Supporting Information

Yao et al. 10.1073/pnas.1007625107

SI Materials and Methods

Exercise Endurance Determination. Exercise endurance in mouse was measured using a motorized rodent treadmill with an electric grid at the rear of the treadmill (Columbus Instruments) as described previously (1). Run duration (in min), run distance (in m, calculated from the run time and speed of the treadmill), and vertical work performed (in kg/m) were used as the parameters to reflect exercise tolerance. Vertical work was calculated as the product of body weight (in kg) and vertical distance (in m), where vertical distance = (distance run) (sinθ), where θ is equal to the angle of the treadmill from 0° to 15°. Mice were placed on the treadmill and allowed to adapt to the surroundings for 3 to 5 min before starting the exercise. The treadmill was started at a speed of 8.5 m/min with a 0° incline. After 9 min, the speed and incline were raised to 10 m/min and 5°, respectively. Speed was increased by 2.5 m/min every 3 min to a maximum of 40 m/min, and the incline increasing 5° every 9 min to a maximum of 15°. Exercise continued until mouse exhaustion, which is defined as an inability to maintain running despite repeated contact with the electric grid. At this stage, each mouse was immediately returned to its home cage.

Measurement of Lung Mechanics. Lung mechanics including lung compliance, R_L, and Rn were determined as described previously (2, 3). Briefly, the mouse was weighed, deeply anesthetized by an i.p. injection of pentobarbital (90 mg/kg) and pancuronium (0.5 mg/kg), and tracheostomized. The trachea was cannulated, and the cannula was connected to a computer-controlled small animal ventilator (FlexiVent; SCIREQ). Estimated lung compliance, R_L, and Rn at 3 cm H_2O positive end expiratory pressure were obtained by fitting a model to each impedance spectrum. The calibration procedure removed the impedance of the equipment and tracheal tube within this system.

Oxygen Saturation Determination. A pulse oximeter (Starr Life Sciences) was used to measure blood oxygen saturation on an awake mouse after 6 mo of CS exposure (4). Briefly, the pulse oximeter sensor clip was placed on the back of mouse neck after depilation, and mouse was put in a STARR-Dust cage until calm. The oxygen saturation was determined and collected using MouseOx software (Starr Life Sciences).

Inflammatory Cells Influx in BAL Fluid. Inflammatory cells influx in BAL fluid were counted on cytospin-prepared slides stained with Diff-Quik (Dade Behring) (5).

Lung Morphometry and Immunohistochemical Staining for Macrophages and SOD3. Mouse lungs (which had not been lavaged) were inflated with 1% low melt agarose at a pressure of 25 cm H_2O, and then fixed with 4% neutral buffered paraformaldehyde (6). Fixed lung was dehydrated and embedded in paraffin, and sectioned into 4-μm sections using a rotary microtome (Microm). H&E staining was performed on lung midsagittal sections to determine Lm of airspace (6). Digital photomicrographs were taken across the widest transect of each section and a grid was superimposed. Lm was calculated using the formula $Lm = 2L_T / I_W$, where L_T is the total length of the grid in micrometers and I_w is the number of times the grid intersects an alveolar wall for each sample based on 10 random fields observed using a cross-line. For macrophage immunohistochemical staining, the deparaffinized and rehydrated lung sections were exposed to 3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. Nonspecific binding of antibodies to the tissue sections was blocked by incubating the sections with 5% normal goat serum in PBS solution for 30 min. Rat antimouse Mac-3 monoclonal antibody (BD Pharmingen) at a titer of 1:50 was used to detect macrophages. Sections were incubated with primary antibodies overnight at 4 °C. After being washed, sections were incubated with secondary antibody biotinylated goat antirat Ig (DAKO) for 1 h. 3,3′-Diaminobenzidine (DAKO) was used as peroxidase substrate. Tissues were counterstained with hematoxylin. The number of Mac-3–positive cells in the lung sections (five random microscopic fields per lung section in three different sections) were counted manually under microscope with 200× magnification and averaged (6). Similarly, anti-SOD3 antibody (Assay Design) was used to study the distribution of SOD3 in mouse lung by immunohistochemistry.

