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Sustained Lung Activity of a Novel Chimeric Protein, SOD2/3, Following Intratracheal Administration

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Abstract

Delivery of recombinant superoxide dismutase to lung is limited by its short half life and poor tissue penetration. We hypothesized that a chimeric protein, SOD2/3, containing the enzymatic domain of manganese superoxide dismutase (SOD2) and the heparan binding domain of extracellular superoxide dismutase (SOD3) would allow for the delivery of more sustained lung and pulmonary vascular antioxidant activity compared to SOD2. We administered SOD2/3 to rats by intratracheal, (IT) intraperitoneal (IP), or intravenous (IV) routes and evaluated the presence, localization and activity of lung SOD2/3 1 day later using Western blot, immunohistochemistry and SOD activity gel. The effect of IT SOD2/3 on the pulmonary and systemic circulation was studied *in vivo* in chronically catheterized rats exposed to acute hypoxia. Active SOD2/3 was detected in lung 1 day following IT administration but not detected following IP or IV SOD2/3 administration or IT SOD2. The physiologic response to acute hypoxia, vasoconstriction in the pulmonary circulation and vasodilation in the systemic circulation, was enhanced in rats treated 1 day earlier with IT SOD2/3. These findings indicate that IT administration of SOD2/3 effectively delivers sustained enzyme activity to the lung as well as pulmonary circulation and has a longer tissue half-life compared to native SOD2. Further testing in models of chronic lung or pulmonary vascular diseases mediated by excess superoxide should consider the longer tissue half-life of SOD2/3 as well as its potential systemic vascular effects.

Keywords

superoxide dismutase; acute hypoxic pulmonary vasoconstriction; SOD2/3

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Introduction

Numerous studies have tested enzymatic and non-enzymatic antioxidant strategies to protect against oxidative stress in both *in vitro* and *in vivo* injury models, including lung and pulmonary vascular diseases. One therapeutic approach is to deliver the antioxidant enzyme superoxide dismutase (SOD), which will scavenge excess superoxide by catalyzing the dismutation of superoxide to hydrogen peroxide. Many studies have shown protective effects following the delivery of SOD, which can protect direct targets of superoxide as well as limit the inactivation of nitric oxide by superoxide, thus enhancing nitric oxide bioavailability. There has been considerable interest in developing delivery strategies to augment SOD activity to protect against superoxide-mediated toxicity in the lung and circulation. We are particularly interested in augmenting extracellular SOD activity, due to the accumulating evidence in multiple clinically relevant models of chronic lung and pulmonary vascular disease that there is a loss of extracellular SOD, and that over-expression of extracellular SOD protects against lung and vascular diseases [1–6].

The three known mammalian SOD isoforms have each been evaluated as therapeutic agents. Recombinant cytosolic Cu, Zn SOD (SOD1) and mitochondrial manganese SOD (SOD2) have been tested previously in models of lung injury. However, the utility of SOD1 was limited by a net negative precluding contact with the negatively charged cell surface and limited movement into the interstitium; a short plasma half-life of minutes with rapid renal elimination and a narrow therapeutic window [7–10]. While SOD2 has a modestly improved half life of 4 hours, the third isoform, a Cu, Zn containing extracellular SOD (SOD3) has a potential advantage over the SOD1 and SOD2 isoforms due to its heparan binding domain, which imparts a positive charge and enables it to bind to cell surfaces and extracellular matrices. A recent study showed that adenoviral delivery of SOD3 protected against monocrotaline-induced pulmonary hypertension (PHTN) [11]. However, recombinant SOD3 has proven difficult to express for high yield production. Thus, a chimeric protein was developed that combined the SOD2 enzymatic domain to the positively charged SOD3 heparan binding domain [12].

The resultant chimeric protein, SOD2/3, has been used in a series of acute injury models. SOD2/3 given as a single IV dose protected in several models of organ injury, including liver or mesenteric ischemic/reperfusion injury and LPS-induced pulmonary vascular leak [12–15]. These studies implicate a specific role for the heparan-binding domain in the SOD2/3 protein since human recombinant SOD2 at an equivalent dose did not confer protection. In addition, a wide range of SOD2/3 doses, from 0.1–24 U/gbw, provided protection, resulting in a more shallow dose response curve than those of SOD1 or SOD2. In this study we sought to establish the best route of delivery to the lung and determine the length of time a single dose of SOD2/3 would be detectable in the lung tissue. Our long-term goal will be to use this information to develop a rationale strategy to test SOD2/3 in chronic models of lung and pulmonary vascular injury.

