

Extracellular Superoxide Dismutase Overexpression Can Reverse the Course of Hypoxia-Induced Pulmonary Hypertension

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Hypoxia leads to free radical production, which has a pivotal role in the pathophysiology of pulmonary hypertension (PH). We hypothesized that treatment with extracellular superoxide dismutase (EC-SOD) could ameliorate the development of PH induced by hypoxia. *In vitro* studies using pulmonary microvascular endothelial cells showed that cells transfected with EC-SOD had significantly less accumulation of xanthine oxidase and reactive oxygen species than nontransfected cells after hypoxia exposure for 24 h. To study the prophylactic role of EC-SOD, adult male wild-type (WT) and transgenic (TG) mice, with lung-specific overexpression of human EC-SOD (hEC-SOD), were exposed to fraction of inspired oxygen (FiO₂) 10% for 10 d. After exposure, right ventricular systolic pressure (RVSP), right ventricular mass (RV/S + LV), pulmonary vascular wall thickness (PVWT) and pulmonary artery contraction/relaxation were assessed. TG mice were protected against PH compared with WT mice with significantly lower RVSP (23.9 ± 1.24 versus 47.2 ± 3.4), RV/S + LV (0.287 ± 0.015 versus 0.335 ± 0.022) and vascular remodeling, indicated by PVWT (14.324 ± 1.107 versus 18.885 ± 1.529). Functional studies using pulmonary arteries isolated from mice indicated that EC-SOD prevents hypoxia-mediated attenuation of nitric oxide-induced relaxation. Therapeutic potential was assessed by exposing WT mice to FiO₂ 10% for 10 d. Half of the group was transfected with plasmid containing cDNA encoding human EC-SOD. The remaining animals were transfected with empty vector. Both groups were exposed to FiO₂ 10% for a further 10 d. Transfected mice had significantly reduced RVSP (18.97 ± 1.12 versus 41.3 ± 1.5), RV/S + LV (0.293 ± 0.012 versus 0.372 ± 0.014) and PVWT (12.51 ± 0.72 versus 18.98 ± 1.24). On the basis of these findings, we concluded that overexpression of EC-SOD prevents the development of PH and ameliorates established PH.

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INTRODUCTION

Pulmonary hypertension (PH) is characterized by an increase in pulmonary vascular resistance that impedes ejection of blood by the right ventricle and subsequently leads to right ventricular failure. Both adults and pediatric patients with lung diseases complicated by alveolar hypoxia are at risk for developing PH, a process that significantly increases mor-

bidity and mortality (1). In adults, PH is a common complication of chronic respiratory conditions such as chronic obstructive pulmonary disease. In addition, PH and right ventricular dysfunction are major well-recognized cardiovascular complications of chronic lung disease of neonates (2). PH contributes significantly to the morbidity and mortality rates of infants with chronic lung disease, pro-

longing hospitalization, limiting life expectancy and increasing long-term adverse neurodevelopmental outcomes (3). Hypoxia plays a pivotal role in the pathogenesis of PH (1), leading to pulmonary vessel constriction, proliferation of endothelial cells, smooth muscle cells and adventitial fibroblasts and vessel narrowing (1,4). Each of the cells described above contribute to pulmonary vascular remodeling, which in turn increases resistance in the pulmonary circulation, leading to right ventricular failure (5).

The role of increased generation of reactive oxygen species (ROS) in the pathogenesis of experimental PH is evidenced by studies performed in adult rats and adult humans with idiopathic PH (6–8). The injured pulmonary vasculature is known to be a major source of ROS, par-

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ticularly superoxide anion ($O_2^{\bullet-}$) (9,10). In humans, increased vascular $O_2^{\bullet-}$ production, during chronic hypoxic exposure, results in impaired nitric oxide (NO) signaling and the development of pulmonary vascular remodeling (7,11).

As shown in our previous studies, extracellular superoxide dismutase (EC-SOD) overexpression decreases lung inflammation, preserves lung development and angiogenesis in both neonate (12) and adult mice (13) and increases NO bioavailability during exposure to oxidative stress (14). Increasing EC-SOD expression, specifically in the airway epithelium, delivers protection against $O_2^{\bullet-}$ -induced cellular injury. Endothelial expression of EC-SOD is necessary to regulate NO metabolism for appropriate regulation of vasomotor tone (15) and regulation of nuclear factor (NF)- κ B activation (16). Because $O_2^{\bullet-}$ reacts rapidly with NO ($\sim 6.7 \times 10^9$ /mol/s) and EC-SOD catalyzes $O_2^{\bullet-}$ at less rapid rates, higher enzyme concentrations through overexpression may be necessary for rapid removal of excess $O_2^{\bullet-}$ produced during oxidative stress. Formation of peroxynitrite by $O_2^{\bullet-}$ represents another potential source of oxidant tissue injury in pulmonary endothelial cells. It may be that the ultimate effects of $O_2^{\bullet-}$ on NO availability in the vascular compartment are more damaging, especially in newborns, who may be more dependent on NO regulation of vasomotor tone and suppression of NF- κ B activation. EC-SOD, therefore, has a unique role in protecting both epithelial and endothelial compartments against hyperoxia-induced injury.

Although, the prophylactic role of EC-SOD to prevent PH has previously been shown (17–20), superoxide dismutase (SOD) mimetics have also been shown to be useful in preventing PH in an adult animal model (21). In this study, the overall goal was to evaluate the possible prophylactic and therapeutic potentials of EC-SOD overexpression in an animal model with PH induced by hypoxic exposure. We believe that EC-SOD may be uniquely positioned to oppose PH be-

cause of its ability to catalyze the dismutation of superoxide radicals, increase NO bioavailability and decrease the inflammatory response associated with oxidative stress.

