Deletion of LR11 Attenuates Hypoxia-Induced Pulmonary Arterial Smooth Muscle Cell Proliferation With Medial Thickening in Mice

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Objective—We aimed to determine whether LR11 (low-density lipoprotein receptor with 11 binding repeats) is a potential key regulator of smooth muscle cell (SMC) proliferation during the progression of hypoxia-induced medial thickening in mice and whether sLR11 (soluble LR11) can serve as a biomarker in patients with pulmonary arterial hypertension.

Approach and Results—The role of LR11 in pulmonary arterial hypertension was investigated using mouse and cell models of induced hypoxia. The expression of LR11 and of hypoxia-inducible factor-1α was significantly increased in lung tissues from C57Bl/6 mice after 3 weeks of exposure to hypoxia compared with normoxia. Serum sLR11 levels were also increased. Physiological and histochemical analyses showed that increased right ventricular systolic pressure, right ventricular hypertrophy, and medial thickening induced under hypoxia in wild-type mice were attenuated in LR11−/− mice. The proliferation rates stimulated by hypoxia or platelet-derived growth factor-BB were attenuated in SMC derived from LR11−/− mice, compared with those from wild-type mice. Exogenous sLR11 protein increased the proliferation rates of SMC from wild-type mice. The expression of LR11 and hypoxia-inducible factor-1α was increased in cultured SMC under hypoxic conditions, and hypoxia-inducible factor-1α knockdown almost abolished the induction of LR11. Serum sLR11 levels were significantly higher in patients with, rather than without, pulmonary arterial hypertension. sLR11 levels positively correlated with pulmonary vascular resistance and mean pulmonary arterial pressure.

Conclusions—LR11 regulated SMC proliferation during the progression of hypoxia-induced medial thickening in mice. The findings obtained from mice, together with those in humans, indicate that sLR11 could serve as a novel biomarker that reflects the pathophysiology of proliferating medial SMC in pulmonary arterial hypertension. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.116.307900.)

Key Words: atherosclerosis ■ biomarkers ■ disease progression ■ pulmonary hypertension ■ vascular smooth muscle

Pulmonary arterial hypertension (PAH) is a critical condition with a median survival of 2.8 years if left untreated.1 Novel drugs with vasodilator action, such as endothelin-1 receptor antagonist, phosphodiesterase-5 inhibitors, soluble guanylate cyclase stimulator, and prostacyclin analogues, have recently improved symptoms, exercise capacity, and hemodynamics among patients with PAH.2–5 Although the initial phases of PAH are clinically silent, the EARLY study showed that mildly symptomatic PAH when left untreated progressively deteriorates both clinically and hemodynamically, regardless of exercise capacity.2 Therefore, the early detection of initially silent PAH pathophysiology is important to improve treatment outcomes. In this context, specific biomarkers of the initial progression of the disease are needed, and BNP/N-terminal pro b-type natriuretic peptide has served as a sensitive marker in this capacity.6 Thus, novel circulating molecules based on a pathophysiology that differs from that of BNP/N-terminal pro b-type natriuretic peptide are required to increase the ability to detect initial disease progression and improve individual treatment strategies. One candidate marker might be a circulating molecule that represents the proliferation of smooth muscle cells (SMC) during the progress of medial thickening. These cells proliferate abnormally under crosstalk with dysfunctional endothelial cells and other components of the pulmonary system.
vascular wall including myofibroblasts, pericytes, and circulating immune cells. Therefore, the mechanism underlying SMC proliferation is complicated, and a key feature of PAH pathogenesis. Vascular smooth muscle cells (VSMCs) proliferate in the media of the pulmonary artery of patients with PAH, and several growth factors, such as platelet-derived growth factor, act as potent mitogens and chemoattractants for VSMC and also cause vascular remodeling.

LR11 (also called SorLA or SORL1) is a low-density lipoprotein receptor that is expressed in intimal SMCs during the development of atherosclerosis. LR11 released in a soluble form (sLR11) from the intimal SMC membrane by proteolytic shedding during the phase of rapid SMC proliferation induces SMC migration. Recent clinical studies have suggested that serum sLR11 could serve as a circulating marker of intimal SMC and reflect cell functions, particularly those of medial SMC proliferation and migration after phenotype alteration.