We hypothesized that SOD2/3 would be optimally delivered to the lung via intratracheal (IT) administration and be active for a longer duration than SOD2. To test our hypothesis, we treated normal adult rats with similar doses of recombinant SOD2/3 or SOD2 by three different routes, intratracheal (IT), intravenous (IV), intraperitoneal (IP). We tested the appearance and activity of SOD2/3 in the lung by Western blot analysis, immunohistochemistry and an SOD activity gel. In addition, to demonstrate that SOD2/3 had a more sustained biologic activity than SOD2 in the pulmonary circulation *in vivo*, we used acute hypoxic pulmonary vasoconstriction as a physiologic endpoint to test SOD2/3 activity in the pulmonary circulation 1 day after IT delivery. The data presented in this manuscript provides a strong foundation for future experiments to test SOD2/3 in chronic

models of oxidant-mediated lung and pulmonary vascular injury using daily airway dosing and new insight into the impact of superoxide and SOD on vascular tone in acute hypoxia.

Materials and Methods

Rat Model

Experiments were performed in male Wistar-Kyoto rats (Harlan Sprague-Dawley Inc.), ranging from 8 – 14 weeks old at 200–300 gm body weight. Animals were allowed ad libitum access to food and water. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at University of Colorado, Denver, Anschutz Medical Campus.

SOD2/3 treatment strategy

To determine which route provided the best sustained lung delivery of SOD2/3, animals were treated with SOD2/3 at 6 units/gram body weight (U/gbw) by three different routes: (1) intravenous (IV), (2) intratracheal (IT), and (3) intraperitoneal (IP), and tissue was harvested 1 day later. To determine the kinetics of IT delivery, a single dose of IT SOD2/3 was administered and tissue harvested at 1, 3, and 5 days. The dose of SOD2/3 was extrapolated from published studies [12,14–18]. The study was performed with a dose of 6 U/gbw for SOD2/3 as well as for experiments with SOD2. The dose was selected as an intermediate dose based on literature that reported acute protection using between 0.1 – 24 U/gbw SOD2/3.

Tissue harvesting

Animals were euthanized by IP pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge IA) 0.2 mL followed by exsanguination. Lungs were flushed via puncture through right ventricle with 20 ml of cold saline. The right lung, heart, kidney, and liver were flash frozen and stored at -80°C until use. The left lung was inflation-fixed at 20 cm H_2O pressure with 4% paraformaldehyde for 15 min, dissected from the chest cavity and placed in 4% paraformaldehyde at 4°C overnight, transferred to 70% ethanol and paraffin embedded for immunohistochemistry.

Western blot

Tissues were homogenized in radioimmunoprecipitation (RIPA) buffer containing 150mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0 and protein concentration was measured by Precision Red (Cytoskeleton Inc, Denver, CO). 30ug protein was loaded on a 12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and separated by gel electrophoresis. Each gel contained a human recombinant (hr) SOD2 and SOD2/3 protein standard. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp, Bedford, MA). Blots were blocked with Superblock (Pierce Laboratories, Rockford, IL) for 5 min followed by incubation with primary antibody, a polyclonal rabbit anti-rat SOD2 (1:8000) (Upstate, Lake Placid, NY) or β -actin (1:1,000) (Sigma) in 5% milk protein in TBST overnight at 4°C . PVDF membrane was then probed with secondary antibody, a goat anti-rabbit HRP-conjugated IgG antibody (1:20,000) (Chemicon, USA). Blots were developed with Luminol Enhancer Solution (Thermo Scientific, Rockford, IL).

SOD activity gel

Tissues were homogenized in a 1mM EDTA potassium phosphate buffer (pH 6.8) then centrifuged at 4°C for 10 min. Samples were then mixed with a native sample buffer (62.5mM Tris-HCl, 40% glycerol, 0.01% bromophol blue at pH 6.8). Proteins were

separated by gel electrophoresis using a native polyacrylamide electrophoresis gel (12% Tris-HCL BioRad, Hercules, CA) with a native running buffer of tris-glycine (25mM Tris, 192mM glycine in dH₂O at pH 8.3) at 120V for 90 min.

The gel was then placed in a photocatalyzed riboflavin/EDTA superoxide- generating system along with nitroblue tetrazolium (NBT) to visualize SOD activity on the gel as previously described. [19]. Briefly, we mixed 40 mls of solution A [Solution A: 0.05mM Kphos buffer, pH 7.8 and 1 mM EDTA, 0.25mM NBT, and 1mM cyanide] to 2 mls of solution B [Solution B: riboflavin 0.05mM into dH₂O]. Cyanide in Solution A inhibited Cu, Zn SOD (SOD1 and SOD3) to enable a clearer image of SOD2 and SOD2/3. The gel was incubated in the dark for 20 mins. The gel was then removed from solution and placed under fluorescent lighting, 6 inches from source to produce superoxide. The reduction of NBT by superoxide stained the entire gel with a purple compound, formazan, except at the sites of SOD activity. SOD2/3 and hrSOD2 standards from 0.2–3.0 standard units were included on each gel to differentiate between SOD2/3, native rat SOD2 or hrSOD2. The gel was scanned and a reversed image presented in this paper.

Immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded lung sections were immunostained with the polyclonal rabbit anti-rat SOD2 antibody (1:1000) (Upstate, Lake Placid, NY). The low SOD2 antibody concentration was selected to detect exogenous SOD2/3 in the lung but being too low a concentration to detect endogenous SOD2. Lung sections were deparaffinized, rehydrated, and unmasked with Trilogy (Cell Marque, Rocklin, CA) for 25 min in a pressure cooker at high setting per manufacturer's protocol (Cell Marque). The slides were blocked with 2.5% horse serum and incubated with the SOD2 antibody at 1:1000 in block for 1 hour at room temperature, followed by the secondary antibody and ABC reagent in the RTU Universal Elite ABC Kit per the manufacturer's protocol (Vector Laboratories, Burlingame, CA). The slides were then rinsed and developed with ImmPact DAB diluent for 30 sec (Vector Laboratories) and counterstained for 15 sec with hematoxylin. Slides were dehydrated with ethanol followed by citrosolve (Fisher Scientific, Pittsburgh, PA) before placement of coverslips. Tissue sections were examined by light microscopy (Carl Zeiss International, Axiovert S100, Thornwood, NY) and photographed using a final magnification of X100.

RNA isolation and real-time PCR

RNA was isolated from flash-frozen lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and analyzed by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA) to determine concentration. RNA was reverse transcribed with the Maxima® First Strand cDNA Synthesis Kit. (Fermentas International Inc, Glen Burnie, MD) PCR was performed on the MyiQ Detection System (Bio-Rad, Hercules, CA) with the RT² Real-Time™ SYBR Green/Fluorescein PCR master mix (SABiosciences, Frederick, MD). Reactions were run in triplicate and results were analyzed with Bio-Rad iQ5 Standard Edition optical system software 2.1.97.1001. Gene copy numbers were normalized to HPRT. Primers were designed with NCBI Primer-BLAST software. SOD2 forward: CGCTGGCCAAGGGAGATGTT SOD2 reverse: TCCACCACCCTTAGGGCTCA HPRT forward: TCCTCCGCCAGCTTCCTCCT HPRT reverse: TCACTAATCACGACGCTGGGA.

Hemodynamic monitoring

Animals were weighed, anesthetized with IM ketamine: xylazine; (75mg/kg: 6 mg/kg), and then administered either saline, SOD2/3, or SOD2 by IT or IP route on the day of surgical catheter insertion. Under aseptic conditions, vessels in the neck were surgically exposed and

the right internal jugular was cannulated with a PV-1 catheter (Becton Dickinson, Franklin Lakes, NJ) [20]. The catheter was inserted into the right ventricle and guided into main pulmonary artery for mean pulmonary arterial pressure measurements (MPAP). A PE-50 catheter (Becton Dickinson) was placed in the superior vena cava for drug administration and central venous pressure measurements. A second PE-50 catheter was placed in the right carotid artery for cardiac output (CO) and mean systemic blood pressure (MSBP) measurements. Pressure tracings confirmed placement in the pulmonary artery and carotid artery. All catheters were then flushed with heparinized saline, tied off, tunneled subcutaneously to dorsal neck region, and exteriorized at back of neck. Caps were placed to protect the catheters. Animals were allowed to recovery for 1 day before hemodynamic monitoring. Catheter placement was reconfirmed at time of hemodynamic measurements. For experiments with an acute treatment, catheters were placed 1 day ahead of time and saline, SOD2/3, or SOD2 were delivered via the IV route into the carotid arterial catheter 30 mins before hemodynamic measuring.

Acute hypoxic pulmonary vasoconstriction

Rats were exposed to acute hypoxia to produce pulmonary vasoconstriction, a well-characterized and reproducible physiologic response which functions in the normal lung to divert blood from poorly ventilated regions to improve ventilation/perfusion matching. Rats were placed in custom-designed Plexiglas chambers with a portal through which catheters were passed and a gas source to control oxygen tension. Catheters were flushed with normal saline then connected to fluid filled pressure transducers. Initial measurements were taken over 10 minutes in normoxia at Denver altitude (5280 ft). Rats were exposed to hypoxia for 15 minutes by flushing the chamber continuously with a hypoxic gas mixture (10% oxygen). MPAP and MSBP were recorded every minute for the experimental period. Cardiac output was measured in triplicate first in normoxia and then after 15 mins of hypoxia using Cardiogreen dye-dilution method (Sigma Aldrich) as previously described [20].

