Extracellular Cyclophilin A, Especially Acetylated, Causes Pulmonary Hypertension by Stimulating Endothelial Apoptosis, Redox Stress, and Inflammation

Chao Xue, Mark Sowden, Bradford C. Berk

Objective—Oxidative stress and inflammation play key roles in the development of pulmonary arterial hypertension (PAH). Cyclophilin A (CypA) is secreted in response to oxidative stress and promotes inflammation and cardiovascular disease. Endothelial cell (EC) dysfunction is an early event in the pathogenesis of PAH. We evaluated the role of extracellular CypA in PAH and compared the effects of acetylated CypA (AcK-CypA, increased by oxidative stress) and CypA on EC dysfunction.

Approach and Results—In transgenic mice that express high levels of CypA in EC specifically, a PAH phenotype was observed at 3 months including increased right ventricular systolic pressure, α-smooth muscle actin expression in small arterioles, and CD45-positive cells in the lungs. Mechanistic analysis using cultured mouse pulmonary microvascular EC and human pulmonary microvascular EC showed that extracellular CypA and AcK-CypA stimulated EC inflammatory signals: increased VCAM1 and ICAM1, phosphorylation of p65, and degradation of iκB. Extracellular CypA and AcK-CypA increased EC apoptosis measured by TUNEL staining, Apo-ONE assay, and caspase 3 cleavage. Oxidative stress stimulated CypA and AcK-CypA secretion, which further promoted EC oxidative stress. AcK-CypA, compared with CypA, stimulated greater increases in apoptosis, inflammation, and oxidative stress. MM284, a specific inhibitor of extracellular CypA, attenuated EC apoptosis induced by CypA and AcK-CypA.

Conclusions—EC-derived CypA (especially AcK-CypA) causes PAH by a presumptive mechanism involving increased EC apoptosis, inflammation, and oxidative stress. Our results suggest that inhibiting secreted extracellular CypA is a novel therapeutic approach for PAH. (Arterioscler Thromb Vasc Biol. 2017;37:00-00. DOI: 10.1161/ATVBAHA.117.309212.)

Key Words: acetylation  □ cyclophilin A  □ endothelial cell  □ hypertension, pulmonary  □ oxidative stress
showed that plasma levels of CypA in patients with PAH were elevated compared with age-matched healthy individuals.

CypA can be post-translationally modified by acetylation. Acetylation modifies the functions of CypA in immunity and viral infection by inhibiting its peptidyl-prolyl cis-trans isomerase activity. Our laboratory previously reported acetylation of lysines K82, and K125 of CypA was important for angiotensin II–mediated CypA secretion in VSMC. In the present study, we tested the hypothesis that EC-derived CypA, especially AcK-CypA, is a novel mediator of PAH through induction of reactive oxygen species (ROS) production, EC dysfunction, and VSMC growth.

Materials and Methods

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

Results

PAH Phenotype in EC-Specific CypA Overexpression Mouse

To define the role of extracellular CypA in the pathogenesis of PAH, we established both EC-specific and VSMC-specific CypA overexpression (ecCypA-Tg and smcCypA-Tg) mice by crossing FLAG-tagged CypA mice with Tie2-Cre or SM22-Cre mice as we previously published. Western blot and immunofluorescence showed increased expression of both FLAG-CypA and total CypA (2.4-fold) in lungs of 3-month-old ecCypA-Tg mice (Figure IA; Figure IA through ID in the online-only Data Supplement). Importantly, ELISA showed increased CypA and AcK-CypA in plasma (Figure IE through IF in the online-only Data Supplement). Additionally, ELISA showed no increase of CypA in plasma (Figure IF in the online-only Data Supplement). The fact that CypA overexpression in vascular smooth muscle cells (SM22-Cre) did not develop any features of PAH suggested the essential role for EC-derived CypA in PAH. To further explore the difference between EC- and VSMC-derived CypA, we detected the basal expression of AcK-CypA in various cell types including rat lung pulmonary artery SMC (PAC1), rat aortic smooth muscle cell (RASMC), and rat pulmonary microvascular endothelial cell. Interestingly, rat pulmonary microvascular endothelial cell showed about 4-fold higher AcK-CypA expression than PAC1 (Figure IG and IH in the online-only Data Supplement).

