Superoxide Dismutase 3 R213G Single-Nucleotide Polymorphism Blocks Murine Bleomycin-Induced Fibrosis and Promotes Resolution of Inflammation

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Abstract

Loss of extracellular superoxide dismutase 3 (SOD3) contributes to inflammatory and fibrotic lung diseases. The human SOD3 R213G polymorphism decreases matrix binding, redistributing SOD3 from the lung to extracellular fluids, and protects against LPS-induced alveolar inflammation. We used R213G mice expressing a naturally occurring single-nucleotide polymorphism, rs1799895, within the heparin-binding domain of SOD3, which results in an amino acid substitution at position 213 to test the hypothesis that the redistribution of SOD3 into the extracellular fluids would impart protection against bleomycin-induced lung fibrosis and secondary pulmonary hypertension (PH). In R213G mice, SOD3 content and activity was increased in extracellular fluids and decreased in lung at baseline, with greater increases in bronchoalveolar lavage fluid (BALF) SOD3 compared with wild-type mice 3 days after bleomycin. R213G mice developed less fibrosis based on pulmonary mechanics, fibrosis scoring, collagen quantification, and gene expression at 21 days, and less PH by right ventricular systolic pressure and pulmonary arteriole medial wall thickening at 28 days. In wild-type mice, macrophages, lymphocytes, neutrophils, proinflammatory cytokines, and protein increased in BALF on Day 7 and/or 21. In R213G mice, total BALF cell counts increased on Day 7 but resolved by 21 days. At 1 or 3 days, BALF pro- and antiinflammatory cytokines

and BALF protein were higher in R213G mice, resolving by 21 days. We conclude that the redistribution of SOD3 as a result of the R213G single-nucleotide polymorphism protects mice from bleomycin-induced fibrosis and secondary PH by improved resolution of alveolar inflammation.

Keywords: bleomycin; fibrosis; superoxide dismutase 3; inflammation; pulmonary hypertension

Clinical Relevance

R213G mice express a naturally occurring human single-nucleotide polymorphism in extracellular superoxide dismutase 3 (SOD3) that causes redistribution of SOD3 from tissues to extracellular fluids. This single-nucleotide polymorphism is protective against chronic obstructive pulmonary disease in humans. We report that R213G is protective against bleomycin-induced fibrosis and pulmonary hypertension by improving resolution of alveolar inflammation in mice. These findings further elucidate how SOD3 distribution impacts inflammation and progression of lung diseases like idiopathic pulmonary fibrosis and pulmonary hypertension.

(Received in original form May 13, 2016; accepted in final form October 13, 2016)

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This work was supported by National Institutes of Health grants T32 HL007171 (G.C.M.), R01HL086880 (E.N.-G.), R01HL111288 (R.P.B.), and P01 HL014985 (K.R.S.).

Author Contributions: Conception and design—G.C.M., R.G., S.P., K.E.K., K.R.S., R.P.B., and E.N.-G.; analysis and interpretation—G.C.M., R.G., S.P., K.E.K., B.H., L.H.-L., K.R.S., R.P.B., and E.N.-G.; drafting the manuscript for important intellectual content—G.C.M., R.G., S.P., K.E.K., K.R.S., R.P.B., and E.N.-G.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 56, Iss 3, pp 362–371, Mar 2017

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Originally Published in Press as DOI: 10.1165/rcmb.2016-0153OC on November 2, 2016

Internet address: www.atsjournals.org

Idiopathic pulmonary fibrosis (IPF) is an incurable and fatal disease in humans (1), characterized by significant lung fibrosis and lung oxidative stress (2, 3). Patients with IPF are also at risk of developing secondary pulmonary hypertension (PH), which worsens outcomes (4). Although the etiology of IPF is incompletely understood, there is strong evidence that lung inflammation, together with loss of antioxidant capacity, contributes to the development of IPF (1, 2).

Extracellular superoxide dismutase 3 (SOD3) is the only extracellular enzymatic defense against the free radical, superoxide. Impaired SOD3 activity is implicated in inflammatory and fibrotic lung and vascular diseases (5–12). For example, SOD3 content is decreased in fibrotic foci within the lungs of humans with IPF (13). Moreover, in rodent models of IPF, mice lacking SOD3 develop exacerbated bleomycin-induced inflammation and fibrosis (6), whereas mice overexpressing SOD3 are protected against bleomycin-induced fibrosis (8).

