome proliferator–activated receptor–γ) axis. Balanced BMP2/BMPR2 and TGFβ1/TGFBR1 signaling is needed for VSMC homeostasis, and PPARγ links both pathways. Recently, we unraveled PPARγ as a major gatekeeper that links vasoprotective BMP2/BMPR2 and detrimental TGFβ1/TGFBR pathways in VSMC, regulating cell proliferation and glucose metabolism.

Others have speculated that LRP1 is a coreceptor and an activator of endothelial BMP21–11—the receptor that is mutated or dysfunctional in many forms of clinical PAH. Experimental evidence suggests that LRP1 associates with BMPER (bone morphogenetic protein endothelial cell precursor) and is required for BMPER endocytosis in mouse endothelial cells.11 An elegant iPSC study suggests that endothelial LRP1 protects from PAH in unaffected BMPR2 mutation carriers (family members), probably by recycling the BMP ligand-receptor complex back to the cell surface.

In VSMC, we showed that PPARγ activation (downstream of BMPR2) protects against PAH by inhibiting canonical and noncanonical TGFβ1 pathways, inhibiting Smad3 and Stat3, inhibiting TGFβ1 downstream signaling, glucose metabolism, and smooth muscle cell proliferation. Furthermore, TGFβ1–overexpressing mice spontaneously develop PAH that can be reversed by the oral PPARγ agonist pioglitazone.

To date, accumulating evidence indicates that LRP1 is vasoprotective, and—by physically interacting with TGFβ1, TGFBR1, and possibly BMPR2—closely involved in VSMC homeostasis, TGFβ1 overexpression induces PAH in transgenic mice, and circulating TGFβ1 levels are heightened in human PAH. However, LRP1 or its deficiency has not been linked to PAH yet. We hypothesized that (1) mice with targeted deletion of LRP1 in VSMC (smLRP1−/−) develop PAH in association with a disinhibited TGFβ1–CTGF axis, all of which may be reversed by PPARγ agonist treatment, and that (2) LRP1 expression is decreased in human pulmonary arterial smooth muscle cell (HPASMC) of patients with PAH.

Methods

This article adheres to the AHA Journal Transparency and Openness Promotion Guidelines. All data and supporting materials have been provided with the published article.

Detailed methods are provided in the Online Data Supplement.

SM22-Cre; LRPlox/−; LDLR−/− (smLRP−/−) With Vascular TGFβ1 Overexpression

Homozygous LDL receptor (LDLR−/−) LRP deficient in VSMCs (SM22-Cre; LRPlox/lox; LDLR−/−), mice referred to as smLRP−/− have been described previously to develop TGFβ1 pathway enhancement. Mice were maintained on normal diet. We divided male and female smLRP1−/− (SM22-Cre; LRPlox/lox; LDLR−/− background) and littermate control mice (LRP1flo/x; LDLR−/−) in 2 sets. A set of 10- to 12-week-old control and smLRP−/− mice (n=3–5) underwent invasive hemodynamic measurement only (catheterization of RV,
Figure 1. The PPARγ (peroxisome proliferator–activated receptor γ) agonist pioglitazone reverses pulmonary arterial hypertension, spontaneously developed in smLRP1−/− mice. A, smLRP1−/− and littermate control mice (LDLR−/− background) were divided into 2 sets: a first set untreated of 10- to 12-wk-old mice (n=3–5) and a second set of 10- to 12-wk-old control and smLRP1−/− mice orally treated for 5 wk with pioglitazone (20 mg/kg per d) and (Continued)
left ventricular [LV], and aorta in closed-chest technique). A second set of 10- to 12-week-old control and smLRP−/− mice (n=9–11) was treated for 5 weeks either with regular chow versus pioglitazone (20 mg/kg per day) incorporated into the chow and sacrificed at 15 to 17 weeks of age. All animal studies were performed according to the institutional animal care and use guidelines and the according state regulations (No. 2015–101088).

**HPASMC Isolation and Culture**
Isolated HPASMCs were cultured in smooth muscle cell basal medium (CC-31–81; Lonza) supplemented with 10 % FBS and growth factors from SmGM-2 SingleQuots (CC-4149; Lonza), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (15140-122; Gibco).