Determination of Oxidative Stress Markers. The occurrence of elastin oxidation in lung was determined by coimmunoprecipitation with an antibody against elastin (Santa Cruz Biotechnology). The immunoprecipitated elastin agarose bead suspension was resolved by SDS/PAGE, and blots were probed with antielastin antibody. After stripping, membranes were equilibrated with 20% (vol/vol) methanol and 80% Tris-buffered saline solution for 5 min, and then incubated with 0.5 mM 2,4-dinitrophenylhydrazine for 30 min at room temperature. The membrane was washed and incubated overnight with anti–2,4-dinitrophenylhydrazine antibody to assess elastin carbonylation (7). The levels of lipid peroxidation products 4-HNE and MDA in lung homogenates were measured separately using a lipid peroxidation kit (Calbiochem) (5, 6). ROS release in BAL cells was determined using 2′,7′-dichlorofluorescein-diacetate, which is an oxidant-sensitive fluorescent probe (6). The fluorescent intensity of cells was assessed with a score between 1 and 3 based on the following criteria: 1, weak staining; 2, moderate staining; 3, intense staining.

Immunoblotting. The preparation of whole cell lysate from lung tissues and cells were described previously (5, 6). Protein level in samples was measured with a BCA kit (Pierce). Immunoblotting was performed with anti-heparan sulfate (MAB2040; Chemicon), antielastin (Santa Cruz Biotechnology), and anti-SOD3 (Upstate) antibodies to determine respective proteins. Equal loading of the samples was determined by quantification of proteins as well as by reprobing membranes for β-actin.

Proinflammatory Mediators Assay. Levels of proinflammatory mediators in lung homogenates were measured by the Luminex 100 using the Beadlyte Mouse Multicytokine Beadmaster kit (Bio-Rad Laboratories) (5). The assay utilizes microspheres as the solid support for immunoassays and permits simultaneous cytometric quantification of multiple chemokines/cytokines with minimal sample volume.

GSH Assay. Intracellular total GSH levels in lung tissues were determined according to the method described previously (5, 8). Briefly, the lungs were homogenized with 0.1 M phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.1% (vol/vol) Triton X-100, and 0.6% (wt/vol) sulfosalicylic acid. The lung debris was collected by centrifugation, the supernatant was incubated with 0.2 mg/mL of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1.67 U/mL glutathione reductase in phosphate buffer-EDTA for 30 s, 0.2 mg/mL β-NADPH was added, and the rate of DTNB reduction was measured spectrophotometrically at 405 nm. The concentration of total GSH in the supernatant was determined by comparison with the reduction of total DTNB by known concentrations of GSH. Results were expressed as the nmol of

GSH per milligram protein. For determining the concentration of GSSG, 0.6% sulfosalicylic acid–extracted lung homogenates were added with 2% 2-vinylpyridine to derivatize GSH. The supernatants of extracted lung homogenates were diluted by 30 times in phosphate buffer before the addition of DNTB, β-NADPH, and GSSG reductase for assay of the level of GSSG via monitoring NADPH spectrophotometrically at 405 nm (8–12). The concentration of reduced GSH was calculated by subtracting GSSG concentrations from the total GSH concentrations (reduced GSH = total GSH − 2GSSG).

SOD3 Activity Assay. SOD3 was separated from intracellular SODs (SOD1 and SOD2) in lung homogenates using a concanavalin A Sepharose column (GE Healthcare) as described previously (13). SOD3 activity was assessed using a SOD assay kit (Dojindo), whereby superoxide anion was generated from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase. A water-soluble tetrazolium salt, WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt], was used in this assay kit to produce a water-soluble formazan dye upon reduction with a superoxide anion, detectable by a colorimetric assay. Hence, SOD3 lowers the rate of WST-1-formazan formation via scavenging superoxide anion (14, 15). SOD3 activity in lung homogenates was expressed in units (U) wherein 1 U of SOD3 was defined as the amount of sample causing 50% inhibition of the colorimetric reaction per milligram of protein.