MATERIALS AND METHODS

Primary human pulmonary microvascular endothelial cells (HPMECs) (Promocell, Heidelberg, Germany) were cultured in endothelial cell growth medium containing fetal calf serum, recombinant human epidermal growth factor, recombinant human basal fibroblast factor, insulinlike growth factor, recombinant human vascular endothelial growth factor 165, ascorbic acid and hydrocortisone. Cells were grown to confluence, in T75 flasks, in a humidified incubator (37°C, 5% CO_2). Cells were then seeded to six-well tissue culture plates at a density of 10,000 cells per well and incubated for 24 h. After incubation, cells were transfected with human EC-SOD (hEC-SOD) cDNA and inserted into a vector plasmid pcDNA3 (5446 nucleotides; Invitrogen Life Technologies, Carlsbad, CA, USA) as previously described (10), using the FuGENE kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Each well received a DNA/FuGENE complex in 100 μ L serum free medium (with a concentration of 1 μ g DNA/100 μ L medium). Control wells received serum free medium alone. Stably transfected cells were selected using Geneticin (Invitrogen Life Technologies). Transfection of HPMECs was confirmed by Western blot analysis using an antibody specific for hEC-SOD (R&D Systems, Minneapolis, MN, USA).

Effect of Hypoxia on Xanthine Oxidase Concentration *In Vitro*

A modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) was used for the cell hypoxia studies. To induce hypoxia, the chamber was tightly closed and flushed with a gas mixture consisting of 95% nitrogen and 5% CO_2 , until the desired ambient oxygen concentration of 1% was reached, as mea-

sured using an oxygen sensor (BioSpherix, Lacona, NY, USA). The whole chamber was then sealed and placed in a standard cell incubator. This method produced a microenvironment within the chamber of 37°C, 1% O_2 , 5% CO_2 and 100% humidity.

To examine the effects of hypoxia \pm EC-SOD overexpression on free radical accumulation, both transfected and nontransfected HPMECs were placed in the hypoxic chamber and incubated for 24 h. A control nontransfected endothelial cell culture was kept in the incubator at fraction of inspired oxygen (FiO_2) 21% for 24 h. Xanthine oxidase (XO) concentration was measured in cell homogenate from all tissue cultures using the Amplex red xanthine/xanthine oxidase assay kit (Invitrogen Detection Technologies, Eugene, OR, USA) (22). Intracellular reactive species were quantified using 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma, St. Louis, MO, USA). Endothelial cells were loaded with 2 pmol/L 2,7-dichlorofluorescein diacetate (DCF) by incubation for 1 h. Before analysis, cells were washed three times in Hanks solution containing 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer. DCF fluorescence in multiwell plates was quantified using a Cytofluor 2350 fluorescence plate reader using 485-nm excitation and 530-nm emission filters (23).

Assessment of EC-SOD Expression on Hypoxia-Induced PH in Mice

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research. Both adult 8- to 10-wk-old male C57BL6 mice (wild-type [WT]) and transgenic adult mice expressing a copy of hEC-SOD (transgenic [TG]) (13) were housed in a pathogen-free environment, under standard light and dark cycles, with free access to food and water. An animal hypoxia chamber system (BioSpherix, Lacona, NY, USA) was used for the *in vivo* studies. With this system, a constant 10% normobaric

hypoxia was achieved for up to 10 d in our study.

Studied animals were divided into four groups (six per group) and housed for 10 d as follows: group A: TG mice FiO₂ 10%; group B: WT mice FiO₂ 10%; group C: TG mice room air; and group D: WT mice room air.

Animals were anesthetized with isoflurane 5% in room air. After the 10-d period, a 26-gauge needle connected to a transducer was introduced into the right ventricle of the heart trans-diaphragmatically. Right ventricular systolic pressure (RVSP) was measured and recorded using a computerized hemodynamic recording system (HAEMODYN, Harvard Apparatus, MA, USA). The mice were then euthanized by exsanguination.

The hearts were removed, dissected and weighed. The heart weights were expressed as a ratio of the weight of the right ventricle to that of the septum plus left ventricle (RV/S + LV) (22). The left lung was ligated and fixed, at inflation, with 4% paraformaldehyde. The right lung was lavaged with three aliquots (0.3 mL each) of phosphate-buffered saline (0.05 mol/L, pH 7.4), bronchoalveolar lavage fluid was centrifuged and bronchoalveolar lavage cells were mixed with 0.5 mL of 0.2% saline for 10 s to lyse any residual erythrocytes, and the cells were then resuspended in 10 mL Hanks balanced salt solution. The total cell number retrieved in the bronchoalveolar lavage was determined with a hemocytometer. Slides were prepared using a Shandon cytocentrifuge (Shandon Scientific, London, UK) and cells were stained with a HEMA 3 Stain Set (Fisher Scientific) for differential cell counting. Counts were made on at least 200 cells per slide by an observer blinded to the grouping.

After lavage, the right lung was frozen and was used to assay XO concentration using the Amplex red xanthine/xanthine oxidase assay kit. Free radical accumulation was assessed using the Oxiselect ROS assay kit (Cell Biolabs, San Diego, CA, USA).

Fixed left lung tissues were immunostained using an α smooth muscle actin

(α -SMA) antibody (Abcam, Cambridge, MA, USA) to reveal the muscular layer of the vessel wall (17). External diameter and internal diameter of 50 alveolar vessels (with an external diameter of 40–100 μ m) per animal were determined and recorded by an independent investigator blinded to the treatment regimen. Vascular wall thickness was expressed as the percentage of total vessel size. Percent wall thickness was calculated as $(2 \times \text{wall thickness})/\text{external diameter} \times 100\%$ (24).

Pulmonary Artery Function

Physiological function assessments were performed on explanted main intralobar pulmonary arteries with intact endothelium. Pulmonary artery segments (~180 μ m internal diameter) were isolated from each animal and maintained in physiological saline. Ring segments were prepared and mounted in microvascular myographs (DMT, Aarhus, Denmark), which were attached to a Power Lab Data Acquisition System from AD Instruments. The arteries were incubated at a passive tension of 0.5 g for 1 h in Krebs-bicarbonate buffer solution containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.5 mmol/L CaCl₂, 25 mmol/L NaHCO₃, 1.1 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄ and 5.6 mmol/L glucose gassed with 21% O₂–5% CO₂–74% N₂. The temperature was maintained at 37°C in the individually thermostated baths containing the rings. After 1 h of incubation, the rings were depolarized with 123 mmol/L KCl containing Krebs-bicarbonate buffer, and the rings were again reequilibrated with normal Krebs-bicarbonate buffer for another 30 min. The pulmonary arterial rings were then contracted with increasing cumulative doses of phenylephrine (PE) (1, 10 and 100 nmol/L) (PE⁻⁹ to PE⁻⁷) doses followed by relaxation with increasing doses of acetylcholine (Ach) (10 nmol/L, 100 nmol/L, 1 mmol/L and 10 mmol/L) (Ach⁻⁸ to Ach⁻⁵). Arteries were again reequilibrated with normal Krebs-bicarbonate buffer for another 30 min. After that, they were contracted

