Exacerbated Pulmonary Arterial Hypertension and Right Ventricular Hypertrophy in Animals With Loss of Function of Extracellular Superoxide Dismutase

Dachun Xu, Haipeng Guo, Xin Xu, Zhongbing Lu, John Fassett, Xinli Hu, Yawei Xu, Qizhu Tang, Dayi Hu, Arif Somani, Aron M. Geurts, Eric Ostertag, Robert J. Bache, E. Kenneth Weir, Yingjie Chen

Abstract—Studies have demonstrated that increased oxidative stress contributes to the pathogenesis and the development of pulmonary artery hypertension (PAH). Extracellular superoxide dismutase (SOD3) is essential for removing extracellular superoxide anions, and it is highly expressed in lung tissue. However, it is not clear whether endogenous SOD3 can influence the development of PAH. Here we examined the effect of SOD3 knockout on hypoxia-induced PAH in mice and a loss-of-function SOD3 gene mutation (SOD3E124D) on monocrotaline (40 mg/kg)-induced PAH in rats. SOD3 knockout significantly exacerbated 2 weeks of hypoxia-induced right ventricular (RV) pressure and RV hypertrophy, whereas RV pressure in SOD3 knockout mice under normoxic conditions is similar to wild-type controls. In untreated control rats at age of 8 weeks, there was no significant difference between wild-type and SOD3E124D rats in RV pressure and the ratio of RV weight:left ventricular weight (0.25±0.02 in wild-type rats versus 0.25±0.01 in SOD3E124D rats). However, monocrotaline caused significantly greater increases of RV pressure in SOD3E124D rats (48.6±1.8 mm Hg in wild-type versus 57.5±3.1 mm Hg in SOD3E124D rats), of the ratio of RV weight:left ventricular weight (0.41±0.01 versus 0.50±0.09; P<0.05), and of the percentage of fully muscularized small arterioles in SOD3E124D rats (55.2±2.3% versus 69.9±2.6%; P<0.05). Together, these findings indicate that the endogenous SOD3 has no role in the development of PAH under control conditions but plays an important role in protecting the lung from the development of PAH under stress conditions. (Hypertension. 2011;58:303-309.) • Online Data Supplement

Key Words: pulmonary artery hypertension • right ventricular hypertrophy • oxidative stress • extracellular SOD

Pulmonary artery hypertension (PAH) is a progressive disease with a very poor prognosis. PAH is characterized by a progressive elevation of pulmonary arterial pressure, ultimately inducing right ventricular (RV) hypertrophy and heart failure. Studies have demonstrated that increased oxidative stress, such as enhanced production of superoxide anions and other reactive oxygen species, may contribute to the pathogenesis and the development of idiopathic PAH in patients and to PAH secondary to high pulmonary vascular flow in lambs. In addition, administration of antioxidants attenuates the development of PAH, suggesting that pulmonary oxidative stress regulates the development of PAH. Superoxide dismutase (SOD) is a first line of defense against free radical attack. Three SOD isozymes have been identified, including a copper/zinc-containing SOD (SOD1), which is primarily cytosolic in location; a mitochondrial manganese SOD (SOD2); and an extracellular SOD (SOD3). SOD3 is a glycoprotein secreted into the extracellular fluid by fibroblasts that binds to sulfated polysaccharides, such as heparin and heparan sulfate, as well as to other matrix components. As a result, SOD3 binds to the surface of endothelial cells and the extracellular matrix, which has a high abundance of heparan sulfate. The lung is one of the organs with a relatively high SOD3 expression. Previous studies have demonstrated that overexpression of SOD3 attenuates hypoxia-induced PAH in mice and monocrotaline (MCT)-induced PAH in rats. Overexpression of SOD3 also attenuates bleomycin-induced lung injury. However, because SOD3 has a minimal impact on total tissue SOD activity, it is uncertain whether endogenous SOD3 can