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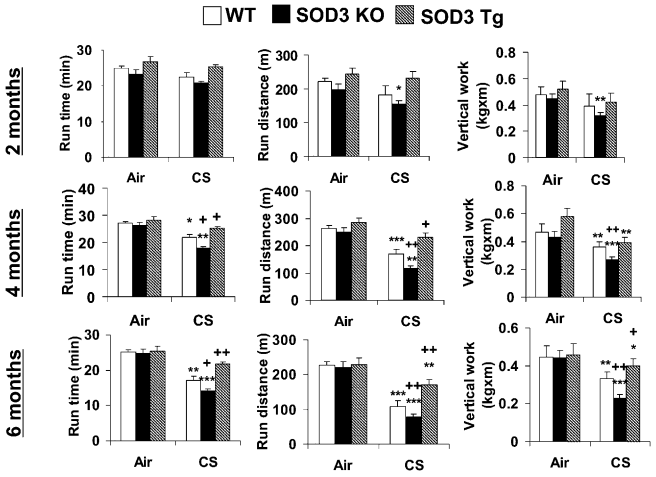

Fig. S1. Subchronic (2 and 4 mo) and chronic (6 mo) CS-induced decrease in exercise endurance was improved by SOD3 in mice. SOD3 KO, SOD3 Tg, and WT mice were exposed to CS for 2, 4, and 6 mo, and exercise capacity (i.e., run time, run distance, and vertical work) was assessed using a motorized rodent treadmill. All CS exposures (2, 4, and 6 mo) decreased exercise tolerance, which was significantly improved by SOD3 in mice. Data are shown as mean ± SEM (n = 3–4 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus corresponding air-exposed groups; $^{+}P < 0.05$ and $^{++}P < 0.01$ versus corresponding CS-exposed WT mice.

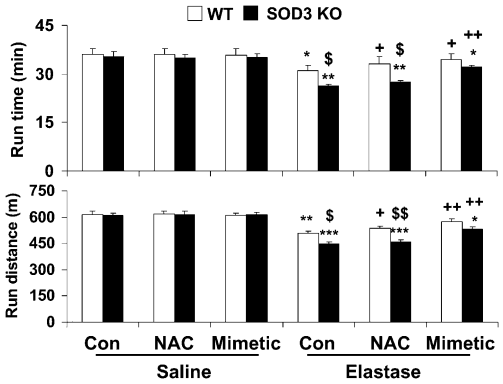

Fig. S2. SOD mimetic improved elastase-induced reduction in exercise endurance in WT and SOD3 KO mice. s.c. injection of SOD mimetic (5 mg/kg/d) significantly improved elastase-induced reduction in exercise tolerance in both WT and SOD3 KO mice. However, administration of NAC (2.0 g/kg/d) attenuated elastase-induced reduction in exercise capacity only in WT, but not in SOD3 KO, mice. Data are shown as mean ± SEM (n = 3–4 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus corresponding saline solution–exposed groups; $^{+}P < 0.05$ and $^{++}P < 0.01$ versus corresponding elastase-exposed control mice; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ versus corresponding elastase-exposed WT mice. Con, control; Mimetic, SOD mimetic.