with increasing doses of serotonin (5-HT; 10 nmol/L, 100 nmol/L, 1 mmol/L and 10 mmol/L) (5-HT⁻⁹ to 5-HT⁻⁵) followed by reequilibration with normal Krebs-bicarbonate buffer for another 30 min. Then the rings were contracted with 100 nmol/L phenylephrine followed by relaxation with an increasing dose of a NO donor spermine NONOate (1 nmol/L, 10 nmol/L, 100 nmol/L, 1 mmol/L and 10 mmol/L) (NO⁻⁹ to NO⁻⁵). Because of the effects of hypoxia on force generation to contractile agents, all data are reported as the actual grams of active force that were measured (25).

EC-SOD Amelioration of Hypoxia-Induced PH

WT male mice C57BL6 (8–10 wks old) were divided into four groups (six animals per group).

Group A. WT mice were exposed to hypoxia at FiO₂ 10% for 10 d. This group was then transfected with *hEC-SOD* by direct instillation into the trachea. *hEC-SOD* cDNA was used for transfection using Ad.CMV.LacZ as the vector. The plasmid *hEC-SOD* cDNA was mixed with a FuGENE 6 transfection reagent (Roche Diagnostics, Branchburg, NJ, USA). This mixture was instilled into the mouse airway after creating a tracheostomy and artificially ventilating the animal using volume ventilation. After instillation, the animal was ventilated for 5 min to flush the DNA down to the terminal airway. The tracheostomy was sutured and the animal was kept in room air to recover. One hour later, the animal was placed back in the hypoxic chamber for a further 10 d.

Group B. WT mice were exposed to hypoxia at FiO₂ 10% for 10 d. This group was then transfected with plasmid alone (empty vector), mixed with FuGENE 6 transfection reagent, using the same procedure described above. One hour later, the animal was placed back in the hypoxic chamber for a further 10 d.

Group C. WT mice were kept in room air for 20 d.

Group D. WT mice were kept in room air for 10 d. This group was then trans-

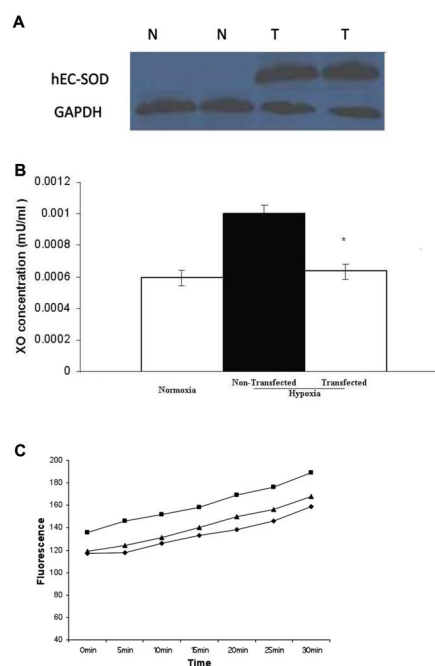


Figure 1. HPMECs nontransfected (N) and transfected (T) with EC-SOD after exposure to hypoxia for 24 h compared with a cell line incubated with FiO_2 21% for 24 h. (A) Western blot for hEC-SOD protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. (B) XO concentration. (C) Intracellular reactive species and hydrogen peroxide measuring using DCF fluorescence over time. *Transfected cell line versus nontransfected cell line, $P < 0.05$. —◆—, RA; —■—, hypoxic nontransfected; —▲—, hypoxic transfected.

fectured with plasmid alone (empty vector) mixed with FuGENE 6 transfection reagent as described above. This group was kept in room air for a further 10 d. All studied groups were euthanized after a total of 20 d, and heart and lungs were studied as described above.

Statistics

Data are expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using SigmaStat (Jandel Scientific Software, San Rafael, CA, USA). Unless otherwise indicated, a one- or two-way analysis of variance followed by Fisher test was used to assess significance ($P < 0.05$).

RESULTS

Hypoxia *In Vitro*

Under normal conditions, xanthine oxidoreductase exists in dehydrogenase form and uses nicotinamide adenine dinucleotide (NAD)⁺, and there is no or little production of superoxide anion. Under hypoxic conditions, depletion of adenosine 5'-triphosphate leads to loss of membrane Ca^{2+} gradient. Increased Ca^{2+} level activates Ca^{2+} -dependent proteases, which cause selective proteolysis of the dehydrogenase and convert the dehydrogenase form into XO. XO acts both on hypoxanthine and xanthine to produce $\text{O}_2^{\cdot-}$. Thus, we measured both XO concentration and $\text{O}_2^{\cdot-}$ in transfected and nontransfected endothelial cells to assess the effect of EC-SOD overexpression. Figure 1A shows the expression of hEC-SOD protein in transfected versus nontransfected HPMECs after exposure to hypoxia for 24 h. Hypoxia leads to increased production of ROS as evident by the increased XO concentration. However, in transfected endothelial cell lines with hEC-SOD, the XO concentration was significantly lower than in nontransfected endothelial cells after exposure to hypoxia for 24 h ($P < 0.05$) (Figure 1B). The effect of EC-SOD overexpression on intracellular reactive species accumulation was studied using the DCF assay, which showed that basal level and total accumulation of ROS were significantly lower in the transfected than the nontransfected endothelial cells after exposure to hypoxia for 24 h and did not significantly differ from the room air control cell culture (Figure 1C).