SOD3 is highly expressed in the lung and vasculature. It retains localization to the lung and the extracellular matrix via its positively charged heparin-binding domain (HBD) within the C terminus of the enzyme (14). A single-nucleotide polymorphism (SNP), rs1799895, identified within the HBD of SOD3 in humans results in an amino acid substitution at position 213 (R213G) with a frequency of 4–6% in Asian populations (15) and 2–3% in European populations (16). The R213G SNP lowers the HBD binding capacity of SOD3 to the extracellular matrix, causing redistribution of SOD3 from lung tissue into extracellular fluids, including the alveolar epithelial lining fluid and plasma. Although SOD3 is redistributed due to the SNP, the SNP does not alter activity (11). In humans, although the redistribution of SOD3 is associated with increased risk for cardiovascular disease, it is conversely associated with decreased risk for lung disease (chronic obstructive pulmonary disease [COPD]) (17).

We recently demonstrated that mice harboring the human SOD3 R213G SNP had less intratracheal LPS-induced alveolar inflammation, but worse chronic hypoxic PH (11), highlighting the importance of SOD3 localization in response to different lung and cardiovascular diseases. Although published data indicate that insufficient SOD3 promotes the development of

inflammation and fibrosis in the pathogenesis of IPF, it remains unclear if and how the redistribution of SOD3 from the lung tissue to extracellular fluids affects the progression of IPF. The intratracheal bleomycin model and associated pathogenesis is well established, and is characterized by an early airway inflammation that, if ameliorated, prevents the secondary development of lung fibrosis (Days 14–28). Therefore, using R213G mice, we tested the hypothesis that alveolar inflammation measured in the lavage fluid would be attenuated in R213G mice after intratracheal bleomycin, similar to the protection observed after intratracheal LPS. Furthermore, we hypothesize that amelioration of early alveolar inflammation will interrupt the pathogenesis of intratracheal bleomycin, and therefore protect against bleomycin-induced pulmonary fibrosis compared with wild-type (WT) mice. We provide compelling new data that the R213G SNP protects mice from bleomycin-induced fibrosis as well as PH, which may be driven by enhanced resolution of alveolar inflammation.

Materials and Methods

Mouse Model

Animal studies were approved by the University of Colorado Denver (Aurora, CO) and National Jewish Health (Denver, CO) Institutional Animal Care and Use Committees. Genetically engineered mice homozygous for the R213G SNP in SOD3 on the C57BL/6 mouse background (R213G) (11) and C57BL/6 WT mice were treated with a single dose of intratracheal bleomycin (100 μ l at 1 U/ml) or PBS, and killed on Days 1, 3, 7, 21, or 28.

Pulmonary Mechanics and Pulmonary Hemodynamics Assessment

Pulmonary mechanics were tested 21 days after treatment. Mice were killed with Fatal-Plus (0.1 ml/lb of body weight; Dearborn, MI). Static lung compliance and elastance were measured with a flexiVent (Scireq, Montreal, PQ, Canada) in tracheostomized mice. PH was evaluated at 28 days after treatment. Mice were anesthetized with 1.5% isoflurane. Right ventricular (RV) pressures were measured by direct RV puncture, as described previously (11).

Bronchoalveolar Lavage Fluid and Lung Tissue Processing

Bronchoalveolar lavage fluid (BALF), lung, and heart were processed as described in the online supplement.

Western Blot Analysis

Western blots were probed with anti-mouse antibodies against SOD1 (1:1,000; Abcam, Cambridge, MA), SOD2 (1:1,000; Millipore, Billerica, MA), SOD3 (1:1,000; Santa Cruz, Dallas, TX), and β -actin (1:1,000; Sigma, St. Louis, MO), as previously described (12).

SOD Activity Assay

SOD3 activity was measured in cell-free BALF, plasma, or lung protein using a SOD assay kit-WST after glycoprotein isolation using concavalin A beads to separate SOD3 from the intracellular SODs (Dojindo Molecular Technologies, Santa Clara, CA; see the online supplement for details).

Glutathione Assay

Reduced glutathione (GSH) and total glutathione levels were measured in lung tissue using a glutathione assay kit (Cayman Chemicals, Ann Arbor, MI; see the online supplement for details).

Collagen Assay

Total collagen was measured in lung tissue using a Sirius red assay kit (Chondrex, Redmond, WA).