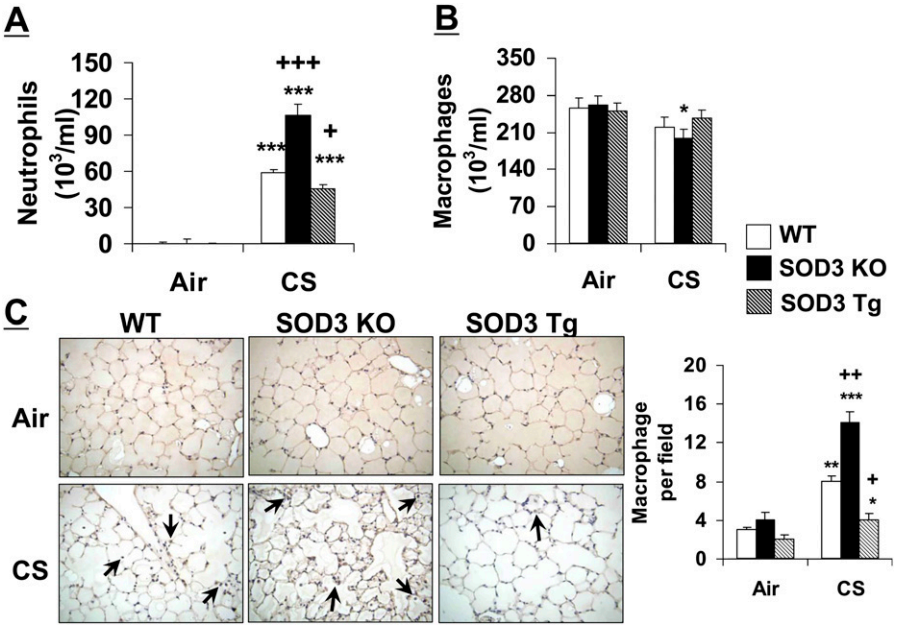

Fig. S3. Acute (3-d) CS-induced inflammatory cell influx into the lungs was attenuated by SOD3. (*A*) Increased neutrophil influx was significantly reduced by SOD3 in mice exposed to CS for 3 d. (*B*) No change of macrophage influx into BAL fluid was observed in WT and SOD3 Tg mice after 3-d CS exposure. However, KO of SOD3 reduced the number of macrophages in BAL fluid compared with WT mice exposed to CS for 3 d. (*C*) The infiltration of macrophages in lung interstitium was increased in WT and SOD3 KO mice, which was significantly attenuated in SOD3 Tg mice. Data are shown as mean ± SEM (n = 3–4 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus corresponding air-exposed groups; $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ versus the corresponding CS-exposed WT mice. Original magnification, 200×.

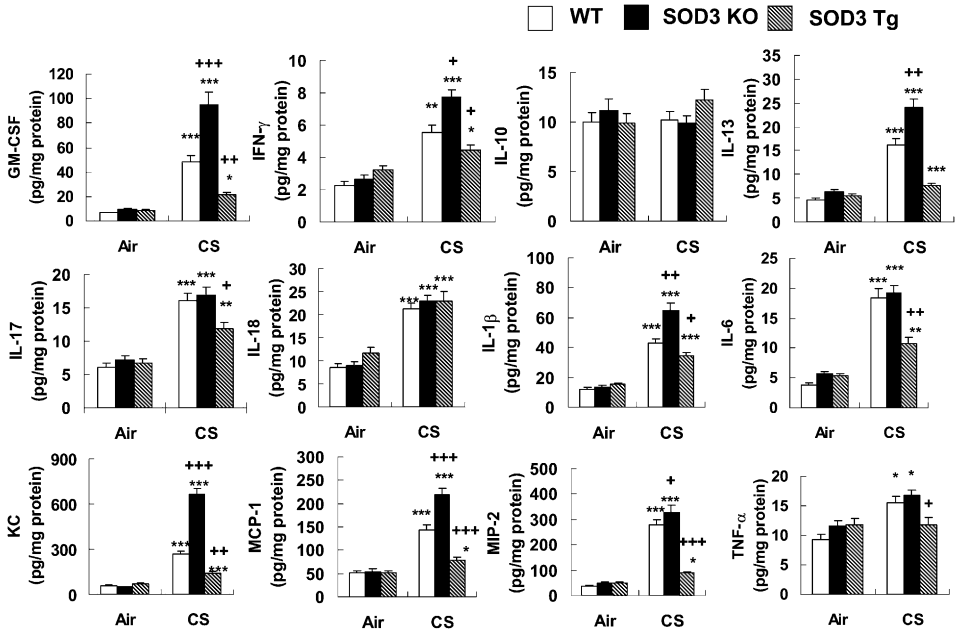

Fig. S4. SOD3 attenuated acute CS-induced proinflammatory mediators release in mouse lung. Proinflammatory mediator levels were determined by the Luminex assay in lung homogenates of WT, SOD3 KO, and SOD3 Tg mice exposed to CS for 3 d. SOD3 protected against CS-induced release of proinflammatory mediators in mouse lung. Data are shown as mean ± SEM (n = 4–5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus corresponding air-exposed groups; $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ versus corresponding CS-exposed WT mice.