Statistical analysis

Data are expressed as mean $\pm$ SE. For hemodynamic data, a two-way ANOVA with repeated measures (MPAP and MSBP) or one-way ANOVA (CO) followed by Bonferroni post hoc analysis were performed using Prism software (GraphPad, CA). Statistical significance was defined as $p < 0.05$.

Results

SOD2/3 was present and active in lung homogenates 1 day after a single IT administration of 6U/gbw SOD2/3

Though IV SOD2/3 has been shown to protect acutely in a model of lung injury, it is not known if SOD2/3 remains in the lung for extended periods of time to justify its use in chronic models of injury. Furthermore, the efficacy of different routes of administration for lung delivery has not been tested. We therefore first tested whether the half-life of SOD2/3 in the lung was longer than SOD2 and evaluated the best route for lung delivery. Figure 1A shows a representative Western blot probed with the anti-SOD2 antibody, which differentiates the chimeric protein SOD2/3 from the native rat (r) or human recombinant (hr) SOD2 based on its higher molecular weight. SOD2/3 was detected in the lung by Western blot 1 day following IT delivery, but not IP or IV administration. The blot included lung homogenates from sham rats (Sh) receiving IT phosphate buffered saline (PBS) and from rats receiving 6 U/gbw of IT SOD2. SOD2/3 activity in the lung was evaluated by an SOD activity gel, which separates the isoforms of the enzyme by both size and charge (Figure 1B). SOD2/3 and hrSOD2 standards demonstrate the ability to differentiate not only SOD2/3 activity from hrSOD2, but also native rSOD2 from hrSOD2. SOD2/3 activity was

detected 1 day after treatment in lung samples from rats receiving IT SOD2/3 but not in rats that received IV or IP SOD2/3. Sham treated rats served as a positive control for native rat SOD2 and did not contain SOD2/3 activity. IT hrSOD2 activity was not detected in lung samples 1 day after treatment. Both the Western blot and activity gel were repeated with at least 3 different rats for each treatment group with similar findings. Though IT SOD2/3 was present and active in lung tissue at 1 day following a dose of 6 U/gbw (or 2 µg/gbw), it was not detected by Western blot or SOD activity gel at 3 or 5 days post IT treatment. (Online Supplement Figure S1) SOD 2/3 was not detectable in the heart, lung, or kidney 1 day following IT SOD2/3 administration (Online Supplement Figure S2). Though the density of the native rat SOD2 band was equal in density to the band from SOD2 treated lungs, administration of IT SOD2/3 appeared to increase the SOD2 band. Uneven protein loading in the blots did not account for the increased SOD2 band in the IT SOD2/3 treated lungs, as shown by equal β -actin expression. (Figure 1A) Furthermore, SOD2/3 did not upregulate endogenous SOD2 mRNA, as demonstrated by similar SOD2 mRNA expression in lungs of sham and IT SOD2/3 treated rats. ($p > 0.1$ by one-way ANOVA, $n=3$) (Figure 1C)

SOD2/3 was immunolocalized to airway epithelium and alveoli as well as pulmonary artery endothelium following IT administration

We performed immunohistochemistry with a SOD2 antibody to identify the site of SOD2/3 deposition in the lung. Diffuse brown staining was easily observed within the airway epithelium and alveoli in rat lungs one day post IT SOD 2/3 treatment. There was intermittent staining observed on the endothelial surface of medium sized pulmonary arteries, as shown in the enlarged vessel in the bottom left corner of Figure 2A, suggesting SOD2/3 delivered to the airway could reach the circulation. (Fig 2A). IHC staining 1 day following IP administration of SOD2/3 (Fig 2B) or IT administration of SOD2 (Fig 2C) was absent or very faint compared to parallel slides for IT SOD2/3. In the IP treated lungs, the faint staining was localized to inflammatory cells and in the adventitia, where the heparan-binding domain of SOD3 is known to bind avidly to matrix proteins, but not in the alveoli or vascular endothelium. Using this low antibody concentration, we did not detect any notable staining of native rat SOD2, as shown by the lack of brown DAB staining in a sham lung (Fig 2D). These findings were reproduced in at least 3 different rats from each group.