Trichrome Staining and Immunohistochemistry

Lung sections stained with Masson's trichrome (Sigma) were imaged using a ScanScope Microscope (Aperio, Buffalo Grove, IL), and fibrosis was scored by a single, blinded investigator, similar to previous reports (18). Lung sections were costained with α -smooth muscle actin (1:1,500; Sigma) and Factor VIII (1:1,500; Sigma) antibodies and pulmonary arteries less than 200 μ m in diameter and adjacent to a terminal bronchiole were analyzed for medial wall thickness (MWT), as previously described (8).

Cytokine Analysis

Cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, keratinocyte chemoattractant/human growth-regulated oncogene (CXCL1) (KC-GRO), TNF- α , and IFN- γ) were analyzed from BALF in an electrochemical-based multiplex assay,

mouse proinflammatory V-plex kit (MSD, Rockville, MD), as previously described (11).

Quantitative PCR

Fold change in mRNA transcripts relative to hypoxanthine guanine phosphoribosyltransferase was assessed with quantitative PCR as described previously (8). Taqman probes (Life Technologies, Grand Island, NY) are listed in Table E1 in the online supplement.

Flow Cytometry and Cell Quantification

BALF cells were isolated and prepared for either cell differential analysis or flow-assisted cell sorting using a sequential gating strategy (see the online supplement for details).

Statistical Analysis

Data are expressed as mean ($\pm$ SEM) and analyzed by two-way ANOVA with the recommended *post hoc* analyses using Prism V6.05 (GraphPad Software, La Jolla, CA). A *P* value less than 0.05 was considered significant.

Results

SOD3 Content and Activity in BALF and Plasma of WT Mice and Mice Expressing the R213G SNP in SOD3 Varies between Strain, and Increases Only in the BALF after Bleomycin

To understand how bleomycin altered the distribution of SOD3 content and its activity, we measured SOD3 amount and activity in BALF and plasma in PBS control mice and at 3 and 21 days after bleomycin

treatment. In BALF, bleomycin increased SOD3 protein levels at 3 days in both WT and R213G mice, with a significantly higher release of SOD3 into BALF in the R213G mice (Figure 1A). By 21 days, both mice had similarly high levels of SOD3 in their BALF compared with baseline (Figure 1B). There was a corresponding increase in SOD3 activity in the R213G mice at both 3 and 21 days, whereas SOD activity in BALF increased in WT mice only at 21 days (Figure 1C). Though SOD3 protein content increased in the BALF by 10- to 40-fold in response to bleomycin, the activity increased at most threefold, indicating that much of the secreted enzyme was inactivated. In plasma, SOD3 expression and activity did not change in response to bleomycin in either strain, and SOD activity was consistently higher in the R213G mice compared with WT mice,

Figure 1. Extracellular superoxide dismutase 3 (SOD3) content and SOD activity levels in plasma and bronchoalveolar lavage fluid (BALF) of wild-type (WT) mice and mice expressing the R213G single-nucleotide polymorphism (SNP) in SOD3 after treatment with bleomycin (Bleo); strain- and time-dependent changes. SOD3 protein content was evaluated by Western blot analysis in 25 μ l plasma or BALF 3 and 21 days after treatment. (A and B) Representative Western blots are shown above graphs of relative optical density, normalized to WT PBS. BALF SOD3 protein expression 3 and 21 days after treatment ($n = 3$). (C) BALF SOD activity per milliliters of BALF 3 and 21 days after treatment ($n = 4-8$). (D and E) Plasma SOD3 protein expression 3 and 21 days after treatment ($n = 3$). (F) Plasma SOD activity per milliliters of BALF 3 and 21 days after treatment ($n = 3-7$). SOD activity was measured using the SOD assay kit-WST. Data are presented as mean ($\pm$ SEM). * $P < 0.05$ versus WT PBS, # $P < 0.05$ versus R213G PBS, \$ $P < 0.05$ versus WT Bleo (time-matched), by two-way ANOVA.

Figure 2. Mice expressing the R213G SNP in SOD3 demonstrate consistently low SOD3 protein expression, SOD3 activity, and oxidative stress in lung tissue after Bleo treatment. SOD3 and β -actin protein expression in lung tissue were evaluated by Western blot analysis 3 and 21 days after treatment. (A and B) A representative Western blot is shown along with relative optical density normalized to WT PBS ($n = 3-5$). SOD3 was separated from the intracellular SODs using a conA Glycoprotein separation kit, and SOD3 activity measured 3 and 21 days after Bleo. (C) SOD activity is expressed as units per milligram of protein. Reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio (D) and total lung glutathione (E) in lung tissue 21 days after treatment ($n = 6$). Data are presented as mean ($\pm$ SEM). * $P < 0.05$ versus WT PBS, # $P < 0.05$ versus R213G PBS, \$ $P < 0.05$ versus WT Bleo (time-matched), by two-way ANOVA.

similar to our previous report (11) (Figures 1D–1F).