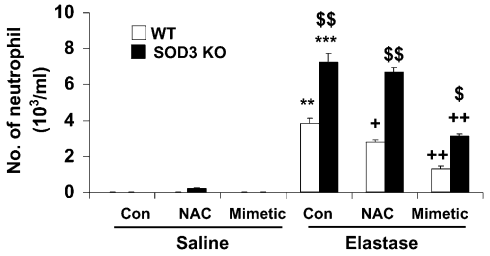

Fig. S5. SOD mimetic decreased elastase-induced neutrophil influx into BAL fluid of WT and SOD3 KO mice. Neutrophil influx into BAL fluid was significantly increased after elastase administration in WT and SOD3 KO mice, which was ameliorated by SOD mimetic. Administration of NAC attenuated elastase-induced neutrophil influx into BAL fluid of WT, but not in SOD3 KO, mice. Data are shown as mean ± SEM (n = 3–4 per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ versus corresponding saline solution–exposed groups; $^{+}P < 0.05$ and $^{++}P < 0.01$ versus corresponding elastase-exposed control mice; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ versus corresponding elastase-exposed WT mice. Con, control; Mimetic, SOD mimetic.

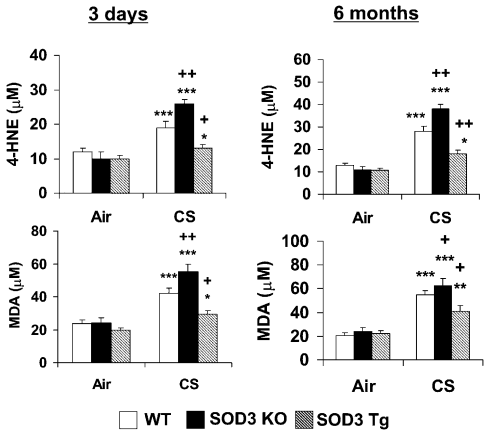

Fig. S6. SOD3 overexpression attenuated CS-induced increase in lipid peroxidation products in the lung. Exposure of WT mice to CS increased the levels of 4-HNE and MDA in lungs of WT mice, which was significantly attenuated in SOD3-overexpressing mice. Data are shown as mean ± SEM (n = 4–5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus corresponding air-exposed groups; $^{+}P < 0.05$ and $^{++}P < 0.01$ versus corresponding CS-exposed WT mice.

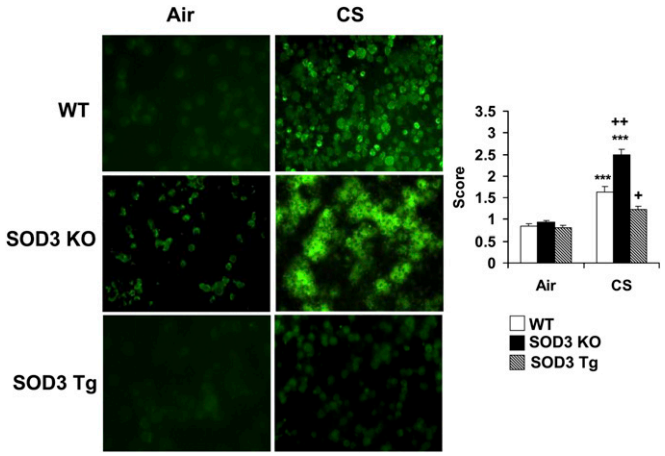

Fig. S7. ROS release in BAL cells was reduced by SOD3 in mice in response to 3 d of CS exposure. 2′,7′-Dichlorofluorescein-diacetate staining showed that SOD3 protected against 3-d CS–induced ROS release in BAL cells from mice. Data are shown as mean ± SEM (n = 4–5 per group). $^{***}P < 0.001$ versus corresponding air-exposed groups; $^{+}P < 0.05$ and $^{++}P < 0.01$ versus corresponding CS-exposed WT mice.