The ability of IT SOD2/3 to impact pulmonary vascular function *in vivo* was confirmed by measuring hemodynamic parameters in chronically catheterized rats upon exposure to acute hypoxia

Acute hypoxic pulmonary vasoconstriction is a reproducible pulmonary vascular response that, although complex and not fully understood, can be modulated by $O_2^{\bullet-}$ and SOD [21–23]. Furthermore, accumulating data implicate reactive oxygen species (ROS) in the pathogenesis of pulmonary hypertension, a chronic and highly lethal disease. Therefore, we were particularly interested in testing whether IT delivery of SOD2/3, which deposited in both the airways and in the vessel by immunohistochemistry (Figure 2), would modulate pulmonary vascular tone. We tested the effect of SOD2/3 on an acute hypoxic challenge in live catheterized rats following IT SOD2/3 treatment compared to sham, IP SOD2/3 or IT SOD2 treated rats. MPAP was measured at baseline with room air breathing and for 15 minutes following the onset of hypoxic breathing (10% oxygen). IT SOD2/3 (solid triangle, $n=10$) given 1 day prior to hemodynamic monitoring significantly increased MPAP in response to acute hypoxia when compared to Sham, with no difference in baseline MPAP (solid square, $n=10$). In contrast to IT SOD2/3, IP SOD2/3 (grey triangle, $n=6$) had no effect on hypoxic pulmonary vasoconstriction. (Fig 3A). To confirm that SOD2/3 provided a more sustained biologic activity in the lung than SOD2, the response to acute hypoxia was compared between IT SOD2/3 and IT SOD2, both dosed at 6 U/gbw given 1 day prior to

hemodynamic testing. IT SOD2 (open triangle, n=7) did not augment acute hypoxic pulmonary vasoconstriction (Fig 3B).

We also found that IT SOD2/3 altered the systemic vascular response to hypoxia, despite our inability to detect the drug in other organs by Western blot or SOD activity gel 1 day after administration by any route. It is well established that the systemic circulation, in contrast to the pulmonary circulation, vasodilates in response to hypoxia. Consistent with this, we measured a decrease in MSBP following hypoxia in sham treated rats (solid square, n=6). Rats treated 1 day earlier with IT SOD2/3 (solid triangle, n=9) showed enhanced vasorelaxation in response to hypoxia compared to sham (Fig 3C). The MSBP following treatment with IP SOD2/3 was similar to sham treated rats and cardiac outputs (CO) measured in normoxia and 15 minutes after the onset of hypoxia were similar between all seven groups of rats (data not shown).

In another series of experiments, IV SOD2/3 was given 30 minutes prior to hypoxia. This IV dosing strategy has been used in several published studies of acute injury models to deliver SOD2/3 to the lung and other organs [12,15,16] and enabled us to address several additional key points. We first confirmed that active SOD2/3 delivered by the IV route (solid circle, n=4) also enhanced acute hypoxic pulmonary vasoconstriction, similar to IT SOD2/3 given 1 day earlier. (Figure 4A). There was no difference in sham rats that received IV PBS 30 minutes before hypoxia compared to IT PBS 1 day earlier. (data not shown).

To determine if the C-terminus heparan-binding domain of SOD3 impacts the effect of SOD2/3 on hypoxic pulmonary vasoconstriction, the experiments were repeated with the same dose of IV SOD2 also given 30 minutes before hypoxia. IV hrSOD2 (open circle, n=4) augmented HPV compared to sham treated rats (solid square), but to a lesser extent than IV SOD2/3 (Fig 4B). We also observed enhanced hypoxic systemic vasodilation with both IV administration of SOD2/3 (solid circle, n=4) and SOD2 (open circle, n=4) (Figure 4C).

To test the contribution of hydrogen peroxide, IV catalase was given concomitantly with IV SOD2/3 30 minutes prior to hypoxia. The addition of IV catalase (CAT/SOD, grey diamond, n=4) to IV SOD2/3 fully reversed the augmented pulmonary vascular response to acute hypoxia. The addition of catalase to IV SOD2/3 did not abolish acute hypoxic vasoconstriction, but instead was similar to the response seen in sham treated rats (Figure 5A). Catalase did not reverse the effect of IT SOD2/3 on the vascular tone with hypoxia in the systemic circulation. Instead, hypoxic vasodilation was enhanced by catalase in rats pretreated with SOD2/3. (Figure 5B)

Discussion

Increased production of reactive oxygen species (ROS) contributes to the pathogenesis of numerous lung and pulmonary vascular diseases. Current antioxidant strategies are limited in part by the difficulty in directing the agents to the site of injury for a sufficient period of time. In this study, we hypothesized that treatment with the chimeric protein SOD2/3, due to the presence of the positively charged SOD3 heparan-binding domain, would allow for a longer half-life of the antioxidant in the lung and pulmonary circulation compared to the native recombinant SOD2 protein. We report that intratracheal administration was the most effective route to deliver SOD2/3 to the lung and led to more sustained SOD activity in the lung and pulmonary vasculature compared to SOD2. We then confirmed the longer drug half-life with SOD2/3 compared to SOD2 following IT administration in an *in vivo* model of acute hypoxia. The data presented in this manuscript provides a solid foundation to base future experiments using daily airway dosing to test the impact of SOD2/3 in chronic models of oxidant-mediated lung or pulmonary vascular injury.