Hypoxia *In Vivo*

Prophylactic study. *In vivo*, the XO concentration was significantly higher in WT adult mice after exposure to hypoxia (FiO_2 10% for 10 d) compared with TG mice (0.051 ± 0.002 versus 0.044 ± 0.001) (Figure 2A). ROS was also significantly higher in WT adult mice than in TG mice (245 ± 35 versus 138 ± 4.2 , respectively) under the same hypoxic conditions (Figure 2B). There was no significant differ-

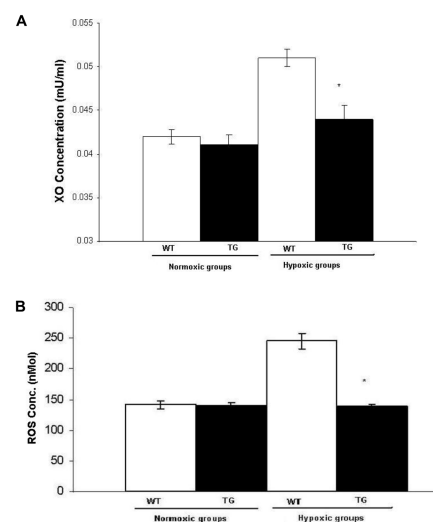


Figure 2. Evaluation of ROS concentration after exposure to hypoxia (FiO_2 10% \times 10 d) in WT and TG adult mice compared with normoxic groups. (A) XO concentration. (B) Total ROS concentration. Data are the mean of six animals per group \pm SEM. *TG hypoxic versus WT hypoxic group, $P < 0.05$.

ence between the hypoxic TG and the normoxic groups.

The existence of PH was assessed by measuring right ventricular hypertrophy and RVSP. There was a significant increase of RV/S + LV ratio in both TG and WT after exposure to hypoxia (FiO_2 10% for 10 d) compared with normoxic groups ($P < 0.05$). The hypoxic TG group also showed a significant decrease in RV/S + LV ratio (0.287 ± 0.015) compared to the hypoxic WT mice (0.335 ± 0.022) ($P < 0.05$) (Figure 3A).

RVSP showed a similar trend to RV/S + LV ratio in the four groups. RVSP was significantly lower in the hypoxic TG group (23.9 ± 1.24) than the hypoxic WT group (47.2 ± 3.4) ($P < 0.05$) (Figure 3B).

Immunostaining for α -SMA showed there was a significant increase in pulmonary vessel wall thickness in the hypoxic WT (18.885 ± 1.529) compared with the hypoxic TG group (14.324 ± 1.107) ($P < 0.05$). There was no significant difference between the TG hypoxic and normoxic groups ($P > 0.05$) (Figures 3C, D).

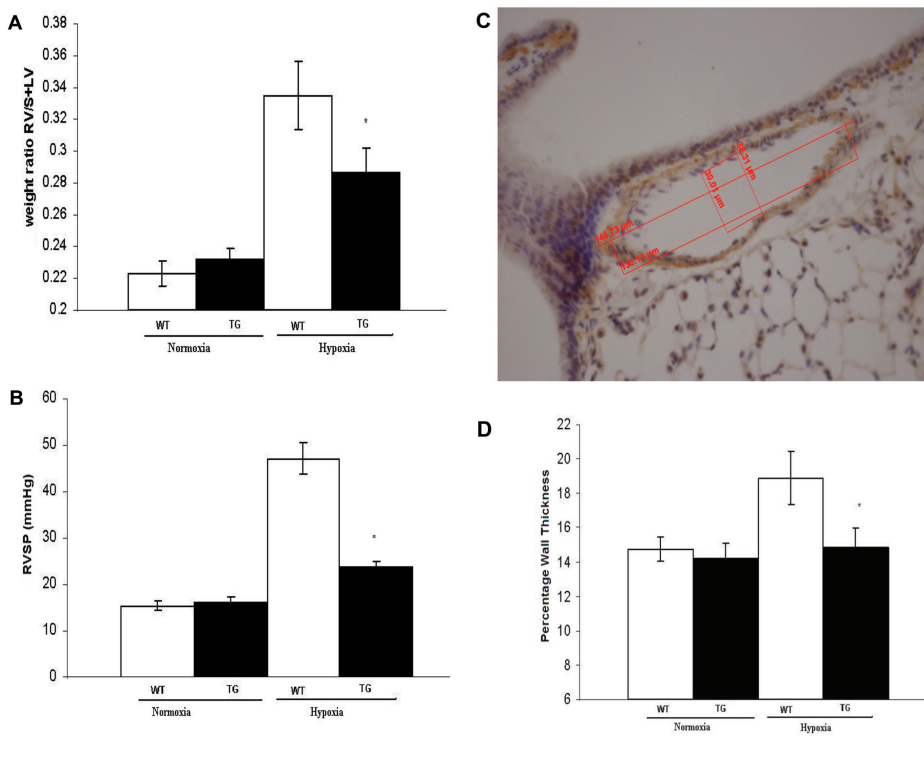


Figure 3. Evaluation of PH and cardiac hypertrophy after exposure to hypoxia (FIO₂ 10% × 10 d) in WT and TG adult mice compared with normoxic groups. (A) RV/S + LV ratio. (B) RVSP. (C) Images of BV stained for α-SMA and measurement showing the method of measurement of wall thickness. (D) Wall thickness of medium-sized pulmonary arteries assessed by immunostaining for anti-α-SMA. Data are the mean of six animals per each group ± SEM. *TG hypoxic versus WT hypoxic group, *P* < 0.05.

Hypoxia is associated with increased generation of free radicals that trigger inflammatory reactions. Accordingly, there was an increase in both total number of white blood cells and polymorphonuclear neutrophils (PMNs) in bronchoalveolar lavage from hypoxic WT animals (1,115 ± 124 × 1,000 and 223 ± 23 × 1,000, respectively), which was significantly higher than the hypoxic TG group (425 ± 45 × 1,000 and 49 ± 5.6 × 1,000, respectively) after exposure to hypoxia (FIO₂ 10% for 10 d) (*P* < 0.05) (Figure 4).

