Disruption of the Apelin-APJ System Worsens Hypoxia-Induced Pulmonary Hypertension

Suparna M. Chandra, Hedi Razavi, Jongmin Kim, Rani Agrawal, Ramendra K. Kundu, Vinicio de Jesus Perez, Roham T. Zamanian, Thomas Quertermous, Hyung J. Chun

Objective—The G-protein–coupled receptor APJ and its ligand apelin are highly expressed in the pulmonary vasculature, but their function in this vascular bed is unclear. We hypothesized that disruption of apelin signaling would lead to worsening of the vascular remodeling associated with pulmonary hypertension (PH).

Methods and Results—We found that apelin-null mice developed more severe PH compared with wild-type mice when exposed to chronic hypoxia. Micro-computed tomography of the pulmonary arteries demonstrated significant pruning of the microvasculature in the apelin-null mice. Apelin-null mice had a significant reduction of serum nitrate levels. This was secondary to downregulation of endothelial nitric oxide synthase (eNOS), which was associated with reduced expression of Kruppel-like factor 2 (KLF2), a known regulator of eNOS expression. In vitro knockdown studies targeting apelin in human pulmonary artery endothelial cells demonstrated decreased eNOS and KLF2 expression, as well as impaired phosphorylation of AMP-activated kinase and eNOS. Moreover, serum apelin levels of patients with PH were significantly lower than those of controls.

Conclusion—These data demonstrate that disruption of apelin signaling can exacerbate PH mediated by decreased activation of AMP-activated kinase and eNOS, and they identify this pathway as a potentially important therapeutic target for treatment of this refractory human disease. (Arterioscler Thromb Vasc Biol. 2011;31:814-820.)

Key Words: endothelial function ■ nitric oxide synthase ■ pulmonary hypertension ■ AMP-activated kinase ■ apelin

Pulmonary hypertension (PH) is a rare clinical disorder with elevation of the pulmonary arterial pressures.1 Clinical symptoms include dyspnea, cyanosis, and right-sided heart failure. A number of vasoactive factors have been described to play important roles in the progression of PH in both experimental and clinical settings, including prostacyclins, endothelin-1, and serotonin.2–5 Therapies targeting endothelial cell dysfunction have proven to be effective and are now used in the clinical setting.6,7 Apelin is a recently described ligand for the G-protein–coupled receptor APJ (APLNR).8,9 Apelin has been shown to be a potent regulator of cardiovascular function. Two main hemodynamic effects described for apelin are vasodilation and augmentation of cardiac inotropy.10–12 The apelin-APJ signaling paradigm represents an intriguing target in the pulmonary vasculature. Both apelin and APJ are highly expressed in the lungs, especially in the endothelium of the pulmonary vasculature.13–15 The vascular effects of exogenous apelin administration have been described in a number of studies to date, the majority of which have documented a vasodilatory effect.11,16 Prior studies have also implicated nitric oxide (NO) as a downstream target of apelin.17,18 Given the breadth of evidence for the role of NO and endothelial nitric oxide synthase (eNOS) in PH,19–21 we hypothesized that the apelin pathway and its targeting of NO has a direct implication in the pathogenesis of PH.

Here we demonstrate that mice lacking the apelin gene develop worsening PH in response to hypoxia, mediated by downregulation of eNOS. We describe a novel mechanism involving AMP-activated kinase (AMPK) and Kruppel-like factor 2 (KLF2) as intermediaries that are critical mediators of apelin-APJ signaling in pulmonary artery endothelial cells (PAECs). We also found that patients with PH have significantly reduced levels of serum apelin, suggesting a potentially important relevance in the human disease.

Methods

A Supplement (available online at http://atvb.ahajournals.org) provides complete details on the methods used in this study.

Animal Studies

All studies were performed under an approved protocol by the Animal Care Committee at Stanford University following the guide-
lines of the American Physiological Society. The apelin-null mice had been described previously.\textsuperscript{13,22} Mice on the SV129 background were used for the studies. Ten- to 12-week-old male mice were used for the studies.