There is a strong rationale to develop pharmacologic agents like SOD2/3 that possess similar properties to SOD3. Studies from our laboratory and others have found that endogenous SOD3 expression in the lung can be decreased in response to injury, while mice overexpressing SOD3 are protected in models of lung and pulmonary vascular disease due to hyperoxia, hypoxia, asbestos, virus and LPS [1–6]. SOD2/3 was initially engineered to address limitations of SOD1 and SOD2 and difficulty producing high quantities of recombinant SOD3. SOD1 is negatively charged, which renders it unable to bind effectively to negatively charged cell surfaces and limits its ability to penetrate the interstitial space. It also has a short (6 – 15 minute) plasma half-life. Though SOD2 is larger than SOD1, it is slightly positively charged under physiologic conditions and equilibrates more quickly with interstitial fluid, but it still only has a half-life of several hours, and like SOD1, has a narrow therapeutic window. SOD2/3 has the advantage of having a small size, similar to SOD2, and carrying the heparan-binding domain of SOD3, which imparts a greater positive charge and improves rate of extravasation into the tissues. [12] Alternative strategies that have been tested include SOD3 adenoviral vectors, liposomes or nanoparticles containing SOD, and SOD conjugated to the endothelial cell-specific PECAM antibody. [11,24,25] SOD2/3 has been used in at least 8 different acute *in vivo* injury models with a wide variety of end points to assess oxidative stress-dependent injury. The degrees of protection observed have ranged from 62 to 98%. [12,15,18]. Our study extends prior work with this chimeric protein to evaluate the ideal route of delivery to the lung and interrogate whether the advantageous biochemical properties in fact led to a longer biologic activity in the lung and pulmonary circulation.

Intratracheal administration of SOD2/3, which provided the optimal lung delivery when compared to other routes of administration, had a more sustained presence and activity in the lung compared to an equivalent dose of intratracheal hrSOD2. This observation was consistent both in the Western blot analysis and SOD activity assay with lung tissue as well as in the *in vivo* studies showing an impact of IT SOD2/3 on acute hypoxic pulmonary vasoconstriction. It was not unexpected that direct intratracheal administration to the lung provided the highest lung drug delivery compared to other routes of delivery. IV administration can deliver SOD2/3 to the lung, as documented in published studies and our *in vivo* experiments in which acute IV delivery of SOD2/3 produced a similar physiologic effect on acute hypoxic pulmonary vasoconstriction as IT SOD2/3. This suggests that either the increased volume of distribution of SOD2/3 following systemic administration led to lower amounts of drug in the lung, or the half life was less when given IV or IP. If the SOD3 tail was cleaved from some of the protein in the circulation, it could have been cleared more rapidly by the kidney. We note that on Western blot, the SOD2 band in lungs of rats treated with IT SOD2/3 was increased compared to SOD2 protein content in the other groups. Since we found no evidence to support an effect of SOD2/3 on SOD2 mRNA expression, we propose that chimeric protein, similar to native SOD3, is susceptible to proteolytic cleavage of the heparan binding domain and the increased band is due to cleaved SOD2/3, which essentially becomes SOD2 (with perhaps a few additional C-terminal amino acid residues). The experiments in which SOD2/3 and SOD2 were given IV immediately before the hypoxic challenge confirmed that the effects of SOD activity were similar regardless of the route of delivery, and the presence of the heparan binding domain in SOD2/3 compared to native SOD2 leads to a more robust effect on the pulmonary circulation than SOD2, despite similar enzyme activity levels. These data provide important new information demonstrating improved drug half-life in the lung using the chimeric recombinant protein, SOD2/3 compared to SOD2.

The ability of SOD2/3 delivered to the airway to penetrate the lung tissue and reach the pulmonary circulation, our second key finding, was demonstrated by both immunohistochemical analysis and the *in vivo* hemodynamic studies. Though we could not

detect SOD2/3 in other organs after IT administration, it is possible that administration of SOD2/3 by IT, IV or IP routes could impact the function of other organs. In fact, despite lack of detection in systemic organs, we could demonstrate enhanced systemic vasodilation following IT SOD2/3. SOD2/3 may actually have a therapeutic advantage over SOD3, as SOD2/3 has a slightly weaker heparin affinity compared to SOD3, which, together with its smaller size and more positive net charge, may improve mobility of the enzyme into tissue. Decreased heparin affinity of SOD3 is seen in humans with a common R213G mutation in the SOD3 heparan-binding domain, and is known to protect against certain diseases such as chronic obstructive pulmonary disease and acute lung injury [26,27]. The observation that SOD2/3 was localized to the pulmonary artery endothelium, and our laboratory's interest in pulmonary vascular disease, had provided the rationale for the use of acute hypoxic pulmonary vasoconstriction as an *in vivo* model to confirm SOD2/3 activity 1 day after IT administration.

