Pulmonary Hypertension Secondary to Left-Heart Failure Involves Peroxynitrite-Induced Downregulation of PTEN in the Lung

Yazhini Ravi, Karuppaipay Selvendiran, Shan K. Naidu, Sarath Meduru, Lucas A. Citro, Balázs Bognár, Mahmood Khan, Tamás Kállai, Kálmán Hideg, Periannan Kuppusamy, Chittoor B. Sai-Sudhakar

Abstract—Pulmonary hypertension (PH) that occurs after left-heart failure (LHF), classified as Group 2 PH, involves progressive pulmonary vascular remodeling induced by smooth muscle cell (SMC) proliferation. However, mechanisms involved in the activation of SMCs remain unknown. The objective of this study was to determine the involvement of peroxynitrite and phosphatase-and-tensin homolog on chromosome 10 (PTEN) in vascular SMC proliferation and remodeling in the LHF-induced PH (LHF-PH). LHF was induced by permanent ligation of left anterior descending coronary artery in rats for 4 weeks. MRI, ultrasound, and hemodynamic measurements were performed to confirm LHF and PH. Histopathology, Western blot, and real-time polymerase chain reaction analyses were used to identify key molecular signatures. Therapeutic intervention was demonstrated using an antiproliferative compound, HO-3867. LHF-PH was confirmed by significant elevation of pulmonary artery pressure (mean pulmonary artery pressure/mm Hg: 35.9±1.8 versus 14.8±2.0, control; P<0.001) and vascular remodeling. HO-3867 treatment decreased mean pulmonary artery pressure to 22.6±0.8 mm Hg (P<0.001). Substantially higher levels of peroxynitrite and significant loss of PTEN expression were observed in the lungs of LHF rats when compared with control. In vitro studies using human pulmonary artery SMCs implicated peroxynitrite-mediated downregulation of PTEN expression as a key mechanism of SMC proliferation. The results further established that HO-3867 attenuated LHF-PH by decreasing oxidative stress and increasing PTEN expression in the lung. In conclusion, peroxynitrite and peroxynitrite-mediated PTEN inactivation seem to be key mediators of lung microvascular remodeling associated with PH secondary to LHF. (Hypertension. 2013;61:593-601.) • Online Data Supplement

Key Words: HO-3867 ■ left-heart failure ■ peroxynitrite ■ phosphatase-and-tensin homolog on chromosome 10 ■ pulmonary hypertension

Pulmonary hypertension (PH) that occurs after left-heart failure (LHF) is a debilitating disease that is associated with significant morbidity and mortality.1−3 LHF-PH originates from a sustained increase of left-atrial pressure (pulmonary venous hypertension) because of left-ventricular (LV) dysfunction resulting in chronic increase of pulmonary arterial pressure, which promotes remodeling of the arterial wall, including abnormalities of elastic fibers, intimal fibrosis, and medial hypertrophy. These changes, which are largely progressive and irreversible, lead to vascular stiffness and decrease in pulmonary vasodilatory responsiveness to therapies.1 In addition, the fixed PH with pulmonary vascular resistance >3 Wood units is linked with a higher posttransplantation mortality and hence considered a contraindication for heart transplantation.3 Of the total number of deaths after heart transplantation, ≈20% are attributed to fatal right-ventricular failure.3 Because PH is usually diagnosed at a late stage, a proper understanding of the underlying pathobiology of the disease progression is necessary for designing suitable interventional approaches at an early stage before irreversible pathological changes occur in the pulmonary vascular bed. Currently available treatment options, such as inhaled NO, nitrates, calcium-channel blockers, prostanoids, endothelin-receptor antagonists, statins, peroxisome proliferator activator receptor-gamma (PPARγ) activators, and phosphodiesterase inhibitors, despite their beneficial effects on PH, have adverse systemic effects.4,5

A phosphatase-and-tensin homolog on chromosome 10 (PTEN) is a modulator of phosphatidylinositide 3-kinase (PI3K) activity associated with PH-mediated vascular remodeling.6,7 PTEN is a dual-function phosphatase capable of dephosphorylating both lipids and proteins, and it negatively regulates PI3K pathway by dephosphorylating...
phosphatidylinositol-triphosphate and pAkt, and hence regulates apoptosis and cell-proliferation pathways. Downregulation of PTEN is linked to smooth muscle cell (SMC) proliferation and vascular remodeling in animal models of arterial restenosis, PH, and lung fibrosis. Recently, we have reported a significant reduction in PTEN levels in the lung tissues of rats with PH induced by monocrotaline or exposure to hypoxia. Reduction in PTEN inversely correlated with pAkt suggesting the involvement of PTEN-mediated modulation of PI3K pathway in the progression of PH. A link between PH and PTEN has been reported in a patient diagnosed with a mutation in PTEN gene (Cowden syndrome). This case report speculated that the patient’s disposition to developing PH was secondary to the loss of PTEN activity and function. However, the precise role and involvement of PTEN in PH-associated vascular alterations in the general population, especially in PH secondary to LHF has not been determined.