Lung tissue from PBS-treated R213G mice had significantly less SOD3 protein content and activity compared with WT mice at baseline (Figures 2A–2C), recapitulating previous reports (11). In WT mice, lung SOD3 content was unchanged 3 days after bleomycin treatment, although it decreased by 21 days, a time point corresponding with pulmonary fibrosis (19). In R213G mice, the SOD3 content remained low compared with WT mice, without a significant change in response to bleomycin at either 3 or 21 days (Figures 2A and 2B). Lung SOD3 activity corresponded with SOD3 protein expression; although the SOD3 activity tended to decrease in the WT lungs at 21 days after bleomycin, the R213G lung SOD3 activity levels remained significantly lower than in WT mice, and did not change

with bleomycin (Figure 2C). The expression of the other SOD isoforms did not significantly change with bleomycin in either strain (see Figures E1A–E1C). To measure the effects of the SOD3 R213G SNP on the lung redox state, GSH and oxidized glutathione (GSSG) levels were determined in the 21-day lung tissue. In parallel with the decrease in SOD3 expression, bleomycin-treated WT mice had a significant reduction in the lung GSH:GSSG ratio. In contrast, in the R213G mice, the GSH:GSSG ratio did not significantly decrease after bleomycin treatment, and was significantly higher than in the WT bleomycin-treated mice (Figure 2D). Total glutathione was similar between strains in both PBS- and bleomycin-treated mice. There was a modest increase in total lung glutathione after bleomycin that reached statistical significance only in the R213G mice

(Figure 2E). The protection against the bleomycin-induced loss of GSH in the R213G mice suggests a protective effect due to the high BALF and plasma SOD3 content conferred by the R213G polymorphism.

R213G Mice Are Protected from Bleomycin-Induced Pulmonary Fibrosis

To further understand how the distribution of SOD3 influences the progression of lung and pulmonary vascular diseases, we tested the impact of the SOD3 R213G polymorphism on the development of bleomycin-induced pulmonary fibrosis. We measured pulmonary mechanics, fibrosis score, total lung collagen, and gene expression of fibrotic markers to evaluate lung fibrosis at 21 days. In WT mice, at 21 days, bleomycin decreased lung compliance and increased lung elastance,

Figure 3. Mice expressing the R213G SNP in SOD3 are protected against Bleo-induced fibrosis. Static lung compliance (Cst) (A) and static lung elastance (Est) (B) in WT and R213G mice 21 days after PBS and Bleo treatment measured by flexiVent ($n = 6-10$). (C) Representative trichrome staining of 5- μ m-thick lung tissue sections. Scale bar, 100 μ m. (D) Fibrosis scoring of trichrome stained lung sections ($n = 5-7$). (E) Total collagen in lung tissues expressed per milligram tissue weight measured by a sircol assay (Chondrex; $n = 3-5$). Lung transforming growth factor β (TGF- β) (F) and collagen type I α 1 chain (G) mRNA expression relative to hypoxanthine guanine phosphoribosyltransferase and normalized to WT PBS ($n = 3-6$). Gene expression was measured by TaqMan quantitative RT-PCR. Data are presented as mean ($\pm$ SEM). * $P < 0.05$ versus WT PBS, \$ $P < 0.05$ versus WT Bleo by two-way ANOVA.

whereas bleomycin had no effect on lung function of R213G mice. (Figures 3A and 3B). Assessment of fibrosis by the validated fibrosis score showed an increase in lung fibrosis in response to bleomycin in WT mice, but no change from baseline in R213G mice (Figures 3C and 3D). Correspondingly, total collagen levels increased after bleomycin treatment in WT mouse lungs, but were unchanged in R213G mice (Figure 3E). Furthermore, mRNA transcripts of transforming growth factor- β 1 and collagen type I α 1 chain

were elevated in the lung tissue of WT mice treated with bleomycin at Day 21. In contrast, both transforming growth factor- β 1 and collagen type I α 1 chain mRNA transcripts were low at baseline, and did not increase in response to bleomycin in the R213G mice (Figures 3F and 3G).