Received November 4, 2010; first decision November 25, 2010; revision accepted June 6, 2011.
From the Lillehei Heart Institute and Cardiovascular Division (D.X., H.G., X.X., Z.L., J.F., X.H., R.J.B., Y.C.), University of Minnesota Medical School, Minneapolis, MN; Department of Cardiology (D.X., Y.X.), Shanghai Tenth People’s Hospital of Tongji University, Shanghai, China; Department of Cardiology (H.G., Q.T.), Renmin Hospital of Wuhan University, Wuhan, China; Pediatric Critical Care Medicine (A.S.), University of Minnesota, Minneapolis, MN; Human and Molecular Genetics Center (A.M.G.), Department of Physiology, Medical College of Wisconsin, Milwaukee, WI; Peking University People’s Hospital (D.H.), Beijing, China; Transposagen Biopharmaceuticals (E.O.), Lexington, KY; Department of Microbiology, Immunology, and Molecular Genetics (E.O.), University of Kentucky and Department of Pathology and Laboratory Medicine, University of Kentucky Chandler Hospital, Lexington, KY; Department of Medicine (E.K.W.), University of Minnesota and Veterans’ Affairs Medical Center, Minneapolis, MN. D.X. and H.G. contributed equally to this work. Correspondence to Yingjie Chen, Lillehei Heart Institute and Cardiovascular Division, University of Minnesota, 420 Delaware St SE, MMC 508, Minneapolis, MN 55455. E-mail chenx106@umn.edu

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.110.166819
influence the development of PAH. To address this question, we examined the effect of SOD3 knockout on hypoxia-induced PAH in mice and the effect of SOD3 gene mutation (SOD3<sub>E124D</sub>) on MCT-induced PAH in rats. Here we report that both SOD3<sub>E124D</sub> in rats or SOD3 knockout in mice had no effect on PAH and RV hypertrophy under control conditions but resulted in significantly greater increases of RV pressure and pulmonary vascular remodeling, as well as greater RV hypertrophy in response to MCT or chronic hypoxia. The findings indicate that the endogenous SOD3 plays an important role in protecting the lung from the development of PAH and RV hypertrophy under stress conditions.

Materials and Methods

SOD3 Knockout Mice

SOD3 knockout mice and control wild-type mice used in the present study are described previously. This study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

SOD3<sub>E124D</sub> Rats

SOD3<sub>E124D</sub> (SS-So<sub>d3</sub> <sup>nm1Mcwi</sup>) rats were identified as a mutation in an ethyl nitrosourea mutagenesis screen by the PhysGen Program in Genomic Applications (http://pga.mcw.edu), backcrossed to the Dahl/salt-sensitive strain (SS/JrHsdMcwi), intercrossed and maintained as a homozygous colony. N2F7-F8 generation animals were used as controls.

Hypoxia-Induced PAH in Mice

Male SOD3 knockout mice and wild-type control mice at ages 10 to 14 weeks were exposed to hypobaric hypoxia, as described by Hampl et al. Briefly, the chamber in the pressure was decreased from 0.8 atmosphere (16.9% O<sub>2</sub>) on day 1 to 0.5 atmosphere (10.5% O<sub>2</sub>) after day 7 and was maintained at 10.5% O<sub>2</sub> for 2 more weeks. The chamber was opened once every week for cleaning and feeding. After exposure to 10.5% O<sub>2</sub> for 2 weeks, mice were removed from the hypoxia chamber for determination of RV pressure and hypertrophy. The sham mice were kept in normobaric conditions.

Induction of PAH in Rats With MCT

Male SOD3<sub>E124D</sub> rats and wild-type control rats (Dahl/salt-sensitive) at age 5 weeks were given IP injections of MCT (40 mg/kg, Sigma, St. Louis, MO) or an equivalent volume of vehicle as a control. The rats were intubated with a 20-gauge Teflon tube attached to MiniVent type 845 mouse ventilator (Hugo Sachs Elektronik).

A 1.2-F pressure catheter (Sciensc Inc, London, Ontario, Canada) was introduced through the right common carotid artery into the ascending aorta for measurement of systolic and diastolic blood pressures, as described previously. For RV hemodynamics, open-chest RV catheterization was performed during anesthesia with 1.5% isoflurane. Data were collected when steady state was reached.

Sample Preparation

After the final hemodynamic assessment, the rats and the mice were euthanized by exsanguination, and the heart, lung, and other major organs were harvested. Lung weight was determined, and the left lung was harvested and snap frozen in liquid nitrogen for biochemical analysis. The airways of the top right lobe were subsequently perfused with and then fixed in 10% buffered formalin for histological analysis. The wet weight of RV and of left ventricle (LV)+septum were weighed, and the ratios of RV weight: LV+septum were calculated as an index of RV hypertrophy.

Histological Staining, Semiquantification of Fibrosis, and Western Blots

For staining of smooth muscle α-actin, tissue sections (5 μm) were deparaffinized, rehydrated, antigen recovered in Tris-EDTA buffer (pH=9.0) for 30 minutes at 95°C to 100°C, and washed in PBS. The sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> in PBS for 20 minutes followed by 1% BSA solution for 1 hour. Sections were then incubated with a monoclonal primary antibody (1:400) against smooth muscle α-actin (Sigma-Aldrich) overnight at 4°C and followed with Alexa Fluor 555 labeled secondary antibody against mouse Ig-G (1:1000) (Invitrogen). The slides were examined using a confocal microscope (Zeiss LSM510). Measurements of ventricular fibrosis and cardiac myocyte size were performed using the method described previously. For methods of semiquantification of pulmonary vascular muscularization, fibrosis, and Western blots, please see the online Data Supplement at http://hyper.ahajournals.org.