Measurement of Serum Apelin Levels
The institutional review board of Stanford University School of Medicine approved the investigational procedures involved in this study. Plasma apelin-12 concentrations were measured with a commercial kit (Phoenix Pharmaceuticals, Burlingame, CA) as per the manufacturer’s protocol.

Results
Pulmonary Apelin and APJ Expression Are Highly Regulated in the Chronic Hypoxia Model of PH
Previous studies have demonstrated robust expression of apelin and APJ in the pulmonary vasculature.\textsuperscript{13,23} In mice subjected to hypoxia, we found that there was a significant increase in total lung expression of apelin and APJ mRNA after 1 week of exposure (4.0±0.18-fold and 3.1±0.24-fold increase, respectively, \(P<0.001\) for both), similar to our previous study (Figure 1A).\textsuperscript{13} However, after 3 weeks of chronic hypoxia exposure, the levels of apelin and APJ expression had returned to the baseline.

Apelin-Null Mice Develop Worsening PH in Response to Hypoxia
We assessed right ventricular systolic pressures (RVSPs) as a surrogate of the pulmonary arterial pressures of the apelin-null mice. At baseline, the apelin-null mice had RVSPs comparable to those of wild-type controls (Figure 1B). However, when these mice were subjected to hypoxia for 3 weeks, they developed more severe PH compared with the wild-type mice (mean RVSP, 34.1±0.9 versus 28.3±0.9 mm Hg, \(P<0.001\)) (Figure 1B). Our previous study demonstrated no significant difference in left ventricular systolic function between the apelin-null and wild-type mice and no difference in the systolic blood pressure.\textsuperscript{22}

Morphometric studies of the lungs from the chronic hypoxia–exposed apelin-null mice and wild-type littermates were performed to assess evidence of vascular remodeling. Muscularization of the alveolar wall arteries was significantly greater in apelin-null mice compared with wild-type mice, as demonstrated by increase in the number of arteries staining positive for smooth muscle \(\alpha\)-actin (32.4±1.9% versus 12.6±1.0%, \(P<0.001\)) (Figure 1C).

Micro-Computed Tomography of Pulmonary Arteries Demonstrates Defective Pulmonary Vasculature in the Chronic Hypoxic Apelin-Null Mice
To evaluate the possibility that apelin-null mice develop abnormalities of the pulmonary vasculature related to the worsening of the PH, we used micro-computed tomography techniques to determine the anatomy of the pulmonary arteries. Normoxia- and hypoxia-treated wild-type and apelin-null mice were imaged. Micro-computed tomography

Figure 1. Apelin-null mice are more susceptible to hypoxia-induced PH. A, Pulmonary apelin and APJ mRNA expression in chronic hypoxia–exposed mice. Graphs depict mouse apelin and APJ expression in response to hypoxia at 1 and 3 weeks (WK). n=5 mice in each group. B, Measurement of RVSPs in wild-type (+/+) and apelin-null (−/−) mice demonstrated a greater increase in RVSP in the apelin-null mice in response to hypoxia. n=7 to 10 mice per group. C, Staining for smooth muscle \(\alpha\)-actin (red) demonstrated increased muscularization in hypoxia-treated apelin-null mice. Counterstaining is for von Willebrand Factor (brown). Graph represents the percentage of muscularization of the alveolar wall arteries.
images demonstrated no significant difference between the wild-type and apelin-null mice under normoxic conditions (Figure 2A). However, when subjected to chronic hypoxia, the apelin-null mice developed a greater loss of distal arteries compared with the wild-type mice. Quantitative analyses of the density of the distal arteries demonstrated no difference in the density of arteries with diameters of less than 75 μm (9888±3168 versus 10,991±475, P=not significant) or between 75 and 100 μm (21,815±7172 versus 24,944±620, P=not significant) under normoxic conditions (Figure 2B). However, after chronic hypoxia, the apelin-null mice had a significantly reduced number of arteries with diameters less than 75 μm in the apelin-null mice compared with wild-type mice (4343±802 versus 6884±617, P<0.05) (Figure 2B). There was no significant difference in the arterial density with diameter between 75 and 100 μm after chronic hypoxia (9797±731 versus 9802±821, P=not significant).