R213G Mice Are Protected from Bleomycin-Induced PH and Vascular Remodeling

We next evaluated whether the R213G mice were protected against PH and vascular

remodeling 28 days after bleomycin. Although RV systolic pressure (RVSP) significantly increased in WT mice 28 days after bleomycin, there was no change in RVSP in bleomycin-treated R213G mice (Figure 4A). We did observe that R213G mice had higher RVSP at baseline (32.1 ± 2.9 mm Hg versus 24.3 ± 1.8 mm Hg in WT mice), similar to the findings reported in our previous report (11). A corresponding trend toward RV hypertrophy was observed only in the bleomycin-treated WT mice (Figure 4B). Finally, bleomycin increased MWT in small (50–200 μ m) peribronchiolar pulmonary arteries in the WT mice, but R213G mice were protected against the increase in MWT (Figures 4C and 4D). Together, these data suggest that the R213G SNP protects from the development of bleomycin-induced PH and vascular remodeling.

The R213G SNP Reduces Alveolar Epithelial Leak and Augments Induction and Resolution of Bleomycin-Induced Alveolar Inflammation

Alveolar-capillary barrier damage is linked with alveolar inflammation, and precedes the development of fibrosis in the intratracheal bleomycin model (19). Therefore, we investigated whether the R213G SNP protected against early alveolar epithelial-capillary barrier damage and alveolar inflammation.

Damage to the alveolar-capillary barrier was measured by leakage of protein into the BALF. In PBS-treated WT and R213G mice, BALF protein content was low, and remained stable over time. In bleomycin-treated WT mice, BALF protein content increased at Days 7 and 21, indicating a compromised alveolar-capillary barrier. In contrast, in R213G mice, the BALF protein content significantly increased on Day 3, then returned to control levels by Day 21, resulting in a significantly lower protein content at 7 and 21 days in R213G mice compared with the WT mice (Figure 5).

To test whether the R213G SNP protected against bleomycin-induced alveolar inflammation, total cell counts and differential counts of lymphocytes, macrophages, and neutrophils were measured in BALF at 1, 3, 7, and 21 days after treatment in WT and R213G mice. The

Figure 4. Mice expressing the R213G SNP in SOD3 are protected against Bleo-induced pulmonary hypertension. (A) Right ventricular (RV) systolic pressure (RVSP) 28 days after PBS ($n = 4-7$) and Bleo ($n = 6-8$) treatment was measured via direct right heart puncture and analyzed using Cardiomax software (Columbus, OH). (B) RV hypertrophy assessed by ratio of RV to left ventricle (LV) plus septum (S) weight ($n = 4-8$). (C) Representative α -smooth muscle actin (α -SMA; purple) staining of smooth muscle cells and Factor VIII (brown) staining of endothelial cells in pulmonary arterioles ($\sim 100 \mu\text{m}$ or less in diameter). Scale bar, $100 \mu\text{m}$. (D) Quantification of medial wall thickness (MWT) of pulmonary arterioles located at the terminal bronchioles was measured in tissue sections stained for α -SMA and Factor VIII ($n = 4-7$). Data are presented as mean ($\pm$ SEM). * $P < 0.05$ versus WT PBS, $^{\$}P < 0.05$ versus WT Bleo, by two-way ANOVA.

total number of BAL cells in bleomycin-treated WT mice was significantly elevated by Day 7, and remained elevated at Day 21. Although R213G bleomycin-treated mice had a similarly significant increase in total cell count on Day 7, by Day 21, the cell count returned to control levels, and was significantly less compared with WT bleomycin-treated mice (Figure 6A). Cell differentials provided further evidence that inflammation was attenuated in the R213G lavage fluid. By cell differentials in Cytospin slides, we measured a significant increase in BALF lymphocytes in bleomycin-treated in WT mice on Day 7 compared with the bleomycin-treated R213G mice (Figure 6B). The number of macrophages significantly increased by Day 21 after bleomycin in WT mice, but not in R213G mice (Figure 6C). Neutrophils were significantly elevated on Day 7 in WT bleomycin-treated mice, whereas R213G mice did not have an influx of neutrophils (Figure 6D).

We then measured the levels of nine cytokines (TNF- α , IL-2, KC-GRO, IL-10 [Figures 6E-6H], IL-6, IL-1 β , IL-12p70, IL-5, and IFN γ [Figures E2A-E2E]) in the BALF by MSD V-plex kit to further interrogate the differences in the inflammatory response between WT and R213G mice over time. Although each cytokine had a different profile in response to bleomycin, we did observe several important patterns. Overall, in WT mice, most cytokines increased within 1 day after treatment with bleomycin, and remained elevated throughout the 21-day time course. In contrast, in R213G mice, the cytokines, including both proinflammatory as well as the antiinflammatory cytokine, IL-10, increased significantly more at 3 days after bleomycin compared with the WT mice, but then returned toward baseline over the 21-day period. Thus, except for IL-12p70, eight of the nine cytokines were significantly

higher in the R213G mice compared with the WT mice at 3 days after bleomycin. Three of the nine cytokines (TNF- α , IL-2, and KC-GRO) were higher in the WT mice at 7 or 21 days after bleomycin compared with the R213G mice, and two additional cytokines (IL-5 and IL-6) tended to be elevated at the late time points in the WT mice.