The present study investigated the functional significance of sLR11 as a regulator of SMC in medial thickening, which is the typical pathophysiology of PAH, using animal models. We then studied the potential of LR11 as a novel biomarker for pathologically proliferating medial SMCs in patients with PAH.

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

### Results

**Hypoxia-Induced LR11 Expression in Murine Lungs**

We assessed LR11 involvement in hypoxia-induced damage to the pulmonary arteries of mice. Western blotting showed significantly increased HIF-1α expression (0.33±0.05 versus 0.53±0.04, *P*<0.05; Figure 1A and 1D), LR11 expression (0.25±0.03 versus 0.65±0.04; *P*<0.05; Figure 1A and 1B), and serum sLR11 levels (Figure 1C) in mice under hypoxia, compared with normoxia. These results indicated that hypoxia induced LR11 expression and the release of sLR11 from the lungs, together with hypoxia-induced intracellular signals via HIF-1α. Therefore, LR11 might be involved in the development of PAH in response to hypoxia.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Western blots of LR11 (low-density lipoprotein receptor with 11 binding repeats) and hypoxia-inducible factor-1α (HIF-1α) protein expression in lungs of mice exposed to normoxia or hypoxia. A and B, LR11 protein expression significantly increased in lungs of wild-type (WT) mice exposed to hypoxia (Hx) compared with normoxia (N). C and D, Expression of HIF-1α protein significantly increased in WT mice under hypoxia compared with normoxia (0.25±0.03 vs 0.65±0.04; *P*<0.05). Lung HIF-1α levels did not significantly differ between WT and LR11−/− mice under hypoxia. Levels of sLR11 (soluble LR11) increased in WT mice after 3 wk of hypoxia compared with those under normoxia (4.6±0.7 versus 6.5±1.0 ng/mL; *P*<0.05). Data in bar graphs are expressed as means±SD (n=5). Statistical significance was determined with unpaired Student t test. KO indicates knockout.
LR11−/− Mice Are Resistant to Induction of Pulmonary Hypertension

We investigated the role of LR11 in the development of PAH in LR11−/− mice that were placed under hypoxia for 21 days to induce experimental PAH. Lung HIF-1α levels did not significantly differ between wild-type (WT) and LR11−/− mice under hypoxia (Figure 1A and 1D). The right ventricular systolic pressure and ratio of RV/LV+IVS were significantly lower in LR11−/− than in WT mice (28.3±2.2 versus 33.3±2.2 mm Hg; *P<0.05 and 30.9±2.2% versus 36.4±2.4%; *P<0.05, respectively; Figure 2A through 2C). These results indicated that LR11-deficient mice are highly resistant to developing PAH induced by hypoxia.

Index of Medial Wall Thickness Is Reduced in LR11−/− Mice

Histological findings showed that the index of medial wall thickness in small pulmonary arteries was significantly lower in LR11−/− than in WT mice (3.6% ± 0.5% versus 5.7% ± 0.4%; *P<0.05; Figure 3A and 3B). The ratios of arteries categorized as muscular, partly muscular, and nonmuscular did not differ between LR11−/− and wild-type mice under normoxia. However, the ratios of partly muscular and muscular arteries in LR11−/− mice were significantly decreased, whereas the ratios of nonmuscular arteries in LR11−/− mice were significantly increased compared with those in WT mice under hypoxia (n=4; *P<0.05; Figure 3C). The results of an immunohistochemical comparison using anti-CD45 antibody, which is a common antigen for leukocytes, revealed significantly fewer CD45-positive cells in LR11−/−, than in WT mice. These findings suggested that decreased inflammatory cell infiltration, in addition to decreased pulmonary arterial smooth muscle cell (PASMC) proliferation, is involved in the decreased medial thickening in the absence of LR11. All these findings indicated that LR11-deficient mice are highly resistant to the development of medial thickening induced by hypoxia (Figure 3C).

