A Common Polymorphism in Extracellular Superoxide Dismutase Affects Cardiopulmonary Disease Risk by Altering Protein Distribution

John M. Hartney, PhD*; Timothy Stidham, MD*; David A. Goldstrohm, PhD*; Rebecca E. Oberley-Deegan, PhD; Michael R. Weaver, BS; Zuzana Valnickova-Hansen, BSc; Carsten Scavenius, PhD; Richard K.P. Benninger, PhD; Katelyn F. Leahy, BS; Richard Johnson, BS; Fabienne Gally, PhD; Beata Kosmider, PhD; Angela K. Zimmermann, PhD; Jan J. Enghild, PhD; Eva Nozik-Grayck, MD*; Russell P. Bowler, MD, PhD*

Background—The enzyme extracellular superoxide dismutase (EC-SOD; SOD3) is a major antioxidant defense in lung and vasculature. A nonsynonymous single-nucleotide polymorphism in EC-SOD (rs1799895) leads to an arginine to glycine amino acid substitution at position 213 (R213G) in the heparin-binding domain. In recent human genetic association studies, this single-nucleotide polymorphism attenuates the risk of lung disease, yet paradoxically increases the risk of cardiovascular disease.

Methods and Results—Capitalizing on the complete sequence homology between human and mouse in the heparin-binding domain, we created an analogous R213G single-nucleotide polymorphism knockin mouse. The R213G single-nucleotide polymorphism did not change enzyme activity, but shifted the distribution of EC-SOD from lung and vascular tissue to extracellular fluid (eg, bronchoalveolar lavage fluid and plasma). This shift reduces susceptibility to lung disease (lipopolysaccharide-induced lung injury) and increases susceptibility to cardiopulmonary disease (chronic hypoxic pulmonary hypertension).

Conclusions—We conclude that EC-SOD provides optimal protection when localized to the compartment subjected to extracellular oxidative stress; thus, the redistribution of EC-SOD from the lung and pulmonary circulation to the extracellular fluids is beneficial in alveolar lung disease but detrimental in pulmonary vascular disease. These findings account for the discrepant risk associated with R213G in humans with lung diseases compared with cardiovascular diseases. (Circ Cardiovasc Genet. 2014;7:659-666.)

Key Words: cardiovascular diseases ■ hypertension, pulmonary ■ lung

In mammals, there are 3 superoxide dismutase (SOD) isoforms that constitute the major enzymatic antioxidant defense against superoxide. The extracellular isoform, EC-SOD or SOD3, is highly expressed in airways and lung epithelium and is the most abundant SOD in blood vessels. By reducing superoxide and oxidative stress, EC-SOD modulates multiple signaling pathways such as nitric oxide, nuclear factor-κB, Egr-1, and RhoA-dependent signal transduction.10-12 In animal studies using knockout and overexpressing mice, EC-SOD attenuates oxidative stress and inflammation in the lungs from multiple inhaled toxins including lipopolysaccharide.4,13-19 Similarly, in the vasculature, EC-SOD preserves nitric oxide signaling, attenuates oxidative injury, and protects against vascular remodeling.3,20-22
4% to 6% in Asian populations and 2% to 3% in European populations. The R213G SNP was first reported to confer increased risk of ischemic heart disease in 9188 participants from the Copenhagen City Heart Study. Paradoxically, the R213G SNP was later found to reduce the risk of chronic obstructive pulmonary disease and acute exacerbations of chronic obstructive pulmonary disease in 3 independent, large population studies. Because the R213G SNP only occurs in humans, we have limited experimental evidence and knowledge to explain this paradox of increased risk of vascular disease and reduced risk of pulmonary disease. From observational studies, we know that carriers of the R213G SNP have increased EC-SOD protein in plasma and that the R213G variant EC-SOD protein has impaired in vitro binding to extracellular matrix elements; however, we do not know how the R213G SNP changes the distribution of EC-SOD in tissue, nor do we understand how the R213G SNP alters the response to lung and vascular injury in vivo.