Pulmonary artery function assessment after exposure to 10% hypoxia for 10 d showed exaggerated contraction, in response to both phenylephrine (PE) and 5-HT at different doses in comparison to room air control groups. Also, there was a marked relaxation effect among arteries from TG mice compared with WT mice, housed under the same

hypoxic conditions, treated with either Ach or an NO donor (spermine NONOate) after induction of contraction by PE or 5-HT (Figures 5A–D). At

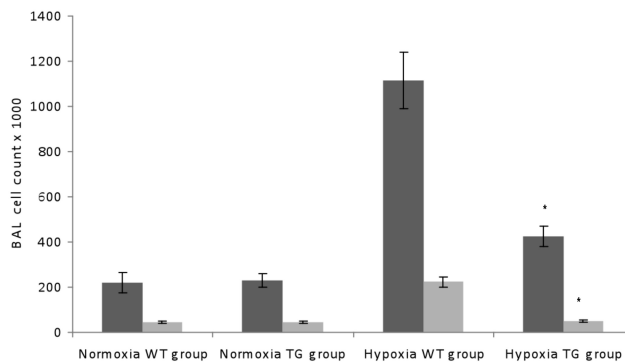


Figure 4. White blood cell counts and PMNs after exposure to hypoxia (FIO₂ 10% × 10 d) in WT and TG adult mice compared with normoxic groups. Data are the mean of six animals per group ± SEM. *TG hypoxic versus WT hypoxic group, *P* < 0.05. BAL, bronchoalveolar lavage; ■, BAL, WBCs count × 1,000; ■, BAL, PMNs count × 1,000.

the higher dose of NO donor (NO⁻⁵ = 10 mmol/L), there was a statistical difference in the relaxation response between the hypoxic TG group compared with the WT hypoxic group (*P* < 0.05).

Therapeutic study. To evaluate the therapeutic potential of EC-SOD, we measured the RV/S + LV ratio after exposure to hypoxia for 20 d in WT mice compared with WT groups kept in room air for 20 d. Both room air and hypoxic groups were transfected with plasmid encoding EC-SOD or empty vector after 10 d of exposure. To confirm successful transfection, Western blot for hEC-SOD protein was performed in both groups and showed the hEC-SOD band only in the group transfected with plasmid encoding the hEC-SOD gene (Figure 6A).

RV/S + LV ratio was significantly higher in WT adult mice transfected with plasmid alone (0.372 ± 0.014) compared with WT animals transfected with plasmid containing the hEC-SOD gene (0.293 ± 0.012) (*P* < 0.05). There was also a significant difference between both hypoxic groups and normoxic control groups (Figure 6B).

RVSP was significantly higher in WT adult mice transfected with plasmid alone (41.3 ± 1.5) compared with groups transfected with plasmid containing the hEC-SOD gene (18.97 ± 1.12) (*P* < 0.05). There was no significant difference between the hypoxic transfected with the

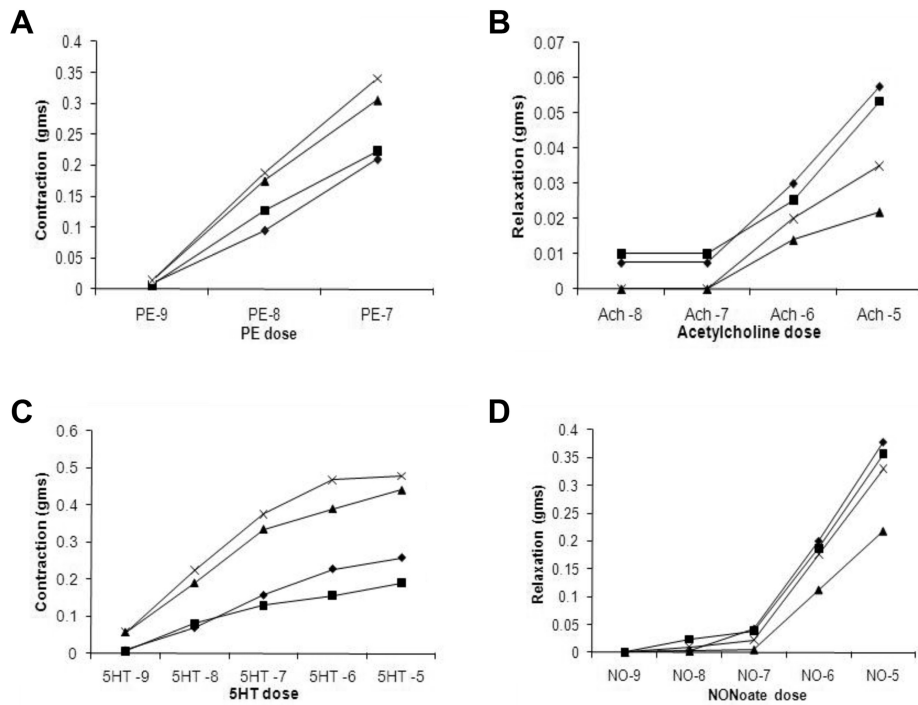


Figure 5. Pulmonary artery function studies were performed on ~180 μm pulmonary arteries isolated from adult WT and TG animals exposed to hypoxia of FiO_2 10% for 10 d compared with normoxic groups. (A) Contraction (grams) induced by PE (1–100 nmol/L = PE⁻⁹ to PE⁻⁷). (B) Relaxation (grams) induced by Ach (10 nmol/L to 10 $\mu\text{mol/L}$ = Ach⁻⁹ to Ach⁻⁵). (C) Contraction induced by 5-HT (10 nmol/L to 10 $\mu\text{mol/L}$ = 5-HT⁻⁸ to 5-HT⁻⁵). (D) Relaxation induced by spermine NONOate (NO, 1 nmol/L to 10 $\mu\text{mol/L}$, NO⁻⁹ to NO⁻⁵). Data are expressed as mean of four animals per each group. RA, room air; —◆—, WT RA; —▲—, WT hypoxia; —x—TG hypoxia.

plasmid and hEC-SOD gene and normoxic control groups ($P > 0.05$). (Figure 6C).

Immunostaining for α -SMA showed significant pulmonary vessel wall thickness in WT animals transfected with plasmid alone (18.98 ± 1.24) in comparison to the WT transfected group with plasmid + hEC-SOD gene (12.51 ± 0.72) ($P < 0.05$). Both hypoxic groups were also significantly higher than the normoxic control groups ($P < 0.05$) (Figure 6D).

DISCUSSION

In this study, we report for the first time an intervention that can reverse the PH induced by hypoxia in an adult mouse model. Pulmonary hypertension is associated with a high morbidity and mortality in all age groups from neonates to the elder population. Al-

though, the prophylactic role of EC-SOD to prevent PH has previously been shown (18–20), this is the first study to show the ability of EC-SOD to rescue animals with established hypoxia-induced PH.