LR11 Is a Potential Key Regulator of SMC Proliferation Under Hypoxia

Soluble LR11 is a phenotype regulator of contractile, to synthetic SMC, and it is closely associated with intimal thickening after femoral artery damage in mice. We assessed the proliferation of PASMC to determine the pathological role of sLR11 in the development of PAH in model mice. Proliferation rates under hypoxia (1% O2 and 5% CO2) for 48 hours and in the presence of platelet-derived growth factor-BB (20 ng/mL) were significantly decreased in PASMC isolated from LR11−/−, compared with those from wild-type mice (0.18±0.02 versus 0.45±0.05 nm; *P<0.05 and 0.16±0.01 versus 0.45±0.07 nm; *P<0.05, respectively; Figure 4). In contrast, the proliferation rates of PASMC from wild-type mice were significantly increased in the presence, compared with the absence, of 10 ng/mL of sLR11 (0.19±0.01 versus 0.42±0.02 nm; *P<0.05). These results suggested that decreased PASMC proliferation contributes at least in part to the mechanism underlying the increased resistance of LR11−/− mice to hypoxia-induced PAH.

Hypoxia-Induced LR11 Expression in Cultured Human Pulmonary Arterial Smooth Muscle Cells Is Dependent on HIF-1α Pathway

The above findings of LR11−/− and WT mice suggested that LR11, a regulator of SMC proliferation and migration that...
causes vascular intimal thickening after injury, is involved in the progression of PAH as an effector gene under HIF1α-mediated intracellular signals. We therefore assessed LR11 and HIF-1α expression in PASMC after incubation for 24, 48, 72, and 96 hours under hypoxic conditions (1% O2 and 5% CO₂). The expression of LR11 peaked at 48 hours under hypoxia compared with normoxia (0.84±0.02 versus 0.31±0.03; \( P < 0.05 \)). The transient increase in LR11 at this time point was similar to that of HIF-1α (0.92±0.05 versus 0.41±0.04; \( P < 0.05 \); Figure 4A and 4B). Thus, we analyzed the effect of HIF1α knockdown on LR11 expression after hypoxia. The sharp increase in LR11 expression at 48 hours was almost completely abrogated in HIF1α-knockdown PASMC under hypoxia (Figure 4C and 4D). These results indicated that LR11 expression is induced via HIF-1α signaling after hypoxia and that it might cause increased PASMC migration and proliferation.

Serum sLR11 Levels Are Increased in Patients With PAH

We investigated whether sLR11 could serve as a marker of PAH in patients because the findings in vitro and in vivo indicated that LR11 is important for PAH progression in mice. We prospectively enrolled 20 consecutive patients with suspected pulmonary hypertension and then compared serum sLR11 levels in patients with and without confirmed PAH whose age, sex, and World Health Organization functional class did not significantly differ (Table 1). Most patients had World Health Organization functional class II PAH. Connective tissue diseases were associated with PAH in 9 of 11 patients and 2 had portopulmonary hypertension. All of the patients without PAH had connective tissue diseases. Levels of sLR11 were significantly higher in patients with, rather than without, PAH (14.2±4.5 versus 9.2±4.1 ng/mL; \( P = 0.019 \)), whereas levels of BNP and uric acid did not significantly differ (Table 1). Pearson correlation coefficient analyses showed that the sLR11 levels positively correlated with mean pulmonary arterial pressure (\( r = 0.633; P = 0.003 \)) and pulmonary vascular resistance (\( r = 0.580; P = 0.007 \)) among all variables (Table 2). Thus, increased sLR11 levels in patients may reflect the pathological status of SMC in PAH.