Though our *in vivo* experiments were designed simply to confirm the presence of SOD2/3 activity in the pulmonary circulation 1 day after IT delivery, we do report intriguing results on the effects of the SOD activity on acute hypoxic responses in the pulmonary and systemic circulation in live, awake and non-diseased animals. In this study, using normal rats and focusing on the immediate pulmonary vascular response, we observed an augmented pulmonary vascular response to acute hypoxia with active SOD, regardless of whether we delivered it one day earlier as SOD2/3 via the airway or acutely as SOD2/3 or SOD2 as an intravenous injection. This finding is consistent with an earlier observation by S. Archer et al, who demonstrated that treatment with IV SOD1 in isolated perfused lung from normal rats increased pulmonary artery pressure [28]. Thus SOD1 and SOD2 activity produce the same pulmonary vascular response to hypoxia under normal conditions despite the known weak peroxidase activity of SOD1. [29] Subsequently, Yamaguchi et al also found that SOD increased acute hypoxic pulmonary vasoconstriction in isolated perfused rabbit lungs in the presence of ROS generated by exogenous xanthine oxidase [30]. Weissman et al reported that inhibition of SOD suppressed acute hypoxic pulmonary vasoconstriction over a 180 minute period in isolated rabbit lungs, consistent with our findings, though they did not observe augmented acute hypoxic pulmonary vasoconstriction with excess SOD. [23]. In contrast, a number of studies performed in disease models of chronic pulmonary hypertension, such as the ductal closure in preterm lambs, group B streptococcal- induced pulmonary hypertension in newborn lambs, or monocrotaline-induced pulmonary hypertension have found that SOD treatment did not alter, or even blunted vasoconstriction [1,31,32]. Other published studies are consistent with our observation that in the systemic circulation, SOD activity can enhance vasodilation. For example, the PECAM-1 antibody conjugated to SOD lowered systemic blood pressure in a model of angiotensin-mediated systemic hypertension. [24] These data support the premise that it is necessary to understand the impact of specific ROS under different physiologic or pathophysiologic conditions and in specific vascular beds to establish the appropriate antioxidant strategies. For instance, though we report in this study that SOD2/3 increased hypoxic pulmonary vasoconstriction in the healthy rat, it is not known if SOD2/3 will lower pulmonary artery pressures in models of pulmonary hypertension similar to the studies using other forms of SOD or further augment pulmonary hypertension. It will thus be essential to consider these possibilities when testing its effect in pulmonary hypertension.

To further illustrate the complexity of the effect of ROS on the physiologic responses, the addition of catalase in our study produced results in the pulmonary and systemic circulation that were very reproducible though difficult to fully interpret. We observed that SOD activity augmented the normal physiologic response to hypoxia in both vascular beds, ie, vasoconstriction in the pulmonary circulation and vasodilation in the systemic circulation. In contrast, the addition of catalase lowered tone in both vascular beds. SOD will limit

reactions of superoxide but would only change the steady state concentration of hydrogen peroxide to a limited extent under very unusual conditions (viz., in the presence of a high concentration of a substance that is avidly reduced by superoxide) [33]. Wolin and Burke have reported that catalase in the presence of its substrate activates soluble guanylate cyclase via a mechanism that may involve the catalase intermediate Compound I. [34] Elevation of cyclic GMP would lead to vasodilation, opposing the SOD2/3 augmented vasoconstriction observed in the pulmonary circulation. It is therefore most likely that SOD2/3 did not augment hypoxia induced responses in the pulmonary or systemic circulation through an increase in hydrogen peroxide, but instead that the effects we observed with SOD2/3 and catalase were due to their effect on different targets in the circulation. The ultimate impact of ROS on vascular tone is determined by the local antioxidant concentrations. When airway delivery of SOD2/3 is tested in chronic models of lung or pulmonary vascular disease, it will be essential to consider for the particular model of injury the contribution of superoxide and hydrogen peroxide in disease pathogenesis as well as the potential for SOD2/3 to increase pulmonary vascular tone. The beneficial effects of SOD2/3 in a variety of acute injuries and its longer half life in the lung compared to native SOD2 makes it an excellent candidate for future studies of chronic lung and potentially pulmonary vascular diseases.