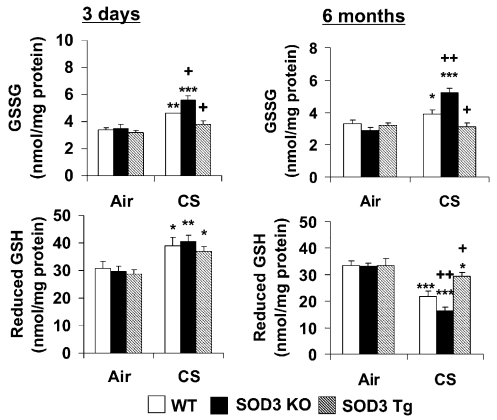

Fig. S8. SOD3 protected against CS-mediated depletion of GSH in mouse lung. SOD3 KO, SOD3 Tg, and WT mice were exposed to CS for 3 d and 6 mo. SOD3 attenuated acute and chronic CS-induced increase in oxidized/disulfide form of GSH (i.e., GSSG). Three days of CS exposure increased the level of reduced GSH in lungs of WT, SOD3 KO, and SOD3 Tg mice. The level of reduced GSH was significantly lowered in WT mice after 6-mo CS exposure, which was attenuated by SOD3. Data are shown as mean ± SEM (n = 4–5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus corresponding air-exposed groups; $^{+}P < 0.05$ and $^{++}P < 0.01$ versus corresponding CS-exposed WT mice.

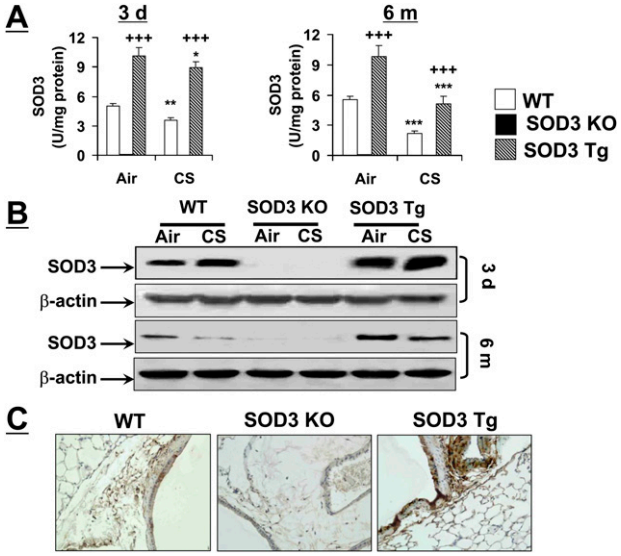

Fig. S9. Effect of CS exposure on level/activity of SOD3 in mouse lung. SOD3 KO, SOD3 Tg, and WT mice were exposed to CS for 3 d and 6 mo. (*A*) Both 3-d and 6-mo CS exposures decreased SOD3 activity in mouse lung. (*B*) Six months of CS exposure significantly decreased whereas 3-d CS exposure increased the level of SOD3 in lungs of WT and SOD3 Tg mice. Gel pictures shown are representative of at least three separate experiments. (*C*) Immunohistochemical staining showed that the distribution of SOD3 in lungs of SOD3 Tg mice was similar to that in WT mice in the presence of ambient air. (*A*–*C*) The levels/activities were significantly increased in lungs of SOD3 Tg, whereas there was no detectable expression/activity of SOD3 in lungs of SOD3 KO mice compared with WT mice. Data are shown as mean ± SEM (n = 4–5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus corresponding air-exposed groups; $^{+++}P < 0.001$ versus corresponding air or CS-exposed WT mice. Original magnification, 200×.