Chemical Analysis

Oxidative stress marker thiobarbituric acid reactive substances content was determined as described previously. Total SOD activity and SOD2 activity of lung tissues were measured using a SOD activity kit (Cayman Chemical) according to the manufacturer’s instructions. For relative lung SOD3 activity assay, a total of 2 μg of primary antibody for SOD3 (Lifespan Biosciences) was added into 500 μL of tissue extract (2 mg/mL) and then incubated for 1 to 2 hours at 4°C. Protein A/G-Agarose of 20 μL was then added to the mixture and incubated at 4°C on a rocker platform overnight. The immunoprecipitates were collected by centrifugation at 3000 rpm for 30 seconds at 4°C. After gently washing with PBS 4 times, the pellets were resuspended in 400 μL of buffer for SOD activity assay with the commercial kit according to the manufacturer’s instructions. Control IgG was used as a negative control.

Total Antioxidant Capacity

Total antioxidant capacity of lung tissues was determined using an antioxidant power assay kit (Oxford Biomedical Research) according to the manufacturer’s instructions.

Statistical Analysis

All of the values are expressed as mean±SE or median (±SE). Data of 2 groups were compared with unpaired t test. Two-way ANOVA was used to test for differences between transgenic and wild-type animals under control conditions and after MCT injection. If analysis of variance demonstrated a significant effect, post hoc pairwise comparisons were made using the Fisher least significant difference test. Statistical significance was defined as P<0.05.
Results

SOD3 Knockout Aggravated the Hypoxia-Induced Increase of RV Pressure and Hypertrophy

To study whether SOD3 dysfunction can affect PAH in other experimental models, we determined the effect of SOD3 knockout on hypoxia-induced PAH in mice. RV pressure and the ratio of RV:LV+septum weight were not different between SOD3 knockout mice and wild-type controls under control normoxic conditions. However, SOD3 knockout significantly exacerbated hypoxia-induced increases of RV pressure (Figure 1A) and RV hypertrophy as indicated by the ratio of RV:LV+S weight (Figure 1B). In addition, hypoxia caused increases of fully muscularized arterioles in both wild-type controls and SOD3 knockout mice, whereas these increases were significantly greater in the SOD3 knockout mice than in the wild-type controls (Figures 1C and S1).

SOD3E124D Mutation Had No Significant Effect on the Animals’ Growth But Exacerbated the MCT-Induced Increase of RV Pressure

SOD3E124D rats grew and developed normally. There were no significant differences in terms of body weight gain (Figure 2A) and left ventricular weight between SOD3E124D rats and wild-type controls at age of 2 months (Table S1). In addition, SOD3E124D mutation had no effect on growth under both control conditions and after MCT injection, respectively (A). SOD3E124D mutation had no effect on RV pressure under control conditions but further elevated the MCT-induced increase of RV pressure, indicating more severe pulmonary artery hypertension (PAH) in SOD3E124D rats (B and C). *P<0.05 vs sham control; #P<0.05 vs corresponding wild-type (WT) rats.

Figure 1. Extracellular superoxide dismutase (SOD3) knockout in mice significantly exacerbated hypoxia-induced increases of right ventricular (RV) pressure (A), of RV hypertrophy as indicated by the ratio of RV:left ventricle (LV)+septum (S) weight (B), and of pulmonary vascular remodeling as indicated by significant increases of fully muscularized small arterioles (C). *P<0.05 vs sham control; #P<0.05 vs corresponding wild-type (WT) mice.

Figure 2. SOD3E124D mutation in rats had no significant effect on growth but exacerbated monocrotaline (MCT)-induced increase of right ventricular (RV) pressure. SOD3E124D mutation had no effect on growth under both control conditions and after MCT injection, respectively (A). SOD3E124D mutation had no effect on RV pressure under control conditions but further elevated the MCT-induced increase of RV pressure, indicating more severe pulmonary artery hypertension (PAH) in SOD3E124D rats (B and C). *P<0.05 vs sham control; #P<0.05 vs corresponding wild-type (WT) rats.
SOD3E124D mutation caused significantly greater pulmonary vascular remodeling. Distribution of nonmuscular, partially muscular, and fully muscularized small arterioles in wild-type (WT) rats and monocrotaline (MCT)-induced pulmonary artery hypertension (PAH) rats (A and B). SOD3E124D significantly aggravated MCT-induced pulmonary vascular muscularization (B). SOD3E124D mutation significantly exacerbated MCT-induced relative medial wall thickness of larger arteries in rats (C). *P<0.05 vs sham control; #P<0.05 vs corresponding WT rats.