Chronic hypoxia leads to both decreased expression and activity of EC-SOD in the WT adult mouse lung but is about five to seven times higher in TG mice that overexpress EC-SOD under the same hypoxic conditions (18). As shown in our study, overexpression of EC-SOD can reverse the pathogenic changes seen in PH. This therapeutic effect can be explained by different mechanisms. First, chronic hypoxia leads to increased free radical production both *in vitro* and *in vivo*. These free radicals are a significant contributors to vascular remodeling (7,10), a process that leads to

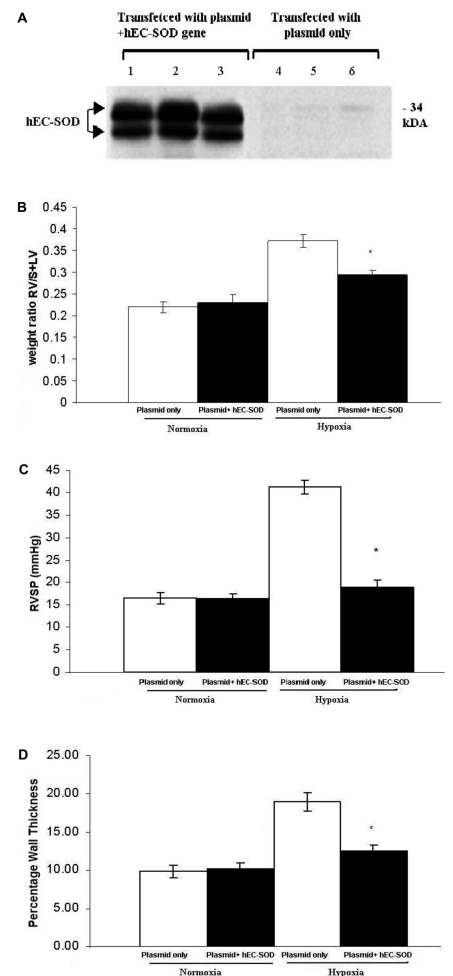


Figure 6. RV/S + LV ratio after exposure to hypoxia (FiO_2 10% \times 20 d) in both WT adult mice groups transfected with plasmid and EC-SOD or plasmid only (after 10 d of the exposure), compared with WT normoxic groups that were treated the same way. (A) Western blot for hEC-SOD protein. (B) RV/S + LV ratio. (C) RVSP (D) Wall thickness of medium-sized pulmonary arteries assessed by immunostaining for anti- α -SMA. Data are the mean of six animals per group \pm SEM. *Hypoxic transfected with plasmid + hEC-SOD versus hypoxic transfected with plasmid alone, $P < 0.05$.

increased pulmonary vascular resistance and increased right ventricular pressure. Thus, interruption of this sequence of events has the potential to interrupt vascular remodeling.

Second, NO is a potent vasodilator, and ROSs inactivate NO by formation of

higher-order nitrogen oxides (7). Previous studies have shown that SOD therapy increases NO bioavailability in experimental PH (20), and in our previous study, we showed that EC-SOD increases NO bioavailability, as evident by increasing cyclic guanosine monophosphate (cGMP) production under oxidative stresses *in vitro* and *in vivo* (13). Increasing cGMP production leads to vasodilation of the pulmonary vascular bed and reduction of both the pulmonary and right ventricular pressure.

Extracellular SOD deficiency exacerbates transverse aortic constriction-induced myocardial oxidative stress, hypertrophy, fibrosis and dysfunction, indicating that the distribution of extracellular SOD in the extracellular space is critically important in protecting the heart against pressure overload and contractile dysfunction (26). On the basis of these findings, we speculate that under hypoxic conditions, relieving pulmonary pressure, decreasing right ventricular pressure and decreasing the oxidative stress by overexpression of hEC-SOD leads to a decrease in the stress pressure and the strain on the right ventricular wall. As a result, the oxidative stress produced within the right ventricular wall because of the combination of ischemia and hypoxia can be alleviated, leading to improvement in right ventricular function and decreased hypertrophy.

In our study, we showed that the process of PH induced by hypoxia and its impact on the right ventricular wall and function is a reversible process, even with the continuous exposure to hypoxia. The concept of reducing the ROS could explain this reversible phenomena that was achieved by overexpression of EC-SOD (27). Our data show that in both *in vitro* and *in vivo* models, hypoxia leads to increased ROS production, which was blunted by overexpression of EC-SOD. Chelating ROS gives both the lung and the heart the chance to recover and restores both their anatomic and physiological function. We speculate that EC-SOD has a particular antiinflammatory advantage that can block the infiltration

of myeloid cells that contribute to PH remodeling, which leads also to recovery and restoration of right ventricle size and function.

Vascular remodeling as a cause of PH induced by hypoxia has been investigated. Experimental studies show that oxidative stress initiates the production of cyclooxygenase-derived contractile factors in blood vessels (25,28). Chronic hypoxia increases ROS levels and impairs endothelial NO-dependent relaxation in mouse pulmonary arteries (29,30). The role of endothelin-1, its vasoconstrictor effect under hypoxic conditions and its role in progression of the PH process have been studied previously (31). Plasma concentration of endothelin-1 correlates with severity of PAH in both animal models (32–34) and patients (35). Endothelin (ET) B receptor, which is expressed abundantly in the lung (36), elicits vasodilation and anti-mitogenic effects through the release of NO and/or prostaglandin 2 (37). Pulmonary ET B clears endothelin-1 from the plasma, limiting the ET A-mediated vasoconstrictor (38). In rats, ET B deficiency exacerbates monocrotaline (39) and hypoxia-induced PH (40). Impaired extracellular ET B-mediated NO/prostaglandin 2 release from pulmonary resistance vessels during hypoxia may have contributed to the development of PAH (41). In both the TG animal model and the transfected model with hEC-SOD, there was a significant decrease of vascular remodeling after exposure to hypoxia compared with the WT animal model. Both models also showed significantly decreased right ventricular pressure as an indication of decreasing pulmonary vascular pressure and resistance as a result of pulmonary vasodilation. The mechanism by which hypoxia downregulates ET B is unknown; also, the interplay between ET B, ROS and EC-SOD is yet to be investigated.