Considering that the marked increase in cytokines at 3 days in the R213G mice preceded the influx of inflammatory cells, we further analyzed the number and type of inflammatory cells in the BALF in the two strains at the early time points using flow cytometry-assisted cell sorting. Similar to our cell differentials, there was no difference between WT and R213G bleomycin-treated mouse cell counts on Day 3 for CD4 $^{+}$ T lymphocytes, CD8 $^{+}$ T lymphocytes, B lymphocytes, neutrophils, and resident (CD11c $^{+}$) and recruited (CD11b $^{+}$) macrophages. In WT mice, bleomycin treatment significantly increased CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes and B lymphocytes by Day 7 compared with PBS-treated mice. Although R213G mice also demonstrated increased CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes and B lymphocytes 7 days after bleomycin, the numbers of cells were significantly lower compared with WT bleomycin-treated mice at 7 days (Figures 7A-7C). Bleomycin treatment significantly increased the number of neutrophils on Days 3 and 7, but there was no difference between WT and R213G bleomycin-treated mice (Figure 7D). The number of resident macrophages was lower in R213G mice compared with WT PBS-treated mice. Resident macrophages were reduced by Day 3 after bleomycin treatment similarly in WT and R213G mice, but, whereas WT bleomycin-treated mice sustained lower levels on Day 7, R213G mice had begun to recover the resident macrophages, with significantly more compared with Day-3 bleomycin-treated mice (Figure 7E). The number of recruited macrophages was significantly higher 3 and 7 days after bleomycin treatment in both WT and R213G mice compared with PBS, with no significant differences between strains (Figure 7F).

Discussion

The human R213G SNP decreases matrix binding affinity, leading to a redistribution

Figure 5. Mice expressing the R213G SNP in SOD3 resolve alveolar edema after Bleo treatment compared with WT mice, which show progressive worsening of edema. BALF total protein concentration at 1, 3, 7, and 21 days after PBS and Bleo treatment ($n = 3-8$) was measured by a standard bicinchoninic acid protein assay. Data are presented as mean ($\pm$ SEM). * $P < 0.05$ versus WT PBS, # $P < 0.05$ versus R213G PBS, \$ $P < 0.05$ versus WT Bleo, by two-way ANOVA.

of SOD3 from the lung and into extracellular fluids. In humans with this genetic polymorphism, this altered

distribution confers a decreased risk for lung injury (COPD) and increased risk for vascular injury (ischemic heart disease)

(11, 17, 20). In this study, we tested the impact of the altered localization of SOD3 using genetically engineered mice with knockin identical to the human R213G SNP in the HBD of SOD3 in a model of bleomycin-induced lung fibrosis and secondary PH. Intratracheal bleomycin is widely used as a model of pulmonary fibrosis, and its pathophysiological process is well documented (19, 21). We report that the R213G SNP in SOD3 protected from the development of bleomycin-induced fibrosis and PH. Furthermore, the R213G SNP promoted resolution of alveolar inflammation after intratracheal bleomycin (see schematic in Figure 8). This study provides unexpected new insight into our current understanding of the impact of the localization of SOD3 in lung and pulmonary vascular diseases.

Our first notable finding was that the R213G SNP modified the distribution of SOD3 in the BALF, plasma, and lung in

Figure 6. Enhanced induction and resolution of airway inflammation in mice expressing the R213G SNP in SOD3. Total cell (A), lymphocytes (B), macrophages (C), and neutrophils (D) were measured using a ViCell counter (Beckman Coulter, Brea, CA) and cell differentials. Cytokine levels (TNF- α , IL-2, keratinocyte chemoattractant/human growth-regulated oncogene [KC-GRO], and IL-10; E-H, respectively) in BALF 1, 3, 7, and 21 days after treatment were measured in 50 μ l of BALF with 1% of bovine serum albumin using a mouse proinflammatory V-plex kit ($n = 4-7$, cells; $n = 5-12$, cytokines). The key is shown in A, with solid lines and squares for WT mice and dashed lines and circles for R213G mice. Data are presented as mean ($\pm$ SEM). * $P < 0.05$ versus WT PBS, # $P < 0.05$ versus R213G PBS, \$ $P < 0.05$ versus WT Bleo, by two-way ANOVA.