Methods

Experimental Animals: R213G SNP Knockin Mouse Creation

The R213G knockin targeting vector was created using high-fidelity Red/ET recombineering methods. Briefly, a 10.5-kb region was cloned from a positively identified C57BL/6 BAC clone using homologous recombination. The short homology arm extended 2.25 kb 3′ to the LoxP-flanked Neo cassette, and the long homology arm extended 6.13 kb to the 3′-end of the single Lox P site. The single Lox P site is inserted upstream of exon 2 in intron 1 to 2, and the LoxP-flanked Neo cassette is inserted downstream of exon 2 in intron 2 to 3. The C→G mutation (amino acid change: R213G) within exon 2 was generated by 3-step polymerase chain reaction mutagenesis. Using conventional subcloning methods, the wild-type (WT) sequence was replaced with the PCR fragment carrying the point mutation. Targeted iT1 BA1 (C57BL/6 Ns129/SvEv) hybrid embryonic stem cells were microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage of agouti coat color were mated to WT C57BL/6 N mice to generate F1 heterozygous offspring. We obtained 2 independent C57BL/6 founder lines to generate homozygote R213G mice. DNA was sequenced periodically to verify lack of new spontaneous mutations. Animal experiments were approved by the Institutional Animal Care and Use Committee.

Southern Blot Analysis

DNA digested with BspEI was electrophoretically separated on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a probe targeted against the 5′ external region of EC-SOD. DNA from C57BL/6, 129/SvEv, and BA1 (C57BL/6x129/SvEv) mice was used as WT controls.

Mouse DNA Genotyping

R213G mice were genotyped using primers (forward: 5′-AGGCTCAAGTCTGTCAGGAAGG-3′, reverse: 5′-TTTCCAATTCATCCACACATGGG-3′) to distinguish WT (357 bp ampiclon) from knockin alleles (419 bp ampiclon). DNA was amplified in genotyping PCR cocktail (10 mmol/L primers, HiFi buffer, 50 mmol/L MgSO4, 10 mmol/L DNTPs, HiFi Taq, water) by a 5-minute 99°C hot start for 30 cycles, 30-second 95°C denaturing, 30-second 55°C annealing, and 3-minute 68°C extending conditions. PCR products were run on a 1.5% agarose gel with a DNA ladder.

Human DNA Genotyping

Lungs, graciously provided by Dr Mason (National Jewish Health), were donated through the National Disease Research Interchange (Philadelphia, PA) and International Institute for the Advancement of Medicine (Edison, NJ) under a Human Subject Research exempt protocol. Isolated lung DNA (Qiagen DNA tissue kit) was screened for the R213G SNP using real-time PCR allelic discrimination assay-by-design service and the TaqMan SNP Genotyping Assay for Human SOD3 (C_2307506_10; Applied Biosystems, Foster City, CA).

DNA Sequencing

A DNA region containing the R213G SNP was sequenced using a forward primer (5′-AGGCTCAAGTCTGTCAGGAAGG-3′) located in the long homology arm upstream of the R213G SNP and a reverse primer (5′-GGAACTCTCCGTAGA CTATGCAGGTG-3′) located within the Neo cassette downstream of the R213G SNP per established protocols (Molecular Research Center, National Jewish Health). DNA was amplified and the PCR product sequenced on an ABI PRISM 3100 genetic analyzer. pGEM-3zf DNA was used as a positive control.

EC-SOD Activity Assays

Pulverized lung (70 mg) was homogenized in SOD activity assay buffer, and total lung protein concentration was determined by bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). EC-SOD was separated from intracellular SOD (SOD1 and SOD2) using the glycoprotein isolation kit (ConA; Pierce Biotechnology) per manufacturer’s instructions with the following minor modifications: spin columns contained 300 μL ConA lectin resin and the intracellular SOD fraction was collected before washing columns. Columns were incubated with 300 μL elution buffer for 15 minutes before collection of flowthrough. Absence of EC-SOD in the intracellular SOD fraction was confirmed by Western blot, whereas eluted fractions containing EC-SOD were pooled and concentrated 10-fold using a centrifprep concentrator (Amicon-ultra-10K; Millipore). Activity levels were measured by the SOD assay kit (WST; Dojindo Molecular Technologies) according to manufacturer’s instructions. Data were expressed as units of EC-SOD activity per milligram of total lung protein.