Peroxynitrite, a potent oxidant formed by the reaction of superoxide with NO, has been shown to stimulate vascular SMC proliferation at low concentrations (<2 μmol/L) through a mechanism involving peroxynitrite-mediated activation of extracellular signal-regulated kinase (ERK) and protein kinase C (PKC). Using immunohistochemistry to localize nitrotyrosine, Bowers et al showed ubiquitous presence of peroxynitrite in the lung tissue of patients with severe PH. This clinical observation along with the in vitro results of Agbani et al on peroxynitrite-induced proliferation of SMCs suggests a possible link between peroxynitrite (oxidant) and the pathogenesis of PH. Despite numerous reports implicating the involvement of reactive oxygen species (ROS) in the pathogenesis of myocardial infarction and heart failure, little is known about the involvement of ROS in the progression of PH with associated heart failure. Hence, we sought to determine whether LHF-PH is mediated by reactive oxidants, leading to a PTEN-dependent vascular SMC proliferation in the lung. We hypothesized that peroxynitrite is a key mediator of SMC proliferation via PTEN signaling. We used an in vivo rat model of LHF-PH induced by permanent ligation of the left anterior descending (LAD) coronary artery. Human pulmonary artery SMCs (PASMC) were used to study the mechanism by which peroxynitrite modulates PTEN and induces cell proliferation. Furthermore, we also tested a novel therapeutic intervention using a unique compound HO-3867, with both antiproliferative and antioxidant properties and known to upregulate PTEN. The results showed the involvement of reactive oxidants including peroxynitrite and PTEN in the development of LHF-PH and demonstrated a potential therapeutic approach for the management of PH associated with LHF.

Materials and Methods
A more detailed Materials and Methods section is provided in the online-only Data Supplement.

Reagents
HO-3867 was synthesized as reported.

Experimental Animals
LHF was induced in male Sprague-Dawley rats (body weight: 225–250 g; Harlan Laboratories, South Easton, MA) by permanent ligation of LAD coronary artery. All procedures were performed with the approval of the Institutional Animal Care and Use Committee of The Ohio State University and conformed to the Guide for Care and Use of Laboratory Animals (National Institutes of Health publication 8th edition, 2011).

Treatment With HO-3867
HO-3867 was administered in the diet (100 ppm; Harlan-Teklad Laboratory Animal Diets, South Easton, MA), beginning day 1 after LAD coronary artery ligation, and continued for the entire treatment period of 4 weeks. The 100-ppm dose of HO-3867 was based on our earlier studies that used oral administration of this compound in mice.

In Vitro Studies
Human PASMCs were obtained from Lonza (Walkersville, CA).

Statistical Analysis
Data were expressed as mean±SD for all groups. Statistical analyses were performed using unpaired Student t test for comparing 2 groups or 1-way ANOVA with Fisher least squares difference (LSD) post hoc test for comparing multiple groups. Differences between groups were considered significant at P<0.05.

Results
LHF Promotes Pulmonary Vascular Remodeling and Downregulation of PTEN in the Lung
LHF was induced in rats by permanent occlusion of LAD coronary artery for 4 weeks. Cardiac dysfunction and development of PH were confirmed by MRI, echocardiography, and hemodynamic measurements (Figure S1 in the online-only Data Supplement). Histopathology and immunohistochemistry were performed to confirm pulmonary vascular remodeling and to identify the involvement of PTEN and other key downstream proteins in the development of PH secondary to LHF. Hematoxylin and eosin (H&E), Masson trichrome, and elasin staining showed the hallmarks of PH-mediated vascular remodeling including medial thickening, luminal narrowing, perivascular fibrosis, muscularization of the peripheral small arteries, and vascular smooth muscle hyperplasia in the tunica media (Figure 1A). Staining for α-smooth muscle actin, which is a specific marker for vascular SMCs, indicated capillary vessel-wall (medial) thickening (Figure 1B). There was a significant reduction in the peripheral distribution of pulmonary small arteries in the lungs of LHF rats (Figure 1C). Immunohistochemical staining of lung tissues identified extensive loss of PTEN and abundance of pAkt in the pericytes of the tunica intima in the LHF group (Figure 1D and 1E). Real-time polymerase chain reaction analysis of lung vascular cells isolated using laser-capture microdissection (Figure 1F) showed a decrease in the expression of PTEN mRNA in the lungs of LHF group (Figure 1G). Overall, histopathology confirmed the development of vascular remodeling and downregulation of PTEN in the lungs of rats with LHF.
Peroxynitrite Induces Cell Proliferation and Downregulation of PTEN in PASMCs

Because PH is associated with abundant production of superoxide in the lung parenchyma,17–19 as well as increased SMC proliferation, we hypothesized that peroxynitrite production in the lungs of LHF-PH modulates the molecular pathways associated with PASMC proliferation. PASMCs were treated with a single bolus dose of 0.5 μmol/L or 1 μmol/L peroxynitrite. At 48 and 72 hours there was an increase in PASMC proliferation and decreased expression of PTEN and pPTEN and an increase in Akt (total) and phospho-Akt (pAkt; Ser473; Figure 2A and 2B). The involvement of PTEN in PASMC proliferation was confirmed using PTEN small interfering RNA and PTEN cDNA transfection studies (Figure 2C). We determined the effect of HO-3867 (Figure 2D), a potent antioxidant and PTEN-stabilizing agent,15 on the peroxynitrite-induced proliferation of PASMCs. In cells, HO-3867 undergoes a 1-electron reversible redox conversion to its nitroxide form in the presence of oxidants, such as superoxide and peroxynitrite (Figure 2E). HO-3867 scavenges peroxynitrite and becomes oxidized to nitroxide (Figure 2F), which is readily detectable by electron paramagnetic resonance spectroscopy. The nitroxide metabolite, in turn, is capable of scavenging superoxide anion radicals (superoxide dismutase-mimetic).14 Addition of HO-3867 to PASMCs in the presence of fetal bovine serum significantly inhibited peroxynitrite-mediated proliferation (Figure 2G).