SOD3E124D Mutation Aggravated the MCT-Induced Increase of RV Hypertrophy and Fibrosis

In sham (no MCT) rats at the age of 2 months, there was no significant difference among WT and SOD3E124D rats in LV+septum weight (641 ± 19.3 mg in wild-type sham versus 640 ± 9.4 mg in SOD3E124D rats), RV weight (162 ± 6.7 mg in wild-type sham versus 157 ± 2.8 mg in SOD3E124D rats), their ratio to body weight or tibia length, and ratio of RV weight:LV+septum weight (0.25 ± 0.02 in wild-type untreated sham rats versus 0.25 ± 0.01 in SOD3E124D untreated sham rats; Table S1 and Figure 2C). Consistent with the significantly greater increase of RV systolic pressure in SOD3E124D rats after MCT, SOD3E124D rats had significantly greater increases of RV weight (239 ± 8.7 mg in wild-type rats versus 285 ± 10.6 mg in SOD3E124D rats; P<0.05) and the ratio of RV:LV+septum (0.41 ± 0.01 in wild-type rats versus 0.50 ± 0.09 in SOD3E124D rats; P<0.05) in response to MCT (Table S1 and Figure 2C), indicating that SOD3E124D mutation exacerbated MCT-induced RV hypertrophy. Histological analysis indicated that MCT caused a significantly greater increase of RV fibrosis (Figures S2 and S3A) and cardiac myocyte cross-sectional area (Figures S2 and Figure S3B), indicating severe RV remodeling in SOD3E124D rats after MCT.

SOD3E124D Mutation Exacerbated the MCT-Induced Pulmonary Vascular Remodeling

To determine the effect of SOD3E124D on pulmonary vascular remodeling, we determined the percentage of nonmuscularized, partially muscularized, and fully muscularized small arterioles in wild-type rats and SOD3E124D rats under sham conditions and 3 weeks after MCT injection (Figure 3A and 3B). MCT caused increases of fully muscularized small arterioles both in wild-type rats and SOD3E124D rats (55.2 ± 2.3% in wild-type rats versus 69.9 ± 2.6% in SOD3E124D rats; P<0.05), but these increases were significantly greater in the SOD3E124D rats than in the wild-type rats (Figure 3). Meanwhile, MCT also caused decreases of nonmuscularized small arterioles both in wild-type rats and SOD3E124D rats (9.4 ± 1.2% in wild-type rats versus 5.2 ± 1.2% in SOD3E124D rats; P<0.05), but these decreases were significantly greater in the SOD3E124D rats than in the wild-type rats (Figure 3). In addition, SOD3E124D rats had significantly exacerbated MCT-induced medial wall thickness (Figure 3C) and medial area (Figure S5) of arteries of 50 to
Together, these data indicate that SOD3E124D significantly exacerbated MCT-induced pulmonary vascular remodeling in rats.

SOD3E124D Mutation Exacerbated the MCT-Induced Pulmonary Oxidative Stress

MCT also caused increases of pulmonary 3'-nitrotyrosine and thiobarbituric acid reactive substances both in wild-type rats and SOD3E124D rats, but these increases were significantly greater in the SOD3E124D rats than in the wild-type rats (Figures 4A, 4B, and S7), indicating a greater degree of pulmonary oxidative stress in SOD3E124D rats than in wild-type rats after MCT. Expressions of lung SOD1 and SOD2 were determined (Figure 4C and 4D). SOD3E124D had no detectable impact on lung overall antioxidant capacity, as indicated by the power of antioxidants (Figure 4E). Lung total SOD activity was significantly lower in SOD3E124D rats only under control conditions, and the difference was small (Figure 4F). Lung SOD2 activity was unchanged in SOD3E124D rats (Figure S8).

SOD mimetic Mn(III)TMPyP rescued SOD3E124D rats from MCT-induced PAH. To further determine the impact of lung oxidative stress on the development of PAH, we determined the effect of Mn(III)TMPyP treatment (6 mg/kg per day). Mn(III)TMPyP significantly reduced MCT-induced RV pressure (Figure 5A), the ratio of RV weight:LV+septum weight (Figure 5B), and lung vascular remodeling in SOD3E124D rats (Figure 5C and 5D).