Heparin–Sepharose Binding

Pooled plasma or lung tissue from 10 mice (WT or R213G) was homogenized and centrifuged and supernatant dialyzed against 20 mmol/L Tris-HCl (pH 7.5). After dialysis, the sample was applied to 1 mL Hitrap Heparin Sepharose (GE Healthcare) and heparin-binding proteins eluted with a linear gradient of NaCl (0 mmol/L–1 mol/L) in 50 mmol/L Tris-HCl (pH 7.5) at a flow rate of 1 mL/min. Fractions were analyzed for EC-SOD activity by inhibition of cytochrome c reduction as described previously. To test if the R213G SNP altered SOD activity, we normalized activity to the relative EC-SOD content in the peak fraction. We determined the relative EC-SOD content by Western blotting using a polyclonal rabbit murine EC-SOD antibody and Cy5-labeled antirabbit secondary antibody (Sigma).

Histological Analysis

EC-SOD immunostaining was performed as previously described using mouse- or human-specific antibodies. Mouse lungs were inflated-fixed at 25 cm H2O pressure with 4% paraformaldehyde. EC-SOD knockout and WT mice were used as negative and positive controls, respectively.

Bronchoalveolar Lavage

Lungs were lavaged with 1 mL PBS, and 20 μL of total bronchoalveolar lavage fluid (BALF) was diluted in 10 mL Isoton II diluent and counted on a Z2 particle count and size analyzer (Beckman Coulter, Fullerton, CA).

Western Blot Analysis

Homogenized lung tissue was run on a 12% Tris/glycine SDS-PAGE gel and transferred to PVDF membrane per standard protocols. Blots were incubated with primary antibody as follows: β-actin 1:20000 (Sigma, St Louis, MO), EC-SOD 1:1000 (8130), 4-hydroxyynonal
1:1000 (Abcam, Cambridge, MA), and nitrotyrosine 1:500 (UpState, Lake Placid, NY). After incubation with secondary antibody, blots were developed with ECL Plus (GE Healthcare), imaged with STORM 860 (GE Healthcare) using the fluorescent blue (430 nm) setting, quantified using ImageQuant software, and expressed relative to β-actin.

Gene Expression
Total RNA was extracted using either TRizol (Invitrogen, Carlsbad, CA) or RNeasy kit (Qiagen) according to the manufacturer’s instructions, and differences in gene expression were determined using real-time reverse transcriptase PCR as previously described.7 Gene expression was determined using comparative threshold cycle (ΔΔCt) as suggested by the manufacturer (Applied Biosystems) normalizing each sample to 18s rRNA. The following primer sets were purchased from Assays on Demand (Applied Biosystems): 18s rRNA (cat#4310893E), SOD3 (Mm01213380_s1), SOD1 (Mm01700393_g1), and SOD2 (Mm0449726_m1).

Proinflammatory Cytokine and Chemokine Measurements
Cytokine and chemokine concentrations in mouse lung tissue and BALF were measured (ELISA TECH, Aurora, CO) using either the Meso Scale Discovery mouse proinflammatory 7-plex ultrasensitive kit or the proinflammatory panel 1 V-PLEX kit (Meso Scale Discovery, Gaithersburg, MD) per manufacturer’s instructions.

Total Differential Cell Counts
Total BALF was centrifuged for 15 minutes at 300g. The cell pellet was resuspended in 200 μL PBS. About 180 μL was spotted to glass slides and allowed to dry overnight. Slides were then stained for differential cell counts using HEMA 3 stain per manufacturer’s instruction (Fisher Scientific, Kalamazoo, MI).