These results established that low levels of peroxynitrite promote PASMC proliferation, via downregulation of PTEN. Furthermore, HO-3867 is capable of scavenging peroxynitrite, and thereby inhibits the peroxynitrite-induced cell proliferation.

HO-3867 Attenuates the Progression of LHF-PH

The effect of HO-3867 on LHF-PH was studied on separate groups (cf, Figure S1) of rats subjected to LAD artery occlusion. Wet-weight measurements showed an increase of lung, heart, and LV mass on LAD occlusion for 4 weeks (Figure S2). HO-3867 treatment significantly blunted the increase in lung weight, LV mass on LAD occlusion for 4 weeks (Figure S2). HO-3867 treatment did not have any significant effect on heart or LV weight. Echocardiography showed that HO-3867 had no effect on heart or LV weight. Echocardiography showed that HO-3867 treatment did not have any significant effect on the LV dysfunction observed in the LHF group (Figure 3). However, hemodynamic measurements showed a significant attenuation of right-ventricular systolic pressure and mean pulmonary artery pressure elevation induced by LHF (Figure 3). However, HO-3867 did not have any effect on LV systolic pressure. Taken together, the functional and hemodynamic data indicated that although HO-3867 had no effect on LV function it attenuated the progression of PH secondary to LHF.

LHF Generates Superoxide and Peroxynitrite in the Lung

Dihydroethidium (DHE) and nitrotyrosine-immunostaining images of lung tissue sections showed an increase in...
superoxide and peroxynitrite production in the LHF group when compared with the control (Figure 5). The intensity data showed a significant increase in the levels of both superoxide and peroxynitrite in LHF group when compared with baseline (control) lungs. HO-3867 almost completely abolished the elevation in superoxide and peroxynitrite levels in lung tissues caused by LHF, further establishing the role of HO-3867 as an antioxidant.

**HO-3867 Inhibits Pulmonary Vascular Remodeling by Restoration of PTEN Activity**

We next determined the effect of HO-3867 on pulmonary vascular remodeling. H&E staining of HO-3867-treated lungs showed a marked decrease in medial thickening and luminal narrowing that was seen in the untreated LHF lungs (Figure 6A). The results suggested the efficacy of HO-3867 in attenuating the pulmonary vascular remodeling. Western blot
analysis of excised whole lung tissues showed a significant decrease in the activity of PTEN in the lungs of LHF rats when compared with control. HO-3867 treatment almost completely restored both the total and active PTEN levels (Figure 6B). It should be noted that the ratio of pPTEN/PTEN or pAkt/Akt did not change (data not shown) on treatment suggesting that both the unphosphorylated and phosphorylated forms are similarly affected. The results showed that loss of PTEN and upregulation of Akt in and treatment with HO-3867 significantly restored PTEN levels and caused downregulation of Akt.

Discussion

PH, regardless of its cause, is a debilitating disease with no effective therapies. The results of the current study provided 2 significant and important conclusions: (1) peroxynitrite and peroxynitrite-mediated PTEN dysregulation seems to be key mediators of

Figure 4. HO-3867 attenuates left-heart failure (LHF)-induced increase in pulmonary artery pressure. LHF was induced in rats by permanent ligation of left anterior descending coronary artery for 4 weeks. HO-3867 was administered in the feed throughout the period. Hemodynamic (pressure) measurements were performed at the end of 4 weeks postligation. A, Representative tracings of the pulmonary artery pressure (PAP). B, Left-ventricular systolic pressure (LVSP). C, Right-ventricular systolic pressure (RVSP). D, Mean pulmonary arterial pressure (mPAP). Data represent mean±SD; n=6 animals/group. *P<0.05. The results show HO-3867 significantly attenuates the increase in mPAP observed in the LHF group.

Figure 5. Left-heart failure (LHF) induces oxidant production in the lung. LHF was induced in rats by permanent ligation of the left anterior descending coronary artery. HO-3867 was administered in the feed throughout the period. Lung tissues, collected at the end of 4 weeks of ligation, were stained for the determination of oxidant (superoxide) and nitrotyrosine (as surrogate of peroxynitrite). A, Representative images of dihydroethidium staining show a higher level of superoxide production in the lungs of LHF group when compared with the control and HO-3867 groups. B, Representative immunostaining images of nitrotyrosine along with nuclear staining by 4',6-diamidino-2-phenylindole (DAPI) show the peroxynitrite levels in the different groups. C, Quantitative analysis of dihydroethidium staining shows a significant increase in superoxide production in the LHF group which is largely attenuated by HO-3867 treatment (mean±SD; n=3 rats with 3 slides/rat). D, Quantitative analysis of the nitrotyrosine images show an increased level of peroxynitrite in the LHF group that is significantly attenuated on HO-3867 (mean±SD; n=3 rats with 3 slides/rat). *P<0.05. The results indicate a substantial production of oxidants in the lungs of the LHF group.
Figure 6. HO-3867 inhibits vascular remodeling by restoration of phosphatase-and-tensin homolog on chromosome 10 (PTEN) activity in left-heart failure (LHF)-mediated pulmonary hypertension (PH). A, Representative images of H&E staining of lung sections show extensive muscularization of the capillary vessel walls in the LHF group indicative of vascular remodeling in comparison with the control group. The images also show a decrease in vascular remodeling on treatment with HO-3867. The results demonstrate the ability of HO-3867 in decreasing the luminal narrowing and vascular smooth muscle cell hyperplasia associated with LHF-PH. B, Western blot assays were performed on lung tissues collected after 4 weeks of left anterior descending artery ligation. Representative Western blot images and densitometric analysis of total (PTEN) and phosphorylated PTEN (pPTEN) and Akt, normalized with respect to loading control (Actin), are shown. The data represent mean±SD; n=5; *P<0.05 vs Control; #P<0.05 vs LHF. The results show a significant downregulation of antiproliferative signaling molecule pPTEN and upregulation of prosurvival protein pAkt in the LHF group which is mitigated by HO-3867.