### Discussion

SOD3 binds to the surface of endothelial cells and the extracellular matrix and plays a critical role in removing extracellular free radical species. SOD3 is highly expressed in...
the lung. However, because SOD3 has a minimal impact on total tissue SOD activity, it is uncertain whether endogenous SOD3 can influence the development of PAH. In the present study, we report that SOD3E124D had no effect on overall pulmonary oxidative stress, PAH, and RV hypertrophy under control conditions but resulted in more severe pulmonary hypertension, more remodeling of the pulmonary arteries, and more RV hypertrophy and fibrosis in the setting of MCT-induced pulmonary hypertension. In addition, SOD3 knockout aggravated hypoxia-induced PAH in mice. The findings indicate that endogenous SOD3 plays an important role in protecting the lung from the development of PAH under stress conditions.

The overexpression of extracellular SOD3 reduces both hypoxia- and MCT-induced PAH. Similarly, an increase in SOD3 activity decreases hypoxic pulmonary vasoconstriction in bovine pulmonary artery rings. Loss of SOD3 could result in increased levels of superoxide anions and downstream radicals such as peroxynitrite, decreased H$_2$O$_2$ in extracellular space, and reduced intercellular diffusion of endothelial NO to surrounding cell types. Decreased NO bioavailability enhances the development of PAH, whereas increased NO or increase of its downstream product cGMP by inhibition of PDE5 attenuates the development of PAH. Thus, it is possible that a contributing factor to the exacerbated PAH in SOD3E124D rats and SOD3 knockout mice is increased scavenging of NO by superoxide and a subsequent reduction in NO/cGMP bioavailability.

The role of endogenous SOD3 in pulmonary vascular physiology and pathophysiology has not been clear. In these studies we show that loss of SOD3 function does not affect the tone or structure of the pulmonary arteries under normoxic control conditions. The finding that loss of function mutation in SOD3E124D rats and SOD3 knockout in mice results in more severe pulmonary hypertension and more RV hypertrophy after MCT or hypoxia (but not under control conditions) is conceptually consistent with previous studies showing that loss of SOD3 function exacerbated infarction or pressure overload-induced left ventricular maladaptive remodeling. Thus, superoxide anions and possibly related downstream radicals play a detrimental role in the pathophysiology of PAH, as well as other pathological conditions, such as ventricular remodeling.

The overall roles of SOD and oxidative stress are of particular interest in the pulmonary vasculature, because the expression of SOD2 is found to be reduced in idiopathic PAH patients and in fawn-hooded rats that spontaneously develop PAH. The decrease in SOD2 precedes the development of PAH in the fawn-hooded rats. The use of an SOD mimetic prevents pulmonary hypertension and reduces RV hypertrophy in rats exposed to chronic hypoxia and in fawn-hooded rats. Similar to the effects of SOD3 depletion, a reduction in SOD2 should also increase superoxide anion levels and downstream radicals, such as peroxynitrite, and decrease levels of H$_2$O$_2$. SOD2 and SOD3 thus appear to play a major role in protecting against the development of PAH by decreasing superoxide anions and increasing H$_2$O$_2$ and NO bioavailability. These results suggest that SOD mimetics or treatments that increase endogenous SOD2 or SOD3 may have therapeutic value in PAH.

**Clinical Perspectives**

PAH is a progressive disease with a very poor prognosis. PAH is characterized by a progressive elevation of pulmonary arterial pressure, ultimately inducing RV hypertrophy and heart failure. Studies have demonstrated that increased oxidative stress may contribute to the pathogenesis and the development of idiopathic PAH. SOD3 plays an important role in attenuating superoxide anion in the extracellular space. However, the effect of the endogenous SOD3 on the development of PAH has not been clear. Here we report that both SOD3 knockout in mice or SOD3E124D mutation in rats resulted in significantly greater increases of RV pressure, RV hypertrophy, and pulmonary vascular remodeling in response to hypoxia (in mice) or MCT (in rats). The findings indicate that endogenous SOD3 plays an important role in protecting against the development of PAH and subsequent RV hypertrophy under stress conditions. These results suggest that SOD mimetics or treatments that increase endogenous SOD3 may have therapeutic value in PAH.

**Sources of Funding**

This study was supported by National Heart, Lung and Blood Institute grants R21HL102597 (to Y.C.), R21HL098719 (to Y.C.), R21HL098669 (to Y.C.), HL079168 (to R.J.B.), and HL65322 (to E.K.W.) from the National Institutes of Health; GRNT2260175 (to Y.C.) and a Scientist Development Award from American Heart Association (to X.H.); and Veterans’ Affairs research funding (to E.K.W.).