Extracellular CypA and AcK-CypA Stimulate EC Inflammatory Signals In Vitro

To investigate the role of extracellular CypA and AcK-CypA in pulmonary EC inflammation, we stimulated EC with 50 nmol/L CypA or AcK-CypA (synthesized as described in Materials and Methods) for varying times and analyzed changes in expression of inflammatory markers (VCAM1, ICAM1, and proteins in the NF-kB pathway). We compared pulmonary EC from 2 different species (mouse and human), and the results were similar in 2 characteristics: (1) The activation of inflammatory signals was similar in both species. (2) AcK-CypA was more potent than CypA. Specifically, as shown in Figure 2A through 2F, CypA and AcK-CypA increased VCAM1 and ICAM1 and also activated the NF-kB pathway in mouse pulmonary microvascular EC (MPMEC). At 24 hours, VCAM1 and ICAM1 were both increased by 1.6-fold in response to CypA and 3.0-fold and 2.2-fold in response to AcK-CypA, respectively. Activation of the NF-kB pathway was measured by the time-dependent changes in expression of phospho-p65 and IkB. Maximum activation of p65 phosphorylation and IkB degradation was at 1 hour. In response to CypA and AcK-CypA, phospho-p65 and IkB showed significantly different changes (at times ≥1 hour), with greater changes induced by AcK-CypA (1.3-fold increase and 1.3-fold decrease versus 2.2-fold increase and 3.7-fold decrease, respectively; Figure 2A through 2F). Similar results were observed using human pulmonary microvascular EC (Figure 2G through 2L). To confirm the role of NF-kB pathway in CypA-induced and AcK-CypA–induced inflammation, MPMEC were transfected with NF-kB p65 siRNA for 48 hours and treated with 50 nmol/L CypA or AcK-CypA. Increased VCAM1 and ICAM1 induced by CypA and AcK-CypA were abolished by transfection with siRNA for NF-kB p65, indicating the essential role of NF-kB pathway in CypA-induced and AcK-CypA–stimulated inflammatory signals (Figure II in the online-only Data Supplement). Phosphorylation of the reduced level of p65 by CypA and AcK-CypA was still apparent (Figure IIB in the online-only Data Supplement).

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AcK-CypA</td>
<td>acetylated cyclophilin A</td>
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<tr>
<td>CypA</td>
<td>cyclophilin A</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<td>MPMEC</td>
<td>mouse pulmonary microvascular endothelial cell</td>
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<td>PAH</td>
<td>pulmonary arterial hypertension</td>
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<td>RASMC</td>
<td>rat aortic smooth muscle cell</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>TNFα</td>
<td>tumor necrosis factor-α</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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AcK-CypA Is More Potent Than CypA for Activation of EC Inflammatory Signals In Vitro

To determine the relative potency of AcK-CypA and CypA, we performed a dose response from 3–100 nmol/L using both MPMEC and human pulmonary microvascular EC. The EC50 value of AcK-CypA in MPMEC was lower for all signals than CypA (Figure 3A through 3D; Table I in the online-only Data Supplement). Specifically, in terms of ICAM1 and VCAM1, the EC50 in MPMEC for AcK-CypA was 1.6 and 9.5 nmol/L, respectively, whereas for CypA, it was 9.0 and 17.8 nmol/L. For IkB, the EC50 value in MPMEC for AcK-CypA was 5.0 nmol/L, whereas for CypA, it was 25.0 nmol/L. For P-p65, there was no CypA response, whereas the EC50 value for AcK-CypA was 17.8 nmol/L. Similar results were observed for human pulmonary microvascular EC. These results suggest that there might be a different receptor for AcK-CypA or the downstream signal transduction pathway differs.