Pulmonary vascular remodeling; and (2) HO-3867, a curcumin analog with novel antioxidant/antiproliferative properties, is effective in targeting the peroxynitrite/PTEN pathway for the treatment of PH and associated vascular alterations.

PTEN has been previously implicated in the negative regulation of SMC proliferation involved with vascular remodeling. PTEN was significantly downregulated in the lung tissues of rats administered with monocrotaline or exposed to chronic hypoxia. In the current study, immunohistochemical staining showed loss of PTEN expression in the lungs of LHF-PH rats. Laser-capture microdissection and real-time polymerase chain reaction results established that the PH-mediated alterations in PTEN were particularly localized to the vascular SMCs of the small- and medium-sized arteries in the lung. Our results are in agreement with a recent study by Nisbet et al, which showed a significant reduction in the expression of PTEN in the lungs of mice with PH induced by exposure to hypoxia. The study further showed that treatment of mice with rosiglitazone, an activator of PPARγ, could largely restore PTEN levels and attenuate PH suggesting the involvement of PTEN in the progression of vascular remodeling. As a corroborative evidence for the role of PTEN, immunohistochemical staining and protein data from our experiments further showed that Akt, a downstream proliferative signaling protein of the PTEN antagonist PI3K, was markedly activated in the lungs of LHF-PH rats. This relation between PTEN and Akt is similar to that observed in our previous studies on the monocrotaline and hypoxia models of PH.

Oxidative stress, characterized by increased production of ROS, has been implicated as a primary contributor to the pathogenesis of PH. Production of ROS has been reported in the lung biopsy samples taken from patients with severe PH and in several rodent models of PH induced by monocrotaline or hypoxia. The causal link between oxidative stress and progression of PH has been established by many studies, which have shown that antioxidant treatment prevented the induction of PH. The current study clearly demonstrated the generation of oxidants in the lungs of heart failure rats. The involvement of ROS is also evident from the fact that LHF rats fed with HO-3867, a scavenger of superoxide and peroxynitrite, showed marked decrease in the oxidant levels in the lung. Nisbet et al, using a mouse model of chronic hypoxia, showed the generation of superoxide radicals via nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase pathway. They further showed that activation of PPARγ by rosiglitazone blunted superoxide radical generation by inhibiting the chronic hypoxia-stimulated Nox4 expression in pulmonary vascular endothelial cells. Taken together, our results and that of others indicate that antioxidant therapy, targeting either the inhibition or scavenging of ROS in the lung vasculature, could be beneficial for the treatment of PH.

Our in vitro results indicate that low doses of peroxynitrite induces proliferation of human PASMCs. Aghani et al have demonstrated that bovine PASMCs stimulated intracellular peroxynitrite formation on exposure to acute hypoxia. They have further shown that peroxynitrite at concentrations <2 μmol/L was capable of stimulating cell proliferation in pulmonary artery endothelial and SMCs. Our Western blot data indicate that peroxynitrite, at low concentrations, downregulates the expression and activity of PTEN and that PTEN negatively regulates cell proliferation. These studies provide a direct link between peroxynitrite and SMC proliferation involving the activation of PTEN. Although our results and
HO-3867 is capable of scavenging superoxide radicals or overexpressing superoxide-scavenging enzymes such as extracellular superoxide dismutase.28,29 A recent study by Chen et al,37 using a mouse model of PH induced by transverse aortic constriction, showed that lung fibrosis, leukocyte infiltration, and vascular remodeling, but not lung edema, were mainly responsible for heart failure-induced PH. This report, as well as those of other groups using different models of heart failure,38,39 highlighted the importance of targeting pathways involved in lung remodeling for the treatment of PH secondary to heart failure. In our study, we did not observe any significant increase in the ratio of lung wet-weight to dry-weight (data not shown) suggesting that lung edema is not a causative factor in the pathogenesis of PH. The substantial increase in vascular wall-thickening and remodeling observed in the LHF group and their attenuation by HO-3867 in our study seem to suggest that HO-3867 treatment targets the mechanisms related to vascular remodeling. Based on the existing knowledge and results of this study, we proposed a mechanistic pathway involved in the progression of PH secondary to LHF (Figure 7).

To our knowledge, this is the first report of the involvement of oxidative stress in the lung in the development and progression of PH secondary to heart failure. We have identified peroxynitrite as a potential inducer of SMC proliferation by downregulation of PTEN and hence may play a causative role in vascular remodeling associated with the progression of PH. This unique pathway is shown to be a potential target for HO-3867, which seems to play multiple roles in attenuating oxidative stress and inhibiting vascular remodeling in LHF-PH. Although the exact mechanism of the effect of HO-3867 on PH is yet to be elucidated, our data indicate the importance of blunting ROS generation in the lung to inhibit this progressive disorder. Nevertheless, the current study has identified a potential therapeutic approach targeting multiple mechanisms in a preclinical model of PH associated with LHF.