The inflammatory process is part of the pathogenesis of PH induced by hypoxia. Perivascular inflammatory cell infiltrates are found in lungs from patients

with PH (42). Our data showed a significant increase of white blood cells in the bronchoalveolar lavage of WT versus TG adult mice after exposure to hypoxia, which indicates that the inflammatory response associated with PH induced by hypoxia is alleviated by EC-SOD overexpression in TG animals. Macrophage inhibitory factor concentration was also found to be elevated in bronchoalveolar lavage of animals and patients with PH induced by hypoxia (43). Compared to healthy controls, patients with idiopathic or associated PH exhibit higher circulating levels and pulmonary expression of various inflammatory cytokines and chemokines including interleukin (IL)-1 β , IL-6 and monocyte chemoattractant protein-1 (MCP-1) (44–46). Lung expression of adhesion molecules such as intracellular adhesion molecule-1 and vascular cell adhesion molecule-1, and of the cytokine MCP-1, were markedly higher with hypoxia than normoxia in WT mice (47,48). The relation between EC-SOD overexpression and cytokines and other inflammatory markers such as macrophage inhibitory factor should be investigated in the future.

Prolonged hypoxia alters aspects of ROS generation and defense mechanisms in animal models with PH, including loss of endothelium-dependent NO-mediated relaxation from increased superoxide and increased Rho kinase activity (49,50) and a decrease in heme oxygenase-1 expression (51). Functional studies of pulmonary arteries extracted from our studied groups showed greater relaxation in the TG hypoxic group than in the WT hypoxic group. This finding supports the protective role of increased EC-SOD expression against the development of pulmonary vasoconstriction after exposure to chronic hypoxia.

Current therapy of PH is on the basis of administration of a high concentration of oxygen, which is a fuel for ROS production; administration of NO, which is oxidized by ROS to toxic metabolites, especially in presence of high oxygen concentration; and administration of systemic vasodilators. Among adult patients

with PH, either idiopathic or secondary to lung disease, none of these therapies change mortality, rate of clinical progression of disease or World Health Organization functional class (52). The limitation of current treatments suggests the necessity to develop new therapeutic targets for this lethal disease. Among neonates with persistent PH of the newborn or PH secondary to chronic lung disease, available therapeutic strategies lead to some relief and limited benefits, and over 50% of patients have a limited or transient response and significant morbidity (53). In this study, we present a novel therapy that targets the main etiology of PH induced by hypoxia.

CONCLUSION

In conclusion, these data provide a foundation of a new therapeutic intervention for PH induced by hypoxia exposure using overexpression of EC-SOD.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- Stenmark KR, Fagan KA, Frid MG. (2006) Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ. Res.* 99:675–91.
- Land RD. (2005) Neonatal chronic lung disease in the post-surfactant era. *Biol. Neonate.* 88:181–91.
- Henderson-Smart DJ, et al. (2006) Prenatal predictors of chronic lung disease in very preterm infants. *Arch. Dis. Child Fetal Neonatal Ed.* 91:40–5.
- Tuder RM, Yun JH, Bhunia A, Fijalkowska I. (2007) Hypoxia and chronic lung disease. *J. Mol. Med.* 12:1317–24.
- Lai YL, Wu HD, Chen CF. (1998) Antioxidants attenuate chronic hypoxic pulmonary hypertension. *J. Cardiovasc. Pharmacol.* 32:714–20.
- Hoshikawa Y, et al. (2001) Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia. *J. Appl. Physiol.* 90:1299–306.
- Lakshminrusimha S, et al. (2006) Superoxide dismutase improves oxygenation and reduces oxidation in neonatal pulmonary hypertension. *Am. J. Respir. Crit. Care Med.* 174:1370–7.
- Liu JQ, Zelko IN, Erbynn EM, Sham JS, Folz RJ. (2006) Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox). *Am. J. Physiol. Lung Cell. Mol. Physiol.* 290:L2–10.
- Grobe AC, et al. (2006) Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 290:L1069–77.
- Konduri GG, Bakhtashvili I, Eis A, Pritchard K Jr. (2007) Oxidant stress from uncoupled nitric oxide synthase impairs vasodilation in fetal lambs with persistent pulmonary hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 292:H1812–20.
- Ahmed MN, et al. (2003) Extracellular superoxide dismutase protects lung development in Hyperoxic newborn mice. *Am. J. Respir. Crit. Care Med.* 167:440–5.
- Folz RJ, Abushama AM, Suliman HB. (1999) Extracellular superoxide dismutase in the airways of transgenic mice reduces inflammation and attenuates lung toxicity following hyperoxia. *J. Clin. Invest.* 103:1055–66.
- Ahmed MN, Codipilly C, Hogg A, Auten RL. (2011) The protective effects of overexpression of extracellular superoxide dismutase on nitric oxide bioavailability in the lung after exposure to hyperoxia stress. *Exp. Lung. Res.* 37:10–7.
- Berrington WR. (2000) Site- and mechanism-targeted interventions for tissue free radical injury. In: *Chronic Lung Disease in Early Infancy*. Bland RD (ed.) Marcel Dekker Press, New York, pp. 883–909.
- Marshall HE, Stamler JS. (2006) Nitric oxide inhibits NF- κ B in the respiratory epithelium through S-nitrosylation of the P50 subunit. *Am. J. Resp. Crit. Care Med.* 161:A244.
- Odaka C. (2009) Localization of mesenchymal cells in adult mouse thymus: their abnormal distribution in mice with disorganization of thymic medullary epithelium. *J. Histochem. Cytochem.* 57:373–82.
- Li FH, Xia W, Li AW, Zhao CF, Sun RP. (2007) Inhibition of rho kinase attenuates high flow induced pulmonary hypertension in rats. *Chin. Med. J. (Engl)*. 120:22–9.
- Nozik-Grayck E, et al. (2008) Lung EC-SOD overexpression attenuates hypoxic induction of Egr-1 and chronic hypoxic pulmonary vascular remodeling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 295:L422–30.
- Kamezaki F, et al. (2008) Gene transfer of extracellular superoxide dismutase ameliorates pulmonary hypertension in rats. *Am. J. Respir. Crit. Care Med.* 177:219–26.
- Farrow KN, et al. (2008) Superoxide dismutase restores eNOS expression and function in resistance pulmonary arteries from neonatal lambs with persistent pulmonary hypertension. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 295:L979–87.
- Elmedal B, de Dam MY, Mulvany MJ, Simonsen U. (2004) The superoxide dismutase mimetic, tempol, blunts right ventricular hypertrophy in chronic hypoxic rats. *Br. J. Pharmacol.* 141:105–13.
- Coffey MJ, Phare SM, Peters-Golden M. (2002) Interaction between nitric oxide, reactive oxygen intermediates, and peroxynitrite in the regulation of 5-lipoxygenase metabolism. *Biochim. Biophys. Acta.* 1584:81–90.
- Shin YJ, et al. (2009) Protective effect of clusterin on oxidative stress-induced cell death of human corneal endothelial cells. *Molecular Vision.* 15:2789–95.
- Reid LM. (1979) The pulmonary circulation: remodeling in growth and disease. *J. Am. Rev. Respir. Dis.* 119:531–46.
- Neo BH, Kandhi S, Wolin MS. (2010) Roles for soluble guanylate cyclase and a thiol oxidation-elicited subunit dimerization of protein kinase G in pulmonary artery relaxation to hydrogen peroxide. *Am. J. Physiol. Heart Circ. Physiol.* 299:H1235–41.
- Zhongbing L, et al. (2008) Extracellular superoxide dismutase deficiency exacerbates pressure overload-induced left ventricular hypertrophy and dysfunction. *Hypertension.* 51:19–25.
- Firth AL, Yuan JX. (2008) Bringing down the ROS: a new therapeutic approach for PPHN. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 295:L976–8.
- Pannirselvam M, Wiehler WB, Anderson T, Triggler CR. (2005) Enhanced vascular reactivity of small mesenteric arteries from diabetic mice is associated with enhanced oxidative stress and cyclooxygenase products. *Br. J. Pharmacol.* 144:953–60.
- Fresquet F, et al. (2006) Role of reactive oxygen species and gp91phox in endothelial dysfunction of pulmonary arteries induced by chronic hypoxia. *Br. J. Pharmacol.* 148:714–23.
- Yanagisawa M, et al. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 332:411–5.
- Stelzner TJ, et al. (1992) Increased lung endothelin-1 production in rats with idiopathic pulmonary hypertension. *Am. J. Physiol.* 262:L614–20.
- Frasch HF, Marshall C, Marshall BE. (1999) Endothelin-1 is elevated in monocrotaline pulmonary hypertension. *Am. J. Physiol.* 276:L304–10.
- Nakanishi K, et al. (1999) Expression of endothelin-1 in rats developing hypobaric hypoxia-induced pulmonary hypertension. *Lab. Invest.* 79:1347–57.
- Cacoub P, et al. (1997) Endothelin-1 in the lungs of patients with pulmonary hypertension. *Cardiovasc. Res.* 33:196–200.
- Li H, et al. (1994) Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia. *J. Appl. Physiol.* 77:1451–9.
- De Nucci G, et al. (1998) Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelin-derived relaxing factor. *Proc. Natl. Acad. Sci. U. S. A.* 85:9797–800.
- Takayanagi R, et al. (1991) Presence of non-selective type of endothelin receptor on vascular endothelium and its linkage to vasodilation. *FEBS Lett.* 282:103–6.