Chronic Hypoxic Pulmonary Hypertension
Four-week-old WT C57BL6 and HM R213G mice were exposed to hypobaric hypoxia (395 torr) for 35 days: chambers simulated altitude (5486 m) equivalent to 10% oxygen. Normoxic control mice were maintained in Denver ambient air (1609 m).

Isolation of Pulmonary Arteries
Lungs were perfused with a slurry of iron particles in warmed agarose and minced; iron-containing pulmonary arteries were separated from lung parenchyma by magnet.4 The pulmonary artery was digested with collagenase to release iron particles, and the isolated pulmonary artery tissue homogenized and sonicated in radioimmunoprecipitation buffer for Western blots.

Assessment of Pulmonary Hypertension
Right ventricular systolic pressure was measured in anesthetized mice by direct right ventricular puncture through a closed chest using the Cardiomax III Cardiac Output system (Columbus Instruments) as previously described.7 Right ventricular hypertrophy was determined as the ratio of right ventricular/left ventricular+septum weights.

Assessment of Pulmonary Vascular Remodeling
To evaluate hypoxia-induced muscularization of small vessels, lung sections were stained with mouse monoclonal α-smooth muscle actin antibody (1:100; Clone 1A4) and the number of positively stained small vessels (<50 μm) determined as previously described.3 Collagen deposition was evaluated using nonlinear second harmonic generation imaging, based on the ability of collagen fibrils to intrinsically generate a second harmonic generation signal.5 Lung sections (100 μm) were deparaffinized and imaged on an LSM510-Meta microscope (Zeiss, Jena, Germany) with a ×63 1.4 NA Plan-Apochromat oil-immersion objective, using a 800-nm mode-locked 100 fs–pulsed Ti:Sapphire laser (Chameleon; Coherent) for nonlinear excitation. Second harmonic generation was detected with a narrow band 390 to 410 nm band pass filter (Chroma), and autofluorescence was detected with a 450 to 700 nm broad band pass filter. Settings were kept constant between experiments. Pulmonary arteries <200 μm diameter associated with bronchioles were imaged (n=5 per mouse). To quantify collagen, the area of second harmonic generation signal around a single pulmonary artery, measured as pixels, was calculated and expressed relative to the vessel perimeter. All image analyses were performed by an investigator blinded to treatment groups using Matlab (Mathworks) and ImageJ (National Institutes of Health, Bethesda, MD).

Statistical Analysis
Results are presented as means±SE. A Kruskal–Wallis test and Wilcoxon rank-sum test were performed to determine whether the groups were significantly different. A value of P<0.05 was considered statistically significant.