Moreover, HPASMCs were isolated from pulmonary arteries obtained from patients with PAH or those with chronic obstructive pulmonary disease, undergoing lung transplantation. Mouse PASMCs were isolated from the pulmonary arteries.

**Statistics**
For cell culture, each condition was tested at least in triplicates, and all experiments were repeated at least 3 times at different passages and in cells from at least 3 different donors (unless specified differently). Values from multiple experiments are expressed as mean±SEM. Normality was tested using the Kolmogorov-Smirnov test. Statistical significance was determined for multiple comparisons using 1-way ANOVA followed by Bonferroni multiple comparison (for normal distribution) or Kruskal-Wallis (for non-normal distribution) test. Student t-test was used for comparisons of 2 groups. P<0.05 was considered significant.

| Table. Hemodynamics, Heart Weight, and Pulmonary Vascular Analysis in smLRP1−/− and Control Mice, Either Untreated or Treated With the PPARγ Agonist Pioglitazone |
|---|---|---|---|---|---|
| 10 to 12 wk old mice | | | | | |
| Body weight, g | 27.5±2.3 | 28.0±2.9 | | | |
| Hemodynamics | | | | | |
| HR, bpm | 328±17 | 337±27 | | | |
| RVSP, mm Hg | 23.6±0.7 | 31.1±0.8* | | | |
| RVEDP, mm Hg | 2.0±0.4 | 2.8±1.2 | | | |
| 15 to 17 wk old mice | | | | | |
| Body weight, g | 24.4±1.1 | 26.3±1.4 | 27.7±1.5 | 26.0±0.8 | |
| Hemodynamics | | | | | |
| HR, bpm | 291±14 | 283±14 | 286±14 | 270±11 | 9–11 |
| RVSP, mm Hg | 23.0±0.7 | 21.5±0.5 | 33.4±2.4* | 22.6±0.6* | C vs L* | 9–11 |
| RVEDP, mm Hg | 3.1±0.5 | 3.5±0.6 | 6.5±0.5* | 3.4±0.5* | C vs L*, CP vs L, L vs LP* | 9–11 |
| LVSP, mm Hg | 100.2±1.9 | 98.0±3.1 | 108.5±3.4 | 103.8±5.1 | | 6–11 |
| LVDP, mm Hg | 9.8±1.4 | 9.9±1.5 | 13.7±3.1 | 11.3±1.7 | | 6–11 |
| SBP, mm Hg | 100.9±1.7 | 98.5±3.4 | 98.6±3.4 | 99.9±3.8 | | 7–13 |
| DBP, mm Hg | 67.3±1.4 | 63.9±2.9 | 55.9±2.4* | 59.0±1.2† | C vs L*, C vs LP† | 7–13 |
| MAP, mm Hg | 78.5±1.4 | 75.4±3.1 | 70.2±2.7 | 72.7±2.0 | | 7–13 |
| Cardiac mass | | | | | |
| RV/BW, mg/g | 0.66±0.03 | 0.73±0.04 | 0.93±0.10* | 0.71±0.07 | C vs L* | 11–14 |
| RV/(LV+S) | 0.22±0.01 | 0.26±0.01 | 0.35±0.02* | 0.24±0.01* | C vs L*, CP vs L, L vs LP* | 11–14 |
| LV+S/BW, mg/g | 2.70±0.07 | 2.83±0.14 | 2.67±0.30 | 2.97±0.19 | | 11–14 |

At 10 to 12 wk of age, smLRP1−/− and control mice received either regular chow or were orally treated with pioglitazone (20 mg/kg per d) incorporated into the chow for 5 wk. Data were generated at the age of 10 to 12 and 15 to 17 wk and are shown as mean±SEM. MAP=(SBP+2×DBP); BW indicates body weight; C, control; CP, control+Pio; DBP, diastolic blood pressure; HR, heart rate; L, smLRP1−/−; LP, smLRP1−/−+Pio; LV, left ventricle; LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; MAP, mean arterial pressure; PPARγ, peroxisome proliferator–activated receptor-γ; RV, right ventricle; RVEDP, right ventricular end diastolic pressure; RVSP, right ventricular systolic pressure.