Discussion

The major finding of this study is that LR11 regulated medial thickening during the process of vascular remodeling in mice with PH. A deletion of LR11 did not result in pulmonary

Figure 3. LR11 (low-density lipoprotein receptor with 11 binding repeats) deletion ameliorates vascular remodeling under hypoxic conditions. A, Ratio (%) of wall thickness of small pulmonary arteries exposed to chronic hypoxia. Representative photomicrographs of vascular remodeling in distal arterioles of lungs exposed to hypoxia for 3 wk. Lung sections were stained with hematoxylin eosin (HE) and Elastic van Gieson. Magnification, ×40; bar=25 µm. B, Medial wall thickness index (%WT) in distal arterioles (diameter, 50–100 µm) of lungs from LR11−/− and wild-type (WT) mice significantly differed after exposure to hypoxia for 3 wk (3.6±0.5% vs 5.7±0.4%; \( P < 0.05 \)). Bar graphs show data expressed as means±SD (n=8). *\( P < 0.05 \). C, Ratios of 3 arteries categorized as muscular, partly muscular, and non-muscular, did not differ between LR11−/− and WT mice under normoxia. Ratios of partly muscular and muscular arteries in LR11−/− mice were significantly decreased and those of nonmuscular arteries were significantly increased in LR11−/− compared with WT mice under hypoxia (n=4 each, \( P < 0.05 \)). D, Number of CD45+ cells in distal pulmonary artery adventitia was significantly reduced in LR11−/− compared with WT mice after exposure to hypoxia. Number of CD45+ cells did not differ between LR11−/− and WT mice under normoxia (n=4 each; \( P < 0.05 \). Data in bar graphs are expressed as means±SD.). Statistical significance was determined with Tukey honestly significant difference. EVG indicates Elastica van Gieson.
LR11 Deletion Prevents Pulmonary Vascular Remodeling via HIF-1α-Mediated Signals

We investigated whether hypoxia induces HIF-1α-dependent LR11 expression in vivo and in vitro to determine the mechanisms of vascular remodeling in PH. We found that deleting LR11 prevented the development of pulmonary hypertension in a mouse model of PH. We previously reported that deleting LR11 in mice with atherosclerosis prevents vascular remodeling such as VSMC proliferation and migration. The present findings showed that an LR11 deletion suppressed vascular thickening induced by hypoxia and that serum sLR11 levels were increased under hypoxia in wild-type mouse models of PH. We also investigated the role of LR11 in the proliferation of PASMC using LR11-deficient PASMC. The proliferation of PASMC from LR11−/− mice under hypoxia was reduced, whereas that from WT mice was increased by exogenous sLR11 protein, suggesting that LR11 deletion regulates SMC proliferation. HIF-1α plays a critical role in the pathological status of PH in these models of hypoxia. We previously reported that HIF-1α knockdown decreased LR11 expression under hypoxia, indicating that HIF-1α plays a key role in LR11 expression under such conditions.

Soluble LR11 as a Biomarker of Proliferative SMC in Patients With PAH

The early phases of PAH are considered to be histologically nonspecific, with medial pulmonary arterial and adventitial thickening and the appearance of muscle in the walls of normally nonmuscular arteries being the only abnormalities. Many current screening modalities depend on detecting an increase in pulmonary arterial pressure, and thus the early stages of pulmonary vascular disease are likely to be overlooked. One mechanism of vascular remodeling is VSMC proliferation. We previously reported that sLR11 is a biomarker of VSMC proliferation in atherosclerosis. That prospective study of a small cohort of naive patients uncovered a relationship between sLR11 and mean pulmonary arterial pressure or pulmonary vascular resistance. Levels of sLR11 were significantly increased in patients with, rather than without, PAH. Serum sLR11 levels were 7.8±1.6 ng/mL in 56 healthy volunteers (data not shown). These levels significantly differed between healthy volunteers and patients with PAH but not between healthy volunteers and patients without PAH. Therefore, sLR11 might serve as a biomarker of pulmonary vascular disease.