Results
Translating human SNP association studies into animals can be challenging for 2 reasons: first, the genetic component for most common diseases are poorly modeled by knockout and overexpressing mice; second, genetic background differences often make a relevant translation of a human SNP into a mouse impossible. For instance, many human disease SNPs do not change protein sequence (ie, are synonymous or in regulatory or unknown element of the gene) and, thus, are difficult to translate onto the mouse genome. Fortunately, the R213G SNP lies in the extracellular matrix binding domain coding region of exon 2, a region that is 100% conserved between mice and humans (Figure 1A). Therefore, we were able to clone a single base cytosine to guanine substitution at the equivalent R213G position (Figure 1B, red asterisk) into the native mouse SOD3 to mimic the naturally occurring R213G SNP in humans. After target insertion, we obtained 4 positive clones that were confirmed for proper homologous integration by Southern blot (Figure 1C). Positive mice and their offspring were further confirmed by genotyping (Figure 1D) and DNA sequencing (Figure 1E). Similar to humans,30 mice with the R213G SNP have high levels of EC-SOD protein and activity in plasma (Figure 1F and 1G; P=0.01). The R213G EC-SOD in plasma has reduced heparin-binding affinity compared with WT protein (Figure 1H); however, the purified WT and R213G EC-SOD had similar SOD activity per unit of purified protein (Figure 1I). Although human studies have shown that the presence of R213G SNP is associated with 2- to 4-fold increased EC-SOD protein in plasma and the R213G variant EC-SOD protein has impaired in vitro binding to extracellular matrix elements,7 there are no reports of the effects of R213G SNP on tissue distribution of EC-SOD. To study tissue distribution of EC-SOD in lung parenchyma and vasculature for the R213G SNP in vivo, we performed both immunolocalization and Western blotting. Furthermore, to determine whether the R213G knockin mice accurately recapitulate R213G human carriers, we screened 78 unique lung samples from a human biobank to identify 2 humans heterozygous for the R213G SNP. By immunolocalization, the R213G SNP in mice was associated with a distinct reduction in staining for EC-SOD in lung parenchyma, pulmonary vasculature (Figure 1J), and aorta (Figure 1K). On quantitative Western blots of lung (Figure 1L), EC-SOD protein was reduced by 72% in the R213G
Figure 1. Characterization of R213G single-nucleotide polymorphism (SNP) knockin mouse. A, Homologous recombination introduces the identical R213G (C→G) SNP resulting in an identical arginine to glycine amino acid substitution in the extracellular matrix-binding domain (ECM-BD) of extracellular superoxide dismutase (EC-SOD). Blue amino acids represent the conserved ECM-BD region, and red represent R213G SNP at position 213. B, R213G SNP homologous recombinant containing a Neo cassette (Neo). Red asterisk is site of R213G SNP. Black triangles are LoxP sites. PB1/2 is probe site for Southern blots. C, Confirmation of R213G-positive clones (asterisks) by Southern blot. D, R213G mouse DNA genotyping. Wild-type (WT) mice produce a 357-bp amplicon; R213G heterozygous (HET) mice produce both a 357- and 419-bp amplicon, and R213G homozygous (HM) mice produce a 419-bp amplicon. E, DNA sequence confirms C→G mutation (red asterisk) changing the codon from CGG (arginine) to GGG (glycine). F, Plasma EC-SOD protein of WT, HET, and HM mice. G, EC-SOD activity in units per milliliter of plasma (n=6 for all genotypes). H, Heparin–sepharose binding using purified EC-SOD from WT (n=1) open squares and dashed line) and R213G HM (R213G; n=1) mouse (black squares and solid line) in units/mL (U/mL) of elution volume. (I) SOD activity normalized to protein (n=3 for both WT and R213G). J) EC-SOD immunostaining in murine lung tissue. Brown staining (arrow indicates airway; arrowhead, vessel) for wild type (WT), R213G heterozygous (HET) and R213G HM mice. EC-SOD knockout mice (KO) were used as background EC-SOD staining controls. (K) EC-SOD immunostaining in mouse aorta. Brown staining (arrows) shows EC-SOD localization for WT, HET and HM mice. EC-SOD immunodepleted (ID) and PBS were used for nonspecific background staining detection. (L) EC-SOD total protein was analyzed in total lung tissue by Western blot from WT (n=3), HET (n=3), and HM (n=3) animals. M, EC-SOD activity in units per milligram of lung tissue protein of WT (n=6), HET (n=6), and HM (n=5) mice. N, EC-SOD protein in lung tissue from WT and R213G human carrier. O, EC-SOD protein in bronchoalveolar lavage fluid (BALF) of WT and R213G homozygous (R213G) mice. P, EC-SOD protein in BALF from WT and R213G human carrier obtained by postmortem. Data are shown as mean±SE. *P<0.05 compared with WT.
mice ($P=0.03$) with corresponding reduction in EC-SOD activity assays (Figure 1M). This marked decrease in lung EC-SOD content in R213G mice was recapitulated in humans who carried the R213G SNP (Figure 1N). Notably, we also discovered that the R213G SNP was associated with higher EC-SOD in BALF in both mice (Figure 1O; $P=0.01$) and humans (Figure 1P). No compensatory gene expression changes were observed in other superoxide dismutase enzymes (SOD1 or SOD2) in the lungs of R213G mice (data not shown). A small but significant increase in SOD3 expression was detected in whole lung tissue but not in isolated vascular tissue (Figure 1A and 1B in the Data Supplement). Thus, these findings demonstrate that the presence of R213G polymorphism is sufficient to alter the distribution of EC-SOD protein from lung and vascular tissue to extracellular fluids such as BALF and plasma.