Extracellular CypA and AcK-CypA Stimulate EC Apoptosis In Vitro

EC apoptosis is believed to be one of the initial events in the development of severe human PAH.27 Our laboratory has previously reported that extracellular CypA induced HUVEC apoptosis in the presence of cycloheximide.12 To measure the relative effects of CypA and AcK-CypA on pulmonary EC apoptosis, MPMEC were treated with vehicle, 50 nmol/L CypA, or 50 nmol/L AcK-CypA in the presence of 5 μg/mL cycloheximide. To inhibit specifically only extracellular CypA and AcK-CypA, we used the novel cyclosporine A analogue, MM284,28 at a concentration of 10 μmol/L. Tumor necrosis factor-α (TNFα) served as a positive control. Cell apoptosis was assayed by TUNEL staining. CypA and AcK-CypA increased EC apoptosis by 1.9-fold and 2.7-fold, respectively, which was significantly different (Figure 4A; P<0.05). MM284 significantly attenuated apoptosis induced by CypA and AcK-CypA but not TNFα (Figure 4A; P<0.05).

To confirm the effect of CypA and AcK-CypA on EC apoptosis, Apo-ONE homogeneous caspase-3/7 assay was performed. The fluorescence intensity was used as an indication of the activity of caspase-3/7. Caspase-3/7 activity was increased by 1.4-fold and 1.9-fold in response to CypA and AcK-CypA, respectively, which was significantly different (Figure 4B; P<0.05). MM284 significantly attenuated caspase-3/7 activity induced by CypA and AcK-CypA (1.1-fold and 1.3-fold, respectively; Figure 4B; P<0.05). To confirm these results, expression of cleaved caspase-3 in
MPMEC treated with CypA or AcK-CypA was measured by Western blot. Expression of cleaved caspase-3 was increased after CypA and AcK-CypA treatment and was attenuated by MM284 (Figure 4C).

Because the JNK and p38 MAPK pathways mediate EC apoptosis, we studied the effect of CypA and AcK-CypA on these pathways. MPMEC were treated with CypA or AcK-CypA (50 nmol/L) from 15 minutes to 3 hours.

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**Figure 2.** Extracellular cyclophilin A (CypA) and acetylated (AcK) CypA stimulate endothelial cell (EC) inflammatory signals in vitro. For all experiments, CypA and AcK-CypA were used at 50 nmol/L. A, Time course of NF-κB pathway activation in mouse pulmonary microvascular EC (MPMEC). B and C, Quantification of phospho-p65 and IkB levels over 3 h shown in (A). D, Expression of adhesion molecules in MPMEC. E and F, Quantification of adhesion molecule expression shown in (B). Data are mean±SEM. *P<0.05. G, Time course of NF-κB pathway activation in HPMEC. H and I, Quantification of phospho-p65 and IkB levels over 3 h shown in (G). J, Expression of adhesion molecules in human pulmonary microvascular EC (HPMEC). K and L, Quantification of adhesion molecule expression shown in (J). Data are mean±SEM. *P<0.05. The data are representative of experiments repeated 3 times, with each experiment in A–F using a separate cell preparation from 3 mice of the same sex; (G–L) using 3 separate lots of commercially available cells from donors of unknown sex.

**Figure 3.** Acetylated cyclophilin A (AcK-CypA) is more potent than CypA for activation of inflammatory signals in vitro. For all experiments, CypA and AcK-CypA were used at 50 nmol/L. A, Dose response of NF-κB pathway activation in mouse pulmonary microvascular endothelial cell (MPMEC) at 3 h. B, Dose response of adhesion molecules expression in MPMEC at 24 h. C, Dose response of NF-κB pathway activation in human pulmonary microvascular endothelial cell (HPMEC) at 3 h. D, Dose response of adhesion molecules expression in HPMEC at 24 h. The data are representative of experiments repeated 3 times, with each experiment in (A and B) using a separate cell preparation from 3 mice of the same sex; (C and D) using 3 separate lots of commercially available cells from donors of unknown sex.
Although CypA and AcK-CypA both activated JNK and p38, AcK-CypA induced significantly greater JNK and p38 phosphorylation (Figure SIII in the online-only Data Supplement).

**Extracellular CypA and AcK-CypA Stimulate EC Oxidative Stress In Vitro**

Oxidative stress has been implicated as a potential mediator of PAH and the associated changes to pulmonary vasculature.4–6

Our laboratory showed that intracellular CypA regulated angiotensin II–induced ROS generation in VSMC by promoting the assembly of NADPH oxidase.15 To further understand the role of extracellular CypA in EC oxidative stress, MPMEC were treated with vehicle or 50 nmol/L CypA and AcK-CypA for 24 hours. DHE staining was performed to detect the generation of ROS. DHE fluorescence indicated a 1.4-fold increase of ROS production in response to CypA and a 2.0-fold increase in response to AcK-CypA. Similar to inflammatory signals and apoptosis, AcK-CypA was significantly more potent than CypA (Figure 5A; Figure SIV A in the online-only Data Supplement; \( P < 0.05 \)).