38. Dupuis J, Goresky CA, Fournier A. (1996) Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ETB receptors. *J. Appl. Physiol.* 81:1510–15.
39. Nishida M, et al. (2004) Role of endothelin ETB receptor in the pathogenesis of monocrotaline-induced pulmonary hypertension in rats. *Eur. J. Pharmacol.* 496:159–65.
40. Ivy DD, et al. (2002) Exaggerated hypoxic pulmonary hypertension in endothelin B receptor-deficient rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 282:L703–12.
41. Kelland NF, et al. (2010) Endothelial ET B limits vascular remodelling and development of pulmonary hypertension during hypoxia. *J. Vasc. Res.* 47:16–22.
42. Savale L, et al. (2009) Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice. *Respir. Res.* 27:1–13.
43. Zhang Y, et al. (2010) Increased lung MIF expression in a hypoxia induced pulmonary hypertension mice model. *Am. J. Respir. Crit. Care Med.* 181:A6328.
44. Tuder RM, Voelkel NF. (1998) Pulmonary hypertension and inflammation. *J. Lab. Clin. Med.* 132:16–24.
45. Humbert M, et al. (1995) Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *Am. J. Respir. Crit. Care Med.* 151:1628–31.
46. Itoh T, et al. (2006) Increased plasma monocyte chemoattractant protein-1 level in idiopathic pulmonary arterial hypertension. *Respirology.* 11:158–63.
47. Sanchez O, et al. (2007) Role of endothelium-derived CC chemokine ligand 2 in idiopathic pulmonary arterial hypertension. *Am. J. Respir. Crit. Care Med.* 176:1041–7.
48. Schober A, Zernecke A. (2007) Chemokines in vascular remodeling. *Thromb. Haemost.* 97:730–7.
49. Dennis, KE, Aschner JL, Milatovic D. (2009) NADPH oxidases and reactive oxygen species at different stages of chronic hypoxia-induced pulmonary hypertension in newborn piglets. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 297:L596–607.
50. Oka M, Homma N, Taraseviciene-Stewart L. (2007) Rhokinase-mediated vasoconstriction is important in severe occlusive pulmonary arterial hypertension in rats. *Circ. Res.* 100:923–9.
51. Achcar ROD, Demura Y, Rai PR. (2006) Loss of caveolin and heme oxygenase expression in severe pulmonary hypertension. *Chest.* 129:696–705.
52. Macchia A, et al. (2007) A meta-analysis of trials of pulmonary hypertension: a clinical condition looking for drugs and research methodology. *Am. Heart J.* 153:1037.
53. Torres F. (2007) Systematic review of randomized, double-blind clinical trials of oral agents conducted in patients with pulmonary arterial hypertension. *Int. J. Clin. Pract.* 61:1756.