**Disclosures**

E.O. has served as the chief executive officer of Transposagen Biopharmaceuticals; A.M.G. has served as a consultant for Transposagen Biopharmaceuticals.

**References**


Exacerbated Pulmonary Arterial Hypertension and Right Ventricular Hypertrophy in Animals With Loss of Function of Extracellular Superoxide Dismutase
Dachun Xu, Haipeng Guo, Xin Xu, Zhongbing Lu, John Fassett, Xinli Hu, Yawei Xu, Qizhu Tang, Dayi Hu, Arif Somani, Aron M. Geurts, Éric Ostertag, Robert J. Bache, E. Kenneth Weir and Yingjie Chen

Hypertension. 2011;58:303-309; originally published online July 5, 2011; doi: 10.1161/HYPERTENSIONAHA.110.166819

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/58/2/303

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2011/07/05/HYPERTENSIONAHA.110.166819.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Exacerbated pulmonary arterial hypertension and right ventricular hypertrophy in animals with loss of function of extracellular superoxide dismutase

Dachun Xu 1,2*, Haipeng Guo 1,3*, Xin Xu 1, Zhongbing Lu 1, John Fassett 1, Xinli Hu 1, Yawei Xu 2, Qizhu Tang 3, Dayi Hu 6, Arif Somani 4, Aron Geurts 5, Eric Ostertag 7,8, Robert J. Bache 1, E. Kenneth Weir 9, Yingjie Chen 1

1 Lillehei Heart Institute and the Cardiovascular Division, University of Minnesota Medical School, Minneapolis, Minnesota, USA
2 Department of Cardiology, Shanghai Tenth People's Hospital, of Tongji University, Shanghai, China
3 Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, China
4 Pediatric Critical Care Medicine, University of Minnesota
5 Human and Molecular Genetics Center, Department of Physiology, Medical College of Wisconsin, Milwaukee, WI, USA
6 Peking University People's Hospital, Beijing, China
7 Transposagen Biopharmaceuticals, Lexington, KY
8 Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky & Department of Pathology & Laboratory Medicine, University of Kentucky Chandler Hospital
9 Department of Medicine, University of Minnesota and Veterans Affairs Medical Center, Minneapolis, Minnesota

* These authors contributed equally to this work.

Running title: endogenous SOD3 and pulmonary hypertension

Yingjie Chen, MD, PhD,
Lillehei Heart Institute & the Cardiovascular Division
University of Minnesota
Tel: 612-624-8970; Fax: 612-626-4411;
email: chenx106@umn.edu
Extended Materials and Methods

**Semiquantification of pulmonary vascular muscularization:** The relative pulmonary vascular muscularization was determined under H&E staining. Briefly, in each rat and mouse, 60 intra-acinar arteries (50-200 μm) were examined and categorized as nonmuscular (NM), partially muscular (PM), fully muscular (FM). The relative percentage of NM, PM and FM arteries was calculated. The outer and inner diameters of the muscular coat identified by the staining for H&E staining were measured and the percentage thickness was calculated as 100x (outer diameter - inner diameter)/outer diameter. When blood vessels had an ovoid shape, percentage thickness was determined for both the long and the short axis and then averaged. In addition, relative pulmonary vascular cross-sectional area was determined: \((\text{area}_{\text{ext}} - \text{area}_{\text{int}}) \div \text{area}_{\text{ext}} \times 100\), where \(\text{area}_{\text{ext}}\) represents the external diameter and \(\text{area}_{\text{int}}\) represents the internal diameter of each vessel. In addition, immunostaining for smooth muscle \(\alpha\)-actin was used to reveal Smooth muscle in the SOD3*E124D* rats and wild type rats.

**Measurement of ventricular fibrosis and cardiac myocyte hypertrophy.** Tissue sections of RV were stained with Sirius Red (Sigma) for detection of fibrosis, and FITC-conjugated wheat germ agglutinin (AF488, Invitrogen) to assist in the evaluation of cardiac myocyte size. For cardiac myocyte size, the cross sectional area of at least 120 cells/sample (from 5 areas) and at least 4 samples of each group were averaged. The percent volume fibrosis was determined.