**Oxidative Stress Stimulates EC Secretion of CypA and AcK-CypA In Vitro**

To examine the effect of oxidative stress on secretion of CypA and AcK-CypA from EC, MPMEC were exposed to varying concentrations of LY83583, which generates intracellular superoxide,31 for 12 hours (Figure SIVB in the online-only Data Supplement). Conditioned medium and total cell lysates were collected, and Western blot was performed to measure the level of CypA and AcK-CypA. LY83583 increased CypA and AcK-CypA levels in the conditioned medium from MPMEC in a dose-dependent manner (Figure 5B). The absence of GAPDH in the conditioned medium indicated that CypA and AcK-CypA came from cell secretion instead of acute necrosis. To confirm the results, TNFα was used to induce oxidative stress, and CypA and AcK-CypA were measured in the conditioned medium. TNFα increased secretion of CypA and AcK-CypA, although less than LY83583 (Figure SIVC in the online-only Data Supplement).

**Extracellular CypA and AcK-CypA Stimulate VSMC Growth Signals In Vitro**

Aberrant proliferation of VSMC in pulmonary arterioles and consequential vessel thickening is a typical feature of PAH.1,3 We previously published that CypA stimulated ERK1/2 activation in VSMC.20,26 However, the effect of extracellular CypA and AcK-CypA on VSMC proliferation is not fully characterized. We treated RASMC and human pulmonary smooth muscle cells with 50 nmol/L CypA or AcK-CypA for various times, and ERK1/2 activation was examined using Western blot. Activation of ERK1/2 started from 5 minutes and peaked at 15 minutes (Figure 6A and 6B) in both cell types. To measure their relative potencies,
the effect of CypA and AcK-CypA (10, 25, and 50 nmol/L) on ERK1/2 phosphorylation at 15 minutes in RASMC was analyzed (Figure 6C). Similar to results for EC inflammation and apoptosis, AcK-CypA was more potent than CypA. The extracellular CypA inhibitor, MM284 (10 μmol/L) successfully blocked the activation of ERK1/2 induced by CypA and AcK-CypA in RASMC at all concentrations (Figure 6D). To rule out the role of intracellular CypA, mouse aortic smooth muscle cells were harvested from WT and CypA−/− mice and treated with 50 nmol/L CypA for varying times. No significant difference was observed in the ability of CypA to activate ERK1/2 in WT and CypA−/− mouse aortic smooth muscle cells (Figure 6E).

Discussion

The 4 major findings of this study are as follows: (1) Extracellular CypA is a novel mediator of PAH as shown by the data that mice which overexpress CypA in an EC-specific
manner (termed ecCypA-Tg here), but not VSMC-specific manner, developed a PAH phenotype. Specifically, ecCypA-Tg mice showed increases in right ventricular systolic pressure, pulmonary arteriole muscularization, lung inflammation, and circulating CypA and AcK-CypA. (2) In vitro, both extracellular CypA and AcK-CypA stimulated EC inflammatory signals, apoptosis, and ROS production, mechanisms known to be involved in the pathogenesis of PAH. (3) The extracellular CypA inhibitor MM284 attenuated CypA-mediated and AcK-CypA-mediated EC and VSMC signal transduction. (4) AcK-CypA was a more potent agonist than CypA for several markers of pulmonary EC dysfunction.