**Perspectives**

Despite numerous medical and surgical therapeutic options for the management of heart failure, treatments for PH that occurs secondary to heart failure are limited. The primary PH, such as pulmonary arterial hypertension, has been the focus of a large number of investigations leading to drug discovery and clinical trials, the secondary PH attributable to heart failure has received little attention. At present, there is limited rationale or hope that the same therapeutic options will be effective for treating heart failure patients with PH. Our study clearly implicates the involvement of oxidative stress in the progression of PH secondary to LHF. We show the generation of peroxynitrite, a potent oxidant, in the lungs of heart failure rats. We further demonstrate downregulation of PTEN in the pulmonary

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**Figure 7.** Illustration of the mechanisms involved in the pathogenesis and progression of pulmonary hypertension (PH) secondary to left-heart failure. Chronic elevation of pulmonary artery pressure, caused by after-load pressure from failing left-ventricle, results in oxidative stress and production of reactive oxidants in the lung microvasculature. The oxidants then trigger a cascade of molecular events involving phosphatase-and-oxidases or overexpressing superoxide-scavenging enzymes such as extracellular superoxide dismutase.28,29

That of Delgado-Esteban et al15 indicate a correlation between peroxynitrite and PTEN activity, the mechanism by which PTEN protein or its activity is degraded by peroxynitrite is yet to be determined. However, it is reasonable to assume that a mechanism similar to that reported12 for hydrogen peroxide-induced reversible inactivation of PTEN could be responsible and operative in the peroxynitrite-PTEN pathway.

HO-3867 is a synthetic analog of curcumin, the principal component of the popular Indian spice turmeric (the rhizome of *Curcuma Longa*). HO-3867 was designed, synthesized, and characterized in our laboratories.16 It is a dual-acting molecule with targeted antiproliferative and antioxidant capabilities.14 Its antiproliferative activity has been evaluated using a number of cancer cell lines,14 murine xenograft tumors,17,33,34 serum-stimulated SMCs,15 and balloon injury-mediated carotid artery restenosis in rats.15 The compound, usually administered as a dietary supplement, is readily bio-absorbed and retained in tissues for 24 hours.15 HO-3867 is capable of scavenging superoxide radicals (SOD-mimetic), peroxyl radicals,14 and peroxynitrite (this work). Under normoxic conditions, such as those occurring in normal (healthy) tissues, HO-3867 functions as an antioxidant and protects cells from ROS-mediated oxidative damage. Most importantly, data from our laboratory have indicated that HO-3867 is nontoxic to healthy tissues, including heart.18 In the current study, we observed that HO-3867 treatment did not have any grossly discernible effect on heart/LV weight or function. This observation is in contrast to the study of Morimoto et al16 that showed curcumin to have a significant effect on improving cardiac function in rats 7 weeks after myocardial infarction (MI). The observed difference on their cardioprotective effect could be a result of the structural modification or differences in dosing and post-MI duration. However, HO-3867 significantly attenuated the elevation of pulmonary arterial pressure, blunted oxidant levels in lung, and restored key signaling proteins involved in the control of vascular remodeling. The causative role of superoxide in inducing hypoxia-mediated PH has been previously determined by administering antioxidants or overexpressing superoxide-scavenging enzymes such as extracellular superoxide dismutase.28,29
arteries resulting in vascular remodeling and vasoconstriction. The findings will enable us in developing targeted therapies for the management of PH. We have also identified a unique multifunctional compound, HO-3867, which attenuates the progression of PH by targeting the antioxidant and antiproliferative pathways. Our future work will explore the specific mechanism of action of HO-3867 in preventing the development of PH and also study the effect of HO-3867 in improving the survival of rats with preexisting PH in comparison with drugs currently used in the management of PH.

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Disclosures
None.

References


**Novelty and Significance**

**What Is New?**

- The study establishes, for the first time, that oxidative stress, particularly peroxynitrite, mediates vascular remodeling by downregulation of PTEN in pulmonary hypertension associated with left-heart failure.
- We further report that, a novel compound, HO-3867, with multifunctional activity including antioxidant and antiproliferative properties attenuates the progression of PH secondary to left-heart failure.

**What Is Relevant?**

- PH is a debilitating disease and involves the interactions of numerous molecular pathways in the development and progression of the disease.
- PH is associated with significant mortality and morbidity as the elevated pulmonary arterial pressure further complicates the management of congestive heart failure.
- There is no known therapy to treat PH.

**Summary**

We conclude that peroxynitrite-mediated downregulation of PTEN induces lung microvascular remodeling in PH secondary to LHF and HO-3867 limits the progression of PH.
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**Supplemental Material**

**Pulmonary hypertension secondary to left-heart failure involves peroxynitrite-induced downregulation of PTEN in the lung**

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**Materials and Methods**

**Reagents:** Dimethyl sulfoxide (DMSO) and antibody directed against actin were obtained from Sigma-Aldrich (St. Louis, MO). Polyvinylidene-fluoride membrane and molecular-weight markers were obtained from Bio-Rad (Hercules, CA). Antibodies directed against Akt (pan), pAkt (Ser473), PTEN, and pPTEN (Ser380 and Thr381/382), were purchased from Cell Signaling Technology (Danvers, MA). Enhanced chemiluminescence reagents were obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). 3-Morpholinosydnonimine hydrochloride (SIN-1) (catalog# M5793) was purchased from Sigma Aldrich (St. Louis, MO) and peroxynitrite (catalog# 81565) was purchased from Cayman Chemicals (Ann Arbor, MI). HO-3867 was synthesized as reported.\(^1\) Stock solutions (20 mM) of the compound were freshly prepared in dimethyl sulfoxide.