Statistically significant differences between groups:
*P<0.01;
†P<0.05; Student t test or ANOVA, Bonferroni post hoc test.
Results

We divided male and female smLRP1−/− and littermate control mice in 2 sets and orally treated a subset with the PPARγ agonist pioglitazone (20 mg/kg per day) orally for 5 weeks (Figure 1A). Both 10- to 12-week-old and 15- to 17-week-old smLRP1−/− mice had spontaneous PAH, based on an elevated RV systolic pressure (Table; Figure 1B and 1C) and RV hypertrophy (increased RV/LV+septum mass ratio; Table; Figure 1E). Over time, the older 15- to 17-week-old smLRP1−/− mice developed mild RV diastolic dysfunction, as indicated by increased RV end diastolic pressure (Table; Figure 1D). In addition, LRP1 deletion in VSMC induced pulmonary artery remodeling as judged by increased muscularization in distal pulmonary arteries (Figure 1F). Intriguingly, oral treatment with pioglitazone fully reversed PAH and RV hypertrophy, prevented diastolic RV dysfunction, and pulmonary vascular remodeling (Figure 1C through 1F). No differences were observed in aortic and LV systolic and diastolic pressures (Table), thus excluding any relevant LV dysfunction or systemic arterial hypertension and hence indicating precapillary pulmonary vascular disease. Of note, no significant differences between male and female mice were observed (data not shown).

We next sought to investigate the basic molecular mechanisms of PAH development in smLRP1−/− mice. On TGFβ1 stimulation, CTGF recently was demonstrated to induce transdifferentiation of myoblasts into myofibroblasts—a major transformation that drives PASMC proliferation and pulmonary vascular disease.13 Thus, we hypothesized that LRP1 deficiency in VSMC induces the mitogenic TGFβ1–CTGF axis that can be inhibited by PPARγ activation. Indeed, phosphorylation of Smad3 (Figure 1G) and the mRNA levels of the TGFβ1 downstream targets CTGF and NOX4 were increased in total lung lysates of smLRP1−/− mice versus controls (Figure 1H). Consistent with our hypothesis, the heightened TGFβ1 signaling in total lung and main pulmonary arteries was inhibited by pioglitazone (Figure 1F and 1G). Of note, smLRP1−/− mice were shown to have increased TGFβ1–dependent nuclear pSmad2/3 accumulation in the aorta that is inhibited by the PPARγ agonist rosiglitazone.4 In summary, our data, together with the published literature, support a major, protective role for LRP1 in PAH by suppressing canonical TGFβ1 pathways.

To confirm the importance of LRP1 in human PAH, we determined LRP1 expression in small pulmonary arteries from control subjects (downsizing donor lungs) or idiopathic PAH patients applying laser capture microdissection/qPCR and immunohistochemistry on lung sections. Although the basal LRP1 expression in pulmonary arteries from idiopathic PAH patients was not (mRNA) or only moderately (protein) decreased versus control, pulmonary plexiform lesions had strongly decreased LRP1 expression at both mRNA and protein level (Figure 3A and 3B). Next, we validated the lowered LRP1 expression in HPASMC explanted from patients with PAH compared with those from patients with chronic obstructive pulmonary disease (used as control). LRP1 was moderately (not significantly) decreased in PAH-PASMC compared with control; with TGFβ1 stimulation, LRP1 protein expression was dampened by 63% (P<0.01) in PAH-PASMC versus nonstimulated PAH-PASMC (Figure 3C). Interestingly, PAH-PASMC responded to a greater degree to TGFβ1 stimulation than control PASMC (chronic obstructive pulmonary disease), as shown by enhanced (1) Smad3 phosphorylation (canonical TGFβ1 signaling), (2) CTGF protein
Figure 3. PASMCs from patients with pulmonary arterial hypertension (PAH) have decreased LRP1 (low-density lipoprotein receptor–related protein) expression and increased TGF (transforming growth factor)β1 sensitivity. A, LRP1 mRNA expression was analyzed by qPCR in laser microdissected pulmonary arteries (inner diameter, <500 mm) or plexiform lesions from idiopathic PAH (IPAH) patients (n=8–9) and control subjects (downsizing donor lungs, n=5). B, Human lung serial sections were analyzed by immunohistochemistry for LRP1 and αSMA expression in peripheral pulmonary arteries or pulmonary plexiform lesions of 3 IPAH patients and 3 healthy donors (scale bar=50 μm). C and D, Human pulmonary artery smooth muscle cells (HPASMCs) isolated from explanted pulmonary arteries of patients with chronic obstructive pulmonary disease (control COPD, n=3) or patients with PAH (n=4). C–F, The cells were seeded (Continued)
expression (TGFβ1 downstream target), and (3) HPASMC proliferation (Figure 3C and 3D). We further confirmed the boosted TGFβ1 response (pSmad3, CTGF) in smLPR1−/− explanted PASMC compared with wild-type control (Figure 2A and 2B). Pioglitazone administration inhibited all the a-bove TGFβ1–mediated effects in control and PAH-PASMC (Figure 3C and 3D). Mechanistically, PPARY activation by pioglitazone blocked TGFβ1–induced Smad3 phosphorylation by direct binding between Smad3 and PPARY (Figure 3E and 3F). Of note, pioglitazone alone did not restore LRPI expression in PAH-PASMC (Figure 3C). LRPI scavenges TGFBR1 (transforming growth factor receptor-β1) to inhibit the TGFβ1 pathway.1–3 (Figure 2A through 2C). According to previous reports on LRPI-binding partners (see Discussion) and our data, we propose that the decreased LRPI expression in PAH-PASMC would release TGFBR1 (transforming growth factor receptor-β1) for mitogenic ligand binding (Figure 3G), explaining the higher sensitivity and response to TGFβ1 in PAH-HPASMC versus chronic obstructive pulmonary disease HPASMC (controls).

