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 morbidity 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, prolonging 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-
particularly superoxide anion (O$_2^-$) (9,10). In humans, increased vascular O$_2^-$ production, 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$_2^-$-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)-κB activation (16). Because O$_2^-$ reacts rapidly with NO (−6.7 × 10^9 /mol/s) and EC-SOD catalyzes O$_2^-$ at less rapid rates, higher enzyme concentrations through overexpression may be necessary for rapid removal of excess O$_2^-$ produced during oxidative stress. Formation of peroxynitrite by O$_2^-$ represents another potential source of oxidant tissue injury in pulmonary endothelial cells. It may be that the ultimate effects of O$_2^-$ 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-κB 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 because 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 basic fibroblast factor, insulinlike growth factor, recombinant human basal fibroblast factor, insulin-like 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 for 24 h. After incubation, cells were transfected with human EC-SOD (hEC-SOD) cDNA and inserted into a vector (Invitrogen Life 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-dichlorodihydrofluorescein 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 multwell plates was quantified using a Cytofluor 2530 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 μm) 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 × wall thickness)/external diameter × 100% (24).

**Pulmonary Artery Function**

Physiological function assessments were performed on explanted main intralobar pulmonary arteries with intact endothelium. Pulmonary artery segments (~180 μm 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⁻⁵ 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⁻⁴ 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⁻⁴) 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⁻³). 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-
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 Ca2+ gradient. Increased Ca2+ level activates Ca2+-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 O2•−. Thus, we measured both XO concentration and O2•− 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 fluorescence over time. *Transfected cell line versus nontransfected cell line, P < 0.05.

Hypoxia In Vivo

Prophylactic study. In vivo, the XO concentration was significantly higher in WT adult mice after exposure to hypoxia (FiO2, 10% for 10 d) compared with normoxic groups (P < 0.05). 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).

Figure 1A. HPMECs nontransfected (N) and transfected (T) with EC-SOD after exposure to hypoxia for 24 h compared with a cell line incubated with FiO2 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.

Figure 1B. In Figure 1B, the mean ± standard error of the mean (SEM) of XO concentration is shown. The transfected cell line (T) shows a significant decrease in XO concentration compared to the nontransfected cell line (N) (P < 0.05). 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).

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Figure 1C. In Figure 1C, the mean ± standard error of the mean (SEM) of intracellular reactive species accumulation is shown. The transfected cell line (T) shows a significant decrease in intracellular reactive species accumulation compared to the nontransfected cell line (N) (P < 0.05). 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 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) (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 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 transfe...
plasmid and hEC-SOD gene and normoxic control groups ($P > 0.05$). (Figure 6C).

Immunostaining for $\alpha$-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. Although, 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 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 bluntly 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 transgenic 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)-1β, 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.

ACKNOWLEDGMENTS
This study was funded by the Department of Pediatrics at the NS-LIJ Health System.

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.

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