**Induction of LHF-PH by ligation of left-anterior-descending coronary artery:** Left-heart failure was induced in rats by permanent ligation of left-anterior-descending coronary artery (LAD). All the procedures were performed with the approval of the Institutional Animal Care and Use Committee of The Ohio State University and conformed to the Guide for Care and Use of Laboratory Animals (NIH publication no. 86-23). Male Sprague-Dawley rats (225-250 g) were intubated orally, placed on a volume-cycled ventilator (Rodent Ventilator, model 683; Harvard Apparatus Holliston, MA), and maintained under general anesthesia with 1 to 2% isoflurane in air. An oblique 12-mm incision was made 8 mm away from the left sternal border toward the left armpit. The chest cavity was opened with scissors by a small incision (10-mm long) at the level of the third or fourth intercostal space, 2 to 3 mm from the left sternal border. The LAD was visualized as a pulsating bright-red spike running through the midst of the heart wall from underneath the left atrium toward apex. The LAD was ligated 1 to 2 mm below the tip of the left atrium using a tapered needle and a 6-0 polypropylene ligature passed underneath the LAD. A double knot was made to occlude the LAD permanently. Occlusion was confirmed by a sudden change in color (pale) of the anterior wall of the left ventricle (LV). EKG changes were recorded, and ST (ventricular depolarization) elevation was observed after LAD ligation. The chest cavity was closed by bringing together the third and fourth ribs with one 4-0 polypropylene silk suture. The layers of muscle and skin were closed with a 4-0 polypropylene suture, and the rats were allowed to recover under a warm light. An age-matched control group underwent sham surgeries and was used for comparison. The study used 8-10 animals per group. No mortality was observed in the 4-week period following LAD occlusion.
**Treatment with HO-3867:** HO-3867 was administered in the diet (100 ppm), beginning day 1 after LAD ligation, and continued for the entire treatment period of 4 weeks. The 100-ppm dose of HO-3867 was based upon our earlier studies that used oral administration of this compound in mice.²,³

**Processing of lung tissues for histopathology and protein analysis:** Before explantation, lungs were flushed with cold saline to remove intravascular blood. The explanted lungs were then, either flash-frozen for mRNA and protein analysis or inflated with 4% paraformaldehyde at 20-cm water pressure followed by immersion-fixation using 4% paraformaldehyde for 48 hours at room temperature for histology.

**Magnetic resonance imaging (MRI):** Rats were anesthetized using isoflurane (1.5%)-carbogen gas mixture and placed on a water-heated imaging bed in the head first, supine position. Two metal EKG (electrocardiogram) pads were then placed on the animal’s front and rear paws and a respiratory pad was placed across the animal’s abdomen. During imaging, respiratory and heart rates were monitored and recorded using a PCSAM (Small Animal Instruments) monitoring program. A Bruker BioSpec 94/30USR 9.4T horizontal bore MRI system (Bruker, BioSpin, Germany), equipped with Paravision 4.0 and was used to acquire images. Multiple cardiac-gated, T1-weighted FLASH-cine images were acquired in the transverse orientation to cover the entire left and right ventricle of each animal, from apex to aortic valve, using the following settings: TR/TE = 16/1.6 ms, slice thickness = 2 mm, α = 10°, FOV = 51.0 mm, 256 x 192 (in-plane pixel size = 0.20 mm x 0.27 mm). All transverse cine images were analyzed using the image analysis program ImageJ (NIH). The end-diastolic (ED) and end-systolic (ES) time points for each acquired cine slice were identified visually. The endocardial and epicardial boundaries of the left and right ventricles were then manually traced for both end-systolic (ES) and end-diastolic (ED) time points of the cardiac cycle and used to calculate LV and RV end-diastolic volume (EDV), end-systolic volume (ESV), LV and LV ES wall thickness (LV ES-WT) and LV ED wall thickness (LV ED-WT). Using Segment (Medviso AB), the ES and ED cardiac timepoints were identified for the mid-ventricular slice of each heart. The epicardial and endocardial boundaries of the LV and interventricular septal (IVS) walls were then manually traced. The LV wall was then divided into eight 45° sectors using the anterior right-ventricular insertion point as the reference, with the first sector further from the RV. Septal wall sectors were then used to calculate fractional IVS wall thickness, while all eight sectors were used to calculate fractional LV wall thickness at both ED and ES. Papillary muscles were excluded from the endocardial boundary of the LV.

**Ultrasound imaging (ECHO):** M-mode ultrasound echocardiography was performed using Vevo 2100 ultrasound system (VisualSonics, Ontario, Canada). Rats, under the influence of 1.5% isoflurane inhalation-anesthesia, were placed in the supine position and EKG limb electrodes were attached to collect physiological data (heart rate, respiratory rate). The chest was carefully shaved and ultrasound gel was applied to the thorax to optimize visibility during the exam. The ultrasonic transducer was applied to the thorax and used to obtain two dimensional, M-mode images from a parasternal short axis view. The images were analyzed to obtain ejection fraction (EF), fractional shortening (FS), LV-ESV and LV-EDV.
Hemodynamic measurements: Rats, anaesthetized with sodium pentobarbital (50 mg/kg, IP), were intubated orally, placed on a volume-cycled ventilator (Rodent Ventilator, model 683; Harvard Apparatus Holliston, MA), and maintained under general anesthesia with 1.5% isoflurane throughout the measurements. A Millar catheter (SPR-1000) (Millar Instruments, Houston, TX) was advanced directly into the LV, RV, or pulmonary artery through RV for pressure measurements using a PowerLab data acquisition system (model ML866; Colorado Springs, CO).