Figure 7. Bleo alters the profile of inflammatory cells in the alveolar fluid, with decreased numbers of specific populations in mice expressing the R213G SNP in SOD3. We performed fluorescence-activated cell sorting (Gallios, Indianapolis, IN) in BALF 3 days after PBS and 3 and 7 days after Bleo treatment in WT and R213G mice. Cell counts of (A) CD4⁺ T lymphocytes, (B) CD8⁺ T lymphocytes, (C) B lymphocytes, (D) neutrophils, (E) resident macrophages (CD11c⁺), and (F) recruited macrophages (CD11b⁺) ($n = 5-10$). WT, solid lines with squares; R213G, dashed lines with circles. Data are presented as mean ($\pm$ SEM). * $P < 0.05$ versus WT PBS, # $P < 0.05$ versus R213G PBS, ^ $P < 0.05$ versus WT and R213G 3 Day Bleo, \$ $P < 0.05$ versus WT Bleo, within time point, by two-way ANOVA.

response to bleomycin, and protected against bleomycin-induced fibrosis and secondary PH. Our data indicate that the substantially lower lung SOD3 content in the R213G mice compared with the WT mice did not increase the risk of injury, but, instead, that the higher SOD3 content and activity in BALF and plasma was responsible for the protection of the R213G SNP against lung fibrosis. Our finding that BALF SOD3 increased after bleomycin is consistent with prior published studies showing increased BALF SOD3 in mice treated with LPS, bleomycin, and asbestos (11, 22), and in the cell culture media of bone marrow-derived macrophages treated with LPS (23). Tan and colleagues (22) demonstrated that the SOD3 released into the airspace in response to asbestos, bleomycin, or bacterial

pneumonia originated from inflammatory cells. In the recent study by Gottsfredsen and colleagues (23), the cellular redistribution of SOD3 in response to LPS was similar in bone marrow-derived macrophages derived from WT and R213G mice, though they did not test the functional responses. We found that BALF SOD3 content increased more than activity, demonstrating that a large portion of the released enzyme was inactivated. This supports other studies that report that SOD3 can be inactivated by reactive oxygen species (24, 25). The BALF SOD3 activity in R213G mice increased at 3 days by roughly twofold, providing enhanced antioxidant activity compared with WT mice, and potentially contributing to the protection in the R213G mice. Other published studies

that tested the impact of altered SOD3 expression on bleomycin-induced lung fibrosis used genetically engineered mice with increased lung SOD3 or lack of SOD3, so that the level of SOD3 expression, rather than the distribution, differed from WT mice (5, 6, 8). We noted in this study that SOD3 content in the BALF did not differ at baseline between the two strains, in contrast to our prior results (11). The discrepancy between the two studies could be accounted for by normalization of SOD3 content to volume in this study, rather than to BALF protein concentrations as in our prior study. We confirmed the finding that baseline BALF SOD3 content was similar between strains by measuring SOD3 activity at baseline in the BALF, which was consistent with the Western blot data. Of

Figure 8. Schematic showing the effect of SOD3 R213G polymorphism on SOD3 distribution and lung fibrosis. In WT mice, SOD3 is highly bound to lung and vascular extracellular matrix (ECM), with virtually no SOD3 in the epithelial lining fluid, measured in BALF, or plasma (*upper left quadrant*). The SOD3 R213G SNP decreases matrix binding affinity and causes redistribution of SOD3 from the lung and vascular ECM into the plasma and epithelial lining fluid (*upper right quadrant*). In response to Bleo treatment, WT mice lose SOD3 in the lung and release SOD3 into the alveolar space, although levels are lower than in the R213G mice. These mice demonstrate a marked inflammatory cell influx to the bronchial lumen and develop lung fibrosis (*lower left quadrant*). In contrast, in mice expressing the SOD3 R213G SNP, there are persistent elevations of SOD3 in the plasma and increased SOD3 in the BALF. These mice exhibit enhanced resolution of inflammation and protection against lung fibrosis (*lower right quadrant*). AEC, airway epithelial cell.

the three compartments, we observed the most striking difference in SOD3 content between strains in the plasma. This suggests that the elevated plasma SOD3 may protect the endothelial barrier, preventing subsequent alveolar injury and leukocyte extravasation. Because bleomycin-induced lung fibrosis is driven by alveolar inflammation, the subsequent set of experiments evaluated cytokine production and inflammatory cell influx into the alveolar space in the two strains of mice.