We sought to validate our findings of the micro-computed tomography analysis by conducting morphometric analysis of lung histology in chronic hypoxia–exposed apelin-null and wild-type mice (Figure 2C). We found a significantly reduced number of vessels less than 75 μm in the apelin-null mice compared with wild-type mice (3.3±0.3 versus 7.3±0.3 vessels per microscope field, P<0.01).

Apelin-Null Mice Have Decreased NO Synthesis
Given a prior study demonstrating a potential link between the apelin-APJ axis and NO,12 we sought to determine the contribution of NO to the worsening PH in apelin-null mice. We measured serum nitrate as a surrogate for NO levels and found significantly lower nitrate levels in the apelin-null mice compared with wild-type mice (29.6±1 versus 129.4±37.7 μmol/L, P=0.01) (Figure 3A). To determine the mechanism of decreased serum nitrate, we evaluated expression of eNOS in the lungs. We found the levels of eNOS mRNA (2.3±0.06-fold decrease, P=0.001) and protein expression to be significantly decreased in the apelin-null mice compared with the wild-type mice (Figure 3B).

In Vitro Knockdown of Apelin in PAECs Leads to Decreased eNOS Levels
Given the decreased expression of eNOS in the lungs of apelin-null mice, we assessed whether disruption of apelin signaling in the endothelial cells can lead to decreased eNOS expression. Using short interfering RNA (siRNA) targeted against apelin, we transfected PAECs and found more than 90% inhibition of apelin expression, as determined by reverse transcription–polymerase chain reaction (Figure 3C). We found a significant reduction in expression levels of eNOS...
mRNA (4.7±0.08-fold reduction, P<0.001) and protein in the cells transfected with apelin siRNA (Figure 3D). In addition, knockdown of APJ also resulted in a statistically significant, albeit more modest, reduction in eNOS expression (Supplemental Figure I).

KLF2 Is a Critical Intermediary for Apelin/APJ Regulation of eNOS Expression

To characterize the mechanism of eNOS downregulation in the context of impaired apelin signaling, we sought to evaluate known upstream regulators of eNOS transcription as a potential mediator. KLF2 is a well-known transcriptional regulator of eNOS, and a recent study has identified it as a known downstream target of AMPK.24 AMPK, in turn, has been found to be a downstream target of apelin signaling in the context of skeletal muscle.25 Indeed, we found that both KLF2 mRNA and protein levels were significantly reduced in the lungs of apelin-null mice (2.5±0.07-fold decrease, P<0.03) (Figure 3E). Knockdown of either apelin or APJ expression in PAECs also resulted in significant reduction of KLF2 expression in these cells (5.0±0.08-fold decrease, P=0.01, and 5.3±0.01-fold decrease P<0.001, respectively) (Figure 3F and Supplemental Figure I).

Apelin Regulates Hypoxia-Mediated AMPK Activation in PAECs

Given the emerging evidence linking apelin with AMPK, as well as the regulation of KLF2 expression by AMPK,24,25 we sought to determine whether apelin targeting of KLF2 expression in PAECs is mediated by AMPK activation. We
confirmed that activation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside is able to induce KLF2 expression in PAECs (Supplementary Figure II). siRNA-mediated knockdown of apelin in PAECs led to a significant decrease in phosphorylation of AMPK and acetyl-CoA carboxylase, a downstream substrate of AMPK (Figure 4A). When PAECs were subjected to 24 hours of hypoxia (1% FiO₂), we found a robust increase in phosphorylation of AMPK in cells transfected with control siRNA, but cells transfected with apelin siRNA did not mount a significant increase in phosphorylation of AMPK or acetyl-CoA carboxylase in response to hypoxia (Figure 4A). In addition, we evaluated the phosphorylation state of eNOS in the setting of apelin knockdown. Previous studies have demonstrated increased eNOS phosphorylation in response to AMPK activation.