Histopathology and immunohistochemistry: Paraffin-embedded tissues were cut into 5-µm thick sections and stained with hematoxylin and eosin (H&E) using standard methods. Immunohistochemical (IHC) staining using PTEN antibody (catalog# 9559, 1:100 dilution; Cell Signaling Technology), Akt antibody (catalog# 9557, 1:100 dilution Cell Signaling Technology) and αSMA (catalog# sc-53015 Santa Cruz Bio-technology) were performed on the tissue sections with 3,3'-diaminobenzidine peroxidase substrate (SK-4100, Vector Labs). The samples were then counterstained with hematoxylin following the manufacturer’s protocol. Representative photomicrographs were taken using an inverted fluorescence microscope (Nikon TE 2000, Japan). To assess the density of muscular arteries, images of lung sections with elastin stain were taken in 10 consecutive fields. The arteries and alveoli in the images were counted, and the number of arteries per 100 alveoli was calculated.

Superoxide and peroxynitrite staining in the lung: Superoxide generation in the lung was assessed using frozen sections of rat lung. Dihydroethidium (DHE) staining was used to evaluate the superoxide production in the lung. Lungs were perfused blood-free, and embedded in optimal cutting temperature (OCT) compound and frozen at -80°C. Sections (5 µm) were then prepared and stained with DHE (10 µM) by covering the section with 30 µl of DHE and a coverslip followed by incubation at 37°C in a humidified, 5% CO₂ atmosphere for 30 minutes. Sections from each treatment group were examined by fluorescence microscopy, and images were acquired at 20x magnification using identical instrument settings. To examine nitrotyrosine protein levels in lung tissue, lungs were pressure perfused, then embedded in OCT and frozen at -80°C. Frozen lung sections (5 µm) were then prepared and stained with primary antibody to nitrotyrosine (1:50) followed by secondary rhodamine red-labeled goat anti-mouse IgG FITC antibody. Sections from each treatment group were examined by fluorescence microscopy, and images were acquired using identical instrument settings. Quantitation of image intensity was performed using ImageJ software.

In vitro studies: Human pulmonary artery smooth muscle cells (PASMC) were obtained from Lonza (Walkersville, CA). The cells were briefly thawed according to the vendor’s protocol and maintained in a humidified incubator at 37°C/5% CO₂. The SmGM-2 “bullet kit” (Lonza CC-3182) containing one 500 ml SMC basal medium, with growth factor supplements (hEGF 0.5 ml; insulin 0.5 ml; hFGF-B 1ml; FBS 10 ml; GA-1000 0.5 ml; penicillin 10 U/ml; streptomycin 1 mg/ml) was used. Cells were trypsinized and passed at 95% confluence. Studies were performed on cells at passage 4 through 6 and at 60% to 90% confluence. The cells were exposed to 0.5 µM, 1 µM, 2 µM and 10 µM peroxynitrite for 24, 48 and 72 h and cells of the same passage were cultured for the same time periods and used as controls. Cell counting assays and protein analysis were performed using these cells. PASMCs were cultured with or
without fetal bovine serum (FBS) and were exposed to 1-μM peroxynitrite and after 24 h were treated with 10-μM HO-3867. Cell counting was performed at 24 or 48 h using an automated cell counter (NucleoCounter, New Brunswick Scientific, NJ).

Transfection of PTEN siRNA: PASMCs were transfected with PTEN siRNA (Invitrogen) and negative control siRNA using FuGene (Roche) according to the manufacturer’s instructions. Gene-silencing was used to inhibit the expression of PTEN gene in PASMCs. Using 6-well plates, cells (1×10^5/well) were seeded in 2 ml of antibiotic-free medium supplemented with 10% FBS, 24 hours before the transfection. The cells were incubated at 37°C until cells reached about 60–70% confluence which was usually attained after overnight incubation. Transfection was done using FuGene. A mixture of FuGene, PTEN siRNA (1 or 2 μg) and serum-free medium were prepared in 3:1, 3:2 and 6:1 dilutions. The FuGene and siRNA mixed together with serum-free medium and was allowed to incubate at room temperature for 20 min and then the entire volume was added to each well along with additional medium prepared with FBS. The FuGene and RNA mixture was added and gently rocked back and forth to ensure adequate mixing. The control well was not treated with the RNA, but only with FuGene and culture medium. Six hours after transfection, the transfection agents were removed and replaced with culture medium containing FBS. The culture plates were returned to the incubator and assayed for target gene activity 24 and 48h after transfection. Cell count was performed on PASMCs transfected with PTEN siRNA using NucleoCounter.

PTEN overexpression using cDNA: PTEN-overexpression experiments were performed using wild-type PTEN cDNA. The FLAG-tagged gene was transfected into PASMC cells using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cell count was performed at 12, 24, and 48 h after the transfection of the PTEN gene using NucleoCounter.

Laser-capture microdissection (LCM) and RNA isolation: Cryopreserved lung specimens were cut into serial 6-8 μm sections and mounted on pre-chilled PEN membrane slides and stored at -80°C. Stored tissue sections were thawed in ice-cold 70% ethanol for 2-3 min prior to staining. A truncated hematoxylin staining procedure was followed: hematoxylin was quick stained for 30 sec, followed by wash in DEPC-treated water for 30 sec twice, dehydrated in 70% and 95% ethanol for 30 sec each, followed by 100% ethanol for 45 sec twice. Slides were air-dried under laminar flow for 10-30 min and immediately processed for LCM. LCM was performed with a PALM MicroBeam IV instrument from Carl Zeiss. Vascular smooth muscle cells and endothelial cells in pulmonary arterioles were identified and captured using a 7.5-μm laser beam at 50-100 mV. Approximately 10000 cells were procured from each lung specimen. Samples were immediately transferred to lysis buffer and stored at -80°C until RNA extraction. Total RNA was extracted from LCM-captured cells with Picopure RNA isolation kit (Arcturus, catalog # 12204-01). One μl of samples was used for RNA quantitative and qualitative analysis and the rest was stored at -80°C prior to running RT-PCR.