**Western Blots:** Protein extracts from different groups of lung were fractionated on a polyacrylamide gel, transferred to nitrocellulose membranes, and probed with various antibodies against SOD1 (Santa Cruz biotechnology inc, CA), SOD2 (Santa Cruz biotechnology inc, CA), SOD3 (LifeSpan BioSciences, Seattle, WA) and 3'-nitrotyrosine (Millipore, Billerica, Massachusetts). Specific protein expression levels were normalized to the GAPDH protein for total cell lysate and cytosolic protein.
<table>
<thead>
<tr>
<th>parameters</th>
<th>WT sham</th>
<th>SOD3E124D sham</th>
<th>WT PAH</th>
<th>SOD3E124D PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>7</td>
<td>10</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Bodyweight (g)</td>
<td>261±6.0</td>
<td>259±2.8</td>
<td>208±4.1*</td>
<td>205±4.6*</td>
</tr>
<tr>
<td>Tibia length (cm)</td>
<td>3.64±0.06</td>
<td>3.64±0.03</td>
<td>3.56±0.03</td>
<td>3.50±0.03</td>
</tr>
<tr>
<td>Right ventricular weight (mg)</td>
<td>162±8.7</td>
<td>157±2.8</td>
<td>239±8.7*</td>
<td>285±10.6†</td>
</tr>
<tr>
<td>Left ventricular + septum weight (mg)</td>
<td>641±19.3</td>
<td>640±9.4</td>
<td>585±12.1*</td>
<td>568±8.1*</td>
</tr>
<tr>
<td>Ratio of RV weight to LV+S weight</td>
<td>0.25±0.02</td>
<td>0.25±0.01</td>
<td>0.41±0.01*</td>
<td>0.50±0.09†</td>
</tr>
<tr>
<td>Lung mass (g)</td>
<td>1.23±0.05</td>
<td>1.20±0.01</td>
<td>1.86±0.04*</td>
<td>1.78±0.04*</td>
</tr>
<tr>
<td>Right atria weight (mg)</td>
<td>16.1±1.21</td>
<td>14.9±1.13</td>
<td>28.5±1.64*</td>
<td>32.2±1.53*</td>
</tr>
<tr>
<td>Left atria weight (mg)</td>
<td>14.8±0.71</td>
<td>16.4±1.12</td>
<td>12.7±0.58*</td>
<td>12.6±0.63*</td>
</tr>
<tr>
<td>Ratio of RA weight to LA weight</td>
<td>1.09±0.09</td>
<td>0.94±0.09</td>
<td>2.27±0.13*</td>
<td>2.62±0.14†</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>10.6±0.42</td>
<td>10.6±0.22</td>
<td>8.38±0.18*</td>
<td>8.90±0.38*</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>2.13±0.05</td>
<td>2.03±0.03</td>
<td>1.65±0.03*</td>
<td>1.76±0.05†</td>
</tr>
<tr>
<td>Ratio of RV weight to body weight (mg/g)</td>
<td>0.62±0.03</td>
<td>0.61±0.01</td>
<td>1.16±0.04*</td>
<td>1.40±0.08†</td>
</tr>
<tr>
<td>Ratio of LV weight to body weight (mg/g)</td>
<td>2.43±0.05</td>
<td>2.49±0.03</td>
<td>2.86±0.10*</td>
<td>2.76±0.05*</td>
</tr>
<tr>
<td>Ratio of lung weight to body weight (mg/g)</td>
<td>4.03±0.76</td>
<td>4.65±0.07</td>
<td>8.90±0.27*</td>
<td>8.70±0.24*</td>
</tr>
<tr>
<td>Ratio of RA weight to body weight (mg/g)</td>
<td>0.06±0.01</td>
<td>0.06±0.01</td>
<td>0.14±0.01*</td>
<td>0.16±0.01*</td>
</tr>
<tr>
<td>Ratio of LA weight to body weight (mg/g)</td>
<td>0.06±0.01</td>
<td>0.06±0.01</td>
<td>0.06±0.01</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>Ratio of liver weight to body weight (mg/g)</td>
<td>40.5±1.02</td>
<td>40.9±0.77</td>
<td>39.9±0.62</td>
<td>43.1±1.23†</td>
</tr>
<tr>
<td>Ratio of kidney weight to body weight (mg/g)</td>
<td>8.14±0.11</td>
<td>7.83±0.15</td>
<td>7.88±0.20</td>
<td>8.59±0.20†</td>
</tr>
<tr>
<td>Ratio of RV weight to tibia length (mg/mm)</td>
<td>4.51±0.22</td>
<td>4.33±0.097</td>
<td>6.81±0.29*</td>
<td>8.17±0.28†</td>
</tr>
<tr>
<td>Ratio of LV weight to tibia length (mg/mm)</td>
<td>17.8±0.69</td>
<td>17.7±0.27</td>
<td>16.6±0.39</td>
<td>16.3±0.28*</td>
</tr>
<tr>
<td>Ratio of lung weight to tibia length (mg/mm)</td>
<td>29.1±4.55</td>
<td>33.0±0.40</td>
<td>52.4±1.33*</td>
<td>51.1±1.29*</td>
</tr>
<tr>
<td>Ratio of RA weight to tibia length (mg/mm)</td>
<td>0.44±0.036</td>
<td>0.41±0.03</td>
<td>0.81±0.05*</td>
<td>0.93±0.05†</td>
</tr>
<tr>
<td>Ratio of LA weight to tibia length (mg/mm)</td>
<td>0.41±0.020</td>
<td>0.45±0.03</td>
<td>0.30±0.02</td>
<td>0.36±0.02*</td>
</tr>
<tr>
<td>Ratio of liver weight to tibia length (mg/mm)</td>
<td>29.2±1.28</td>
<td>29.1±0.66</td>
<td>23.6±0.47*</td>
<td>25.5±1.16*</td>
</tr>
<tr>
<td>Ratio of kidney weight to tibia length (mg/mm)</td>
<td>5.86±0.18</td>
<td>5.56±0.11</td>
<td>4.64±0.08*</td>
<td>5.05±0.16†</td>
</tr>
</tbody>
</table>