In the present study, we determined the correlation of serum LR11 levels with clinical parameters of PH patients. The present study indicates that sLR11 might serve as a biomarker of pulmonary arterial remodeling in PH.
Considering the difficulties involved in trying to identify the major source of cells that regulate circulating sLR11 levels, we analyzed LR11 expression in rat monocrotaline and mouse hypoxia models. Levels of LR11 protein increased in both models, indicating that LR11 expression increased in the present models of PAH under hypoxia and under treatment with monocrotaline (data not shown). The findings suggested that LR11 expression is increased in association with the SMC proliferation during the progress of PAH, and not only under hypoxia. Although we found that sLR11 alone induced the proliferation of cultured PASMC, endothelial cells and immature cells such as HSPC might also regulate serum sLR11 levels in pulmonary hypertension.\textsuperscript{19,26} We previously showed that sLR11 could be a useful biomarker of conditions such as atherosclerosis,\textsuperscript{17} large B-cell lymphoma,\textsuperscript{27} non-Hodgkin lymphoma,\textsuperscript{28} follicular lymphoma,\textsuperscript{29} diabetic retinopathy,\textsuperscript{30} and acute coronary syndrome.\textsuperscript{31} Overall, sLR11 might serve as a potentially noninvasive and objective parameter of responses to therapy, although further investigation using other models of PH is essential to define how much circulating sLR11 levels reflect the pathological conditions of PASMC in patients.

We recently found that plasma cyclophilin A could serve as a marker of VSMC proliferation in PH and that plasma cyclophilin A levels increase in patients with PH according to the severity of pulmonary vascular resistance.\textsuperscript{32} The PAH in the present study was mainly within World Health Organization functional class II, and therefore, we think that sLR11 will be useful to detect PAH. Further investigation is needed to confirm whether sLR11 can serve as a biomarker of the effects of drugs on PAH.

### Table 2. Pearson Correlation Coefficient Between sLR11 Levels and Variables

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BNP indicates brain natriuretic peptide; CI, cardiac index; LR11, low-density lipoprotein receptor with 11 binding repeats; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; PVR, pulmonary vascular resistance; UA, uric acid; and WHO FC, World Health Organization functional class.

### Figure 5. Expression of LR11 (low-density lipoprotein receptor with 11 binding repeats) and HIF-1\textalpha (hypoxia-inducible factor-1\textalpha) in human arterial SMC under hypoxia.

**A**, Levels of LR11 and HIF-1\textalpha protein analyzed by Western blotting after 24, 48, 72, and 96 h under hypoxic conditions (1% O\textsubscript{2} and 5% CO\textsubscript{2}). **B**, Levels of LR11 and HIF-1\textalpha protein expression peaked at 48 h under hypoxia compared with normoxia (0.9±0.02 vs. 0.31±0.03; *P*<0.05 and 1.17±0.04 vs. 0.42±0.07; *P*<0.05, respectively). Statistical significance was determined with unpaired Student t test. **C**, HIF-1\textalpha expression in human pulmonary arterial smooth muscle cell was knocked down using small interfering RNA (siRNA) and then LR11 and HIF-1\textalpha protein expression was compared in cells incubated with 2 specific siRNA and 1 nonspecific control. **D**, Levels of LR11 and HIF-1\textalpha protein expression decreased compared with nonspecific controls (0.54±0.05 vs 0.02±0.01, 0.02±0.01; *P*<0.05 and 0.61±0.05 vs 0.04±0.01, 0.05±0.01; *P*<0.05, respectively). Statistical significance was determined with Tukey honestly significant difference.
The present study found that sLR11 induced the proliferation of PASMC, which could be a therapeutic target of pulmonary hypertension. In this context, the effects of statins on sLR11-mediated PASMC proliferation should be determined, because statins might be effective against PAH and inhibit the LR11-induced migration of intimal SMC.

In conclusion, this description of sLR11 serves as a biomarker of VSMC proliferation in PH. A deletion of LR11 prevents the development of pulmonary hypertension, indicating that LR11 is an important factor in the progression of this disease.

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Disclosures

None.

References


Highlights

- Deleting LR11 (low-density lipoprotein receptor with 11 binding repeats) did not result in pulmonary hypertension and LR11 expression increased dependently on hypoxia-inducible factor-1α under hypoxia.
- LR11 is a potential key regulator of smooth muscle cell proliferation under hypoxia.
- Serum soluble LR11 levels in patients with pulmonary arterial hypertension are increased and serum soluble LR11 was associated with mean pulmonary arterial pressure or pulmonary vascular resistance. Soluble LR11 might serve as a biomarker of pulmonary arterial remodeling.
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