Western blotting: Western blotting was used to determine the expression of proteins in the lung associated with PH. Whole lung tissues were homogenized with nondenaturing lysis buffer (10 mM Tris-HCl pH 7.4; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA; 1 mM EGTA; 0.3 mM phenylmethylsulfonyl fluoride; 0.2 mM sodium orthovanadate; 0.5% NP40; 1 μg/ml aprotinin and 1 μg/ml leupetin). The lysates were centrifuged at 12,500 rpm for 20 min at 4°C, and the supernatant was separated from the solid material. The protein concentration in the lysates
was determined using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Approximately 25 to 50 µg of protein lysate per sample was denatured in 2x sample buffer and subjected to SDS-PAGE on a 4%-12% bis-tris gel. The separated proteins were transferred to a PVDF membrane and the membrane was blocked with 5% nonfat milk powder (w/v) in TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. The membranes were incubated with primary antibodies directed against or known to cross-react with samples of rat origin. Actin was used as the loading control and detected by the corresponding primary antibody. The bound antibodies were detected using appropriate horseradish peroxidase (HRP)-labeled secondary antibodies using an enhanced chemiluminescence detection system. Protein expression was quantified using UN-SCAN-IT gel v6.1 (Silk Scientific, Orem, UT).

**Real-time PCR**: Quantitative real-time PCR was used to measure the RNA transcription level of various genes in LCM cells from lung samples. TaqMan probes were selected to bind specifically to rat cDNA. RNA extracted from LCM-captured cells was reverse-transcribed and amplified using Arcturus™ RiboAmp® PLUS (Applied biosystems, cat# KIT0521). Commercially available Taqman gene expression assays were used to for rat PTEN (Applied Biosystems, Assay Id: Rn00477208-m1). QRT-PCR was performed on Roche Light Cycler 480 system using 2x Taqman Gene expression master mix (Applied biosystems cat# 4370048) with the starting cDNA template of 4 µl (10-15 ng). All real-time reactions had the following profile conditions: 10 min hot start at 95°C followed by 40 cycles (95°C for 15 sec and 60°C for 60 sec). The results were normalized to expression of endogenous 18s Rrna (App. Biosystems, Assay Id: Rn03928990_g1).

**Results**

**LAD coronary artery ligation induces LHF and PH**

After permanent occlusion of LAD coronary artery in rats for 4 weeks, alterations in cardiac function were quantified using MRI and echocardiography. MRI data showed an increase in LV end-systolic and end-diastolic volumes and wall thinning, indicating dilatation of the LV and the onset of end-stage cardiomyopathy and heart failure (S. Figure 1A & 1B). Transthoracic M-mode echocardiography showed a significant decrease in LV ejection fraction and fractional shortening indicative of heart failure post-LAD ligation (S. Figure 1C & 1D). Hemodynamic measurements showed a significant decrease in the left-ventricular systolic pressure (LVSP) indicative of left-heart failure (S. Figure 1E). Significant increases in right ventricular systolic pressure (RVSP) and mean pulmonary arterial pressure (mPAP) confirmed the development of PH in the ligated group. Thus the MRI, echo, and hemodynamic measurements clearly established the development of pulmonary hypertension secondary to left-heart failure.
References


Figure S1. Left-heart failure leads to development of pulmonary hypertension. Left-heart failure (LHF) was induced in rats by permanent ligation of the left-anterior-descending coronary artery. Magnetic resonance imaging (MRI), ultrasound imaging (echo), and hemodynamic (pressure) measurements were performed 4 weeks after induction of LHF. (A) Representative short-axis MR images of the heart at end-systole and end-diastole show substantial remodeling of the left ventricle. Arrows indicate the site of infarction. (B) Quantitative results of the MRI data (mean±SD; n=4) show significant increases in LV end-systolic (LV-ESV) and LV end-diastolic (LV-EDV) volumes and LV mass in the LHF group. The data also show decreases in LV end-systolic (LV ES-WT) and end-diastolic (LV ED-WT) wall thickness. (C) Representative transthoracic M-mode ultrasound images of the hearts show decreased contractility of the LV in LHF. (D) Quantitative results of the echo data (mean±SD; n=4) show significantly decreased ejection fraction (EF) and fractional shortening (FS) in the LHF hearts. (E) Hemodynamic data (mean±SD; n=6) indicate a significant decrease in LV systolic pressure (LVSP) and significant elevation of right ventricular systolic pressure (RVSP) and mean pulmonary artery pressure (mPAP) in the LHF group when compared to the control group. *P<0.05. The imaging and hemodynamic data confirm the development of PH secondary to LHF.
Figure S2. Effect of HO-3867 on lung and heart weight. Left-heart failure (LHF) was induced in rats by permanent ligation of LAD coronary artery for 4 weeks. HO-3867 was administered in the feed throughout this period. Wet weights of whole lung, whole heart, and LV (+ septum) were measured. The data show an increase of lung, heart, and LV mass upon LAD coronary artery occlusion for 4 weeks. HO-3867 treatment significantly blunted the lung weight increase, but had no effect on heart or LV weight.