Given the marked reduction in hypoxia-induced AMPK phosphorylation in the setting of apelin knockdown, we sought to determine (1) whether KLF2 expression is also regulated by apelin. We initially found a robust increase in KLF2 expression in response to hypoxia in PAECs was abrogated with apelin knockdown. *P<0.05, **P<0.01.

Humans With PH Demonstrate Decreased Serum Apelin Levels

Previous clinical studies have demonstrated changes in apelin levels in disease processes such as heart failure and diabetes mellitus. We measured the serum levels of apelin to determine whether patients with PH have lower levels of apelin. Serum was obtained from 23 patients with PH and 17 normal controls (Table). The mean RVSP of patients with PH was markedly elevated (68.7±24.7 mm Hg). We found a significant reduction in serum apelin levels in patients with PH compared with healthy controls (0.89±0.08 versus 1.89±0.18 ng/mL, P<0.01) (Figure 5). Subgroup analyses of the patients by the etiology of PH also demonstrated a significant reduction in serum apelin level, regardless of the subtype of PH population (Supplemental Figure III).

Discussion

The role of apelin signaling in the pulmonary vasculature is beginning to be understood. Our present findings of increased

### Table. Characteristics of Patients With PH

<table>
<thead>
<tr>
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<th>PH</th>
<th>Control</th>
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<tr>
<td>n</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>Age, years (P=NS)</td>
<td>44±11.2</td>
<td>37±10.2</td>
</tr>
<tr>
<td>Female, n (%) (P=NS)</td>
<td>17 (74)</td>
<td>11 (65)</td>
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<tr>
<td>NYHA Class, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (9)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>17 (74)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1 (4)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3 (13)</td>
<td></td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostanoids</td>
<td>11 (48)</td>
<td></td>
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<tr>
<td>ETA</td>
<td>11 (48)</td>
<td></td>
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<tr>
<td>PDE-1</td>
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<tr>
<td>Immunosuppressants</td>
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<tr>
<td>Combination therapy, n (%)</td>
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<tr>
<td>Mono</td>
<td>9 (39)</td>
<td></td>
</tr>
<tr>
<td>Dual</td>
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<td></td>
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<tr>
<td>Triple</td>
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<tr>
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<td>1 (4)</td>
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<tr>
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<tr>
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<tr>
<td>Transplanted</td>
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NS indicates not significant; NYHA, New York Heart Association; ETA, endothelin antagonist; PDE, phosphodiesterase.
RVSP and pulmonary arterial remodeling in hypoxia-exposed apelin-null mice suggest that apelin is an important homeostatic factor in the pulmonary arterial system. Furthermore, the apelin-null mice developed marked pulmonary vascular defects in response to hypoxia and exhibited disruption of eNOS signaling. We corroborated these data by finding that in vitro knockdown of apelin expression in PAECs led to decreased eNOS levels, as determined by reverse transcription–polymerase chain reaction and Western blot. This occurred in the context of decreased AMPK activation, which led to both decreased expression of KLF2 and decreased eNOS phosphorylation. In addition, patients with PH were found to have decreased serum apelin levels, suggesting that disruption of apelin signaling contributes to the pathogenesis of the clinical disease.

Recent studies have explored a potential role for augmentation of apelin signaling in ameliorating rodent models of PH. A study by Falcao-Pires et al suggests a minimal pulmonary vascular effect of exogenous apelin administration in the monocrotaline model of PH and suggests that the beneficial effect of apelin may be primarily myocardial. However, our data suggest a direct effect of apelin signaling in the pulmonary vasculature. A study by Andersen et al relies on ex vivo effects of apelin, the relevance of which to the in vivo animal models and clinical disease is limited. In addition, no prior study has directly linked apelin and eNOS in the context of the pulmonary endothelium, nor defined the mechanism by which apelin regulates eNOS expression and activation. Our current study identifies 2 novel targets of apelin signaling in PAECs—AMPK and KLF2—that function as critical intermediaries upstream of eNOS.