Because human gene association studies suggested the R213G SNP was associated with lower rates of lung inflammation (acute exacerbations of chronic obstructive pulmonary disease) and animal studies using EC-SOD knockout and overexpressing mice have shown that EC-SOD protects the lung from multiple inflammatory and oxidative stress insults, we postulated that the high levels of EC-SOD in BALF associated with the R213G SNP would protect mice from lipopolysaccharide-induced acute lung injury. One day after exposure to lipopolysaccharide, mice with the R213G SNP had a trend toward fewer BALF total cells (Figure 2A; $P=0.02$) and significantly fewer neutrophils (Figure 2B; $P=0.03$). The number of macrophages recovered from BALF did not significantly differ between groups at any time point assessed (Figure 2C). Additionally, there were significantly less proinflammatory cytokines (tumor necrosis factor-α, KC, and IL-12p70) in BALF after lipopolysaccharide exposure (Figure 2D). Measurement of these same mediators in homogenized lung tissue revealed no difference between WT and R213G samples at any of the time points assessed (Figure 2D in the Data Supplement). Similarly, although lipopolysaccharide inhalation induces a significant increase in total BALF protein in both genotypes, the R213G samples were not significantly different from the respective WT samples (Figure 2E in the Data Supplement). Finally, there was a trend toward significantly less oxidative stress in the lung tissue of R213G mice after lipopolysaccharide compared with WT mice as measured by Western blot of 4-hydroxynonenal (Figure 2E; $P=0.1$). The attenuation of oxidative stress occurred despite the decreased content of lung EC-SOD, which suggests that high levels of EC-SOD in airway fluids (rather than lung tissue) are sufficient to reduce oxidative stress from toxic inhalations. These data strongly suggest that people with R213G polymorphism are likely protected from inhalational lung injury because of high levels of EC-SOD in their bronchoalveolar fluid.

In contrast to reduced risk of lung disease, the R213G SNP has been associated with increased risk of cardiovascular disease. The mechanism for this increased risk is unknown, but our findings indicate that the R213G SNP leads to a marked depletion of EC-SOD in the walls of blood vessels (Figure 1K). To determine if the R213G SNP increases susceptibility to vascular injury, we evaluated the impact of R213G SNP on the development of pulmonary vascular disease because of chronic hypoxia (hypoxia-induced pulmonary hypertension [PH]). By Western blot, EC-SOD protein was dramatically reduced in isolated pulmonary arteries from R213G mice (Figure 3A). Compared with WT littermates, mice with R213G SNP exhibited PH at baseline and had worsened chronic hypoxic PH, shown by elevated right ventricular systolic pressure (Figure 3B; $P=0.0007$) and hypertrophy (Figure 3C; $P=0.0007$). The increased PH in R213G SNP mice was associated with increased medial wall remodeling as measured by an increased number of muscularized vessels.

![Figure 2](http://circgenetics.ahajournals.org/)