Neutrophils and macrophages are the first responders in an innate immune response; in our bleomycin model of IPF, we see a similar trend, with a surge in these phagocytes after the bleomycin challenge. Persistent increases in lymphocytes, including CD4⁺ and CD8⁺ T and B lymphocytes in the WT mice treated with bleomycin compared with the R213G counterparts, may suggest a role of plasma SOD3 in the transendothelial migration of inflammatory cells to the lungs. However, we do not know if the SOD3 affected the migration directly or indirectly. More

studies are warranted to understand the effects of SOD3 on the migration and activation of inflammatory cells. Interestingly, inflammatory cytokine levels were much higher in R213G mice compared with the WT mice treated with bleomycin at Day 3. This suggests that the resident alveolar macrophages and structural cells, like airway epithelium, are responsible for the initial inflammatory cytokine surge in R213G mice, and, furthermore, that the low SOD3 levels within the lung may have augmented the early cytokine response. However, the inflammation kinetics change quickly with the higher levels in plasma and BALF SOD3 of R213G mice as they resolve the inflammation more quickly toward baseline levels of multiple cytokines compared with the WT, by Day 7, corresponding to a decrease in neutrophils and lymphocytes by Day 7. The increased resolution of inflammation in the R213G correlated with the protection against lung fibrosis and PH.

The antiinflammatory cytokine, IL-10, plays a seminal role in containing

inflammation by abrogating the immune response to various pathogens and injuries (26). In our model, we saw an increase in the level of IL-10 at Day 3 in the BALF of R213G mice, suggesting a role of the unbound SOD3 in triggering an antiinflammatory signaling cascade. The higher IL-10 level might account for the quick resolution of inflammation in the R213G mice. IL-10 inhibits production of reactive oxygen species from monocytes/macrophages and neutrophils (27). Suppression of superoxide anion production by IL-10 is accompanied by a down-regulation of the genes for subunit proteins of nicotinamide adenine dinucleotide phosphate reduced oxidase (28), and its deficiency has been related to increased superoxide and endothelial dysfunction (29). However, it is not clear how SOD3 may modulate the IL-10 signaling pathway. Future mechanistic studies in inflammatory and structural cells are required to test the direct or indirect effects of SOD3 on IL-10 and the role of IL-10 in the resolution of bleomycin-induced inflammation in the R213G mice.

Another intriguing finding in the present study was that the R213G mice showed protection against bleomycin-induced PH, whereas our previous report showed that the same strain of mice showed worsened chronic hypoxia-induced PH. This indicates that the redox-regulated mechanisms that drive PH and vascular remodeling differ in response to bleomycin relative to hypoxia. In our previous publication, we proposed that the loss of vascular SOD3 due to redistribution away from the vessel wall was responsible for the development of PH, whereas the alveolar inflammation with intratracheal LPS required increased SOD3 in the extracellular fluids. A similar hypothesis was put forth by other investigators to explain the discrepant outcomes in COPD versus ischemic heart disease in humans with the R213G SNP (16, 17). Our results indicate that our understanding has been incomplete, and, for PH, we need to reconsider the pathophysiology driving the vascular disease—in particular, the redox-regulated pathways that result in inflammation, fibrosis, and end-stage pulmonary vascular remodeling in different settings. These findings have important clinical implications, as they emphasize that we need to understand individual variations when designing the optimal therapy for

patients with PH. As we consider a targeted approach to delivering SOD3 to restore antioxidant activity, our results demonstrate that we also need to consider modifications to SOD3 that enhance tissue versus fluid availability and activity.

In conclusion, mice carrying the R213G SNP in SOD3 have an early enhanced induction, followed by improved resolution of the inflammatory response to

intratracheal bleomycin, which ameliorates alveolar damage and thereby protects against the development of fibrosis and secondary PH. Our data emphasize the importance of SOD3 localization on the progression of lung and vascular diseases. Further investigation is warranted to understand how and in what cell types the R213G SNP alters the inflammatory response to promote enhanced resolution in fibrotic diseases. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. David Riches of National Jewish Health (Denver, CO) for expert advice and Crystal Woods (University of Colorado Denver, Aurora, CO), Joanne Maltzahn (University of Colorado Denver, Aurora, CO), and Jason Varasteh (National Jewish Health, Denver, CO) for excellent technical support.

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