- p<0.05 as compared with corresponding control conditions; † p<0.05 as compared with wild type + MCT. RV: right ventricle; LV: left ventricle.
Table S2. Hemodynamic data for wild type (WT) and SOD3$^{E124D}$ rats under sham conditions or 3 weeks after MCT-induced PAH.

<table>
<thead>
<tr>
<th>parameters</th>
<th>WT sham</th>
<th>SOD3$^{E124D}$ sham</th>
<th>WT PAH</th>
<th>SOD3$^{E124D}$ PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Heart rate (beats per minute)</td>
<td>358±7.95</td>
<td>354±12.9</td>
<td>340±7.65</td>
<td>339±15.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>115±4.76</td>
<td>115±5.22</td>
<td>108±2.62</td>
<td>109±3.82</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>84.0±4.05</td>
<td>84.2±4.96</td>
<td>85.9±2.35</td>
<td>86.3±4.02</td>
</tr>
<tr>
<td>Mean aortic pressure (mmHg)</td>
<td>94.4±4.27</td>
<td>94.5±5.02</td>
<td>93.2±2.33</td>
<td>93.9±3.89</td>
</tr>
<tr>
<td>RV end systolic pressure (mmHg)</td>
<td>26.0±0.99</td>
<td>25.1±0.53</td>
<td>48.6±1.84*</td>
<td>57.6±3.10†</td>
</tr>
<tr>
<td>RV end diastolic pressure (mmHg)</td>
<td>2.07±0.11</td>
<td>2.09±0.08</td>
<td>4.23±0.37*</td>
<td>5.09±0.46*</td>
</tr>
</tbody>
</table>

*p<0.05 as compared with corresponding control conditions; † p<0.05 as compared with wild type + MCT.
Figure S1. Representative H&E staining of lung tissues showed that SOD3 knockout in mice exacerbated hypoxia-induced increases of pulmonary vascular remodeling as indicated by increased fully muscularized small arterioles.
**Figure S2.** Representative whole heart, cross section of ventricles, Sirius red staining for RV fibrosis, and H&E staining or WGA staining for myocyte size of RV.
Figure S3. SOD3$^{E124D}$ mutation exacerbated the MCT-induced increase of the right ventricular (RV) fibrosis (A), and RV cardiac myocyte hypertrophy as indicated by an increase of the cross-sectional area of cardiac myocytes (B) in rats. *$p<0.05$ vs sham control; #$p<0.05$ vs corresponding WT rats.

Figure S4. Lung SOD3 expression is unaffected in SOD3$^{E124D}$ rats.
**Figure S5** SOD3^{E124D} mutation significantly exacerbated MCT-induced relative wall medial area in arteries, indicating SOD3^{E124D} mutation exacerbated MCT-induced pulmonary vascular remodeling in rats. *p<0.05 vs sham control; #p<0.05 vs corresponding WT rats.

**Figure S6.** Immunostaining show increased proliferation of smooth muscle cells in rats after PAH.
Figure S7. Alteration of lung 3’-nitrotyrosine, SOD1 and SOD2 expression in wild type and SOD3E124D rats under control conditions or after MCT-induced PAH. The summarized data is presented in Figure 4A-C.

Figure S8. SOD2 activity is unaffected in rats with SOD3E124D mutation.