**Figure 2.** Mice with R213G single-nucleotide polymorphism have attenuated injury to inhaled lipopolysaccharide (LPS). A, Total cell counts in bronchoalveolar lavage fluid (BALF) 0, 4, and 24 h post-LPS (n=4 per group). B, Total neutrophil counts in BALF. C, Total macrophage counts in BALF. D, Cytokines in BALF of wild-type (WT) and R213G animals at 0 h (WT n=4, R213G n=3), 4 h (WT n=8, R213G n=4), and 24 h (WT n=9, R213G n=4) post-LPS inhalation. E, 4-hydroxynonenal (4-HNE) in lung tissue. Data are presented as mean±SE. #P<0.05 compared with samples at 0 h for each genotype. *P<0.05 comparing WT to R213G samples under identical conditions.
(<50 μm) identified by positive α-smooth muscle actin immunostaining (Figure 3D and 3E; P=0.03). The R213G mice also display increased hypoxia-induced pulmonary artery adventitial remodeling, as demonstrated by enhanced peri-vascular collagen deposition (Figure 3F and 3G; P=0.002). These results indicate that increased risk of vascular disease

**Figure 3.** R213G single-nucleotide polymorphism reduces vascular extracellular superoxide dismutase (EC-SOD) and increases susceptibility to pulmonary hypertension. **A,** EC-SOD protein expression in pulmonary artery tissue isolated from normoxic (NX), wild-type (WT), and R213G homozygous mice (R213G). **B,** Right ventricular systolic pressure (RVSP; mm Hg) at baseline (WT n=9, R213G n=6) and after 35 d of chronic hypoxia (HPX) in WT (n=8) and R213G (n=6). **C,** RV hypertrophy (RV/left ventricle+septum weights) at baseline (WT n=8, R213G n=6) after 35 d of HPX (WT n=7, R213G n=8). **D,** Representative lung α-smooth muscle actin (α-SMA) immunostaining (brown signal) with counterstain by hematoxylin (blue) after 35 d of HPX. Bar, 50 μm. Arrows indicate muscularized small vessels identified by positive α-SMA immunostaining. **E,** Quantification of muscularized small vessels (<50 μm) in a ×10 field at baseline (WT n=5, R213G n=6) and after 35 d of HPX (WT n=4, R213G n=5). **F,** Collagen visualized by two-photon excitation second harmonic generation in representative unstained tissue sections. Collagen (red signal) and tissue autofluorescence (green signal). **G,** Quantification of collagen standardized for vessel size (n=6 for all groups). Data are shown as mean±SE. *P<0.01 for WT compared with R213G samples under identical conditions. #P<0.01 for NX compared with HPX.
in people with R213G SNP is because of reduced EC-SOD in blood vessels.

Discussion
The human R213G SNP (rs1799895) is associated with increased risk of cardiovascular disease and reduced risk of airway disease in multiple large genetic association studies. In this study, we show that the SNP results in reduced binding to heparin, but no loss of enzymatic activity. The reduced binding to heparin and other matrix elements is most likely because of changing the codon from arginine to glycine for a key amino acid in the extracellular matrix–binding domain of the protein. We are the first to show that this results in reduced EC-SOD protein and activity in both human lung and vascular tissue, but leads to increased EC-SOD in extracellular fluid such as plasma and lung epithelial lining fluid.

The benefit of a shift of EC-SOD protein from tissue to extracellular fluid such as the lung epithelial lining fluid is that there is reduced lung inflammation after inhalation of pro-oxidants such a lipopolysaccharide. The increased superoxide dismutase activity in the epithelial lining fluid leads to a reduction in proinflammatory cytokines and inflammatory cells. This attenuation in inflammation outweighs the potential loss of EC-SOD activity in the lung tissue. The consequences of reduced EC-SOD in vascular tissue are more significant in oxidative stress–dependent vascular injury models such as hypoxia-induced PH. The increased susceptibility to vascular injury models should not be surprising because the R213G SNP results in marked depletion of EC-SOD in blood vessels, in which EC-SOD is normally the most abundant antioxidant enzyme. Thus, carriers of the human R21G polymorphism and its murine equivalent mouse have a phenotype similar to an EC-SOD overexpressor mouse in the epithelial lining fluid, but an EC-SOD knockout in tissue. These paradoxical effects are consistent with animal models that show that EC-SOD–overexpressing mice are protective of lipopolysaccharide (LPS) and increased susceptibility to vascular injury such as hypoxia-induced PH and ischemia reperfusion.