A key role for oxidative stress in the pathogenesis of PAH has been suggested by many studies. However, no specific agonist that stimulates oxidative stress in the lung vasculature has been identified, to our knowledge. Our laboratory and others have shown a key role for CypA in mediating oxidative stress and inflammation in several diseases including aortic aneurysm, allergic lung inflammation, atherosclerosis, intima formation, and cardiac hypertrophy. Recently, it was shown that the level of circulating CypA correlated with severity of coronary artery disease, strengthening a pathological role for extracellular CypA in cardiovascular disease. Here, we show that EC-specific overexpression of CypA in vivo (ecCypA-Tg mouse) induced a PAH phenotype characterized by multiple parameters. (1) We found increased right ventricular systolic pressure, which is the most typical feature of PAH. (2) There was increased α-smooth muscle actin immunohistochemistry, especially in small artery and pericapillary regions, indicating muscularized distal pulmonary arteries. (3) We observed lung inflammation shown by increased CD45-positive cells and adhesion molecules (VCAM1 and ICAM1). (4) A role for extracellular CypA was suggested by increased levels of circulating CypA and AcK-CypA, similar to data for plasma CypA in human patients with PAH. Surprisingly, VSMC-specific overexpression of CypA in vivo (smcCypA-Tg mouse) showed no phenotype of PAH. AcK-CypA was detected in rat lung pulmonary artery SMC (PAC1), RASMC, and rat pulmonary microvascular endothelial cell. Interestingly, rat pulmonary microvascular endothelial cell showed about 4-fold higher AcK-CypA expression than PAC1 (Figure 9B), suggesting that EC-derived CypA may be a more potent agonist than VSMC-derived CypA. This may, in part, explain the presence of a PAH phenotype in the ecCypA-Tg mice but not in smcCypA-Tg mice at 3-month age. It is possible that PAH will develop in smcCypA-Tg mice at older ages. Future studies using stimuli such as inflammation and others have shown a key role for CypA in mediating oxidative stress and inflammation in several diseases including aortic aneurysm, allergic lung inflammation, atherosclerosis, intima formation, and cardiac hypertrophy. Recently, it was shown that the level of circulating CypA correlated with severity of coronary artery disease, strengthening a pathological role for extracellular CypA in cardiovascular disease. Here, we show that EC-specific overexpression of CypA in vivo (ecCypA-Tg mouse) induced a PAH phenotype characterized by multiple parameters. (1) We found increased right ventricular systolic pressure, which is the most typical feature of PAH. (2) There was increased α-smooth muscle actin immunohistochemistry, especially in small artery and pericapillary regions, indicating muscularized distal pulmonary arteries. (3) We observed lung inflammation shown by increased CD45-positive cells and adhesion molecules (VCAM1 and ICAM1). (4) A role for extracellular CypA was suggested by increased levels of circulating CypA and AcK-CypA, similar to data for plasma CypA in human patients with PAH. Surprisingly, VSMC-specific overexpression of CypA in vivo (smcCypA-Tg mouse) showed no phenotype of PAH. AcK-CypA was detected in rat lung pulmonary artery SMC (PAC1), RASMC, and rat pulmonary microvascular endothelial cell. Interestingly, rat pulmonary microvascular endothelial cell showed about 4-fold higher AcK-CypA expression than PAC1 (Figure 9B), suggesting that EC-derived CypA may be a more potent agonist than VSMC-derived CypA. This may, in part, explain the presence of a PAH phenotype in the ecCypA-Tg mice but not in smcCypA-Tg mice at 3-month age. It is possible that PAH will develop in smcCypA-Tg mice at older ages. Future studies using stimuli such as inflammation and hypoxia may promote PAH in smcCypA-Tg mice. Together, these data strongly suggest a pathogenic role for EC-derived CypA in PAH patients and CypA as a biomarker for PAH.

Evidence for oxidative stress in PAH includes protein, lipid, and DNA oxidation, as well as antioxidant depletion in PAH patients. Our previous publications show that intracellular CypA regulates angiotensin II–induced ROS generation in VSMC through interaction with subunits of NAPDH oxidase and the cell cytoskeleton. Furthermore, oxidative stress induces CypA and AcK-CypA secretion from VSMC. Here, we found extracellular CypA and AcK-CypA also directly induced ROS production in pulmonary EC. Oxidative stress induced by LY83583 and TNFα stimulated CypA and AcK-CypA secretion from EC, suggesting the existence of a positive feedback loop between EC oxidative stress and CypA secretion. In PAH, we propose a 2-step pathogenic mechanism. First, mutations in genes such as BMP2R (bone morphogenetic protein receptor type II) and environmental factors cause EC oxidative stress, leading to secretion of CypA. The increase in extracellular CypA and AcK-CypA then stimulates further oxidative stress of both EC and VSMC, leading to pulmonary vascular pathological changes.