**Discussion**

Based on the above literature, our current work, and previous reports,4,9,14 we suggest that LRPI deficiency in VSMC leads to induction (disinhibition) of the canonical TGFβ1–pSmad3/CTGF pathway, thereby suppressing PPARY activity5,15,16 and thus ultimately leading to PAH in vivo (Figure 3G). We had previously linked enhanced canonical (pSmad3/CTGF) and noncanonical (pSTAT3/pFoxO1) TGFβ1/TGFBRII signaling with suppression of the vasoprotective BMPR2–PPARY axis in VSMC.4,9,14 Our current study introduces a new PAH mouse model of LRPI deficiency in VSMC in conjunction with enhanced canonical TGFβ1–pSmad3/CTGF signaling, all of which can be reversed by PPARY agonist treatment (pioglitazone).

Of note, it is possible that LRPI generates vasoprotection in the context of PAH via other cell types and mechanisms (beyond suppressing TGFβ1 signaling in VSMC): for example, LRPI sustains PPARY activity in non-VSMC cell types such as endothelial cells (LRPI metabolic, transcriptional coactivator of PPARY)5 and pericytes (LRPI-dependent migration).17 Moreover, in macrophages, LRPI tyrosine phosphorylation is required to activate transcription of the cholesterol transporter ABCA1 through PPARY and LXR.16 In addition, it is possible that PPARY interacts with noncanonical TGFβ1 pathways. In VSMC, we have previously described inhibitory PPARY–Stat3 interaction,2 and others have described PPARY inhibiting NF-κB–mediated events,18,19 which likely contribute to the beneficial, anti-inflammatory effects of PPARY activation.

To the best of our knowledge, this is the first study demonstrating the vasoprotective role of LRPI in PAH. From a clinical perspective, pioglitazone has a positive risk–benefit ratio that has been proven in recent large randomized trials (IRIS trial)20,21 and a safety profile that compares favorably with rosiglitazone.20,22 Targeting PPARY with minimal side effect is a new challenge for drug discovery research; strategies to leverage PPARY deacetylation have been recently proposed to develop advanced PPARY agonists.21,22 Further preclinical and clinical studies should explore the efficacy of pioglitazone and other PPARY agonists in the advanced targeted therapy of patients with PAH.

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**Disclosures**

L. Calvier and J. Herz are cofounders of Reelin Therapeutics, Inc. G. Hansmann holds a patent application (USPTO [United States Patent and Trademark Office] No. 1289344) and an investigational new drug application (IND [investigational new drug application] No. 105428) related to the use of PPARY (peroxisome proliferator–activated receptorγ) agonistic agents for the treatment of pulmonary hypertension.

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