The role of eNOS in PH has been studied extensively. The remarkable similarity between apelin-null mice and previously described eNOS-null mice supports our conclusion of decreased eNOS expression as a mechanism of PH exacerbation in our apelin-null mice. Studies of eNOS-null mice have demonstrated that these mice are more susceptible to PH response to hypoxia. In addition, a number of clinical studies have implicated decreased NO levels and eNOS expression in patients with PH. It has also been shown that cell-based eNOS gene transfer was able to restore the microvascular loss in the monocrotaline model of PH. Our current findings provide support for these prior studies, given the demonstrated loss of eNOS expression and microvasculature in the apelin-null mice. Prior studies have also demonstrated that hyperactivation of eNOS may lead to exacerbation of PH, suggesting that a strict, multifactorial regulation of eNOS expression and activation may be critical for maintenance of homeostasis of the pulmonary vasculature.

KLF2 regulation of eNOS expression has been well established. Emerging evidence suggests that KLF2 is dysregulated in rodent models of PH, and restoration of KLF2 expression may be able to ameliorate the severity of PH. Our extension of the previous findings showing AMPK targeting of KLF2 expression and apelin induction of AMPK phosphorylation provides a novel mechanism that ultimately may lead to better understanding of eNOS regulation in PH. Although no prior studies have directly implicated AMPK in PH, metformin, a diabetes drug known to activate AMPK, has been shown to significantly improve the PH severity in both the chronic hypoxia and monocrotaline models of PH.

Our human data demonstrated reduced serum apelin levels in the largest cohort of PH patients to date. This provides an intriguing correlation with the data from our animal and in vitro studies. The exact mechanism by which apelin expression is decreased in patients with PH remains to be elucidated. A prospective study would potentially shed more light on the possible correlations of apelin levels with disease progression and response to therapeutics.

In summary, we found that mice with disruption of apelin signaling develop worsening PH and that this pathway is impaired in patients with PH. These findings point to a therapeutic potential for the apelin pathway in PH that needs further investigation.

Acknowledgments
We thank M. Simons, J. Hwa, and K. Martin for critical reading of the manuscript.

Sources of Funding
This study was supported by Grants HL-095654 (to H.J.C.) and HL-077676 (to T.Q.), HHMI Physician Scientist Early Career Award (to H.J.C.), Intelligenz Award (to H.J.C.), GSK International Competitive Grant Award (to H.J.C.), and Sarnoff Foundation Fellowship (to S.M.C.).

Disclosures
None.

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Articular Thrombosis Vasc Biol  April 2011

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Arterioscler Thromb Vasc Biol. 2011;31:814-820; originally published online January 13, 2011;
doi: 10.1161/ATVBAHA.110.219980
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Methods

Animal studies. For chronic hypoxia (FiO₂ 10%), oxygen levels were maintained for three weeks with the ProOx 110 system (BioSpherix, Lacona, NY). RVSP measurements were performed in non-ventilated mice under isoflurane anesthesia by inserting a catheter (Millar Instruments, Houston, TX) via the right jugular vein as described previously.¹

Immunohistochemical/Western analyses. Lungs were perfused with normal saline, fixed in 10% formalin overnight, and then embedded in paraffin for routine histology as previously described¹. Paraffin-embedded sections were deparaffinized in Xylene followed by rehydration with graded alcohol. Antigen retrieval was performed using a heat-mediated epitope retrieval method using Rodent Decloaker (Biocare Medical, Concord, CA). Sections were incubated with primary antibodies specific for α-smooth muscle actin and von Willebrand Factor (Abcam, Cambridge, MA) for 2 hours at room temperature. Staining was completed using secondary antibodies (rabbit on rodent AP-polymer and rabbit on rodent HRP-polymer) and Vulcan fast red chromogen and Betazoid DAB for substrates (Biocare Medical).

Western blotting of SDS-PAGE gels was performed with standard methodology, and the bands were visualized using an enhanced chemiluminescence system. Antibodies specific for AMPKα, phospho-AMPKα, ACC, phospho-ACC, phospho-eNOS, eNOS and KLF2 were used followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (all from Cell Signaling Technology, Inc. except KLF2 from Santa Cruz). Each western blot is a representative of three independent experiments.