In summary, the overall purpose of this study was to determine whether the novel chimeric antioxidant SOD2/3 could deliver more sustained enzymatic activity to the lung when compared to recombinant native SOD2. We found that intratracheal delivery of SOD2/3 had a longer tissue half life than SOD2, penetrated the lung to distribute both to the alveolar epithelium and pulmonary vascular endothelial cells, and had a more potent in vivo effect on the pulmonary circulation than SOD2 because of its heparan-binding domain. This provides a strong foundation to further explore SOD2/3 as an antioxidant strategy in chronic models of lung and pulmonary vascular injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Active SOD2/3 was detected in lung tissue 1 day following intratracheal (IT) administration but not intravenous (IV) or intraperitoneal (IP) administration

A. The presence of SOD2/3 in lung tissue 1 day following administration was determined by Western blot analysis. Lanes 1–5 are lung homogenates as follows: IT PBS (Sham), IT human recombinant SOD2 (hrSOD2), or SOD2/3 given either by the IT, IP, or IV route of delivery 1 day prior to harvest. The gel was stripped and reprobed with β -actin to confirm equal protein loading. **B.** The presence of active SOD2/3 in lung tissue was detected by an SOD activity gel following cyanide-inhibition of SOD1. Lane 1 contains two standards, hrSOD2 and SOD2/3. Lanes 2–6 are lung homogenates from sham, IT SOD2, or SOD2/3 by the IV, IP, or IT route of delivery 1 day prior to harvest. Selected lanes from a single gel are presented. A reverse black and white image is shown. **C.** Lung SOD2 mRNA expression,

expressed as copies of SOD2 relative to housekeeping gene HPRT by real time RT-PCR, was not increased by SOD2/3.

Figure 2. Localization of SOD2/3 to the airway, alveoli, and vasculature

IT or IP SOD2/3, IT SOD2, or PBS were given 1 day prior to tissue harvesting.

Immunohistochemistry was performed with a low concentration of SOD2 antibody (1:1000) to identify SOD2/3 in the lung with negligible staining of endogenous SOD2. **A.** IT SOD2/3. **B.** IP SOD2/3. **C.** IT SOD2. **D.** IT PBS (Sham). The arrow in Panel A identifies positive staining on the endothelial surface of a medium sized pulmonary artery. A magnification of this pulmonary artery is shown in the bottom left corner of Figure 2A. Minimal staining is apparent in IP SOD2/3 treated lung (B). The lack of staining in the IT SOD2 treated (C) or Sham (D) treated lung indicates that the positive staining is due to SOD2/3 and not endogenous or delivered SOD2. Bar = 50 microns.

Figure 3. IT SOD2/3 augmented the physiologic response upon exposure to acute hypoxia in the pulmonary and systemic circulation *in vivo* in chronically catheterized rats

IT SOD2/3, IP SOD2/3, IT SOD2, and IT PBS were delivered 1 day prior to hemodynamic monitoring. 6 U/gbw of each preparation of SOD was administered per identified route. Mean pulmonary arterial pressure (MPAP, mmHg) and mean systemic arterial pressure (MSAP, mmHg) monitoring was initiated in room air and recorded every minute for 15 minutes upon initiation of hypoxia (10% oxygen). **A.** MPAP in acute hypoxia 1 day after IT SOD2/3 (solid triangle), IP SOD2/3 (grey triangle) or Sham (PBS) treatment (solid square). **B.** MPAP in acute hypoxia 1 day after IT SOD2/3, IT SOD2 (open triangle) or Sham (PBS) treatment. **C.** MSBP in acute hypoxia 1 day after IT SOD2/3, * $p < 0.05$ by two way ANOVA vs Sham rats.

Figure 4. Acute intravenous treatment with SOD2/3 augmented the vascular response to acute hypoxia, similar to intratracheal delivery and the presence of the heparan-binding domain enhanced the hypoxic response compared to native SOD2

A. MPAP in acute hypoxia 30 minutes after IV SOD2/3 (solid circle) compared to SOD2/3 given IT (solid triangle) 1 day earlier. **B.** MPAP in acute hypoxia 30 minutes after IV SOD2/3 compared to IV SOD2 (open circle). **C.** MSBP in acute hypoxia 30 minutes after IV SOD2/3 compared to IV SOD2. * $p < 0.05$ by two way ANOVA vs Sham rats (solid square). # $p < 0.05$ by two way ANOVA vs IV SOD2/3.

Figure 5. IV Catalase prevented additional hypoxic vasoconstriction by IV SOD2/3 in the pulmonary circulation but augmented vasodilation in the systemic circulation in response to acute hypoxia

A. MPAP in acute hypoxia 30 minutes following pretreatment with IV SOD2/3 (solid circle), IV SOD2/3 + IV catalase (CAT/SOD, grey diamond), or PBS (Sham)(solid square).

B. MSBP in acute hypoxia 30 minutes following pretreatment with IV SOD2/3 (solid circle), IV SOD2/3 + IV catalase (CAT/SOD, grey diamond), or PBS (Sham)(solid square).

* $p < 0.05$ by two way ANOVA vs Sham rats. # $p < 0.05$ by two way ANOVA vs IV SOD2/3.