There are many signaling events induced by CypA in vitro that support a pathogenic role for CypA and especially extracellular CypA in PAH. Both extracellular CypA and AcK-CypA stimulated EC inflammatory signals, apoptosis, and ROS production. A key role for extracellular CypA was shown by the ability of the specific extracellular CypA inhibitor MM284 to inhibit CypA-stimulated and AcK-CypA-stimulated signal transduction events in EC and VSMC. Furthermore, these signals were activated in VSMC in the absence of intracellular CypA. Specific pathways stimulated by extracellular CypA include (1) proinflammatory signals, especially via the NF-κB pathway, including phosphorylation of p65 and degradation of IκB, which regulate the expression of adhesion molecules such as VCAM1 and ICAM1; (2) proapoptotic pathways including JNK, p38, and activation of caspase-3/7; and (3) VSMC growth signals (ERK1/2 activation).

An important concept suggested by this work is that drugs, which inhibit the actions of extracellular CypA specifically, represent attractive therapies for diseases such as PAH. MM284 is a cyclosporine A derivative CypA inhibitor, that by virtue of its structure remains extracellular. Recently, MM284 was shown to reduce myocardial inflammation and remodeling in a myocarditis mouse model. The authors suggested that the primary effect of MM284 was to inhibit CypA-induced monocyte recruitment. Here, we show that MM284 also attenuated EC apoptosis induced by both CypA and AcK-CypA, but not by TNFα, demonstrating a high specificity of MM284.

We found that AcK-CypA was a more potent agonist than CypA for several markers of pulmonary EC dysfunction and VSMC growth. Specifically, AcK-CypA had a lower EC50 for inflammatory signaling than CypA. Previously, we showed that both AcK-CypA and CypA stimulated HUVEC adhesion molecule expression and monocyte binding to HUEVEC but did not measure relative potency. The mechanism by which AcK-CypA is more potent than CypA is unclear. CD147 (Basigin or EMMPRIN) is believed to be the receptor of extracellular CypA. AcK-CypA is more potent than CypA is unclear. CD147 did not measure relative potency. Therefore CypA acetylation may increase its binding affinity because of CypA acetylation may increase its binding affinity to CD147, thereby amplifying the downstream signals. In addition, the peptidyl-prolyl cis-trans isomerase activity of AcK-CypA may be greater. However, it is possible that there is another CypA receptor besides CD147 that specifically binds AcK-CypA. This possibility is further supported by the fact that no obvious signaling mechanism for CD147 has been identified. Finally, there may be a positive feedback loop in which EC-derived CypA stimulates SMC inflammation, ROS production, and acetylation of VSMC CypA. Future work...
will be necessary to study the complex effects of EC-derived CypA on pathogenic SMC properties in vivo.

In conclusion, we demonstrated that a novel EC-specific CypA overexpression transgenic mouse showed features characteristic of PAH without any environmental stress (such as hypoxia or monocrotaline). In vitro studies suggest key roles for CypA in EC inflammation, apoptosis, and ROS production. These effects were inhibited by both the specific extracellular CypA inhibitor MM284, suggesting a key role for extracellular CypA. Finally, we found that acetylation of extracellular CypA made it a more potent agonist than CypA for several markers of EC dysfunction. On the basis of these results, we suggest that extracellular CypA is a key mediator of PAH and that targeting extracellular CypA, especially AcK-CypA, is a novel approach to treat PAH.

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Disclosures
None.

References


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**Highlights**

- Extracellular cyclophilin A (CypA) is a novel mediator of pulmonary arterial hypertension as shown by the data that mice which overexpress CypA in an endothelial cell–specific manner (but not vascular smooth muscle cell–specific) developed a pulmonary arterial hypertension phenotype. Specifically ecCypA-Tg mice showed increases in right ventricular systolic pressure, pulmonary arteriole muscularization, lung inflammation, and circulating CypA and acetylated CypA.

- In vitro, both extracellular CypA and acetylated CypA stimulated endothelial cell inflammatory signals, apoptosis, and reactive oxygen species production, mechanisms known to be involved in the pathogenesis of pulmonary arterial hypertension.


- Acetylated CypA was a more potent agonist than CypA for several markers of pulmonary endothelial cell dysfunction.
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