Morphometric analysis. We took the same full section in the mid-portion of the left lung parallel to the hilum and embedded it in the same manner. Pulmonary artery muscularization
was assessed at x200 magnification by calculating the proportion of fully and partially muscularized peripheral pulmonary arteries to total peripheral pulmonary arteries in 5 random fields per lung (1 field = x200 magnification).

The total number of vessels less than 75 μm was expressed as the number of vessels counted per random microscope field (5 random fields per lung at x200 magnification).

**Casting of pulmonary arteries.** The animals were anesthetized using isoflurane and injected with Heparin. After a lethal dose of sodium pentobarbital, the chest cavity was exposed using a thoracotomy and the main pulmonary artery was isolated. PE60 tubing (Becton-Dickinson, Franklin Lakes, NJ) was inserted into the right ventricle and advanced to the level of the main pulmonary artery. A syringe pump was used to inject catalyzed Microfil (Flow Tech Inc., Carver, MA) at physiological pressures. The elastomer was then left at 4°C for 24 hours to allow complete polymerization. The lungs and the heart were then removed en bloc and fixed in formalin.

**Imaging and image analysis.** Lungs were imaged using a high-resolution micro-CT scanner, vivaCT 40 (SCANCO Medical, Brüttisellen, Switzerland), with isotropic pixel size of 12.5mm, 45kVp and 177mA. A three-dimensional level-set segmentation of the pulmonary arteries >400mm in diameter was created from the image data using ITK-SNAP. The segmentation was then subtracted from the original image to leave only pulmonary arteries <400mm in diameter. A skeletonization algorithm was applied to these smaller vessels (AnalyzeDirect, Inc., Overland Park, KS) and custom-made MATLAB (Natick, MA) code was utilized to determine the number of arteries of a given diameter.
**Serum nitrate measurement.** Blood was collected from each mouse at the time of sacrifice. Nitrate levels were measured as a surrogate for NO via the Griess reaction per manufacturer’s protocol (Thermo Scientific, Waltham, MA) using serum.

**In vitro knockdown studies.** Human PAECs (Lonza, Basel, Switzerland) were used. PAECs were transfected with either control siRNA or apelin-specific siRNA (Stealth siRNA, Invitrogen) using Lipofectamine RNAiMax (Invitrogen). Cells were harvested after 72 hours and total RNA and cell lysates were obtained. Real-time PCR and western blots were performed as described previously.²

**Real-time PCR.** Flash-frozen lung tissue and cell culture samples were homogenized followed by RNA isolation using RNeasy Mini Kits (Qiagen, Valencia, CA). Purified RNA was reverse transcribed with SuperScript II (Invitrogen, Carlsbad, CA). RT-PCR was performed on a 7900HT Sequence Detection System with TaqMan Probes (Applied Biosystems, Foster City, CA). Values were normalized to the relative amounts of 18S rRNA for each sample. RT-PCR analyses from cell cultures were performed in triplicates in three independent experiments.

**Measurement of serum apelin levels.** Blood samples were collected from patients with confirmed diagnosis of PH without evidence of left ventricular failure seen at Stanford Hospital. Control subjects without clinical signs and symptoms of pulmonary or cardiovascular disease were also recruited. The control subjects did not receive medical treatment, and all patients and control subjects gave written informed consent for participation. Comparisons were made using Student’s paired t-test. All plasma samples were analyzed in duplicates.

**Statistics.** Data are presented as mean ± SEM. Statistical significance was determined using 1-way ANOVA. When only two groups were compared, Student’s t test was used. A value of $p < 0.05$ was considered statistically significant.
References


Supplemental Figure I. Knockdown of APJ expression results in decreased eNOS and KLF2 expression. Assessment of mRNA expression of eNOS and KLF2 in PAECs transfected with siRNA directed against APJ.
Supplemental Figure II. Induction of KLF2 mRNA expression by AMPK activation with AICAR. 2 mM of AICAR was used to stimulate PAECs for the designated time periods. *p<0.05.
Supplemental Figure III. Serum apelin level is lower in patients with PH irrespective of the PH etiology. IPAH-idiopathic pulmonary arterial hypertension. *p<0.05.