The teleological explanation for the persistence of SNP in northern European (mean allele frequency 2–3%) and Asian populations (mean allele frequency ≈6%) is unknown. We speculate that increased EC-SOD in lung lining fluid may serve to attenuate chronic lung inflammation from indoor air pollution (ie, indoor cooking). This benefit would have to lead to reproductive advantages compared with increased susceptibility to cardiovascular disease.

In summary, the human R213G SNP is one of only a few SNPs that have been reproducibly linked with cardiopulmonary disease and also one of the few SNPs that can be faithfully translated into a mouse model. Our findings demonstrate that humans and mice with the R213G SNP have a shift in distribution of EC-SOD protein from lung and vascular tissue into extracellular fluids. This altered distribution increases the amount of SOD activity in lung lining fluid and reduces SOD activity in lung tissue and blood vessels (Figure 4). This shift explains the paradox of how a single polymorphism can simultaneously reduce the risk for lung disease while increasing the risk of vascular disease. The benefits of reducing the risk of lung injury from inhaled agents may provide a teleological explanation for why the R213G SNP is common in humans despite its concurrent increased susceptibility for vascular disease.

Figure 4. Schematic demonstrating the change in distribution of extracellular superoxide dismutase (EC-SOD) associated with the R213G single-nucleotide polymorphism. The consequence of the change in distribution is a reduction in susceptibility to inflammation from lipopolysaccharide (LPS) and increased susceptibility to vascular disease (hypoxic pulmonary hypertension and remodeling). ECM indicates extracellular matrix; and WT, wild type.

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Disclosures
None.

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Supplemental Methods

Gene expression

Total RNA was extracted using either TRIzol (Invitrogen, Carlsbad, CA) or RNeasy kit (Qiagen) according to the manufacturer’s instructions and differences in gene expression were determined using Real-Time RT-PCR as previously described\(^1\). Gene expression was determined using comparative threshold cycle (ΔΔC\(_T\)) as suggested by the manufacturer (Applied Biosystems, Foster City, CA) normalizing each sample to 18s rRNA. The following primer sets were purchased from Assays on Demand (Applied Biosystems): 18s rRNA (cat #4310893E) and SOD3 (cat# Mm01213380-s1).

Proinflammatory cytokine and chemokine measurements

Cytokine and chemokine concentration in mouse lung tissue were measured using the MSD (MesoScale Discovery, Gaithersburg, MD) Proinflammatory Panel 1 V-PLEX kit.

Total protein in bronchoalveolar lavage

Total protein was quantitated using Coomassie Plus (Bradford) Assay kit (Thermo Fisher Scientific, Rockford, IL) per manufacturer’s instructions.

Supplemental Figures

Figure legends

**Fig 1.** (A) Expression of SOD3 was measured by Real-Time RT-PCR in whole lung tissue from WT (n = 3), HET (n = 3) and HM (n = 3) animals. Gene expression was normalized to 18s rRNA
expression and displayed relative to WT samples. (B) Expression of SOD3 was measured by Real-Time TR-PCR in isolated aortas from WT (n = 3), HET (n = 3) and HM (n = 3) animals. Gene expression was normalized to 18s rRNA expression and displayed relative to WT samples. (C) Proinflammatory cytokines and chemokines in lung tissue from WT and R213G animals at 0 (WT n = 4, R213G n = 3), 4 (WT n = 8, R213G n = 4) and 24 hours (WT n =9, R213G n = 4) post LPS inhalation. Mediators are expressed as pg per 30ug of total protein. (D) Total protein in broncoalveolar lavage fluid (BALF) in WT and R213G animals at 0 (WT n = 3, R213G n = 2), 4 (WT n = 7, R213G n = 4) and 24 hours (WT n = 4, R213G n = 7) post LPS inhalation. Data are presented as mean ± S.E. # P < 0.05 compared to samples at 0 hours for each genotype. * P < 0.05 comparing WT to R213G samples under identical conditions.

Supplemental References
